Ultracryotomy and the corneal stroma

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ULTRACRYOTOMY AND THE CORNEAL STROMA

Thesis submitted for the degree of Doctor of Philosophy in the Discipline of Biophysics

By

Leslie Rees Williams

Open University

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ABSTRACT

Corneal stroma has two fundamentally interesting properties. Firstly, at physiological values of hydration, it is transparent to visible radiation. Secondly when bathed in aqueous solutions it will take up water and can achieve hydrations many times those of the physiological level with a consequent loss of transparency. These two important properties are intimately linked with two other features of corneal stroma. These are the lamellae of collagen fibrils of uniform diameter which make up the greater part of the stroma and the presence within the stroma of glycosaminoglycans which at physiological pH are negatively charged. The organisation of the collagen fibrils and their relationship to the glycosaminoglycans are important factors in understanding both the transparency of the tissue and the way in which it swells.

The work in this thesis falls into two parts. The first is concerned with the development of a technique for cutting ultrathin frozen sections of unfixed biological tissue for transmission electron microscopy. This technique, termed ultracryotomy, offers an alternative preparatory route for ultrastructural study in the electron microscope and in combination with X-ray microanalysis holds great promise for the identification and quantification of chemical distributions at an ultrastructural level. The second part describes experiments to measure the concentration of negatively charged poly-electrolytes within the stroma. This was done indirectly using a purpose built swelling pressure apparatus and then calculating a value for the charge concentration from a theory linking swelling pressure and
charge concentration. As a comparison a direct measure of the charge concentration within the stroma was made using radioisotopes.

Exhaustive development was carried out with the technique of ultracryotomy and two fundamental limitations to the technique were established. These being the ultrastructural damage caused by the rapid freezing of the tissue and the artefacts produced when sectioning the frozen material. The results from the swelling pressure apparatus indicated that the value of charge concentration within the stroma was a function of the ionic strength of the stromal bathing medium. The measures of charge concentration obtained radioisotopically were considerably larger than those from the swelling pressure apparatus and showed no significant differences between the various bathing media. However there was a significant difference in the charge concentration between rapidly frozen and unfrozen corneal stromas as measured by this radioisotopic technique which suggests that rapid freezing was damaging the chemical integrity of the tissue.
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INTRODUCTION

The initial impetus for the work described in this thesis sprang from a desire to resolve the question of how the glycosaminoglycans are distributed within the corneal stroma. Glycosaminoglycans are known to be important in causing the cornea to swell (Heringa et al, 1940; Meyer et al, 1953; Hedbys, 1961). Two models of corneal swelling have been described which offer markedly different viewpoints on the location and consequent contribution that the glycosaminoglycans make to the swelling phenomenon (Hart and Farrell, 1971; Hodson, 1971). The method chosen to investigate this problem offered, at that time, the possibility of not only indicating the physiological situation of corneal glycosaminoglycans but also of opening a new field for biological investigation.

This introduction, then, includes a brief description of X-ray microanalysis, because the description helps to explain why such efforts went into the development of the technique of ultracytotomy, described in the next chapter of this thesis.
Ultracryotomy, as first defined by Hodson and Marshall (1970), is a technique for cutting ultrathin sections of frozen biological tissue for electron microscopy. It provides not only an alternative preparative route for biological tissue that one may wish to examine in the electron microscope but, more importantly because of its directness and potential simplicity, it holds great promise of subsequent quantitative chemical analysis at the ultrastructural level within the tissue. Chemical analysis at this level can be achieved by a combination of ultracryotomy and X-ray microanalysis within the electron microscope.

The interaction of the primary electron beam and the specimen in the electron microscope produces, amongst other things, X-rays which characterise the atomic constituents of the specimen and, by furnishing one's electron microscope with a suitable X-ray detector and analyser, it becomes possible to determine both the structural and elemental composition of one's specimen. X-ray microanalysis has a spatial resolution of the order of 20 - 30 nm and a detection limit of about $10^{-18}$ g under favourable conditions (Hall, 1977). The limiting factor in the use of X-ray microanalysis with biological specimens is specimen preparation and the technique of ultracryotomy was developed to reduce this limitation.

X-Ray Microanalysis in the Electron Microscope

As indicated above the interaction of the primary electron
beam and the specimen in an electron microscope produces, among many other things, an X-ray signal from the atoms in the specimen which characterises those atoms and thus provides a means of identifying the elements within a sample.

This X-ray signal consists of two components: the characteristic X-rays and the background X-rays.

(i) **Characteristic X-Rays**

For the production of characteristic X-rays from an atom the energy of the primary electrons has to be above what is termed the critical excitation potential for that atom. If this condition is satisfied then it is possible for a primary electron to penetrate the outer electron orbitals of that atom and displace an electron from an inner orbital shell. The primary electron loses energy undergoing what is termed inelastic scattering whilst the atom loses the energy it has gained by a series of electron transitions from outer to inner shell vacancies, the energy loss being in the form of X-rays. There is thus produced an X-ray spectrum of the atom consisting of a number of discrete X-ray lines the energies of which are directly related to the electron orbital configuration of the excited atom. This relationship between the energy or frequency of the characteristic X-ray radiation and the atomic number of the element responsible for the emission was first described by Moseley (1913, 1914) and since then all the elements
of the periodic table have been characterised by this relationship and their X-ray spectra tabulated (Bearden, 1964).

Not all the characteristic X-ray radiation escapes from the specimen. In the case of the less energetic or softer X-rays emitted by elements of low atomic number there is considerable absorption and transduction of X-ray energy and only a fraction of the characteristic X-ray energy produced is finally emitted. This fraction is termed the fluorescent yield and is strongly dependant on atomic number, increasing from a small fraction at low atomic number (Z = 10) to approaching unity for Z > 60 when considering the K-lines of the X-ray spectra which are produced by electron transitions from outer electron orbitals to the innermost electron orbit.

(ii) Background Radiation

If a primary electron passes very close to the nucleus of an atom within a specimen it can undergo a large deflection from its original path and also lose energy in the form of X-rays. In theory (Kramers, 1923), the intensity distribution of this energy is continuous and ranges from infinity at zero energy to zero intensity at the energy of the primary electron. In practice, however, at the low energy end of the spectrum, absorption of X-rays within the specimen and also by the window normally fitted to X-ray detectors attenuates the signal.
This type of X-ray radiation is termed background radiation, white radiation, continuum radiation or Bremsstrahlung and the primary electron in producing these X-rays is said to have undergone imperfect elastic scattering.

(iii) X-Ray Detectors

The combination of electron-optical imaging and X-ray microanalysis was first developed in the late 1940's and early 1950's (Castaing and Guinier, 1949; Castaing, 1951). Material scientists and metallurgists quickly took advantage of the potential offered by this combination. Biologists, however, have been very much slower to do so, primarily because of the preparatory problems associated with specimens having a high water content.

At the present time X-ray microanalysis is carried out in the electron microscope with two types of X-ray detectors:

a) Wavelength Dispersive Crystal Spectrometers

b) Energy Dispersive Spectrometers

Wavelength dispersive detectors consist of a diffracting crystal of known lattice spacing and X-rays from the specimen incident upon this crystal produce enhanced reflections from the crystal if the wavelength of the incident X-rays satisfies Bragg's law. The reflections from the crystal are detected
using a proportional counter and in practice the crystal is rotated through a range of angles until a 'peak', or maximum of intensity, is detected in the counter at which point the conditions of Bragg's law are satisfied and the wavelength of the X-rays can be identified.

The energy dispersive detector consists essentially of a silicon crystal. X-ray photons entering the detector ionize this crystal producing negative charges in the form of mobile electrons in the conduction band of the silicon and positive charges or 'holes' in the valence band. The formation of electron-hole pairs requires a precise amount of energy and by collecting the total negative charge the energy of the ionizing X-ray photon can be determined.

Both systems have their advantages. The wavelength dispersive system for example has a much better resolution than the energy dispersive detector and is able to separate X-ray lines which are less than 10 ev apart using a suitable crystal. The energy dispersive system is at present limited to a resolving power of about 150 ev. It is however capable of a very high detection efficiency because the crystal can be positioned very close to the specimen. It is also capable of simultaneous detection and display of all X-ray energies leaving the specimen, whereas the wavelength dispersive system is restricted to analysing one wavelength at a time with a relatively low detection efficiency.
Energy dispersive systems are more widely used in biology because of their ease of operation, high collection efficiency and lower cost. Ideally, though, a combination of the two detector types would be best.

Specimen Preparation for X-ray Microanalysis

Conventional preparatory techniques for the examination of biological material in the electron microscope usually involve a sequence consisting of fixation, dehydration, embedding, sectioning and staining of the material, all carried out in a variety of fluid phases. Major changes can and do occur in the elemental distribution and composition of the specimen during this sequence and these changes severely limit the applicability of specimens prepared in this way for subsequent X-ray microanalysis.

The literature on elemental loss, addition and redistribution has been reviewed by Morgan (1981). Workers in this field have used techniques such as flame spectrophotometry (Vassar et al. 1972; Pentilla et al. 1974), scintillation counting (Mehard and Volcani, 1975; Howell and Tyhurst, 1976) and ion microelectrode techniques (Gupta & al. 1977) to quantify the changes occurring during conventional processing and to obtain comparisons with X-ray microanalytical data.
(i) **Elemental loss during Conventional Processing**

The major loss of elements occurs during fixation primarily because this is the first step in the processing sequence. There is an initial large loss of free electrolytes followed by a slower release from less readily exchangeable compartments (DeFilippis and Pallaghy, 1973). Some idea of the rates at which these losses occur is given by the estimate that 50% of the $K^+$ ions would diffuse out of a 1 nm thick fully hydrated section in $10^{-4}$s (Marshall, 1972).

If losses at the fixation stage could somehow be minimized or prevented the specimen would still be equally vulnerable to the other steps in the preparatory sequence. This point has been graphically illustrated by the work of Harvey et al (1976). In the case of specimens to be examined in the transmission electron microscope (TEM), embedded tissue is sectioned on an ultramicrotome and the sectioned material collected from the surface of a water bath. These workers in preparing material for TEM choose a technique of freeze-substitution as an alternative to conventional fixation and dehydration outlined above. Their tissue was rapidly frozen in a liquid coolant and the ice thus formed was removed by placing the frozen tissue in a solvent which had the property of dissolving ice at temperatures well below 0°C. Having thus dehydrated the tissue it was then embedded and the material sectioned. The loss of
Na and Cl from the sections was measured and it was found that between 40 - 90% of the label was lost during a standardised 2 minute period on the water bath.

Comparable losses have been measured by other workers during the staining of tissue with the two heavy metal stains uranyl acetate and lead citrate used extensively in conventional electron microscopy (Agostini and Hasselbach, 1971; Yarom et al, 1974).

(ii) Elemental Redistribution during Conventional Processing

To assess accurately the redistribution of elements within a specimen during its processing for electron microscopy, one has obviously to know what the original distribution was and this is not easily determined. X-ray microanalysis coupled to a preparatory technique which retains the physical and chemical integrity of the specimen would provide such an answer. In the absence of such a technique an empirical approach is necessary to establish whether redistribution has occurred during specimen preparation.

If a number of different preparatory routes are taken and subsequent X-ray microanalysis reveals differing patterns of elemental distribution then, clearly, redistribution has occurred during specimen preparation. The approach has been used to look at the calcium distribution within cells of the parasite
**Schistosoma mansoni** and marked differences were seen in this distribution after different preparatory procedures (Erasmus and Davies, 1979).

(iii) **Elemental Addition during Conventional Processing**

There are three ways in which elements can be introduced into the tissue during specimen preparation:

a) By contamination from the various processing fluids. Examples are the introduction of chlorine from the embedding media and calcium from glutaraldehyde fixatives (Oschman and Wall, 1972).

b) By translocation from the extracellular fluid of the tissue itself because of changes in membrane permeability during fixation (Krames and Page, 1968).

c) The deliberate introduction of exogenous elements such as lead and uranium from staining solutions.

However exogenous elements are introduced, their presence in the tissue presents a number of difficulties in the interpretation of the X-ray data from the tissue.

As already indicated, the more widely used energy dispersive X-ray detectors are limited to a resolution of about 150 ev. This resolution is in fact a function of X-ray energy and
decreases linearly with increasing X-ray energy so that the resolution is normally quoted as that obtained at 5.9 kev which is the Kα line of manganese. This limited resolution of energy dispersive systems compared to wavelength dispersive systems leads to overlapping of X-ray lines from indigenous and exogenous elements which makes qualitative as well as quantitative interpretation of the data difficult.

If an exogenous element is also naturally occurring within the tissue then any attempt at localisation and quantification of that element is difficult to achieve.

The limitations then of conventional preparatory techniques used in conjunction with X-ray microanalysis in the electron microscope are numerous and often disabling. Although certain problems have yielded to this combination, for reviews see Coleman and Terepka (1974), Chadially (1979), Morgan (1981), a more comprehensive application of X-ray microanalysis in biology demands a specimen in which not only is the physical integrity well preserved but the chemical integrity also.

Alternative Preparatory Techniques for X-ray Microanalysis

There has arisen among biologists a sustained interest in the idea that the physical and chemical integrity of cells and tissues can be preserved by cooling them rapidly.

At this point it is useful to give an approximate working definition of rapid cooling or freezing as the phrases will reoccur...
throughout the remainder of this thesis. For this purpose a specimen will be considered to have been subjected to rapid cooling or freezing if the maximum rates of change of temperature within the specimen are of the order of $10^3 \text{ K.s}^{-1}$. It has been argued (Ververgaert, 1973; Moor, 1972) that structure and viability can only be preserved with freezing rates $10^4 \text{ K.s}^{-1}$ but it is unlikely that these sort of rates can be achieved in anything but very small samples whose usefulness is limited.

One of the major attractions of rapid cooling is the relative instantaneousness of the 'fixation' when compared to chemical alternatives, with the possibility of arresting metabolically interesting phenomena within milliseconds of their occurrence. Considerable practical and theoretical effort has been expended upon this first important step as an alternative preparatory route to the electron microscope.

(i) Rapid Freezing

Numerous and, sometimes, novel have been the attempts to measure and maximize the freezing rates of biological specimens.

The list includes the quenching of specimens into liquid coolants such as liquid air (Gersch, 1932), isopentane (Hoerr, 1936) and propane (Bell, 1952) the latter two being cooled by liquid air. The cooling of inflammable fluids with liquid air
is combined with a risk of explosion (Hoerr, 1936) and with the subsequent widespread availability of liquid nitrogen this practice has been displaced by the use of isopentane (Harvey et al., 1976), propane (Bachmann and Schmitt, 1971), Freons 12 and 22 (Costello and Corless, 1978; Woolley, 1974) all cooled with liquid nitrogen and then used as quenching agents. Nitrogen itself in the form of melting nitrogen has also been used (Baker and Appleton, 1976; Seveus, 1978; Ross et al., 1981). Others have used cooled metal surfaces to rapidly freeze specimens (Eranko, 1954; Van Harreveld and Crowell, 1964; Christensen, 1971; Dempsey and Bullivant, 1976a) taking advantage of the high thermal capacities and conductivities of metals such as copper and silver compared to those of liquid coolants. Riehle (1968) and Moor and Riehle (1968) described a high pressure freezing device for small specimens and a wide range of instruments have been constructed to optimize the heat transfer during the quenching process (Luyet and Gonzales, 1951; Bachmann and Schmitt, 1971; Glover and Garvitch, 1974; Van Harreveld et al., 1974; Moor et al., 1976; Costello and Corless, 1978; Sitte et al., 1977; Handley et al., 1981; Escaig, 1982).

(ii) Frozen-Hydrated Specimens

Theoretically this approach is the one to take to retain the chemical integrity of the specimen. Rapid freezing should
immobilize diffusible ions and electrolytes. Maintaining the specimen in this state through to its examination in the electron microscope would permit the determination, by X-ray microanalysis, of the concentration of diffusible ions within cellular and extracellular compartments, which is a very exciting prospect for biology.

Restricting this discussion to transmission electron microscopy, a frozen-hydrated specimen has somehow to be sectioned and the sections transferred in their hydrated form to the cold stage of an electron microscope. Although there is now available equipment which can be used for the cutting, transfer and examination of frozen-hydrated material in the electron microscope, when this study was begun no such equipment was available.

The alternatives to the frozen-hydrated approach involve dehydrating the specimen at some point in the preparation procedure and this can be done by either freeze-drying or freeze-substituting the specimen.

(iii) Freeze-Dried Specimens

The freezing of a biological specimen removes most of the water from solution and converts it into either vitreous water or into ice crystals, the size of which are a function of the rate of freezing. Freeze-drying is the removal of the vitreous
water or ice crystals by sublimation. The rate at which the sublimation occurs is a function of the temperature of the specimen and of the partial pressure of water vapour above the specimen. The water vapour evolved from the specimen is removed by a cold trap or dessicant. The net loss of water molecules from the specimen is brought about by reducing the water vapour pressure above the specimen. Most frequently this is achieved by housing the frozen specimen in a vacuum chamber the purpose of the vacuum being to facilitate the flow of water vapour from the specimen surface to the cold trap or dessicant by reducing the number of gas collisions between the specimen surface and the trap or dessicant. However, any other method of removing water vapour from above the specimen surface can be used (Meryman, 1959; Edelmann, 1978).

Freeze-drying can be employed either immediately after the specimen has been frozen or after the frozen material has been sectioned.

The first option usually involves a system whereby the tissue can be embedded in the freeze-drying chamber under vacuum where the material can then be sectioned (Frederik and Klepper, 1976). The second option requires some means of producing ultrathin sections of the frozen specimen which are then freeze-dried (Spriggs and Wynne-Evans, 1976).

Dehydrating the specimen inevitably leads to redistribution of the water soluble components within the tissue, impairing the chemical integrity of the tissue. If freeze-drying is accompanied
by resin embedding and sectioning then there is the further possibility of loss of redistribution of these components by solubilization in the resin or by surface tension forces at the advancing resin front. The severe limitations of recovering sectioned material on aqueous baths or troughs has already been discussed.

(iv) Freeze-Substituted Specimens

This technique involves dissolving the ice in the specimen by means of a liquid dehydrating agent at low temperature. Small pieces of the frozen tissue are placed in the dehydrating agent at temperatures of about 193°K to 203°K for several days to attain complete dehydration. Following removal or 'substitution' of the ice the specimens are brought to room temperature and embedded in a plastic embedding material.

As with freeze-drying, the removal of water from the specimen limits the ability to localise diffusible elements. Surprisingly, however, a number of such elements are retained with little or no loss. Furthermore when compared to freeze-dried material the retention of diffusible elements within lumina and extracellular spaces is much improved (Läuchli et al., 1970; Harvey et al., 1976; Van Zyl et al., 1976; Forrest and Marshall, 1976).
(v) **Ultracryotomy**

This technique, as indicated earlier in this introduction, involves the ultrathin sectioning of frozen biological material. The sections are freeze-dried and measures are taken to prevent their rehydration.

At the time this work was begun the option of examining the sections in their hydrated form was not a practicable one. The process requires an electron microscope fitted with a specimen stage which can be accurately maintained at temperatures low enough to prevent freeze-drying of the sections in the microscope vacuum and this facility was not then available. The use of hydrated specimens is still today a specialised research area and the philosophy behind ultracryotomy was that it should become a widely used alternative to the conventional preparatory techniques used for transmission electron microscopy. Using ultracryotomy, ultrathin sections could be examined in the electron microscope within 1 hour of freezing the specimen.

Having frozen the specimen and having opted to dehydrate it the choice between freeze-drying and freeze-substitution was resolved in favour of freeze-drying. At the time the choice was made very little was known of the quantitative effects of freeze-substitution whereas the technology of freeze-drying was well established and, compared to the elaborate procedures associated with freeze-substitution, uninvolved.

The frozen specimen was sectioned using the ice matrix as an embedding medium, thereby avoiding the problems associated
with resin embedding, and then freeze-dried. The dehydrated state was maintained by sealing the freeze dried sections between two carbon films in a development of ultracryotomy discussed more fully in the next chapter.

Corneal Stroma

Two remarkable properties of corneal stroma are firstly its ability to transmit light and secondly its tendency to swell. These two phenomenon are inextricably linked. If the cornea is allowed to swell above its physiological hydration then its transparency is impaired.

(i) Structure and Chemistry

The corneal stroma consists of a number of structural components bathed in an interstitial fluid.

The most important components are collagen fibrils organised in thin sheets or lamellae which lie parallel to each other and to the surface of the stroma. The fibrils are of a uniform diameter, of the order of 25 nm, and within each lamella the fibril axes are orientated parallel to each other. This axial orientation changes from one lamella to the next.

The stroma also contains a small number of cells, keratocytes, lying between and within the lamellae.
The largest constituent of fresh stroma is water which contributes about 75% of stromal weight. Considering the remaining dry weight, then approximately 70% of this is collagen and a further 5% is due to the glycosaminoglycan or acidic mucopolysaccharides, chondroitin sulphate and keratan sulphate (Anseth and Laurent, 1961). Extracellular proteins form about 20% of dry stromal weight and include glycoproteins, proteins attached to the mucopolysaccharides and soluble collagen. Intracellular proteins and salts make up the remainder of the dry weight. Goodfellow (1975) provides a comprehensive review of corneal stromal structure and chemistry.

(ii) Transparency

Prior to the electron microscopical observations of Jakus (1954), the most generally accepted theory of corneal transparency was that all the components of the stroma had the same refractive index. The fine fibrils of uniform diameter seen by Jakus in the electron microscope together with their high degree of orientation throughout the stroma led Maurice (1954, 1957) to propose an alternative theory of corneal transparency.

He first of all showed that the refractive index of interstitial fluid was different from that of the collagen and from these observations calculated a value of the birefringence or double refraction of the stroma. The value was in good
agreement with the experimental work of others (Naylor, 1953). He further estimated that a random arrangement of fibrils in the interstitial fluid would lead to the stroma scattering 95% of incident light and, thus, be opaque. He proposed that corneal stroma was transparent because its fibrils were parallel, equal in diameter, and disposed in a regular two-dimensional lattice. Then the stroma would act as a series of diffraction gratings, corresponding to individual lamellae, and at physiological hydration light scattered by the fibrils would interfere destructively in all directions except that of the incident beam. In the incident direction light would be scattered but would interfere constructively and the stroma would appear transparent.

Should the cornea swell or deturgess then the regular array of the two-dimensional lattice would be destroyed and scattering in the incident direction would become progressively more destructive.

This lattice theory is now widely accepted as the basis of corneal transparency. However others, again using electron microscopical data, have challenged the existence of an ordered array of stromal fibrils (Goldman and Benedek, 1967; Goldman et al., 1969; Smith and Frame, 1969).

Bearing in mind the limitations of conventional electron microscopy already discussed in this chapter it was hoped that by using a radically different preparatory technique, i.e. alternative data on stromal organisation could be obtained.
(iii) Swelling

If an excised cornea is placed in an aqueous solution then it will swell. The degree of swelling is a function of the composition of the bathing solution and under certain conditions the cornea will swell to many times its physiological hydration. This propensity to swell is balanced in vivo by an electrogenic bicarbonate ion pump which has the effect of dehydrating the stroma (Hodson and Miller, 1976).

Heringa et al (1940) found that extraction of the cornea with 0.02% potassium hydroxide greatly reduced this swelling tendency. Since the total sulphur content of the extracted corneas decreased by more than 50% they suggested that the change in the swelling properties was due to the extraction of sulphate containing polysaccharides. This finding was supported by the work of Hedbys (1961) who precipitated the polysaccharides with cetylpyridinium chloride and reduced stromal swelling and also by Meyer et al (1953) who used the enzyme hyaluronidase to digest cornea and produce a decrease in swelling. Chondroitin-4-sulphate is a substrate for hyaluronidase.

The glycosaminoglycans are then most certainly involved in corneal swelling but their precise location in relation to the collagen fibrils is the subject of debate.

Two models of corneal swelling have been described where the positions of these glycosaminoglycans within the stroma is
very different. In the first (Hart et al, 1969; Farrell and Hart, 1969; Langham et al, 1969; Hart and Farrell, 1971) they are considered to extend orthogonally between the collagen fibres whilst in the second (Hodson, 1971) there is no cross-linking between collagen fibrils. In this model the glycosaminoglycans are wrapped around the collagen fibrils.

Much of the debate has centred upon electron microscopical evidence. The use of ultracryotomy could resolve this issue particularly when coupled to X-ray microanalysis with the potential for localising the sulphur containing glycosaminoglycans in the corneal stroma.

Such were the considerations which give rise to the present study.
CHAPTER 2

ULTRACRYOTOMY

Introduction

The reasons for wishing to use ultrathin frozen sections have been discussed in the first chapter of this thesis. This chapter is concerned with the development of the technique of ultrathin frozen sections, termed ultracryotomy by Hodson and Marshall (1969a, 1970).

The technique originally described by them has been modified and expanded in a number of ways. In particular, the methods for handling the frozen sections after they have been cut have been changed. For this purpose I describe the use of a vacuum transfer stage, in which the frozen sections can be freeze dried and transferred to a conventional vacuum unit whilst the sections remain under continuous high vacuum. The frozen sections which have been collected on standard 3 mm copper grids which carry a carbon coated collodion membrane are deposited in the vacuum transfer stage. There, they are freeze dried and transferred under vacuum in this stage to a commercial vacuum coating unit where a top coating of carbon is deposited on the dehydrated sections. The sections are therefore sandwiched between two layers of carbon. The idea behind the technique was to seal the dehydrated sections between two carbon layers and prevent rehydration of the sections and eliminate the attendant problems of interpreting the artefacts produced by rehydration.
METHODS

Instrumentation

The ultracryotome is a modified Cambridge Instrument Co. ultracryotome incorporating a Cambridge Huxley Mk. II ultramicrotome. The instrument is based on the principles given in Hodson and Marshall (1969a, 1969b, 1970), further modifications and development having been carried out by myself, with the cooperation of the Cambridge Instrument Co.

The assembled ultracryotome is shown in Figure 1. Details of the interior of the cryoattachment are shown in Figure 2. The cryoattachment has a heated window at the top to prevent frost forming and impairing visibility within the attachment. The window is double glazed, the heating element being sandwiched between two glass panels. The window and its frame slide fit into the top of the cryoattachment. Electrical connections for the heating element are made from the window frame to the main body of the attachment as shown in Figure 3.

The knife assembly (Figure 1) is advanced or retracted using the vernier control A, and lateral positioning is accomplished with control B. Assembly C is a trimming device (Hodson and Marshall, 1969b). Dewar flasks to the right supply liquid nitrogen to cool the knife and specimen holders. On the left is the control unit which regulates heating coils in the knife base, heating coils in the Dewar flasks, and the heating element in the window. Figure 2
shows the interior of the cryoattachment as viewed from above. A glass knife (A) is clamped into an invar steel knife holder which in turn is clamped into an invar steel knife base assembly via the bolt (O). This knife holder assembly is alternately cooled by liquid nitrogen from a liquid nitrogen reservoir (B) or warmed by a heating element (C). A platinum resistance thermometer (D) is situated between the liquid nitrogen reservoir and the heater. It is wired into a bridge circuit which either passes current through the heating element in the knife base or switches on an immersion heater in the liquid nitrogen dewar which supplies the knife base liquid nitrogen reservoir.

The knife base is shown schematically in Figure 4 and its design and construction involve three important principles. First, the whole knife base is made of invar steel which has a very low linear coefficient of thermal expansion \((10^{-5}/^\circ C)\) at working temperatures thereby minimizing linear expansions or contractions associated with temperature changes. Second, the knife and its holder are mounted over the null plane of expansion (YOX) of the knife base, minimizing lateral movement due to movement of its base. Thirdly it has been accepted in designing the instrument that it is practically impossible not to have temperature fluctuations within the knife base when there is liquid nitrogen within the knife base a few centimetres from room temperature outside the cryoattachment. The on/off method of cooling the knife base is intended to produce a deliberate temperature oscillation within the knife base. The temperature of the knife
at equilibrium will then include a slight oscillation which may be minimized by judicious positioning of the platinum resistance thermometer.

Specimen Holder Assembly

The specimen cooling assembly is shown in Figure 5 and the specimen holder itself is shown in Figure 6. The specimen is cooled by a flow of liquid nitrogen into the specimen reservoir and the level of liquid nitrogen within the reservoir is controlled by the level sensing diode within the reservoir (Klemperer and Snaith, 1968), the circuit diagram for which is shown in Figure 7. The frozen specimen itself is placed in the phosphor bronze jaws which are in turn slotted into the specimen holder and clamped via the screw (A). The complete specimen holder then fits over the screw (B) and this is tightened onto the cooling assembly via lever (C). The specimen holder and cooling assembly form the front end of the boom arm of the ultramicrotome and between the cooling assembly and the major portion of the boom arm is a ceramic insert (Cervit) which insulates the cooling assembly from the rest of the boom arm as well as itself having a low coefficient of thermal expansion. The ceramic insert is designed to minimize boom arm cooling and hence movement of the specimen away from the knife. Ideally the boom arm itself would be made of invar steel to further reduce this problem and in a subsequent commercial development of this instrument this suggestion has been incorporated (Spriggs and Wynne-Evans, 1976). In the
instrument described here however a compromise has been made.
The original microtome arm forms an inner core about which lies an outer core made of invar steel. The boom arm enters the rear of the cryoattachment through a large hole which is sealed using a flexible, transparent Mylar film. This film is fixed to the cryoattachment via a clamping ring, which forms the periphery of the large hole, and the film is further clamped to the arm of the microtome between the cooling assembly and the ceramic insert (Figure 8). This allows the arm to fall through its cutting stroke and be recovered whilst still partially insulating the cryoattachment from its surroundings.

Liquid nitrogen for the specimen cooling reservoir and the knife base reservoir are supplied from two ten litre Dewars (Figure 1). These Dewars are fitted with pressure tight heads which carry immersion heaters and liquid nitrogen feed tubes. A current through the heaters causes the liquid nitrogen to boil and the resultant gaseous nitrogen being unable to escape from the vessel drives out liquid nitrogen via the feed tubes. Both reservoirs within the cryoattachment are fitted with sponges to eliminate splashing of liquid nitrogen. The level of liquid nitrogen in the specimen reservoir is controlled as already indicated, by the level sensing diode positioned within the reservoir, whilst that in the knife base reservoir is effectively controlled by the platinum resistance thermometer in the knife base. By adjusting the resistances in the bridge circuitry the knife temperature could be made to oscillate about a given temperature.
The cryoattachment is fitted with a device for trimming the block of frozen tissue. This device (Hodson and Marshall, 1969a) is manipulated from outside the cryoattachment and allows the specimen to be trimmed on the specimen holder after the lid of the cryoattachment is closed.

All the other controls associated with the ultracryotome are those of a standard Cambridge Huxley Mk. II ultramicrotome.

Transfer Stage

Having cut sections of frozen hydrated tissue one then has two options for viewing the sections in the electron microscope.

1) With a suitable cold stage on the electron microscope the sections can be viewed in the frozen hydrated form.

2) The sections can be freeze dried and viewed in the dehydrated form.

Not having ready access to an electron microscope fitted with a cold stage the second approach has been adopted in this work. For this method, a purpose built transfer stage has been used. In this transfer stage frozen sections can be freeze dried and then transferred under vacuum to a conventional vacuum coating unit where the sections are then coated with a carbon film. The transfer stage is shown diagramatically in Figure 9. The idea of the transfer stage is to sandwich the dried sections between two layers of carbon;
the initial layer deposited upon the collodion film which covers the grids and the final layer deposited after the sections have been freeze dried. These two layers of carbon prevent rehydration of the sections when they are finally exposed to air. This latter step is omitted by many workers in this field, and hence the potential rehydration of the sections when exposed to air is ignored.

Section recovery

In the main, sections were recovered using an electrophorous (Hodson and Marshall, 1969a). The device is shown schematically in Figure 10. It consists of an aluminium base (A) fitted with an insulating perspex handle (B). Connected to the base via a length of coaxial cable is a pair of insulated fine forceps. The aluminium base sits on a sheet of expanded polysterene. When this sheet is rubbed with a piece of fur it becomes electrostatically charged. Placing the aluminium base upon the charged sheet induces charge of an opposite polarity on the base. Earthing the base and then lifting it of the polysterene sheet causes the induced charge to flow from the aluminium and off the ends of the fine forceps.

With the forceps holding a copper grid partially immersed in the floatation medium contained in the knife boat the charge streams off the forceps/grid system and onto the frozen sections. By replacing the aluminium base on the polysterene sheet the potential of the grid/forceps system relative to the charged
frozen sections is reduced and the sections are attracted to the grid and recovered. The mutual repulsion between the sections spreads the sections over the grid.

Knives

Initially 45° glass knives were used for cutting the frozen sections, the knives being prepared with the aid of a conventional knifemaker. The glass knives were fitted with troughs to contain a floatation medium. Initially, the floatation medium used was cyclohexene, (Hodson and Marshall, 1970) but later isopentane was used. The troughs were constructed from strips of polythene glued to the knife with two tube Araldite.

Tissue freezing

Freshly excised tissue was cut with a razor blade into wedge shaped pieces about 5 mm long x 1 mm thick tapering down to 1 mm wide. Just prior to excision of the tissue, Freon 22 (chlorodifluoromethane) was dispensed into a small vial fitted with cotton thread handles. This vial containing the Freon 22 was then transferred to a Dewar flask containing liquid nitrogen, the Freon 22 and its vial being gently immersed into the liquid nitrogen and left suspended there by means of a pencil passing through the cotton handles and resting across the mouth of the Dewar. With a freezing point of approximately 127°K the Freon 22 solidifies in the liquid
nitrogen and can be left there. Just before the Freon 22 is used to quench the tissue it is removed from the liquid nitrogen and placed carefully on the bench. Using a large thermal mass e.g. the blade of a fairly large screwdriver, a central hole is melted in the solid Freon 22. Then using a pair of insulated forceps, the wedge shaped tissue piece is plunged rapidly into the liquid core of the Freon 22. Usually the tissue would stick to the insulated forceps but could easily be dislodged using a second pair of, pre-cooled, insulated forceps. The tissue is then rapidly transferred from the liquid Freon 22 to liquid nitrogen for storage.

**Tissue mounting**

The tissue was mounted, under liquid nitrogen, using the jig shown in Figure 11, care being taken to maintain the level of liquid nitrogen above the top of the jig during the clamping of the tissue in the phosphor bronze jaws of the specimen holder. Care is also necessary in the clamping of the tissue since overtightening of the clamping bolt inevitably leads to shattering of the tissue. With the tissue clamped in the specimen holder, the whole system of jig and holder is kept under liquid nitrogen until the working temperatures within the cryoattachment have been attained and stabilised. The specimen holder is then transferred to the arm of the ultramicrotome by removing the lid of the cryoattachment,
sitting the holder in position on the end of the arm, locking the holder on the arm, and finally closing the lid of the cryo-attachment.

Complete Procedure

Having described the instrumentation and the procedures used in the technique of ultracryotomy details will now be given of a complete run through the technique.

The source of tissue was normally rat liver though on other occasions rat cornea or kidney have been used. Prior to sacrificing the animal, solid Freon 22 stored in liquid nitrogen was prepared and placed on one side of the bench. The animal was sacrificed by breaking its neck and pieces of its liver excised. These, in turn, were cut using a razor blade into smaller wedge shaped pieces. Whilst this was happening, the solid Freon 22 was removed from the liquid nitrogen and a central well of Freon 22 was melted using the blade of a screwdriver. These two operations were synchronised so that freshly excised and shaped tissue could be plunged immediately into liquid Freon 22 that had just been produced from solid Freon 22. After freezing, the tissue was removed from the Freon 22 and stored in liquid nitrogen.

Having quenched and stored the tissue, the heaters in the two liquid nitrogen dewars supplying the knife base and specimen holder were switched fully on for 60 seconds and 30 seconds respectively and then set to their equilibrium values, previously determined, to give a knife temperature of say 148 K and a specimen holder
temperature of 138 K. The timings of the initial full scale bursts on the Dewar heaters were such as to bring the ultracryotome down to its operational temperatures in a rapid but controlled fashion. Too long on a full scale setting for either the knife base or specimen holder Dewar heater would bring the ultracryotome down to and beyond its working temperatures and set up a violent oscillation about these temperatures; too short an initial burst on the heater full scale settings and the ultracryotome would never achieve operational temperatures. With optimum setting of the heater controls, previously determined using copper-constantan thermocouples in the knife base and in the specimen holder, the ultracryotome could be ready to cut within 20 minutes of the initial heater bursts. The window heater of the ultracryotome was normally switched on some 10 minutes after starting up.

Whilst the ultracryotome was reaching working temperatures the frozen tissue was transferred to the jig shown in Figure 11, the jig having already been filled with liquid nitrogen. Using an Allen key and insulated forceps, the frozen tissue was clamped within the phosphor bronze jaws of the specimen holder. This operation was timed to coincide with the ultracryotome reaching working temperatures. The specimen holder, under liquid nitrogen was then transferred from the jig to the specimen arm of the ultracryotome through the opened window on the cryoattachment. The specimen holder was then locked onto the specimen arm and the window of the cryoattachment closed.
The specimen could then be trimmed using the trimming device. Suitable positioning about the specimen could be achieved by a combination of height movement of the specimen arm and lateral and rotational movement of the trimmer blade.

Having trimmed the frozen specimen, the cutting knife was advanced to the block face and the face further trimmed using the right hand side of the knife. The knife was then moved sideways and positioned so that the extreme left hand side of the cutting edge was just in front of the trimmed tissue block face. The knife trough was then carefully filled with isopentane from the syringe in the side of the cryoattachment. With the advancement of the specimen arm set at 100 nm and with a cutting speed of 0.5 mm.s\(^{-1}\), the microtome arm was set cutting.

Sections were then recovered using the electrophorus. The sections were picked up on standard 3 mm diameter copper grids used in conventional transmission electron microscopy. The grids had been previously coated with a collodion membrane to provide support for the recovered frozen sections and this collodion membrane in turn had been coated with a film of evaporated carbon. Before recovery, the grid was pre-cooled in liquid nitrogen using the insulated electrophorus forceps and the expanded polysterene base of the electrophorus was electrostatically charged by rubbing or beating with fur. The aluminium disc was then placed upon the expanded polysterene base, earthed, the window of the cryoattachment was then slid open and the pre-cooled grid was partially immersed in the floatation medium in the knife boat. The aluminium disc
was then lifted off the expanded polystyrene base and the excess charge in the copper grid - forceps - aluminium base system streamed off the forceps tips and charged the frozen sections. The aluminium disc was then replaced upon the polystyrene base lowering the potential of the grid - forceps - base system thereby attracting the charged sections to this lower potential and hence recovering the sections onto the grid. The sections thus recovered were placed upon the mushroom of the transfer stage which was housed within the cryoattachment.

The transfer stage itself was mounted on a stand to one side of the ultramicrotome. After the sections had been recovered and placed on the transfer stage mushroom, the well of the transfer stage was filled with liquid nitrogen in order to cool it. The lid of the cryoattachment was opened and using cooled insulated forceps the mushroom was taken from the cryoattachment and placed within the transfer stage. The rotary pump connected to the transfer stage was switched on, the lid of the transfer stage seated and the vacuum valve (Figure 9) was carefully opened and left open whilst the transfer stage was allowed to warm to room temperature over a period of about 20 minutes. The valve to the rotary pump was then closed and the vacuum line to the transfer stage disconnected. The transfer stage was then taken to and placed within a vacuum coating unit. The coating unit was then evacuated. When the vacuum within the unit was equal to or better than that within the transfer stage the lid of the transfer stage could be removed using a magnet controlled from outside the vacuum. The grids
within the transfer stage were then coated with carbon from the evaporation source of the coating unit after which the unit was vented to air and the grids with the carbon coated sections were collected.

Having outlined the general procedure for the operation of the ultramicrotome, the variations and modifications made to improve the performance of the system will now be discussed. These come under four general headings, i.e. freezing, cutting, specimen mounting and transfer stage modifications.

**Freezing Modifications**

As well as the method of rapidly freezing tissue already described in the protocol given above a number of other methods were evaluated in attempting to improve the rate of transfer of heat from the specimen to the quenching medium.

1) **Freezing in isopentane**

Liquid isopentane ($\text{C}_5\text{H}_{12}$, Boiling Point 301 K, Melting Point 113 K) was used in the same fashion as Freon 22, i.e. approximately 10 ml of isopentane was dispensed into a 15 ml glass vial. The vial containing the isopentane was then carefully immersed in liquid nitrogen causing the isopentane to freeze and then, when the tissue was ready to be frozen, the vial and solid isopentane was removed from the liquid nitrogen and a central well of liquid
isopentane was produced within the vial using a large bladed screwdriver which served as a large thermal mass to melt the solid isopentane. The freshly dissected tissue pieces were then plunged into the liquid isopentane using a pair of insulated forceps.

2) Freezing in propane

Liquid propane (C₃H₈) (Boiling Point 231 K, Melting Point 85 K) dispensed from a pressurised gas storage vessel was used in exactly the same way as Freon 22 and isopentane.

3) Freezing in pumped nitrogen

Largely due to the hazards associated with the use of hydrocarbons and to certain difficulties inherent in the use of the safe but possibly less effective fluorocarbons, repeated attempts have been made to develop procedures by which inorganic fluids, particularly liquid nitrogen might be made more effective. Luyet and Kroener (1960), Sjöstrand and Elfin (1964) used partially frozen nitrogen, obtained by prolonged boiling at reduced pressures. MacKenzie (1969) describes an apparatus for the production and storage of the mixture of solid and liquid nitrogen and gives results of freezing tissue slices in the partially frozen nitrogen. He found that thin slices of rat kidney froze three times faster in freezing nitrogen than in boiling nitrogen.
A simple variation for preparing partially frozen nitrogen was tried. Liquid nitrogen was placed in a standard 1 litre Dewar almost filling the vessel. This flask was then placed within a vacuum dessicator, normally used for dessicating photographic materials. The dessicator was then pumped by a rotary pump for about 1 hour. Tissue was then rapidly quenched in the resultant mixture as soon as the lid of the dessicator was removed.

**Pistol Injection**

In a further effort to improve the freezing rate of the tissue the injection of tissue pieces into liquid refrigerants was tried. A modified commercial air pistol was used for this purpose (Diana Model 2). With this pistol a spring loaded barrel is compressed and an air pellet is normally placed in the bore of the barrel, the trigger is then pressed, the spring loaded barrel shoots forward, is checked, and the pellet is ejected from the barrel. Fortuitously it was found that the front portion of the spring loaded barrel was threaded. A specimen holder was constructed which screwed onto this thread, specimens could then be placed on the end of this holder and 'fired' into the quenching medium, (Figure 12).

**Injection and Stirring**

The work of Glover and Garvitch (1974) indicates that, with specimens injected into a liquid refrigerant, heat transfer appears
to be mainly through forced convection and logically the more one can 'force' this convection the higher the rate of heat transfer from specimen to refrigerant should be. Combinations of injection and stirring of tissue and refrigerant respectively were then tried.

Using small metal chips, pre-cooled in liquid nitrogen, and inserted into the well of liquid refrigerant one could, by placing the refrigerant container upon a magnetic stirrer, stir the quenching medium during the injection of the tissue into the refrigerant. Using a larger container than the normal glass vial e.g. a 250 ml plastic beaker, insulated in a polystyrene collar, and sitting upon a magnetic stirrer one could use the pistol injection system to rapidly freeze specimens. Both of these methods were tried.

**Cutting Modifications**

Figure 13 defines the angles and directions involved in sectioning material. As already described, the ultracryotome was used initially with conventional glass knives having a knife angle of 45° with a clearance angle of 4° - 6° these knives being fitted with troughs made of librafilm, a polythene frequently used in protective covering for books. The fitting of these boats has already been described. As well as knife angles of 45° glass knives with knife angles ranging from 30° - 70° were used. Workers in the field of the physics of machining have shown that a plane of high shear stress exists from the tool tip to the outer surface of the
chip or section (Ernst, 1938; Merchant, 1950). Figure 14 illustrates this. The shear stress arises because the material being cut from the block has to be transferred from the plane of cutting to the rake face of the knife. The shear stress is greater the smaller the rake angle. In view of the hardness of ice when compared to that of the plastic embedding materials normally used for the ultrathin sectioning of biological materials, knives having large rake angles were used in an attempt to minimize the shear stress on the sections and the distortions within a section. In theory the plastic embedding media used with biological material is capable of deforming elastically and when the stress is removed at the end of the cut the deformation disappears. In practice however most biological sections are shorter than the face from which they are cut indicating that the deformation they have undergone was not wholly elastic. For minimum distortion of the section during cutting and for maximum resistance of the knife edge to fracture and wear the clearance angle should be a minimum. Therefore a combination of a small clearance angle (1°) and a relatively small knife angle (30°) giving thus a large rake angle and minimum shear stress would appear the best method of obtaining sections in which the distortions produced during the cutting action were kept to a minimum when using conventional glass knives. These alternatives were tested with the ultracryotome.

Diamond knives are indispensable for cutting metals and hard biological materials. A standard commercial diamond knife was used in the ultracryotome. This diamond knife had previously
been used for sectioning a variety of plastic embedded biological materials and during this time had been damaged, about 10% of the knife edge having been chipped away. The knife was placed in the ultracryotome knife holder and clamped using invar spacers and the bolt B (Figure 15).

As well as the trials with the above diamond knife system a new diamond knife was purchased and mounted on a purpose-made invar steel holder. This knife with a knife angle of 45° and a cutting edge of 4 mm together with its holder is shown in Figure 16. The holder was machined from a solid block of invar steel - NILO 36 (Henry Wiggin & Co., Holmer Road, Hereford, U.K.). NILO 36 is a binary Ni-Fe alloy used for its low coefficient of thermal expansion. Work on binary Ni-Fe alloys has shown that an anomalously low coefficient of linear expansion exists in the region of 36% nickel and at room temperature it is almost negligible, Figure 17. Some of its chemical and physical properties are shown in Figure 18. NILO 36 has a low but unstable coefficient of thermal expansion in the cold worked condition and for critical applications such as the knife holder the minimum coefficient of expansion can be obtained by a three stage stabilising heat treatment. The machined knife holder was subjected to a procedure thus: 30 minutes at 830°C - air cooled to 315°C - 1 hour at 315°C - air cooled to 100°C - 48 hours at 100°C and then air cooled to room temperature. The knife was mounted onto its holder using an epoxy resin recommended by the knife manufacturers, Du Pont De Nemours & Company, which was Armstrong A-4 adhesive with Armstrong A activator supplied by
Armstrong Products, Warsaw, Indiana, U.S.A. A mix of 4 parts activator to 100 parts by weight of adhesive and then a cure at 75°C gave the necessary adhesion. With a knife angle of 43° mounting the knife on a 60° base as in Figure 16 gives a clearance angle of 5°. At one side of the knife holder there was a space for mounting a glass trimming knife. This knife was cut from 4 mm thick plate glass and had a knife angle of 45° and a clearance angle of 6°. The trimming knife is clamped by the screw X, (Figure 16). This diamond knife assembly was then used in the ultracryotome.

Specimen Mounting Modifications

The initial system used for specimen mounting, i.e. phosphor bronze jaws clamped via an allen bolt (Figure 6) was used extensively during the use of the ultracryotome and in the main the system worked well, its limitation being that if one was not particularly careful the allen bolt could be overtightened and shatter the frozen tissue. To try to overcome this problem a number of invar steel stubs were made (7 mm long x 6 mm wide x 2 mm thick) which fitted within the existing specimen holder system (Figure 19). Before using these stubs, they were thoroughly cleaned by ultrasonating them in a detergent solution followed by ultrasonication in a de-greasing agent such as Inhibisol. The tissue was then rapidly dissected out and touched onto the stub which was then plunged into the quenching medium with the aid of a pair of insulated forceps. The stub/tissue system could then be mounted, under liquid nitrogen, into the existing
specimen holder and clamped via the allen bolt. With the original system it was difficult to tell whether the tissue was clamped or not because frequently the allen would tighten not on the tissue but because of icing of its threads. This could be overcome by applying a little more torque to the allen bolt but if this tightening was in fact the bolt on the tissue then one could easily shatter the tissue. With the tissue on the invar stubs and the allen bolt tightening on the invar stub one could carry on tightening until one was sure that the stub was firmly clamped.

One commercially available instrument for producing ultrathin frozen sections used a silver pin as a method of specimen mounting. The tissue to be frozen is placed on the silver pin and the tissue then rapidly frozen by ones chosen method upon the pin and the pin is then clamped into a specially designed specimen pin holder. Using a modified form of the invar stubs already described above (Figure 20), the silver pin system was tried with the ultracryotome. The pins were housed within the invar steel stub push fitting into the cylinder (Figure 20). The stub was then clamped as in the previous modification.

Transfer Stage Modifications

During the operational lifetime of the ultracryotome a number of modifications were made to the original transfer stage described above. These modifications will now be described.
Problems had been experienced with sealing the lid of the transfer stage and the existing spring/ball bearing system was suspected. The modification to test this is shown in Figure 21. The connector from the transfer stage was fitted with an "O" ring onto which seated a ball bearing. The ball bearing ran in a short length of vacuum tubing of sufficient diameter to permit suction around the bearing and consequent evacuation of the vacuum chamber of the transfer stage. When the rotary pump was running, the buffer system prevented the ball bearing from blocking the glass vacuum line. After sections had been placed in the pre-cooled well of the transfer stage, the stage was evacuated and, whilst still being pumped by the rotary pump, the transfer stage was allowed to warm to room temperature. The rotary pump was then switched off and the ball bearing was positioned on its "O" ring seal by means of a bar magnet run parallel to the vacuum tubing. The rubber bung was then quickly removed and the glass vacuum line separated from the rubber vacuum line. The transfer stage was then taken to the vacuum coating unit.

Further uncertainties about the sealing of the transfer stage lid and difficulties in manipulating the grid holding disc when taking it from the ultracryotome to the transfer stage lead to two further modifications to the transfer stage system, which are shown in Figure 22. The magnet-ball bearing- "O" ring seating was replaced by a Quickfit Teflon valve system and the grid holding disc was replaced by a mushroom and insert system. The mushroom was stored as per the original system inside the
ultracryotome during the frozen sectioning. The insert was suspended, initially, in a Dewar of liquid nitrogen. When sections had been cut and placed upon the mushroom within the ultracryotome the well of the transfer stage was cooled with liquid nitrogen and the insert was then removed from liquid nitrogen and slotted into the transfer stage well. The mushroom was then taken from the ultracryotome and fitted to the insert. The lid was then placed upon the transfer stage and with the rotary pump running the valve was opened carefully to evacuate the transfer stage chamber. When the sections had been freeze dried and the transfer stage allowed to warm to room temperature the valve was closed and the transfer stage and valve were detached from the vacuum line. The transfer stage plus the valve could then be taken to the vacuum coating unit.

Having made two modifications to the vacuum valve sealing the transfer stage, problems were still occurring in maintaining the vacuum within the transfer stage. One potential source of difficulty was the seal between the "O" ring in the lid of the transfer stage and the cold upper surface of the transfer stage itself. To resolve the situation, a neoprene annulus was stuck to the upper surface of the transfer stage to act as an insulator and thus provide a less cold, less polished surface on which to obtain a seal.

One further variation on the handling of the sections after they had been cut was to freeze dry them inside the cryoattachment. This was achieved by leaving the sections in the base of the
cryoattachment for about an hour, keeping the ultracryotome running as if still sectioning so that the ambient temperature, as measured with copper-constantan thermocouples, where the grids were sitting was between 173 K and 183 K. The ultracryotome was then allowed to warm to room temperature whilst the cryoattachment was carefully flushed with dry nitrogen gas at room temperature in order to avoid condensation of moisture. The grids were stored in a dessicator over silica gel.

Results

The first and most important point to emphasize about the technique of ultracryotomy as described here are the many problems encountered in obtaining ultrathin frozen sections. It was not possible to vary one parameter during a cutting session and be sure that some other parameter would not affect the end result. For example, consistently obtaining a seal on the transfer stage was usually a problem as was section recovery and to establish the effect of changing one parameter involved repeated trials with the ultracryotome. Even after a lengthy series of trials one could still be unsure of the effect that changing the parameter was having.

The same format has been adopted in describing the results as was used with the methods section. For the most part the results section is concerned with what came to be the basic operational procedure for the ultracryotome. Briefly this is freshly excised
tissue rapidly frozen in Freon 22, clamped in the phosphor bronze jaws of the specimen holder assembly, sections of 100 nm cut using a diamond knife and collected via the electrophorus on carbon coated grids the sections then being freeze dried in the transfer stage before finally being top coated with carbon in a vacuum coating unit. The results will show the potential of the technique and indicate the problems encountered and the probable limitations.

Figures 23 and 24 represent the best results consistently attainable by the technique described here. To illustrate clearly the limitations of the technique as demonstrated by Figures 23 and 24, a micrograph of rat liver, prepared conventionally for electron microscopy is shown in Figure 25. The first and encouraging point to note is that the micrographs are comparable. One can identify nuclei for example in the frozen sections and their size and distribution corresponds well with the size and distribution of nuclei in conventional sections. Another very noticeable feature of the frozen sections are the many electron dense objects throughout the cell cytoplasm and again from the size and distribution of these objects they are certainly mitochondria.

The striking differences between the micrographs are the large tears in the frozen sections and the background of electron transparent regions or 'holes' in the frozen sections. These points are illustrated in Figure 24. This background impairs the resolution of any fine structure such as the Golgi apparatus, endoplasmic reticulum and glycogen granules as indicated in the conventional micrograph in Figure 25.
I believe that these holes in ultracryotome sections represent the ghosts of ice crystals, the crystals having been formed when the tissue was frozen and the ice subsequently removed by sublimation when the frozen sections were freeze dried. That this damage to the tissue was produced during freezing can be reasoned simplistically as follows: It is noticeable from Figures 23 and 24 that the size of the holes is greater within the nucleoplasm as compared to the holes within the cell cytoplasm. It is known that the size of ice crystals produced when biological tissue is frozen is directly proportional to the rate at which the tissue is frozen (Stephenson, 1956; Dempsey and Bullivant, 1976a; Van Venrooij et al., 1975; Frederik and Busing, 1981; Handley et al., 1981). It is estimated that with a cooling rate of the order of 10,000 K/s the rate of removal of heat would be rapid enough to vitrify the ice and not produce ice crystals (Moor, 1972). The deeper inside a tissue block the more difficult it is to remove heat quickly from that region because of its greater insulation from the quenching medium. Hence one would expect that the ice crystal size would increase as one penetrated more deeply into a frozen tissue block, which has been shown to be so (Dempsey and Bullivant, 1976a; Frederik and Busing, 1981). The increase in hole size between nucleoplasm and cytoplasm as observed in Figures 23 and 24 could be attributed to the extra impedance, in the form of the nuclear membrane, presented to heat conduction from the nucleus and the consequent lowering of the rate of cooling and its attendant increase in ice crystal size. Further and substantial
evidence that the damage seen in tissue sections prepared by the technique of ultracryotomy as described here is in fact due to ice crystals is provided by many workers using the technique of freeze substitution (Whittaker, 1974; Karow and Shlafer, 1975; Walter et al., 1975; Dempsey and Bollivant, 1976). This technique has only one step common to that used in ultracryotomy and that is the initial rapid freezing of the tissue. Thereafter the two techniques differ in that freeze substitution involves the dissolving away of the ice formed during the freezing using a suitable solvent and then warming the tissue to room temperature and embedding it in a plastic embedding material prior to sectioning as one does with material prepared conventionally for electron microscopy. The point of interest is that tissue prepared by the technique of freeze substitution and that prepared in this work by the technique of ultracryotomy show a striking similarity. With only the initial rapid freezing as a common step the experimental evidence strongly suggests that damage seen in ultrathin sections prepared by these two techniques is due to the growth of ice crystals during the rapid freezing step.

The reasons for the large tears in the sections are less clear. One was constantly aware whilst trying to cut ultrathin frozen sections of the difficulty in achieving consistent sectioning, first because of the innate hardness of the material being sectioned and second because of the technical problems involved in obtaining controlled specimen advancement with some parts of the instrument at liquid nitrogen temperatures and other parts at
room temperature. These large tears could be produced at the cutting stage of the technique and represent material being torn or chipped from the frozen tissue block rather than sectioned. Alternatively the freeze drying of the ultrathin sections could produce these tears. The removal by sublimation of the ice from the ultrathin frozen sections must leave behind a very fragile skeleton. In a volume of frozen section consisting mainly of large ice crystals, sublimation of the ice could leave behind a very fragile, potentially unstable, structure. One can imagine that the large tears or gaps in freeze dried ultrathin sections were originally regions of the section consisting of a small number of large volume ice crystals, the removal of which precipitated the collapse of the remaining skeleton. The problem then being caused by a combination of less than rapid freezing of a region producing large volume ice crystals which when removed leave behind an unstable skeleton which collapses producing the tear or gap. Alternatively the explanation of the tears/gaps could be some complex combination of the technique, problems produced by less than rapid freezing being further exacerbated by cutting and subsequent freeze drying. Reiterating then, Figures 23 and 24 represent the best results consistently obtainable. The limitation of these sections appears to be ice crystal artefacts produced during the initial freezing of the tissue block giving at best a constant background which masks all but two of the intracellular organelles and which at its worst leaves large holes in the section surrounded by strands of cytoplasm.
A description will now be given of the spectrum of results produced by the technique of ultracryotomy as detailed here before discussing the effects of the variations and modifications made to the basic technique.

Figures 23 and 24 illustrate the primary problem of ice crystal damage but apart from the possible involvement of cutting action in producing the tears seen in Figures 23 and 24, these sections would seem to be free of any problems associated with cutting. That ultracryotome sections can reflect the artefacts associated with thin sectioning is shown by Figure 26. Here a knife mark is seen running across the section exactly as one sees in conventional plastic embedded material when sectioning with a damaged knife. One of the commonest problems encountered when sectioning ultrathin frozen sections is illustrated in Figures 27, 28 and 29. Ultrastructure can be obliterated by striations or "ripple". The striations always lie parallel to each other with a pitch in the range 50 - 200 nm. As already described a plane of high shear stress exists from the knife edge to the outer surface of the section. The shear stress arises because the material being cut from the block has to be transferred from the plane of cutting to the rake face of the knife and is greater the smaller the rake angle. It is probable that the striations represent slip lines formed in the relief or partial relief of shear stress. It is an immediate consequence of this deformation during cutting that the section is usually thicker than the amount of specimen
advance and that the material is shorter in the direction of cutting than it was before cutting. The amount of thickening and shortening increases as the rake angle decreases (Phillips, 1961).

Another major difficulty was the failure to freeze dry the sections consistently in the transfer stage because of the problems in obtaining a seal with this stage. Failure to dehydrate the frozen sections leads to melting of the ice upon warming the sections to room temperature and this melt-back produces a characteristic effect upon the sections which is shown in Figure 30. The edges of the section are indistinct. The point at which the section ends and the supporting film begins is almost indeterminate. It is impossible with these partially or wholly hydrated sections to obtain Fresnel fringes at the section edge in contrast to the sections which have been dehydrated completely about whose edges Fresnel fringes could always be obtained when the electron microscope was taken through focus. This criterion, the ability to obtain Fresnel fringes off the edges of sections, may be used to determine whether or not a section had been properly freeze dried.

Within the category of hydrated sections, i.e. sections which had only been partially freeze dried or had not been freeze dried at all there existed a spectrum of results which is illustrated by Figures 30, 31, 32, 33 and 34. At one end of this spectrum we see examples such as Figure 30 all having ill-defined edges to the sections and a uniformly amorphous background devoid of any
obvious detail. Sometimes, however, although not having a defined edge a section would have the suggestion of detail within it, e.g. Figures 31, 32 and 33 and in some quite rare cases one could plainly see detail comparable to that normally seen with plastic embedded unstained material (Figure 34). Initially, Figure 34 caused much excitement. The section had been cut with a diamond knife on its second trial, after several years of using a glass knife. This result suggested that the answer to successful ultrathin frozen sections lay in using diamond knives. However, a number of pertinent comments can be made about Figure 34. It appears at first that the left hand edge of the section is well defined and should produce Fresnel fringes although this was not checked at the time the section was first viewed. It is more probable, however, that the section has in fact folded over on itself along this left hand side and that the true edge of the section is somewhat inward of the edge shown in Figure 34. The increase in electron density along the left hand edge further suggests folding about the left hand edge. The other edges of the section are ill-defined, which is characteristic of a section that has melted, as is the uniform amorphous background to the section. The interesting thing about this section is that the cristae are visible within the mitochondria. In nearly all other sections, what were assumed to be mitochondria (the assumption being based upon the observed size distribution of the particles when compared to the size and distribution of mitochondria in conventional electron microscopy sections) appeared shrunken and electron dense. If in both cases we are looking at mitochondria,
why should there be such remarkable differences? Two reasons suggested themselves. Firstly, some difference in the initial rapid freezing of the tissue, i.e. in the case of the section which shows the relatively electron transparent mitochondria with visible cristae, Figure 34, then the section could have been cut from a region of the tissue block where the freezing rate was high enough to prevent the growth of observable ice crystals, even, possibly, complete vitrification of the ice occurred. In the case of Figure 24, the freezing rate in the region of the block from which this section was cut could have been less rapid than that achieved in Figure 34. Water is then drawn osmotically from the intracellular mitochondria causing them to shrink and condense which could account for their enhanced electron density seen in Figures 23 and 24.

Alternatively the section seen in Figure 34 could have undergone some degree of melting when it was warmed to room temperature during the freeze drying stage, the freeze drying being either wholly or partially incomplete. Hence the melt-back characteristics of the section, i.e. ill-defined edges and uniform amorphous background. It was only in this one section, out of thousands in total, that such detail was seen. It seems improbable that in just this one case the retention of such morphology as the mitochondrial cristae should be due only to, say, partial freeze drying of the section. I suspect that the section was from a region of the tissue block frozen rapidly enough to prevent ice crystal damage or even to have no ice crystals at all and there was also melt-back of some degree because of incomplete freeze drying.
Summarising then this descriptive part of the results section; it was possible to cut ultrathin sections of rapidly frozen tissue but subsequent freeze drying of these sections and their examination in the electron microscope revealed that in the great majority of cases (> 95%) the ultrastructure of the tissue had been impaired by the presence of ice crystal cavities, formed by the sublimation of the ice crystals produced during the initial freezing of the tissue. Frequently the picture was further complicated by a defect in the cutting action, i.e. the inability of the frozen tissue section to elastically absorb and then release the shear stress to which it is subjected. This manifested itself as a fine line structure or 'ripple' representing slip lines formed in the relief or partial relief of this shear stress.

The first two categories of this results summary has dealt with sectioned material which had been successfully freeze dried. The characteristic used to establish successful freeze drying was the existence of a well defined edge to the section which would give Fresnel fringes about the edge when the electron microscope was taken through focus on the section. The other category of sections are those where there had been a failure, partial or complete, to freeze dry the sections and subsequently when viewed in the electron microscope the sections had ill-defined edges and no sign of ice crystal damage or cutting artefacts. It is suggested that the failure to remove the ice by freeze drying results in the
ice melting when the sections are warmed to room temperature, and if the conclusions already drawn are substantially correct, then prior to warming of the section to room temperature one has material which has been altered firstly by the presence of ice crystals and secondly by the cutting process. If the ice in the section then melts because of a failure to freeze dry then one can imagine that any residual ordering or structure not impaired by freezing or cutting damage is highly likely to be lost when the ice melts and the section is then forcibly dehydrated when placed within the vacuum of the electron microscope. This category of sections is characterised by a uniform amorphous background, ill-defined edges to the section and in most cases absence of any kind of structure or ordering, (Figure 30).

However, in some cases (Figures 31, 32 and 33) there are signs of structure and in other much rarer cases (Figure 34) very definite structure. These latter cases are probably sections which were well frozen initially, free of cutting artefacts, but which melted when warmed to room temperature because of a partial failure at the freeze drying stage.

Variations

1) Freezing variations

Figure 35 shows cooling curves of copper-constantan thermocouples in various liquid quenching media (Isopentane, Freon 22, Propane, Liquid Nitrogen and Melting Nitrogen).
a) **Isopentane** \((C_5H_{12})\)  Boiling Point = 301°K; Melting Point = 113°K.

The cooling curves in Figure 33 demonstrate the relative efficiency of heat transfer of 5 quenching media. Isopentane is seen to be significantly better than liquid nitrogen as are the remainder of the group but of these four, i.e. (propane, melting nitrogen, Freon 22 and isopentane) isopentane appears the least efficient. When using isopentane as a quenching agent in conjunction with the ultracryotome no qualitative differences were seen with the resulting sections when compared to sections which had been cut from tissue blocks frozen in the routinely used Freon 22. Those sections in which the characteristic ice crystal damage appeared were comparable to sections produced when using Freon 22 as a quenching medium.

b) **Propane** \((C_3H_8)\)  Boiling Point = 231°K; Melting Point = 85°K

No qualitative differences were seen when using liquid propane as a quenching medium. Figure 35 implies that it has no great advantages over Freon 22 and there are certain disadvantages in its usage in the laboratory.

**Pumped Nitrogen**

Pumping of liquid nitrogen in a vacuum desiccator resulted in a viscous 'slush' of liquid and solid nitrogen. The cooling curve
of a copper-constantan thermocouple in such a mixture is shown in Figure 35. A series of quenchings of tissue in this mixture and subsequent sectioning of the frozen material with the ultracryotome showed no qualitative improvement upon Freon 22.

**Pistol Injection**

Tissue pieces were fired into Freon 22, Isopentane and Propane quenching liquids. The process was difficult to control, the distance between the quenching liquid and the specimen mounted on the end of the sprung barrel (Figure 12) needed to be quickly and accurately gauged otherwise when the trigger was pressed the tissue would come to rest short of the quenching medium either staying on the end of the specimen mount or just falling into the quenching medium. The resulting sections showed no effect of this deformation and no obvious differences from the sections produced by immersing tissue by hand into Freon 22.

**Injection and Stirring**

The addition of stirring to the pistol injection method produced no discernable benefits in terms of minimizing or eradicating ice crystal artefacts in the resultant sections.
Cutting Variations

About 70% of the sectioning on the ultracryotome used glass knives, with all of the initial work being done with 45° glass knives fitted with librafilm boats. Sectioning with glass knives was never consistent or regular. Frequently the glass knife would section well for a short time (approximately 6 - 10 cycles of the ultracryotome) and then its performance would deteriorate rapidly after which, the knife was able only to produce fragments of tissue from the frozen block. Repositioning of the knife rarely led to an improvement of the cutting action which suggested that the glass knives were being blunted very quickly and extensively. Using smaller knife angles (30° - 40°) proved unproductive. Knives with these angles always failed to cut sections. Similarly, knives of larger angles (60° - 70°) would not produce sections from the frozen tissue block.

a) Diamond Knife

The damaged diamond knife first used in the ultracryotome was an improvement upon 45° glass knives. It cut sections more consistently than glass knives but still fell short of the kind of sectioning achieved with plastic embedded material. The early results with this damaged diamond knife included the 'mitochondrial' micrograph of Figure 34 and led to the purchase of a new diamond knife and to the design and construction of an invar steel holder for this knife.
b) Diamond Knife and Holder

After designing, constructing and heat treating the Invar steel knife holder and then mounting the diamond knife on this holder with the recommended epoxy adhesive it was disappointing to find that its ability to cut frozen sections was as limited as conventional glass knives. The knife and holder were then used to cut material embedded in a plastic, Araldite, as used for conventional ultrathin sectioning. The results indicated that the knife was effectively shredding the material. There were no obvious faults with the knife edge when viewed under a dissecting microscope at x 50. Possibly there was some problem with the knife holder or the mounting of the knife on the holder or some combination of the two. Whatever the reason the knife would not cut sections and it was decided to return it to the manufacturers in the U.S.A. for re-sharpening. Unfortunately because of poor communication between Dupont in the U.K. and the parent company in the U.S.A. it was twelve months before the knife and holder were returned by which time the limiting factors in the technique of ultracryotomy as described here were evident.

c) Cerama Glass Knives

These were obtained from Polaron U.K. Ltd. and had a knife angle of 45°. On the majority of occasions these knives were used they sectioned consistently well and visibly better than any
other type of knife used. Ribbons of sections could be cut even with a dry knife. Why these knives should prove so superior to any of the other types of knives used is not clear. Possibly the conditions within the ultracryotome were conducive to good sectioning. However the coincidence of a series of good cutting sessions each time with the same type of knife makes this seem improbable. The only disadvantage of these knives was that it was easy to damage, i.e. shatter, the whole knife by overtightening the clamping system. Both of the two knives used were subsequently destroyed in this way. This suggested that either there was a large discrepancy in coefficients of thermal expansion between the knife and knife holder materials with the holder contracting more than the knife and hence overtightening on the knife, or that the clamping of the Cerama glass knife and the cooling of the knife through as much as 150°K produced stresses which caused the knife to crack and shatter. The latter explanation seems the more likely since the knife holder was constructed from material specifically chosen to have as low a coefficient of thermal expansion as possible. Retrospective enquiries to the suppliers about the source and nature of these Cerama glass knives proved unhelpful. The original source had subsequently gone out of business and even when supplying the material the company would reveal very little detailed information about the knives. Having used two of these Cerama glass knives it was not possible to obtain further examples because of difficulties at the source.
After the period with Cerama glass knives and the diamond knife and holder system the ultracryotome was used with 45° glass knives and the damaged diamond knife already described. In all cases, sections were cut with a dry knife. The quality of the sectioning, as already indicated, was poor.

Specimen Mounting Variations

The first variation tried, that of touching the dissected tissue on to a clean invar stub and then quenching the stub/tissue combination into liquid coolant and then mounting the combination into the existing chuck, proved unsuccessful. Although one could be sure of clamping securely upon the invar stub the frozen tissue was dislodged either when attempting to trim or if this step was omitted then the tissue would be dislodged during the cutting process.

The same problem occurred using the silver pin system; the tissue being lost either during the trimming step or the cutting process.

Transfer Stage Variations

First Modification

Although intended to improve the sealing of the transfer stage and hence the reliability of freeze drying of the sections, the
frequency of good seals was in fact slightly reduced.

**Second Modification**

The modification to the grid holding system improved the handling and transfer of grids from the ultracryotome to the transfer stage. The further modification to the vacuum valve sealing the transfer stage, although an improvement on the two former valves, was still not completely reliable.

**Third Modification**

An addition of a neoprene upper to the transfer stage further improved the sealability of the system but failure to achieve a seal was still occurring 10 - 15% of the time.

**Freeze Drying in the Ultracryotome**

Using this method of freeze drying the resulting sections always showed the characteristics of 'melt-back', i.e. indistinct edges, amorphous background and, at most, vague suggestions of structure within the section.
Discussion

The work described in the results section of this chapter highlights two of the problems which limit the technique of ultracryotomy as described here. These are:

a) Less than 'rapid' freezing.

b) Cutting artefacts.

The use of a transfer stage illustrates the problems that are encountered in handling frozen sections after they have been cut.

Less than 'rapid' Freezing

Figures 24 and 25 illustrate what I believe to be the end point of the technique of ultracryotomy as described in this chapter. The morphology within the sections is comparable to that seen in sections of biological tissue embedded in plastic. Whether one should be aiming toward the kind of morphology seen in conventional sections is debatable. Quite possibly the procedures described here could be expected to produce a markedly different picture from that seen with conventional sections. However, when one considers the results obtained with ultrathin sections cut from biological material embedded in a plastic and compares them with results from techniques such as freeze substitution, freeze etching and freeze fracturing one sees that there is in fact a good deal of complementarity
between the techniques, suggesting in fact that conventional plastic embedded sections do represent a good guide to what is achievable with ultrathin frozen sections.

The two limitations of sections such as those shown in Figures 24 and 25 are the large tears in the section and the electron transparent background or 'holes' in the section.

There are a number of possible explanations for the 'holey' appearance of freeze dried ultrathin frozen sections. Among them are:

a) Growth of ice crystals during the initial freezing step.
b) Cutting damage during sectioning.
c) Growth of ice crystals during the cutting of the sections as some of the potential energy of the microtome arm is dissipated into the ultrathin section causing warming of the section.
d) Damage caused during freeze drying.
e) Some combination of a - d.

Initially working with the ultracryotome produced a whole range of different results and it was not until much data had been accumulated that the significance of sections such as those shown in Figures 24 and 25 became apparent. As has already been stated, sections with this 'holey' appearance were the most commonly occurring and revealed the most morphological detail. Having established the 'holey' sections as a baseline for the technique
of ultracrystotomy it was striking to see the similarity between the results obtained with this technique and those obtained by many other workers using the technique of freeze substitution (Whittaker, 1974; Karow and Shlafer, 1975; Walter et al., 1975). The only common step in both these techniques is that of the initial tissue freezing implying that the damage seen in both cases is that due to the growth of ice crystals during the freezing step. Before discussing the point, further consideration will be given to the three other potential sources of the damage listed above.

Cutting Damage

At first, cutting damage was thought to be the source of all the damage seen in the sections the reasoning being that the knife, possibly damaged itself, was shredding the sections from the frozen tissue block. We varied the knife angle of glass knives from $30^\circ - 70^\circ$, used diamond knives, Cerama glass knives or tungsten coated glass knives. None of these alternatives produced any differences in the 'holey' sections. As indicated above these 'holey' sections, because of the consistency with which they were obtained and also the relatively good morphological preservation present, were used as baseline against which any changes in experimental protocol were measured. Although not conclusive evidence, our experience suggests that cutting is not the primary factor in producing the 'holey' appearance seen in the sections.
Thawing of Sections During Cutting

It is possible that as frozen sections are cut from the tissue block the knife impact may be sufficiently energetic to cause thawing in the section (Thornburg and Mengers, 1957; Hodson and Marshall, 1972). Calculations of the extent of section thawing by Hodson and Marshall, 1972, making the most disadvantageous approximations, gave a maximum of 10 nm of section thawing in a total section thickness of 100 nm with their system. With the work described in this chapter the ultracryotome was normally operated with a knife temperature of 148 K and a tissue block temperature of 138 K so that heat generated during the cutting stroke would flow away from rather than into the sectioned material. Many variations were tried with knife and block temperatures, the temperature gradient between knife and block were also reversed, but whatever the settings of knife and block within the values explored no difference was seen in the 'holey' appearance of the freeze dried sections. This evidence argues against substantial crystallization or recrystallization of water during the cutting stroke.

Freeze Drying Damage

Appleton (1978) claims that the 'holey' appearance seen in ultrathin frozen sections which have been freeze dried is an artefact produced by the freeze drying processes. He demonstrates
the apparent differences between sections which had been freeze
dried in different ways. In the first method the sections were
left inside the chamber of the cryoultramicrotome and the
temperature of the chamber was maintained at its cutting
temperature for some 3 hours with the sections being at
atmospheric pressure throughout this period. After this
period, the sections were gently warmed to room temperature and
viewed in the electron microscope. The alternative method
of freeze drying was comparable to that used in the work
described in this chapter, i.e. to rapidly freeze dry the
sections under vacuum. Looking at the sections which had been
freeze dried at atmospheric pressure one sees all characteristics
of sections that have either melted or rehydrated, i.e. ill-
deﬁned edges and poor resolution of ultrastructure. Again one
would return to the similarities between freeze substituted
material and material produced using the technique of ultra-
cryotomy or cryoultramicrotomy with freeze drying under vacuum
as a final step. Now unless the forces in play during the
substitution of the ice by the relevant substituting ﬂuids
are similar to the corresponding forces involved during freeze
drying then the only step these two techniques have in common
is that of the initial rapid freezing of the tissue. This, it
seems to me, indicates that the freeze drying under vacuum is
not the stage at which the damage is being produced. More recent
work (Frederik and Busing, 1981), supports this view. After
quench freezing tissue they then subjected it to a number of
alternative preparatory routes for viewing in the electron microscope. They concluded that 'the ultrastructure of cells is neither damaged by the growth of ice crystals (migratory recrystallization) at 193 K, nor by freeze drying at temperatures warmer than 193 K. The observed ice crystal damage to the ultrastructure of frozen kidney therefore depends only on the freezing conditions.'

**Growth of Ice Crystals during the Initial Freezing Step**

The weight of evidence, then, points to the initial freezing step as the source of the damage seen as 'holes' in the freeze dried ultrathin sections. These 'holes' are the cavities left behind by the subliming ice crystals. The ice crystals having been formed during the freezing of the tissue. Much work has now been published concerning the problems associated with rapid freezing (Luyet and Gonzales, 1951; Stephenson, 1956; Cowley et al 1961; MacKenzie and Luyet, 1967; MacKenzie, 1969; Bachmann and Schmitt, Van 1971; Vennrooij et al, 1975; Costello and Corless, 1978; Van Harreveld and Trubatch, 1978; Handley et al, 1981) and it is now established that unless certain criteria are fulfilled in terms of sample size and the means of freezing, then ice crystals will be produced during 'rapid' freezing and subsequently the morphology of the tissue will be impaired by the cavities left behind when the ice is removed (Riehle, 1968; Moor, 1972; Dempsey and Bullivant, 1976 a, b; Frederik and Busing, 1981).
Cutting Artefacts

Many sections were marred by the 'ripple' effect shown in Figures 28, 29 and 30. This would seem to be characteristic of a cutting problem, i.e. the slip lines formed in the relief or partial relief of shear stress. The shear stress arising because the ultrathin section being cut from the frozen tissue block has to be transferred from the plane of cutting to the rake face of the knife. It was not possible to correlate the appearance of the 'ripple' with any of the variables involved in the cutting process. One might expect for example that the colder the tissue block the harder it would be (Barnes and Tabor, 1966) and consequently the more difficult it would be to section and in this situation cutting artefacts would be more probable. This was not demonstrably so. However, one has to qualify this by considering the difficulties in obtaining consistent results with the ultracryotome system.

Transfer Stage

Having cut ultrathin frozen sections one has two alternatives for viewing the sections in the electron microscope. If the electron microscope has a cold stage for the specimen then the possibility exists of viewing the sections in their hydrated form. Without a cold stage the sections have to be dehydrated usually by some form of freeze drying.
Work with hydrated ultrathin frozen sections has shown that there is little or no image contrast in the sections when viewed in the electron microscope \cite{Rose et al., 1981; Zierold, 1982}. Furthermore, the characteristic X-ray spectra of frozen hydrated sections is poor and it is found that at least partial freeze drying is necessary for the detection of characteristic X-ray peaks in frozen hydrated sections \cite{Zierold, 1982}. As the end point of ultracryotomy was, and still is, the preparations of biological material for subsequent X-ray analysis, and not having a specialised cold stage on the electron microscope used, it was decided to freeze dry the sections before examination in the electron microscope. A vacuum transfer stage was designed and built for this purpose. In this vacuum transfer stage frozen sections could be freeze dried at low temperatures, warmed to room temperature whilst still under vacuum, transferred again under vacuum to a coating unit where a layer of carbon was evaporated upon the sections. Having initially collected the frozen section on a grid covered with a carbon coated collodion membrane and then finally coated the freeze dried sections with carbon the sections could then be exposed to air. In theory the sections were protected from rehydration by their carbon 'seal', in practice, however, it was found that once the sections were freeze dried then whether they were finally carbon coated or not made no difference to their appearance in the electron microscope. It is possible that with minimal exposure to water
vapour little or no rehydration will occur (Spriggs and Wynne-
Evans, 1976). However, Frederik and Busing, 1981, have shown
that rehydration of ultrathin sections, freeze dried under
vacuum, does occur and this rehydration is sufficient to
obscure the products of freezing damage, i.e. the holes in
the section. It is difficult to resolve this contradictory
evidence. Possibly the deliberate rehydration described by
Frederik and Busing was successful whereas minimal exposure
to water vapour is insufficient to achieve rehydration.

Finally I would return to the possibility of freeze drying
damage. I would suggest that the differences seen in sections
freeze dried under vacuum and those freeze dried in nitrogen gas
are the differences between a section which is dehydrated in
the former case and one which has rehydrated in the latter case.
Figure 1

The ultracryotome.
Figure 2
Plan view of the cryo-attachment.

Specimen liquid nitrogen reservoir

Knife liquid nitrogen reservoir

Specimen holder

Heater

Platinum resistance thermometer

Figure 3
Detail of cryo-attachment window heater connections

Heating element

Window
Figure 4
Schematic of knife base

Figure 5
Detail of specimen cooling assembly

Figure 6
Specimen holder

Figure 7
Circuit diagram for liquid nitrogen level sensing diode.
Figure 8. Detail of boom arm entry into cryo-attachment.

Figure 9

The transfer stage
Figure 10. The electrophorus.

- Perspex handle
- Aluminium base
- Polysterene sheet
- Insulated forceps

Figure 11. Frozen tissue mounting jig.

Figure 12. Pistol specimen holder

- Specimen
- Quenching medium
Figure 13. Schematic of cutting system.

Figure 14. Schematic of sectioning
Figure 15.
Diamond knife clamping system

Figure 16.
The modified diamond knife assembly
Figure 17
Expansion coefficients of Nickel-Iron alloys at 20°C and at -100°C.

![Coefficient of thermal expansion graph]

Figure 18
Some physical and chemical properties of Invar steel (specification Fe-Ni36) at 20°C.

Composition (w/w %): Fe 62.35; Ni 36; Mn 0.5; Si 0.5; Cu 0.5; C 0.15.

<table>
<thead>
<tr>
<th>Property</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean coefficient of thermal expansion</td>
<td>$1.5 \times 10^{-6}$ °C$^{-1}$</td>
</tr>
<tr>
<td>Density</td>
<td>8.13 kg/dm</td>
</tr>
<tr>
<td>Thermal conductivity</td>
<td>10 W/m °C</td>
</tr>
<tr>
<td>Melting point</td>
<td>1430 °C</td>
</tr>
<tr>
<td>Tensile strength</td>
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</tr>
<tr>
<td>Young's modulus</td>
<td>$138 \times 10^3$ N/mm$^2$</td>
</tr>
<tr>
<td>Poisson's ratio</td>
<td>0.25</td>
</tr>
</tbody>
</table>
Figures 19 & 20.

Modifications to the specimen holder

Figure 19

Figure 20

Invar steel stub

Silver pin

Figure 21. The modified vacuum transfer stage

Figure 22. Detail of the seal to the vacuum transfer stage

Teflon valve

Mushroom

Insert

Neoprene annulus
Fig. 23. Ultracryotome section of rat liver. (x 8,200).

Fig. 24. Ultracryotome section of rat liver. (x 17,000).
Fig. 25. Rat liver in conventional electron microscopy. (X 9,500)
Fig. 26. Knife marks (arrowed) in ultracryotome sections of rat liver (x 7,200).

Fig. 27. Striations in ultracryotome sections of rat liver (x 13,500).
Fig. 28. Striations in ultracryotome sections through rat kidney cortex (x 9,800).

Fig. 29. Striations in ultracryotome sections through rat liver (x 15,400).
Fig. 30. A rehydrated ultracryotome section of rat liver (x 12,400).

Fig. 31. Rehydrated rat liver (x 12,400).
Fig. 32. Rehydrated rat liver (x 7,400).

Fig. 33. Rehydrated rat liver (x 18,500).
Figure 34. Mitochondria in rat liver (x 21,000).
Fig. 35. Cooling curves for a thermocouple immersed in various quenching agents.
CHAPTER 3

INTRODUCTION

The work described in the previous chapter serves to illustrate one of the major limitations of the technique of ultracryotomy, and this is the problem of damage to the tissue by ice crystals produced by the initial rapid freezing of the tissue. It is a problem common to a number of techniques which employ rapid freezing of biological tissue as a starting point (Bachmann and Smitt, 1971; Christensen, 1971; Frederik and Klepper, 1976; Dempsey and Bullivant, 1976a,b; Nei, 1976; Spriggs and Wynne-Evans, 1976; Zierold, 1982). Cryoprotective agents reduce the presence of ice crystals, but their action would be counter-productive to my aim of using ultracryotomy as a means for obtaining reliable data from X-ray microanalysis. To have some understanding of the factors which affect the appearance and growth of ice crystals in rapidly frozen tissue one needs to know something of the thermal history of the specimen through the freezing process.

Early work of this nature was carried out by Luyet and Gonzales (1951) and Stephenson (1956) using small thermocouples as sensors and a cathode ray oscilloscope as a means of recording. Subsequent work by MacKenzie, again using small thermocouples and a high-speed galvonometer, provided interesting data on the rapid freezing of biological material and on the experimental techniques for acquiring
this data (MacKenzie, 1969). Of especial interest are the plateau temperatures shown by MacKenzie on his cooling curves from biological material cooled by plunging into liquid nitrogen or other similar coolant. Figure 1 illustrates this phenomenon and shows a relatively long hold in the temperature of the specimen at or about the freezing point of the tissue. This hold would seem a highly probable source of ice crystal nucleation and subsequent growth. Is there some way in which the position of the plateau could be modified? Could the water in the specimen be significantly supercooled and the plateau lowered to a level at which ice crystal growth is reduced and then quickly halted? Or could the plateau be raised above its supercooled level so that a volume of tissue about to be frozen is held above the freezing point of the tissue water until the much faster freezing rates demonstrated in the portion XY of Figure 1 can be applied to the volume of tissue?

Hence, it was decided to investigate the freezing rates of biological tissue using microthermocouples. Both the freezing rate as a function of block size and the freezing rate at different depths within a block were of interest and so it was decided to work with a system of tissue slices where more slices could be sandwiched together to increase the size of the block and the microthermocouple could be placed between different slices to investigate freezing rates at different depths within the composite block. The size and distribution of ice crystals within the block were examined in the microscope using the preparative techniques of freeze substitution (Feder and Sidman, 1958).
METHODS

Microthermocouples

Initial microthermocouple work was carried out using thin slices of rat liver and kidney. The slices were prepared using a Sorvall TC-2 tissue sectioner.

A suitable sample-holding material such as a 7% aqueous solution of Agar was spread upon a filter paper attached to the specimen stage assembly. A strip of the specimen was then placed in the Agar and completely coated with additional Agar. The coating provides specimen rigidity, prevents the knife blade from lifting the specimen strip, and ensures that the sections do not scatter under the rapid action of the knife blade. The specimen, mounted on the filter paper, was then firmly clamped to the specimen-stage assembly via a spring clip and locking ring. A new razor-blade, cleaned with 70% alcohol, was fitted to the cutting arm of the tissue-sectioner. The instrument was then set to section and bundles of sections were placed in a suitable storage medium and gently shaken to separate the sections from each other.

The thermocouples were constructed from copper and constantan wire (SWG 42) approximately 100 µm thick. The wires were then further flattened between metal plates, the copper wire then having a thickness of 50 µm and the constantan a thickness of 60 µm. One junction was placed in ice/water and the other carried the tissue slices.
The emf generated by the thermocouple was displayed on a Textronics Storage Oscilloscope (Type 564B, using a Type 2B67 Time Base and a Type 3A9 Differential Amplifier). The resulting cooling curves were photographed using Polaroid film.

Initial experiments with 200 µm slices of rat kidney proved difficult, the flexibility of the sections being such that building an assembly of these sections was almost impossible. As an alternative it was decided to work with thin slices of storage taproot tissue of carrot (Daucus carota). The relative rigidity of 200 µm carrot slices considerably eased the problem of building section assemblies.

**Labelled Slices**

Early experiments with these carrot slices showed considerable variations in their freezing rates when they were plunged into liquid nitrogen. These variations arose, it was assumed, from differences in the water content and differences in the levels of adsorbed water, due to over- or under-blotting of the slices prior to sandwiching them around the microthermocouples. Figure 2 illustrates this point. If experiments with these slices were to have any validity then it was obvious that greater accuracy was required to produce assemblies of tissue slices with a consistent water content. To this end it was decided to monitor the amount of water in each preparation by exchanging tissue water with tritiated water.
A preliminary experiment indicated that fresh carrot slices were isotonic with 0.9% saline. A carrot cylinder (10 mm diameter, 1.8 mm long) was loaded in tritiated water having an activity of $10^6$ dpm.ml$^{-1}$. The carrot cylinder was placed in 5 ml of the labelled loading solution within a glass vial and shaken in this solution on a flask shaker for 30 minutes. The carrot cylinder was then blotted and unloaded into 5 ml of 0.9% saline once for 2 minutes and then a further nine times for 10 minutes. The unloading was carried out using the flask shaker.

Analysis of the activity in the unloading solutions was carried out using a Packard Model 3380 Tri-Carb Liquid Scintillation Spectrometer at 5°C using the preset tritium channel. The scintillation fluid included 5 grms of PPO (2,5 Diphenyloxazole) as a primary solute/scintillator and 0.2 grms of POPOP (5, phenyloxazole) as a secondary solute/scintillator. The solvent was 1 l of toluene with 500 ml Triton X-100. One ml of the solution whose activity was required was added to 9 ml of the scintillation fluid in a plastic scintillation vial, well shaken, and then placed in the spectrometer to be counted.

Although the quenching of the isotope activity was about the same in all the samples, a quenching correction was made by means of the automatic external standard within the spectrometer and of a set of tritium standards. A curve of A.E.S. ratio versus efficiency was produced for each set of samples and this curve was then used to correct the samples for quenching. Subsequently,
each preliminary experiment involved determining the efficiency curve using tritium standards, counting the activity in (a) the loading solution, (b) the unloading solution, and (c) the unlabelled loading solution, to provide a measure of the background from the spectrometer. Wherever practicable the samples were counted to at least 10,000 counts to reduce the statistical counting error to 1%. Details of these experiments given in the results section of this chapter show that labelled water exchanged fully with tissue water and hence labelled water could be used to monitor tissue water.

The experimental procedure consisted of the following steps:

a) Carrot cylinders 3.6 mm in diameter were punched out axially from the parenchyma of whole carrots using a cork borer. The resultant cylinders were embedded in 7% Agar and sectioned along their axes on the tissue sectioner to produce 200 μm thick circular slices.

b) The slices were loaded with labelled solution (0.9% saline including tritiated water with a specific activity of $10^6 \text{ dpm.ml}^{-1}$) by shaking them in 5 ml of the loading solution on a flask-shaker for 20 minutes.

c) The loaded slices were then blotted, mounted around a microthermocouple and gently compressed together to form a good seal.

d) The assembly was plunged into liquid nitrogen and the temperature within the assembly was recorded using the storage oscilloscope.
e) The frozen slices were unloaded by shaking them for 20 minutes in 5 ml of 0.9% saline on a flask-shaker to back extract the tritiated water.

f) Actual tissue water was then calculated from the back extracted activity.

Plunging of the assemblies into liquid nitrogen was standardised using the apparatus shown in Figure 3. This consisted of a hollow weight running on a retort stand, the bore of the hollow weight and the retort stand having been polished to achieve a smooth descent of the weight down the stand. The weight carried the thermocouple tissue assembly and also a magnet which activated a reed relay which in turn triggered the oscilloscope sweep. The trigger circuit is shown in Figure 4.

Using the basic 200 µm slices, tissue assemblies could be built of varying thicknesses and for a given assembly the thermocouple could be incorporated at one of a variety of depths. In the experiments described a standard notation is used to define a given tissue assembly in terms of its thickness and the position of the thermocouple. For example 400/600 refers to a tissue package 1000 µm thick made up from 5 x 200 µm slices, two on one side of the thermocouple and three on the other.

The following assemblies underwent the experimental procedure given above.
Series A

(i) 200 / 200
(ii) 200 / 400
(iii) 200 / 600
(iv) 200 / 800

In this series the depth of the thermocouple from the surface of the tissue assembly was held constant while the total tissue mass was varied.

Series B

(i) 200 / 1000
(ii) 400 / 800
(iii) 600 / 600

Here the total tissue mass was held constant while the depth of the thermocouple from the surface was varied.

Series C

(i) 200 / 200
(ii) 400 / 400
(iii) 600 / 600

Here both the mass of the tissue and depth of the thermocouple from the surface is varied but the thermocouple remained at the centre of the assembly.
Freeze-Substitution

This work was done with rabbit cornea. The rabbit cornea consists primarily of water and collagen, 75% approximately of the wet weight being water and 70 - 75% of the dry weight being collagen. Collagen is the principal structural component of the corneal stroma which is bounded on the posterior side by a multi-layered epithelium and on the anterior side by a single-layered endothelium. For the freeze-substitution work described here the epithelium and endothelium were removed using a rotating brush comprised of radially oriented bristles (Hodson, 1974).

Corneas were rapidly frozen in liquid nitrogen either singly, in assemblies of two, or in assemblies of three. These tissue assemblies were then freeze-substituted. Biological freeze-substitution involves the rapid freezing of tissue and its subsequent dehydration by dissolving away the ice at temperatures well below 273K using a suitable solvent. The ice within the tissue is thus, over a period of days, replaced or substituted by the solvent. The tissue can then be warmed to room temperature and processed in the same fashion as material conventionally processed for light and electron microscopy by embedding and subsequent sectioning.

Freeze-substitution was first described by Simpson (1941) as an excellent method of preparing tissue for study in the microscope. Subsequent modifications to the technique (Feder...
and Sidman, 1958; Fernández-Morán, 1957) have involved the use of chemical fixatives in the substituting fluids to improve the quality of tissue preservation. The technique has been used, as was originally intended, as a means of rapidly arresting metabolic processes with the retention of labile components of cell cytoplasm (Hereward and Northcote, 1972; Pallaghy, 1973; Harvey et al, 1976) for the visualization of transient ultrastructural arrangements (Woolley, 1974; Van Harreveld and Fifkova, 1975; Barlow and Sleigh, 1979) as well as a means of investigating the effects of rapid freezing on biological systems (Walter et al, 1975; Dempsey and Bullivant, 1976a) the latter being its application in the present study.

The method of freeze-substitution used was based upon that described by Karow and Shlafer (1975) which in turn was modified from the techniques of Sherman and Kim (1967) with the inclusion of osmium tetroxide as suggested by Feder and Sidman (1958).

**Freeze-Substitution Protocol**

Corneas were obtained from 6-month-old Dutch rabbits, the rabbits being sacrificed by an intravenous injection of sodium pentobarbitone. The whole eyeball was carefully removed from the animal and the cornea dissected out. Corneal pieces approximately 5 mm² were cut from whole corneas and these pieces attached to a dummy thermocouple lead. This system was then quenched into liquid nitrogen using the apparatus described previously (Figure 3). The
tissue was kept immersed in liquid nitrogen whilst a 20 ml glass serum bottle, previously stored in liquid nitrogen, was transferred to the quenching Dewar. The tissue was then transferred into the serum bottle by cutting the dummy thermocouple lead. The tissue could then be stored in the serum bottle under liquid nitrogen ready for the freeze substitution procedure.

The substitution fluid was a 1% solution of osmium tetroxide in equal volumes of absolute acetone and ethanol. Since osmium tetroxide reacts with acetone at room temperature (Feder and Sidman, 1958), it was necessary to prepare the substitution fluid by pre-cooling the acetone-ethanol mixture to 203K and then adding the osmium tetroxide. This preparatory work was done in the cold cabinet of a cryostat ("Pearse" Cold Microtome Cryostat) from which the microtome had been removed. The compartment had a lower temperature of 203K which was monitored with an alcohol thermometer placed inside the compartment. The substitution fluid was prepared two days before it was due to be used. A volume of 100 ml of acetone-ethanol mixture was made up in a glass stoppered bottle at room temperature. This was then placed in an insulating container packed with dry ice and stored at 193K in a chest type of deep-freeze cabinet (Cliffco Model No. 1080).

On the day prior to the freezing of the tissue the substitution fluid was transferred to the cryostat compartment for the addition of the osmium tetroxide. A 1 gram vial of osmium tetroxide was scored with a glass cutter and placed together with a glass rod within the
cryostat compartment to equilibrate. The vial of osmium tetroxide was then dropped into the acetone-ethanol mixture and broken open using the glass rod. All these and subsequent manipulations within the cryostat compartment were carried out with the hinged window of the compartment ajar using an inner pair of disposable gloves and an outer pair of domestic rubber gloves for protection. After carrying out any given manoeuvre, the window was closed and the compartment and its contents were allowed to re-equilibrate at 203K. The substitution fluid was shaken and transferred back to the deep freeze at 193K ready for use the following day.

After freezing the tissue, the Dewar containing the serum bottles, frozen tissue and liquid nitrogen was transferred to the cryostat compartment at 203K. The substitution fluid together with two all glass syringes and two rubber serum caps were also transferred to the cryostat compartment. The rubber serum caps were pierced by two needles one a 25 mm x No. 25 gauge and the second a 60 mm No. 1 serum needle. The needles served as vents through which vapourised nitrogen could egress and through which substituting fluid could be added or removed. The serum bottle and tissue were then removed from the Dewar and were allowed to stand uncapped in the cryostat compartment to allow the liquid nitrogen to evaporate. When the liquid nitrogen level had fallen to just above that of the tissue in the serum bottle the bottle was capped and substitution fluid was aspirated from the stock bottle with a glass syringe and added to the tissue in the serum bottle via the 60 mm needle in the bottle.
cap. The bottles were then quickly swirled and the needles stoppered with cotton wool to prevent moist air or frost from entering the bottles.

The bottles were then transferred to the deep-freeze cabinet using an insulated container packed with dry ice. The bottles were left in this container within the deep-freeze for one week with the serum bottles shaken gently each day. At the conclusion of this week the old substitution fluid was removed and replaced with fresh identical solution using the cryostat chamber at 203K to make these solution changes. The bottles were then returned to the deep freeze for a further week and each day the bottles were gently shaken for a brief period. At the end of this week the serum bottles were transferred back to the cold chamber of the cryostat and the substitution fluid was withdrawn and replaced with an equal volume of a solution that was identical, except that it contained no osmium tetroxide. The bottles were then allowed to warm to 250K in a standard freezer and then to 277K in a refrigerator and finally to room temperature.

The acetone-ethanol mixture was then replaced with three changes of propylene oxide and embedded in Araldite. Sections 1 μm thick were cut on a Huxley Mk II ultramicrotome and stained with toluidine blue for light microscopy and, when possible, ultrathin sections were cut for electron microscopy and stained with uranyl acetate and lead citrate.
Theory of the labelled tissue

Considering the efflux of labelled water from cells washed in a large volume of unlabelled solution we have for simple surface or membrane-limited exchange,

\[
\frac{dC_i}{dt} = \frac{P_d A}{V} C_i \quad \text{(1) House (1974)}
\]

where \( C_i \) is the concentration of labelled water inside the cell at some time \( t \)

\( A \) is the surface area of the cells
\( V \) is the volume of the cells
\( P_d \) is the diffusional permeability of the cells to water

Integrating (1) we have,

\[
\ln C_i = -\frac{P_d A t}{V} + B
\]

where \( B \) is a constant of integration

if at \( t = 0 \) \( C_i = C_0 \)

where \( C_0 \) is the concentration of labelled water in the leading solution then

\[
\ln C_0 = B
\]

which gives,

\[
\ln C_i = -\frac{P_d A t}{V} + \ln C_0 \quad \text{(2)}
\]
Thus a plot of $\ln C_t$ versus $t$ should yield a straight line whose slope is related to the diffusional permeability of the cells to water.

This method of measuring $P_d$ rests on several assumptions:

Firstly, it is assumed that tritiated water is an ideal tracer for water. In respect of water transport across a biological membrane it is impossible to predict what sort of transport rates might result from the properties of different isotopic forms of water. Nevertheless, it is notable that no significant difference has been demonstrated between the permeability coefficients of frog skin for deuterated and tritiated water (King, 1969) and, therefore, it seems unlikely that an 'isotope' effect is a serious source of error in determinations of $P_d$ for biological membranes.

Secondly, it is assumed that the labelled water, by virtue of its chemical and physical properties, does not interfere with the normal behaviour of the carrot cell membranes. For instance tritiated water emits weak radiation which might damage these membranes. However, the radiation dose delivered to the cells is generally found to be negligible (Paganelli and Solomon, 1957) and any damage caused is hence assumed to be insignificant.

The third and final assumption is that both the cellular and external medium are well mixed. In practice this condition is the most difficult to satisfy and is a potential source of error (House, 1974).
RESULTS

Labelled Tissue

An efflux plot for a carrot cylinder is shown in Figure 5. On the basis of equation 2 (which relates to surface limited exchange) the plot is expected to be linear. In the event, however, the plot is curvilinear and this curvilinearity is interpreted as either a small fraction (less than 1%) of water slowly exchanging from subcellular compartments or possibly proton exchange. Over the linear portion of the plot during which time most (about 99%) of the label effluxes from the carrot, the gradient m is given by,

\[ m = \frac{P_d A}{V} \ s^{-1} \]

The measured value of m is 1.75 x 10^{-3} on the plot and with,

A = 2\pi r (r + h) and V = \pi r^2 h

where r = 5 mm and h = 1.8 mm then,

\[ P_d = 1.16 \times 10^{-4} \text{ cm.s}^{-1} \]

Also from the experiment it is possible to compare the activity recovered from a carrot cylinder during the efflux with the expected activity in the carrot cylinder thus :

If \( A_L \) = Measured activity of the loading solution

\[ V_L = \text{Volume of carrot cylinder} = \pi r^2 h = 141 \ \mu l \]
and with a carrot water content of 89%,

Volume of water in a carrot cylinder = 125 μl

Hence the expected activity of water in a carrot cylinder $A_{CX}$ is given by,

$$A_{CX} = A_L V_W$$

Such calculations indicate that in these efflux experiments 97 ± 1% of total tissue water was labelled.

**Thermocouple Experiments**

The results of plunging a 200 / 200 assembly into liquid nitrogen are shown in Figure 6. The carrot slices used in this experiment were of 5 mm diameter as were the slices used in the other A Series experiments illustrated in Figure 7. Smaller slices (3.6 mm diameter) were used in the B and C Series experiments to reduce the overall weight of the larger assemblies used in these experiments.

Considering Figure 6, trace a) was obtained from a 200 / 200 assembly which had been overblotted until the tissue slices were visibly dry.

Trace b) was obtained from a 200 / 200 assembly which was 'optimally' blotted i.e. excess surface water was removed but the tissue slices were still wet.

Trace c) was obtained from a 200 / 200 assembly from which most but not all of the excess water had been removed.
The expected activity in dpm., knowing the volume of the slices and the activity of the loading solution was $638 \pm 2$ dpm. The measured values for traces a), b) and c) are given in Figure 6 and when compared to the traces themselves show the resolving power of the technique.

The results of an experiment from Series A are shown in Figure 7. When one considers the portion 'x - x' of each of the cooling curves i.e. post the freezing plateau and prior to the 'knee' characteristic of freezing in liquid nitrogen it is seen that as the mass of the tissue assembly was increased the rate of freezing at a fixed depth from one surface decreased.

Figure 8 shows the results of an experiment from Series B where the mass of the assembly was kept constant whilst the depth of the thermocouple from the surface was varied. Within the limits of the experimental protocol it is seen that the rates of freezing are comparable.

Results for an experiment from Series C (Figure 9) show that where the thermocouple is at the centre of the tissue assemblies of varying masses increasing mass leads to a decrease in the freezing rate as measured by the centrally placed thermocouple.

Having developed the labelled slice technique because of the variability both of water content and of adsorbed water levels of the tissue slices initially encountered, the experience gained during the development and subsequent application of the technique enabled the assemblies to be put together with such a consistent
water content that labelled slices were included on only one side of the thermocouple as a monitor. In the A Series the single 200 μm slice was labelled, in the B and C Series the 200, 400 and 600 μm assemblies on one side were labelled. Table 1 summarises the expected and measured activities for the labelled slices used in the A, B and C Series experiments described above and illustrated in Figures 7 - 9. In this particular set of experiments the slices were labelled as described above and using Table 1 quantitative interpretations can be made of the associated cooling curves. For example the difference in the cooling curves for the 600 / 600 assemblies in the B and C Series experiments (shown in Figures 8 and 9) is due to the excess water that was frozen in the first case.

Freeze-Substitution

Ice crystal cavities (iccs) were present in all the 1 μm thick sections of rapidly frozen cornea viewed under the light microscope. Figure 10 shows an unfrozen control cornea and is to be compared with Figures 11 - 13 showing corneas which had been rapidly frozen in liquid nitrogen. Figure 11 shows the results of rapidly freezing a single cornea, Figure 12 that of a cornea rapidly frozen as one of a pair, and Figure 13 that of a cornea rapidly frozen as one of a triplet and being on the outside of the triplet to afford a direct comparison with the corneas in Figures 11 and 12. In describing the results reference will be made to round/elliptical iccs and
elongated iccs. Looking at the unfrozen control shown in Figure 10 it is possible to envisage how these two types of iccs are formed. Regions such as $Y$ in Figure 10 would produce elongated iccs. assuming that the region $Y$ once contained water. Regions such as $Z$ would produce rounded/elliptical iccs as viewed in the sectioned material, the shape of the iccs being determined by the spacing between the corneal lamellae and being influenced by the orientation of the collagen fibrils within the lamella.

With single corneas the iccs were of the order of $1 \mu m$ or less in diameter. The ice crystal cavity (icc.) distribution was complex, regions of rounded iccs. $1 \mu m$ in diameter being interspersed with regions having smaller iccs. these latter being in the majority. There was no obvious change in size distribution across the thickness of any cornea, nor any marked differences occurring between the icc. sizes at the surface and those at the centre.

The results for two corneas frozen together are shown in Table 2. Reference is made in this table to single and double corneas because, although it was usually possible to separate the corneas making up an assembly, in one case this was not possible. The corneas were separated by gently pulling the lead, around which they had been mounted, from between the corneas. However in one case with two corneas frozen together, and in one with three frozen together, this was not possible and the corneas still adhered to each other after the lead was removed. The freeze-substitution of two corneas frozen together thus gave eight single corneas and one double assembly.
As with the single corneas the icc. distribution was often very complex and it should be understood that the descriptions of these distributions are qualitative, serving to emphasize the absence of any obvious icc. size distribution. There was however a dramatic increase in the mean icc. size when two corneas were frozen together as compared to corneas frozen singly (Figures 11 and 12). In spite of differences between corneas there was no obvious variation in icc. size across the thickness of the preparation.

The results of three corneas frozen together (thickness about 1200 µm) are shown in Table 3. From the five triplets there were obtained, four single corneas, four double corneas, and a triplet. In the latter case removing the mounting lead from the triplet failed to separate the assembly into its single and double components. These results showed similar characteristics to those of two corneas rapidly frozen together i.e. the overall pattern is of a uniform distribution of icc. sizes with the exception of one of the single corneas. As can be seen from Tables 2 and 3 the maximum icc. dimensions seen in single corneas increased as the size of the assembly increased from two to three corneas.

There are two exceptions to the results described above for two and three cornea frozen together. These are for the unseparated doublet in Table 2 and the unseparated triplet in Table 3. In both assemblies the iccs. were smaller than in the rest of the corresponding group, with the doublet having large areas free of iccs. Why this should be so is not immediately obvious.

It seems likely from the above results that icc. size is a complex function of cooling rate, possibly showing a saturation
phenomenon at slower cooling rates (compare Tables 2 and 3, and Figures 12 and 13). Nevertheless in all cases examined the ice crystal cavity size did not systematically increase or decrease throughout the thickness of the preparation.

DISCUSSION

The results from the thermocouple experiments and from the freeze substitution work described above demonstrate two points about the rates of freezing achieved when tissue was quenched in liquid nitrogen.

Firstly and most obviously the rate of cooling achieved was a direct function of the tissue mass. The results from the A and C Series thermocouple experiments show this and if one uses ice crystal size or the cavity left behind by the ice crystals as an inverse measure of the freezing rate then generally the freeze-substitution work confirmed this, ice crystal cavities increased in size as the mass of tissue frozen increased.

Secondly under the conditions of rapid freezing pertaining in these experiments it appears that the rate of freezing, measured directly with the microthermocouples and indirectly from the icc. size, was independent of position for a given mass of tissue. Results from a B Series experiment shown in Figure 8 illustrates this as do Tables 2 and 3 and Figures 11, 12 and 13 for the freeze-substitution work. The latter show that icc. size did not systematically increase or decrease throughout the thickness of the preparation.
Considering the freezing of thin slices of tissue, where effectively the freezing processes operate in only one spatial direction i.e. at right angles to the parallel sides of the tissue, two simplified models of ice crystal formation can be visualised. In the first a cold front, the temperature of which is below that at which ice crystal growth is taking place, forms at the surface of the tissue and then sweeps inwards. This could be termed the 'moving front' model in which water deep in the tissue freezes and continues to cool relatively slowly with the consequent growth of ice crystals, a process which does not stop until the passage of the cold front.

Alternatively one can envisage a second model in which the temperature gradients within the thickness of a tissue slice are small or negligible, and the whole system then cools at approximately the same rate. This could be termed the 'isothermal' model. The results from this work support the 'isothermal' model of cooling under the conditions of tissue freezing described here.

The results contrast with other work on the measurement of freezing rates and ice crystal sizes in specimens frozen under varying conditions. Van Venrooij et al (1975) described the freezing of cylinders of glycerol solution 2 mm in diameter by immersion into Freon 22. They used thermocouples placed at varying depths within the samples. Measuring the change of temperature at the centre of the sample, at its outer edge and halfway between these two positions they produced cooling curves for each of these
positions. These they then compared to theoretical curves generated by a model system. Also they compared ice crystal sizes in freeze-etch replicas to the experimental and theoretical freezing velocities, and showed the ice crystals at the centre and at the outer surface of the sample to be small compared to the ice crystal sizes halfway into the sample. With their experimental and theoretical work on cooling rates they defined freezing velocity as the decrease in temperature per unit time after the moment that freezing had started and on this basis their results support the freeze-etch work with the freezing velocity higher at the centre and at the outer surface than halfway into the sample.

Comparable results were obtained by Van Harreveld and Trubatch (1979) using gelatin sections and also tissue sections 50 - 60 µm thick and a form of 'slam' freezing onto a cooled silver surface (Van Harreveld and Crowell, 1964). They determined the rate of freezing with a method based upon the large difference in dielectric constants between water and ice. They found that freezing started at a high rate which declined during the first 5 milliseconds of the freezing process but then increased and usually became high at the end of fusion. Typically with one of their 60 µm gelatin samples, 3 µm of the sample would be frozen in the first millisecond, the freezing rate then declined during the next 5 milliseconds, increased gradually during the next 10 milliseconds and became quite large during the final 5 milliseconds of the freezing process. Whereas this particular study was confined to monitoring the progress of
fusion within the gelatin sample a previous study (Van Harreveld and Crowell, 1964), used essentially the same freezing procedure with specimens of mouse liver. After 'slam' freezing and then freeze-substituting the tissue they found that only a narrow surface layer (10 μm) was free of ice crystal cavities and as one proceeded into the tissue block ice crystal disruption increased, ultimately destroying the ultrastructure of the tissue. However, the size of the samples frozen, 60 μm thick gelatin Van Harreveld and Trubatch (1979) and 2–3 mm thick mouse liver specimens Van Harreveld and Crowell (1964), invalidates any detailed comparison between the two studies. Nevertheless it would have been interesting to have seen the results of freeze-substitution on the frozen gelatin sections in the former study.

Many other studies describe the gradation of ice crystal/ice crystal cavity size seen when biological or simpler material used as an alternative to biological material, is subjected to rapid freezing (Stephenson, 1956; Staehelin and Bertaud, 1971; Frederik and Klepper, 1976; Nei, 1976; Dempsey and Bullivant, 1976 a, b), the ice crystals/ice crystal cavities within a narrow surface layer, (10–15 μm), of the frozen specimen being either so small as to be beyond the resolution of the electron microscope or possibly absent altogether. Beyond this region their sizes are seen to increase, probably exponentially (Dempsey and Bullivant, 1976a), with increasing depth within the specimen.

Much of the work on rapid freezing supports the simple 'moving front' model rather than the 'isothermal' model. The
differences between the 'isothermal' results described in this chapter and the 'moving front' results of others outlined in this discussion must lie with the method of freezing used here i.e. freezing in liquid nitrogen compared to freezing in Freon 22 or onto a cold metal surface. The former is a much less satisfactory way to freeze biological tissue since upon quenching into liquid nitrogen at its boiling point a zone of gaseous nitrogen of poor thermal conductivity forms about the sample and inhibits heat transfer from the sample. This inhibition is quite probably the cause of the 'isothermal' results obtained with the microthermocouples and the freeze-substitution work described here.
Fig. 1. Typical cooling curve of hydrated tissue (in this case kidney) quenched in liquid nitrogen.

Fig. 2. The variation in cooling of nominally identical biopsies of carrot tissue quenched in liquid nitrogen.
Fig. 3. Plunger for freezing slices.

Fig. 4. Trigger circuit.

Fig. 5. Efflux of tritiated water from carrot slices.
The variation in cooling rates of (a) under-hydrated (b) normally hydrated and (c) over-hydrated slices of carrot tissue. Normally hydrated tissue would contain 638 cpm.

Cooling curves for (from left to right) assemblies of 200/200, 200/400, 200/600, 200/800.
Fig. 8. Cooling curves for (from left to right at the lower ends of the traces) assemblies of 200/1000, 600/600, 400/800.

Fig. 9. Cooling curves for (from left to right) assemblies of 200/200, 400/400, 600/600.
Fig. 10. Section through a control (unfrozen) rat cornea.

Fig. 11. Section through a rat cornea frozen in liquid nitrogen and freeze substituted.

Fig. 12. Section through a rat cornea frozen in liquid nitrogen whilst pressed against another rat cornea.

Fig. 13. Section through a rat cornea frozen in liquid nitrogen whilst pressed against two other rat corneas.
<table>
<thead>
<tr>
<th></th>
<th>Expected activity (dpm)</th>
<th>Measured activity (dpm)</th>
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</thead>
<tbody>
<tr>
<td><strong>Series A</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>310 ± 1</td>
<td>200 ± 13</td>
</tr>
<tr>
<td></td>
<td>310 ± 1</td>
<td>328 ± 13</td>
</tr>
<tr>
<td></td>
<td>310 ± 1</td>
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<td>310 ± 1</td>
<td>255 ± 13</td>
</tr>
<tr>
<td><strong>Series B</strong></td>
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<tr>
<td></td>
<td>329 ± 1</td>
<td>409 ± 10</td>
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<td></td>
<td>658 ± 2</td>
<td>853 ± 13</td>
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<td>1291 ± 15</td>
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<tr>
<td><strong>Series C</strong></td>
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<td></td>
<td>317 ± 1</td>
<td>398 ± 10</td>
</tr>
<tr>
<td></td>
<td>634 ± 2</td>
<td>730 ± 13</td>
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<tr>
<td></td>
<td>951 ± 3</td>
<td>1080 ± 95</td>
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Table 2
Maximum ice crystal cavity dimensions in doublet corneas

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Round ics (µm)</th>
<th>Elongated ics</th>
<th>Distribution</th>
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<tbody>
<tr>
<td>single</td>
<td>5</td>
<td>20</td>
<td>Complex</td>
</tr>
<tr>
<td>single</td>
<td>10</td>
<td>150</td>
<td>Uniform</td>
</tr>
<tr>
<td>single</td>
<td>15</td>
<td>65</td>
<td>Uniform</td>
</tr>
<tr>
<td>single</td>
<td>10</td>
<td>20</td>
<td>Uniform</td>
</tr>
<tr>
<td>double</td>
<td>All rounded of size 1µm</td>
<td></td>
<td>Large areas free of ics</td>
</tr>
<tr>
<td>triple</td>
<td>Rounded of size 5µm</td>
<td></td>
<td>Uniform</td>
</tr>
</tbody>
</table>

Table 3
Maximum ice crystal cavity dimensions (µm) in triplet corneas

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Round ics</th>
<th>Elongated ics</th>
<th>Distribution</th>
</tr>
</thead>
<tbody>
<tr>
<td>single</td>
<td>15</td>
<td>100</td>
<td>Complex</td>
</tr>
<tr>
<td>single</td>
<td>10</td>
<td>70</td>
<td>Uniform</td>
</tr>
<tr>
<td>double</td>
<td>10</td>
<td>60</td>
<td>Uniform</td>
</tr>
<tr>
<td>double</td>
<td>15</td>
<td>100</td>
<td>Uniform</td>
</tr>
<tr>
<td>triple</td>
<td>Rounded of size 5µm</td>
<td></td>
<td>Uniform</td>
</tr>
</tbody>
</table>
CHAPTER 4

SWELLING PRESSURE OF THE CORNEAL STROMA

INTRODUCTION

The technique of ultracryotomy as described in Chapter 2 has, at the moment, two practical limitations:

1) The ultrastructural damage caused by 'rapid' freezing.

2) The failure to produce consistently ultrathin frozen sections free of cutting artefacts.

In view of these limitations it was decided to continue with the investigations of corneal stroma using more conventional techniques.

It is widely held that the cornea swells because of the presence of fixed charge groups upon the acidic mucopolysaccharides or glycosaminoglycans within the corneal stroma (Loeven and van Walbeek, 1954; Hedbys, 1961; Hart and Farrell, 1971; Hodson, 1971; Davson, 1972). The charge groups involved are the carboxylic acid groups and sulphonic acid groups on chondroitin-4-sulphate and keratan sulphate (Goodfellow, 1975). This fixed charge concentration generates a potential - the Donnan potential - between the stroma and its bathing medium as well as producing an asymmetrical distribution of permeant ions - the Donnan distribution - between bathing medium and stroma. This Donnan distribution gives rise to an osmotic pressure difference which causes the stroma to hydrate and swell.
This osmotic swelling force is balanced \textit{in vivo} by an ion pump located in the single layer of endothelial cells on the posterior surface of the stroma (Hodson and Miller, 1976; Hull et al, 1977; Mayes and Hodson, 1979). Ions are transported from the stroma and into the aqueous chamber of the eye. Coupled to this ion flux is a flux of water which counteracts the innate tendency of the stroma to hydrate. The nature of this coupling has yet to be fully elucidated (Mayes and Hodson, 1978; Mayes, 1979).

The work in this chapter is concerned with the swelling pressure measurements of corneal stroma bathed in media of varying ionic strength. The data thus obtained was then fitted to a previously described model of corneal swelling (Hodson, 1971) to obtain an estimate of the fixed charge concentration within the stroma.

\textbf{METHODS}

Figure 1 shows an outline of the instrument which was designed for measuring corneal swelling pressure. It utilised a simple optical microscope.

Figure 2 is a block diagram which illustrates the principle of the instrument.

A cornea is constrained to swell against a beam mounted at each end on supports. The deflection of the beam is sensed by a strain gauge stuck beneath the beam which in turn suffers a change in resistance $\Delta R$. The strain gauge is arranged to be part of a Wheatstone bridge circuit, the balance of which is disturbed by the
change in strain gauge resistance. The potential difference from
the out of balance bridge network $\Delta V$ is fed to an instrumentation
amplifier the output voltage from which is recorded on an
electrometer and is a measure of the original force applied by
the cornea to the beam.

The beam B, (Figure 1), made of Perspex, sat across the slide
table of a modified optical microscope. It was located on the table
by three locating screws. About the centre of the beam was mounted
a nylon well W to contain the corneal bathing medium. At the centre
of the beam and on its lower surface was mounted a strain gauge G.
A corneal button C, whose swelling pressure in a bathing medium of a
certain ionic strength and at a certain hydration one wished to
measure, was placed centrally over the strain gauge on the upper
surface of the beam within the well.

The original microscope objective was modified by removing its
lens system and fitting in its place a stainless steel ram, R,
the ram being secured to the objective body O by a bolt. The
lower diameter of the ram was 3 mm and great care was taken to
ensure that the lower surface of the ram, that in contact with the
cornea, was perfectly flat. The cornea was thus positioned in a
parallel-sided (rather than a wedge-shaped) gap and the thickness
of the gap was an accurate measure of corneal thickness and
consequently its hydration (Hedbys and Mishima, 1966). The
corneal button was constrained by lowering the ram/objective
system upon it using the rack and pinion of the microscope.
An important criterion in the design of the instrument was that there should only be small displacements of the beam so that there would be a correspondingly small volume changes experienced by a given cornea which would then equilibrate relatively quickly. Obviously one had to balance minimum displacement against a workable output from the strain gauge/amplifier system.

The perspex beam was initially 81 mm long x 34 mm wide x 6 mm thick. This, however, proved too rigid and its thickness was halved to give a beam of 81 mm x 34 mm x 3 mm. The beam located on the microscope table via three screws, one 10 mm from one end and on the centre line of the beam and two at the other end again 10 mm from the end and 10 mm either side of the centre line. The table of the microscope was drilled at the appropriate points to provide locations for the screws and by suitable adjustment of these screws the beam could be positioned upon the table free from rotational motion.

The strain gauge was a Type No. 2A-1A-350P (Sasco Ltd., Crawley, Sussex) with a gauge factor of 108 the gauge factor GF being defined as:

\[
GF = \frac{\Delta R}{\Delta l} = 108
\]

where \( \Delta R \) is the change in resistance when the gauge is strained

\( R \) is the unstrained resistance of the gauge

\( \Delta l \) is the change in length of the gauge under strain
\[
R = 347 \, \Omega \\
1 = 10 \, \text{mm} \\
\Delta l = 1 \, \mu \text{m}
\]

then

\[
\frac{\Delta R}{R} = GF \times \frac{\Delta l}{l} = 108 \times 10^{-6} \times 10^{-2}
\]

\[
R = 1.08 \times 10^{-2} R = 1.08 \times 10^{-2} \times 347 = 3.75 \, \Omega
\]

Hence a deflection of the order of 1 \(\mu\text{m}\) of the beam would be expected to produce a change in resistance of the strain gauge of about 4 \(\Omega\).

The strain gauge was stuck on the underside of the beam using Araldite epoxy resin.

The bridge circuit is shown in Figure 3. \(R_3\) and \(R_4\) were precision wire wound resistors of 3.75 k\(\Omega\) with a tolerance of 0.02\%.

\(R_2\) was the unstrained tolerance of the strain gauge (347 \(\Omega\)) and \(R_1\) was a combination of two trimming potentiometers one of 500 \(\Omega\) and one of 10 \(\Omega\) for coarse and fine adjustment respectively of the bridge balance. The input voltage to the bridge was supplied from a voltage regulator.

The output from the bridge circuit \(\Delta V\) was fed to a purpose built instrumentation amplifier shown in Figure 4. \(A_1\) and \(A_2\) were two operational amplifiers (Type IM 308 AH - L.T.T. Comp. Ltd., Bristol). All the resistors were 100K \(\Omega\) and again were
precision wire wound with a tolerance of 0.02%. The capacitors were of a ceramic type ensuring low leakage current and high stability. \( R_a \) was an adjustable 10K \( \Omega \) potentiometer which allowed the gain of the amplifier to be varied.

The electronics associated with the beam/strain gauge was completed by a two-stage low pass filter shown in Figure 5 which had a cut-off frequency of 10Hz to block unwanted ripple from such sources as the mains.

Finally the output from the filter was measured on an electrometer (Model 33B-2 Vibron Electrometer - Electronic Instruments Ltd., Richmond, Surrey), having input ranges covering 0 - 10 mV to 0 - 1000 mV.

Corneas from rats were used for the swelling pressure experiments. The animals used were 3 month old female Wistar rats having a body weight between 175 and 190 grams. The animals were sacrificed by having their necks broken. Incisions were made in the upper and lower orbital portions of the eyelids and the eyeball removed from its orbit by severing the optic nerve and supportive musculature. The remainder of the eyelids plus the conjunctiva were then removed. The epithelium was removed using a rotating bristle brush and the cornea was then dissected out from the eyeball at the corneal/scleral junction. The endothelium was then removed using a piece of filter paper and a corneal button 2.6 mm in diameter was punched out from the whole cornea using a purpose made trephine. One corneal button was used immediately whilst
the other from the second eye was stored at 4°C in a moist chamber containing a saline swab for 24 hours.

The corneal button was placed within the well on the beam and the well filled with 5 ml of the initial bathing fluid (154 mM NaCl). The stainless steel ram was then lowered into the bathing medium until it was approximately 10 mm from the upper surface of the beam as judged by a mark on the side of the well. The bathing fluid was then topped up by 1 ml of corn oil to prevent evaporation of the bathing medium during the course of the experiment.

The measurement circuitry was switched on and allowed to stabilize for 30 minutes after which the electrometer was accurately zeroed.

The ram was then further lowered using the rack and pinion coarse and vernier controls respectively until it just displaced the beam as indicated by a deflection on the electrometer. The position of the vernier control was noted and the ram was then retracted 2 mm (20 turns of the vernier control) from this position.

The corneal button was manoeuvred into the 2 mm gap between the ram and the beam using two pairs of fine forceps. The ram was then lowered carefully upon the corneal button and the button further compressed by the ram down to its starting thickness of the experiment (150, 125, 100 or 75 μm). This latter operation was carried out using the vernier control of the rack and pinion, lowering 10 μm at a time and monitoring the
output from the strain gauge on the electrometer to ensure that the relatively high transient deflection of the beam had fallen back to a lower level before lowering the ram further. When the final thickness was reached the system was left to equilibrate as indicated by a stable output from the strain gauge displayed on the electrometer.

When the electrometer reading had stabilised, usually within 20 minutes of reaching the working thickness, the ram was swiftly released by winding back the vernier control of the rack and pinion. The difference in electrometer voltage readings between the initial deflected beam and the final undeflected beam was used as a measure of corneal swelling pressure. The ram was then re-positioned upon the cornea at the original thickness setting and the electrometer reading was once again allowed to stabilise. This usually took less than 5 minutes when the electrometer reading returned to within 5 mV of its original value. The ram release sequence was then repeated to give another set of readings from the beam. This process was further repeated to give a total of 5 readings for the beam deflection in the bathing medium being used.

After a set of five readings were obtained the bathing medium and cooking oil were withdrawn from the well and the well flushed three times with fresh medium of a different ionic strength. The well was then refilled with this fresh medium (5 ml) and topped with 1 ml of corn oil.

Swelling pressure measurements were made at 4 corneal thicknesses 150, 125, 100 and 75 μm. In total 8 experiments were performed using one pair of eyes at each thickness. Each corneal
button was bathed in NaCl of a range of ionic strengths beginning in 154 mM through 120, 85, 51, 17 mM NaCl and then distilled water. This series was then reversed returning from distilled water back through to 154 mM NaCl with five readings of swelling pressure taken at each step.

Before each experiment the beam was checked by deflecting it 10 μm at a time using the microscope rack and pinion. In total the central portion of the beam was deflected about 100 μm. This was to ensure that the beam and associated electronics were giving a linear output, that there had been no change in the system since the previous experiment, and also to enable the nominal thickness settings, i.e. 150, 125, 100 and 75 μm to be corrected to actual thickness.

At the end of each experiment the beam was calibrated using a dummy ram and a series of weights. The dummy ram had the same diameter as the corneal buttons (2.6 mm). This could be used to determine the swelling pressure of a corneal button knowing the deflection in millivolts the button produced. One thus had a series of swelling pressure measurements obtained from corneal buttons at a number of thicknesses and bathed in media of a range of ionic strengths.

A model of corneal swelling has been described based upon the Donnan potential associated with the acidic groups of the tissue mucopolysaccharides (Hodson, 1971). This model relates the swelling pressure of corneal stroma to the concentration of fixed charge within the stroma at a given stromal hydration and
in a given bathing medium. The swelling pressure data obtained as described above was used to calculate an experimental value for the fixed charge concentration via the relationship derived from this model. One thus had values for the fixed charge concentration at a number of different corneal hydrations. The next step was a form of normalisation to obtain a measure of the fixed charge concentration at physiological hydration. The mathematics of this situation is described in the next section of this chapter but for practical purposes one needs to know or measure a number of properties of the corneal stroma being used.

It is established (Hedbys and Mishima, 1962; Ytteborg and Dohlman, 1965; Hedbys and Mishima, 1966) that there exists for steer, human and rabbit cornea a linear relationship between thickness and hydration and preliminary experiments indicated that this was so for rat corneal stroma (Williams, unpublished). Knowing the constants in the thickness hydration relationship one can, for a given thickness determine the corresponding hydration. These constants can be calculated if one knows the dry thickness and the dry density of the stroma (Hedbys and Mishima, 1966). One needs then to know one further factor, the physiological thickness of the stroma, to be able to relate the fixed charge concentration at some hydration $H_x$ to that at physiological hydration $H_p$.

The dry thickness of the rat cornea was measured using a Surfometer (Type SF100 - G.V. Planer Ltd., Sunbury-on-Thames, England). This instrument is designed to provide the contour
display and centre line average roughness measurement of surfaces.
The equipment uses a displacement transducer to monitor the
movement of a diamond stylus which is made to traverse the surface
to be evaluated. The resultant movement is indicated on a built-in
meter and may be displayed on a chart recorder.

Whole rat corneas obtained as described above were de-epithelialised and de-endothelialised and then rinsed briefly in
physiological saline and the excess fluid removed by blotting the
corneas. Individual corneas were then laid on glass microscope slides
and using two sets of fine forceps were stretched upon the slide so
that the cornea was as wrinkle free as possible. They were then
dehydrated over silica gel in an oven at 30°C for one week. Ten
corneas were thus prepared and profiles were then obtained across
a central line of the dried cornea using the Surfometer. The
instrument was zeroed upon the glass slide and the diamond stylus
traversed across the cornea to produce a profile of the corneal
surface (Figure 6), which was displayed on an associated chart
recorder. The dry thickness was estimated from this profile.

The density of dry corneas was determined by a hydrostatic
weighing technique (Bauer, 1945). The weighings were carried out
using a Torsion balance with a capacity of 10 milligrams. The
draught cage and weighing pan normally fitted to the balance were
both removed. Dried corneas, those prepared for the previous
thickness determination, were pierced with small holes using a
heated needle and then hung via these holes upon a length of thin
wire which in turn was suspended from the weighing arm of the
balance. Four separate weighings were carried out, firstly the weight of the suspended wire alone, secondly the weight of the suspended wire plus dried corneas (usually two or three), thirdly the weight of the wire plus corneas with the corneas immersed in toluene, and finally the weight of the wire alone immersed in toluene. The weighing was carried out by positioning a small vial of toluene on a stand beneath the balance weighing arm. From these four weighings and knowing the density of toluene the density of dried cornea could be calculated. Each set of four weighings was performed ten times.

An estimate of the physiological thickness of the rat cornea was obtained using a corneal specular microscope. This instrument, first introduced for the high magnification observation of the corneal endothelium in vivo (Maurice, 1968), has been used extensively for the in vitro measurement of corneal thickness (Dikstein and Maurice, 1972; Hodson and Miller, 1976; Bowman and Green, 1976; Mayes and Hodson, 1978).

In essence the instrument consists of an objective lens the aperture of which is divided across its centre. Light in the form of an illuminated slit is shone down one side of the aperture and is reflected from the focal plane of the objective back up the other side of the aperture and can be imaged by a suitable eyepiece. Enhanced reflections can be obtained from the endothelial and epithelial surface of the cornea and the difference in objective positions between these enhanced reflections gives a measure of corneal thickness.
The specular microscope used for the measurement of rat corneal thickness described here was fitted with a mounting/perfusion system plus a water jacket for the in vitro thickness measurement of excised rabbit cornea (Dikstein and Maurice, 1972).

Whole rat eyeballs obtained as described above were seated in a hollowed perspex cylinder which in turn fitted into the aperture of the water jacket normally occupied by the mounting/perfusion system used for rabbit corneas. The eyeball was covered with silicone oil and was examined under a x 40 water immersion objective. Specular reflections were obtained from the endothelial and epithelial surfaces and then the epithelium was removed using a rotating bristle. The procedure was then repeated, focussing on the upper stromal surface and again on the endothelium. The two sets of measurements gave a measure of stromal and epithelial thickness and were normally performed within 10 minutes of the animal being sacrificed.

Although not truly physiological, these measurements enabled an approximation of the physiological thickness of rat cornea to be made.

THEORY

(i) Beam Deflection

The maximum deflection experienced by a beam supported at its ends when a load W is applied to its centre is given by
\[ y = \frac{Wl^3}{48EI} \]  \hspace{1cm} (1)

where \( E \) is the modulus of elasticity of the beam material

\( W \) is the load on the beam

\( l \) is the length of the beam

\( I \) is the moment of inertia of the cross section of the beam (Oberg and Jones, 1970)

For a beam of rectangular cross-section

\[ I = \frac{bd^3}{12} \]  \hspace{1cm} (2)

where \( I \) is the moment of inertia about the axis

\( b \) is the width of the beam

\( d \) is the thickness of the beam

Considering the published data on the swelling pressure of steer, rabbit and human corneal stroma (Hedbys and Dohlman, 1963; Ytteborg and Dohlman, 1965), a figure of 1000 mm Hg was used as the probable upper limit of pressure that would be applied to beam.

Now \( 1000 \text{ mm Hg} = 1.33 \times 10^5 \text{ N.m}^{-2} \)
With corneal buttons of 2.6 mm diameter the area over which this pressure would be applied is $5.31 \times 10^{-6} \text{ m}^2$.

" Maximum load at the centre of the beam is likely to be

$$1.33 \times 10^5 \times 5.31 \times 10^{-6} N$$

Considering a beam of dimensions 80 mm long x 30 mm wide x 6 mm thick, dimensions determined in part by designing the beam around an optical microscope system then

$$I = \frac{b \cdot d \cdot t^3}{12} = 30 \times 10^{-3} \times (6 \times 10^{-3})^3$$

$$I = 5.40 \times 10^{-10} \text{ m}^4$$

For steel (3% C)

$$E_s = 21 \times 10^{10} \text{ Nm} \quad (\text{Kaye and Laby, 1968})$$

Then from (1) the maximum deflection expected from a steel beam of the above dimensions is

$$y_{\text{max}} = \frac{7.06 \times 10^{-1} \times (80 \times 10^{-3})^3}{48 \times 21 \times 10^{10} \times 5.4 \times 10^{-10}}$$

$$y_{\text{max}} = 6.6 \times 10^{-8} \text{ m} = 66 \text{ nm}$$
For an aluminium beam with the above dimensions

\[ E_{Al} = 7.03 \times 10^{10} \text{ Nm}^{-2} \quad \text{(Kaye and Laby, 1966)} \]

and the maximum deflection to be expected is

\[ y_{\text{max}} = 1.98 \times 10^{-7} \text{ m} = 198 \text{ nm} \]

For a 'Perspex' beam

\[ E_p = 2.94 \times 10^9 \text{ Nm} \quad \text{(ICI Plastics, 1974)} \]

and the maximum deflection to be expected from a 'Perspex' beam of the above dimensions is

\[ y_{\text{max}} = 4.74 \times 10^{-6} = 4.74 \mu\text{m} \]

For a 'Perspex' beam with the same length and width as that above but 3 mm thick then

\[ I = \frac{bd^3}{12} = \frac{30 \times 10^{-3} \times (3 \times 10^{-3})^3}{12} \]

\[ I = 6.75 \times 10^{-11} \text{ m}^4 \]

and the maximum deflection expected is

\[ y_{\text{max}} = 37.95 \mu\text{m} \]
As indicated earlier the essence of the beam design was one of minimum displacement, the experimental cornea would then undergo only small volume changes which would allow it to equilibrate quickly. With this in mind it was decided initially to use a beam made of 'Perspex' having a thickness of 6 mm which in theory would give a maximum deflection under extremely dehydrated conditions of a few microns. It was reasoned that the deflections to be expected from steel or aluminium beams of 6 mm thick would be insufficient to provide a workable output from the strain gauge.

(ii) Fixed Charge Concentration

Considering isolated stroma bathed in physiological saline then the osmotic pressure difference between the stroma and the external bathing medium can be determined using van't Hoff's equation:

\[
\Delta \Pi = R T \Delta C \hspace{1cm} (3)
\]

where \( \Delta \Pi \) is the osmotic pressure difference
\( R \) is the Gas Constant
\( T \) is the Absolute Temperature
\( \Delta C \) is the concentration difference between the two phases

which in this case becomes

\[
\Delta \Pi = R T \left( [\text{Na}]_i + [\text{Cl}]_i - [\text{Na}]_o - [\text{Cl}]_o \right) \times 10^{-3} \hspace{1cm} (4)
\]
where square brackets indicate concentrations in mequiv. \( ^{-1} \) of fluid, suffix \( i \) refers to inside the stroma, suffix \( o \) means in the bathing medium and all solutions are considered to act ideally, i.e. all activity coefficients are unity.

For electrical neutrality inside and outside the stroma,

\[
[Na]_o = [Cl]_o \quad \text{.................................. (5)}
\]

\[
[Na]_i = [Cl]_i + F \quad \text{.................................. (6)}
\]

where \( F \) is the concentration of charge within the stroma. This fixed charge concentration produces a Gibbs-Donnan equilibrium of the permeant sodium and chloride ions which are distributed according to the Gibbs-Donnan ratio, i.e.

\[
[Na]_i \cdot [Cl]_i = [Na]_o \cdot [Cl]_o \quad \text{.................................. (7)}
\]

To obtain an experimental value for \( F \) one has to express \( \Delta \Pi \) in terms of readily measurable variables, e.g. \([Na]_o\).

Thus from (6)

\[
[Cl]_i = [Na]_i - F \quad \text{.................................. (8)}
\]

and substituting for \([Na]_i\) from (7)

\[
[Cl]_i = \frac{[Na]_o \cdot [Cl]_o}{[Cl]_i} - F \quad \text{.................................. (9)}
\]
which becomes

$$[Cl]_i^2 + 2 [Cl]_i F = [Na]_o [Cl]_o$$  \hspace{1cm} (10)$$

substitution for $[Cl]_o$ from (5) and (10) becomes

$$[Cl]_i^2 + F [Cl]_i - [Na]_o^2 = 0$$

from which

$$[Cl]_i = \frac{-F \pm \sqrt{F^2 + 4 [Na]_o^2}}{2}$$

as only positive solutions exist for $[Cl]_i$ then

$$[Cl]_i = \frac{-F + \sqrt{F^2 + 4 [Na]_o^2}}{2}$$  \hspace{1cm} (11)$$

substituting (5) and (6) into (4) gives

$$\Delta \Pi = RT \left(2 [Cl]_i + F - 2 [Na]_o\right) \times 10^{-3}$$

and substituting for $[Cl]_i$ from (11) gives

$$\Delta \Pi = RT \left\{\left(F^2 + 4 [Na]_o^2\right)^{\frac{1}{2}} - 2 [Na]_o\right\} \times 10^{-3} \ldots \ (12)$$

which enables $F$ to be determined from experimental values of the swelling pressure, knowing $[Na]_o$.
Now $F$ is the charge concentration at some hydration $H$, $H$ being defined here as the ratio of the weight of water to the dry weight of the cornea, i.e.

$$H = \frac{\text{Wet weight of cornea} - \text{Dry weight of the cornea}}{\text{Dry weight of the cornea}}$$

and $F$ and $H$ are inversely related if the number of negative changes in a corneal stroma is not dependent upon the hydration. i.e. $F \cdot H = \text{Constant}$

or

$$F \cdot H = F_p \cdot H_p \tag{13}$$

where $H_p$ is physiological hydration and $F_p$ is the charge concentration at physiological hydration. Hence having obtained an experimental measure of $F$ using (12) a value for $F$ can be determined if one knows $H$ and $H_p$.

(iii) **Thickness - Hydration Relationship**

It has been shown for steer, human and rabbit cornea that there exists a linear relationship between thickness and hydration (Hedbys and Mishima, 1962; Ytteborg and Dohlman, 1965; Hedbys and Mishima, 1966), which is of the form

$$H = A \cdot q - b$$
where \( H \) is the corneal hydration as defined above
\( q \) is the corneal thickness in millimeters
\( A \) and \( b \) are constants for a given body weight range
within a given species.

With certain assumptions these constants can be determined experimentally. These assumptions are:

1) the cornea swells only across its thickness, with no shrinkage or expansion along its plane during the change in hydration.

2) that the volume of the dry cornea and of the water in the hydrated state are simply additive. Then,

\[
V = q \cdot S \quad \text{................................................. (14)}
\]

where \( V \) is the volume of the cornea
\( q \) is the thickness of the cornea
\( S \) is the surface area of the dry cornea

and also,

\[
V = V_w + V_d \quad \text{................................................. (15)}
\]

Where \( V_w \) is the volume of water in the cornea
\( V_d \) is the volume of the dry cornea

Now by definition,

\[
H = \frac{W_w}{W_d} \quad \text{................................................. (16)}
\]
where $W_w$ is the weight of water in the hydrated cornea

$W_d$ is the weight of the dry cornea

With the density of water $\rho_w$ equal to unity and the density of the dry cornea $\rho_d$, (16) becomes,

$$H = \frac{\rho_w V_w}{\rho_d V_d} = \frac{V_w}{\rho_d V_d} \quad \text{......................... (17)}$$

Equating $V_w$ from (15) and (17) we have,

$$V - V_d = H \cdot (\rho_d V_d)$$

or

$$H = \frac{V}{\rho_d V_d} - \frac{V_d}{\rho_d V_d} = \frac{V}{\rho_d} - \frac{1}{\rho_d}$$

Substituting for $V$ from (14) gives,

$$H = \frac{q_d S}{\rho_d V_d} - \frac{1}{\rho_d} \quad \text{......................... (18)}$$

Now

$$V_d = S \cdot q_d$$

where $q_d$ is the thickness of the dry cornea and on substituting (18) becomes,

$$H = \frac{q_d S}{\rho_d S \cdot q_d} - \frac{1}{\rho_d}$$
then \[ H = \frac{q}{\rho _d \cdot q_d} - \frac{1}{\rho _d} \]  \hfill (19)

which is of the form

\[ H = A \cdot q - b \]  \hfill (20)

where \( A = \frac{1}{\rho _d \cdot q_d} \) and \( b = \frac{1}{\rho _d} \)

(iv) Density via Hydrostatic Weighing

Four weighings were carried out to determine the density of dry corneas. These were:

\[ W_1 = \text{Weight of wire in air} \]
\[ W_2 = \text{Weight of wire + corneas in air} \]
\[ W_3 = \text{Weight of wire + corneas in toluene} \]
\[ W_4 = \text{Weight of wire in toluene} \]

Then \( W_0 = W_2 - W_1 = \text{Weight of corneas} \)

and \( W_L = W_3 - W_4 = \text{Weight of corneas in toluene} \)

and the apparent loss of weight \( W_0 - W_L \) is equal to the mass of displaced toluene. The volume of displaced toluene is then,

\[ V_T = \frac{W_0 - W_L}{\rho _T} \]
where $P_T$ is the density of toluene. This volume is equal to the volume of the corneas hence the density of the dry corneas $P_c$ is given by,

\[ P_c = \frac{W_o}{W_o - W_L} P_T \]

RESULTS

(i) Voltage Outputs

Table 1 shows the voltages $V$ produced by corneas at the four experimental thicknesses (150, 125, 100 and 75 μm) through the range of ionic strengths of the saline bathing medium. This medium was successively diluted from 154 mM (0.9%) saline down to distilled water and then the series was reversed back to 154 mM saline giving two sets of readings at each ionic strength. The time taken to achieve steady state in these experiments was usually less than twenty minutes.

(ii) Deflection Calibration

Table 2 gives a typical beam deflection calibration showing the electrometer reading in millivolts produced by deflecting the beam 10 μm at a time with the ram objective system driven by the microscope rack and pinion. The calibration was performed
before each experiment and was effectively unchanged throughout all the experiments so that Table 2 was used to apply thickness corrections to each cornea.

(iii) **Weight Calibration**

Table 3 shows a weight calibration for the beam using the dummy ram (cross sectional area 5.31 mm²) loaded with weights. Again this calibration was performed in conjunction with each experiment and remained unchanged throughout the experiments.

(iv) **Pressure and Voltage**

Figure 7, derived from Table 3, gives the pressure in mmHg and the corresponding output in millivolts shown on the electrometer. The best straight line fit to these parameters is given by,

\[ P = 10.5D - 1.8 \]

(21)

where \( P \) is the pressure in mmHg and \( D \) is the electrometer reading in millivolts.

Using this equation and Table 1, Table 4 has been constructed which gives the swelling pressure measurements of rat corneal stroma under the various conditions tested.
(v) Charge Concentration, $F_x$

Using equation (12), i.e.

$$
\Delta \Pi = R \cdot T \cdot \left\{ (F^2 + 4 [Na]_o^2)^{1/2} - 2 [Na]_o \times 10^{-3} \right\} \ldots (12)
$$

where $R = 62.4 \text{ mmHg}.1.\text{K}^{-1}.\text{mol}^{-1}$

$$
T = 293 \text{ K}
$$

plus the values of $\Delta \Pi$ and saline concentration $[\text{Na}]_o$ from Table 4, the fixed charge concentration $F$ at a thickness $q$ (150, 125, 100 or 75 $\mu$m) can be calculated. These values are shown in Table 5.

(vi) Dry Thickness

A typical trace from the Surfometer is shown in Figure 6. The estimated dry corneal thickness $q_d$ from ten such traces was averaged at 30 $\mu$m and used to determine $A$ in equation (19).

(vii) Dry Density

The results of the hydrostatic weighings are shown in Table 8 from which a mean value for $\rho_d$, the dry density of the corneas used was obtained,

$$
\hat{\rho}_d = 1.39 \pm 0.02 \text{ g.cm}^{-3}
$$
Hence using,

\[ A = \frac{1}{\rho_d q_d} = \frac{1}{1.39 \times 0.03} \quad \text{and} \quad b = \frac{1}{1.39} \]

then \( A = 23.98 \) and \( b = 0.72 \)

and thus for the rat corneas used,

\[ H = 24q - 0.72 \quad \text{................................. (20a)} \]

(viii) **Thickness Correction**

Table 6 shows the results of applying thickness corrections to the voltage readings in Table 1. It is seen from Table 2 that deflecting the beam 1 \( \mu m \) gives a corresponding output from the strain gauge electronics of approximately 5 mV. Using this figure and the values of \( V \) in Table 1 the preset thickness was corrected to an actual thickness and these values are given in Table 6.

(ix) **Charge Concentration and Hydration**

By substituting the corrected thicknesses from Table 6 into equation (20a) Table 7 has been constructed showing the calculated charge concentration \( F_x \) at a hydration \( H_x \) at each ionic strength for each cornea.
(x) **Physiological Thickness and Hydration**

The specular microscope results were averaged \((n = 8)\) and from these it is estimated that the physiological thickness of the rat corneas used was \(-165\ \mu m\). Substituting this value into equation (20a),

\[
H_p = 24 (0.165) - 0.72 \quad \text{................................. (20b)}
\]

giving a value of \(H_p = 3.24\).

(xi) **Charge Concentration at Physiological Hydration**

Using equation (13), i.e.

\[
F_{xx} = F_{xp} \quad \text{........................................ (13)}
\]

and the values of \(H_x\) and \(F_x\) from Table 7 plus the estimate of physiological hydration obtained above, a value for the charge concentration at physiological hydration \(F_p\) can be calculated for each cornea in the different bathing media. These results are shown in Table 9.
It is seen from these results that the calculated charge concentration at physiological hydration is a function of the ionic strength of the bathing medium. Its value is seen to increase with increasing ionic strength and spans a range from approximately 20 m.equiv.\textsuperscript{-1} to 35 m.equiv.\textsuperscript{-1}. 
Fig. 1. A diagram of the instrument used to measure corneal swelling pressure. B is the beam whose elastic deformation is detected by the change in the resistance of the strain gauge, G. The cornea, C, is immersed in saline contained by well W and is constrained at its top surface by a ram, R, which is bolted to the objective, O, of an optical microscope. For clarity of illustration, the diagram is exploded in the region of the cornea.
Fig. 2. A block diagram of the swelling pressure detector

Beam Deflect → Strain Gauge → ΔR → Bridge Circuit → ΔV → Pre-Amp → V → Electrometer

Fig. 3. The bridge circuit.

Fig. 4. The instrumental amplifier.

Fig. 5. The filter circuit.
Fig. 6. A typical profile of dried rat corneal stroma when measured with the surfometer.
Table 1. Mean potentials (mV) recorded from the swelling pressure instrument (± Standard Error, n = 10) for rat corneal stroma held at various nominal thicknesses in various strength saline solutions.

<table>
<thead>
<tr>
<th>NaCl solution (% w/v)</th>
<th>0.9</th>
<th>0.7</th>
<th>0.5</th>
<th>0.3</th>
<th>0.1</th>
<th>0.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal corneal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>thickness (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>4.2 ± 0.4</td>
<td>4.9 ± 0.5</td>
<td>5.5 ± 0.4</td>
<td>6.5 ± 0.5</td>
<td>10.2 ± 0.5</td>
<td>40.6 ± 0.6</td>
</tr>
<tr>
<td>125</td>
<td>7.1 ± 0.4</td>
<td>7.3 ± 0.7</td>
<td>6.6 ± 1.1</td>
<td>12.5 ± 0.5</td>
<td>21.7 ± 1.3</td>
<td>58.1 ± 1.1</td>
</tr>
<tr>
<td>100</td>
<td>9.8 ± 0.8</td>
<td>13.2 ± 0.6</td>
<td>9.3 ± 0.6</td>
<td>13.3 ± 0.9</td>
<td>22.8 ± 1.1</td>
<td>60.1 ± 1.1</td>
</tr>
<tr>
<td>75</td>
<td>20.7 ± 1.3</td>
<td>21.2 ± 2.5</td>
<td>23.3 ± 0.9</td>
<td>41.0 ± 2.0</td>
<td>54.2 ± 2.0</td>
<td>88.1 ± 1.7</td>
</tr>
</tbody>
</table>
Table 2.
Output of the swelling pressure detector as the beam is vertically displaced

<table>
<thead>
<tr>
<th>Displacement (µm)</th>
<th>Output (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>50</td>
</tr>
<tr>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>30</td>
<td>147</td>
</tr>
<tr>
<td>40</td>
<td>207</td>
</tr>
<tr>
<td>50</td>
<td>278</td>
</tr>
<tr>
<td>60</td>
<td>353</td>
</tr>
<tr>
<td>70</td>
<td>412</td>
</tr>
<tr>
<td>80</td>
<td>472</td>
</tr>
<tr>
<td>90</td>
<td>522</td>
</tr>
<tr>
<td>100</td>
<td>592</td>
</tr>
<tr>
<td>110</td>
<td>640</td>
</tr>
<tr>
<td>120</td>
<td>695</td>
</tr>
<tr>
<td>130</td>
<td>750</td>
</tr>
<tr>
<td>140</td>
<td>815</td>
</tr>
<tr>
<td>150</td>
<td>870</td>
</tr>
<tr>
<td>160</td>
<td>910</td>
</tr>
<tr>
<td>170</td>
<td>960</td>
</tr>
</tbody>
</table>

Table 3.
A typical calibration of the output of the detector when loaded with weights

<table>
<thead>
<tr>
<th>Weight (gram)</th>
<th>Output (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1 ± 0.2</td>
</tr>
<tr>
<td>2</td>
<td>3 ± 1</td>
</tr>
<tr>
<td>3</td>
<td>4 ± 1</td>
</tr>
<tr>
<td>5</td>
<td>6 ± 1</td>
</tr>
<tr>
<td>10</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>20</td>
<td>28 ± 3</td>
</tr>
<tr>
<td>50</td>
<td>66 ± 2</td>
</tr>
<tr>
<td>100</td>
<td>131 ± 2</td>
</tr>
</tbody>
</table>

Fig. 7. Electrometer displacement as a function of pressure on the beam.
Table 4. Calculated rat corneal stromal swelling pressures* (± Standard Error, n = 10) held at various nominal thicknesses in various strength saline solutions

<table>
<thead>
<tr>
<th>NaCl solution (% w/v)</th>
<th>0.9</th>
<th>0.7</th>
<th>0.5</th>
<th>0.3</th>
<th>0.1</th>
<th>0.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal corneal thickness (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>42.3 ± 2.4</td>
<td>49.7 ± 3.4</td>
<td>56.0 ± 2.4</td>
<td>66.5 ± 3.4</td>
<td>105 ± 5</td>
<td>424 ± 5</td>
</tr>
<tr>
<td>125</td>
<td>72.8 ± 2.4</td>
<td>74.9 ± 5.5</td>
<td>67.5 ± 9.7</td>
<td>129 ± 3.4</td>
<td>226 ± 12</td>
<td>608 ± 9.7</td>
</tr>
<tr>
<td>100</td>
<td>101 ± 6.6</td>
<td>137 ± 5.5</td>
<td>98.6 ± 5.5</td>
<td>139 ± 7.6</td>
<td>238 ± 10</td>
<td>629 ± 10</td>
</tr>
<tr>
<td>75</td>
<td>216 ± 12</td>
<td>221 ± 24</td>
<td>243 ± 10</td>
<td>429 ± 19</td>
<td>567 ± 20</td>
<td>923 ± 16</td>
</tr>
</tbody>
</table>

* Units: mm Hg
Table 5. Stromal fixed negative charge (Milliequivalents / litre stromal fluid) calculated from the Donnan theory of corneal swelling.

<table>
<thead>
<tr>
<th>NaCl solution (mM)</th>
<th>154</th>
<th>120</th>
<th>85</th>
<th>51</th>
<th>17</th>
<th>—</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal corneal thickness (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>41.4 ± 9.1</td>
<td>36.5 ± 5.8</td>
<td>31.9 ± 5.3</td>
<td>28.5 ± 3.6</td>
<td>21.2 ± 2.8</td>
<td>23.0 ± 0.3</td>
</tr>
<tr>
<td>125</td>
<td>54.0 ± 7.3</td>
<td>44.4 ± 6.9</td>
<td>37.7 ± 6.8</td>
<td>38.2 ± 5.0</td>
<td>30.3 ± 4.0</td>
<td>34.8 ± 0.8</td>
</tr>
<tr>
<td>100</td>
<td>53.6 ± 8.6</td>
<td>61.2 ± 8.0</td>
<td>41.5 ± 7.1</td>
<td>39.7 ± 5.0</td>
<td>32.1 ± 3.9</td>
<td>34.5 ± 1.0</td>
</tr>
<tr>
<td>75</td>
<td>102 ± 10</td>
<td>80.3 ± 8.0</td>
<td>68.0 ± 7.2</td>
<td>70.1 ± 8.2</td>
<td>56.3 ± 5.8</td>
<td>49.7 ± 0.7</td>
</tr>
</tbody>
</table>
Table 6. The correction of nominal corneal thickness to actual corneal thickness (μm).

<table>
<thead>
<tr>
<th>NaCl solution (mM)</th>
<th>154</th>
<th>120</th>
<th>85</th>
<th>51</th>
<th>17</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal corneal thickness</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>151</td>
<td>151</td>
<td>151</td>
<td>151</td>
<td>152</td>
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<tr>
<td>125</td>
<td>126</td>
<td>126</td>
<td>126</td>
<td>127</td>
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<tr>
<td>100</td>
<td>101</td>
<td>102</td>
<td>101</td>
<td>102</td>
<td>104</td>
</tr>
<tr>
<td>75</td>
<td>80</td>
<td>79</td>
<td>79</td>
<td>81</td>
<td>84</td>
</tr>
</tbody>
</table>
Table 7 The calculated fixed negative charge ( m.eq / l ) of rat corneal stroma at various hydrations in different strength saline solutions

<table>
<thead>
<tr>
<th>Salt solution ( mM )</th>
<th>154</th>
<th>120</th>
<th>85</th>
<th>51</th>
<th>17</th>
<th>—</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.90</td>
<td>$41.4 \pm 9.1$</td>
<td>$36.5 \pm 5.8$</td>
<td>$31.9 \pm 5.3$</td>
<td>$28.5 \pm 3.6$</td>
<td>$21.2 \pm 2.8$</td>
<td>—</td>
</tr>
<tr>
<td>2.93</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$23.0 \pm 0.3$</td>
</tr>
<tr>
<td>3.05</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2.30</td>
<td>$54.0 \pm 7.3$</td>
<td>$44.4 \pm 6.9$</td>
<td>$37.7 \pm 6.8$</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>2.33</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$38.2 \pm 5.0$</td>
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<tr>
<td>2.35</td>
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<td>$30.3 \pm 4.0$</td>
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<tr>
<td>2.52</td>
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<td>$34.8 \pm 0.8$</td>
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<tr>
<td>1.70</td>
<td>$53.6 \pm 8.6$</td>
<td>—</td>
<td>$41.5 \pm 7.1$</td>
<td>—</td>
<td>—</td>
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<tr>
<td>1.73</td>
<td>—</td>
<td>$61.2 \pm 8.0$</td>
<td>—</td>
<td>$39.7 \pm 5.0$</td>
<td>—</td>
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</tr>
<tr>
<td>1.78</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$32.1 \pm 3.9$</td>
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<tr>
<td>1.92</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$34.5 \pm 1.0$</td>
</tr>
<tr>
<td>1.18</td>
<td>—</td>
<td>$102 \pm 10$</td>
<td>$68.9 \pm 7.2$</td>
<td>—</td>
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<tr>
<td>1.20</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
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<tr>
<td>1.22</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$70.1 \pm 8.2$</td>
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<tr>
<td>1.30</td>
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<td>—</td>
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<td>$56.3 \pm 5.8$</td>
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<tr>
<td>1.42</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>$49.7 \pm 0.7$</td>
</tr>
</tbody>
</table>
Table 8. The determination of the density of dehydrated rat corneal stroma. All weights in mg.

<table>
<thead>
<tr>
<th>Weight of wire $W_1$</th>
<th>Weight of wire and cornea $W_2$</th>
<th>Their weight in toluene $W_3$</th>
<th>Weight of wire in toluene $W_4$</th>
<th>Density of dry cornea</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.06</td>
<td>6.35 (2)*</td>
<td>6.55</td>
<td>5.65</td>
<td>1.42</td>
</tr>
<tr>
<td>4.03</td>
<td>7.22 (3)</td>
<td>6.83</td>
<td>5.64</td>
<td>1.38</td>
</tr>
<tr>
<td>4.04</td>
<td>7.13 (3)</td>
<td>6.88</td>
<td>5.64</td>
<td>1.41</td>
</tr>
<tr>
<td>3.98</td>
<td>7.17 (3)</td>
<td>6.80</td>
<td>5.75</td>
<td>1.29</td>
</tr>
<tr>
<td>4.58</td>
<td>7.57 (3)</td>
<td>7.26</td>
<td>6.15</td>
<td>1.38</td>
</tr>
<tr>
<td>4.59</td>
<td>7.62 (3)</td>
<td>7.28</td>
<td>6.14</td>
<td>1.39</td>
</tr>
<tr>
<td>4.60</td>
<td>7.66 (3)</td>
<td>7.30</td>
<td>6.10</td>
<td>1.42</td>
</tr>
<tr>
<td>4.55</td>
<td>6.76 (2)</td>
<td>6.92</td>
<td>6.03</td>
<td>1.45</td>
</tr>
<tr>
<td>4.57</td>
<td>7.72 (3)</td>
<td>7.20</td>
<td>6.15</td>
<td>1.30</td>
</tr>
<tr>
<td>4.59</td>
<td>7.49 (3)</td>
<td>7.28</td>
<td>6.05</td>
<td>1.50</td>
</tr>
</tbody>
</table>

*Number in parentheses indicates number of rat corneas pooled for each determination of density.

Mean dry stromal density is estimated at $1.39 \pm 0.1 \text{ g/ml}$. 

Table 9 The fixed negative charge (m.eq / l) of rat corneal stroma corrected to physiological hydration (3.24)

<table>
<thead>
<tr>
<th>Saline solution (mM)</th>
<th>154</th>
<th>120</th>
<th>85</th>
<th>51</th>
<th>17</th>
<th>0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nominal corneal thickness when swelling pressure was recorded (μm)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>150</td>
<td>37.0 ± 8.1</td>
<td>32.7 ± 5.2</td>
<td>28.5 ± 5.0</td>
<td>25.5 ± 3.2</td>
<td>19.2 ± 2.6</td>
<td>21.6 ± 0.3</td>
</tr>
<tr>
<td>125</td>
<td>38.3 ± 5.2</td>
<td>31.5 ± 4.9</td>
<td>26.7 ± 4.8</td>
<td>27.4 ± 2.9</td>
<td>22.0 ± 2.8</td>
<td>27.1 ± 0.7</td>
</tr>
<tr>
<td>100</td>
<td>28.1 ± 4.5</td>
<td>32.6 ± 4.7</td>
<td>21.7 ± 3.7</td>
<td>21.1 ± 2.2</td>
<td>17.6 ± 2.1</td>
<td>20.4 ± 0.8</td>
</tr>
<tr>
<td>75</td>
<td>37.7 ± 3.7</td>
<td>29.2 ± 2.9</td>
<td>25.0 ± 2.6</td>
<td>26.4 ± 2.7</td>
<td>22.5 ± 2.2</td>
<td>21.8 ± 0.4</td>
</tr>
<tr>
<td>Average</td>
<td>35.2 ± 2.4</td>
<td>31.5 ± 0.8</td>
<td>25.5 ± 1.4</td>
<td>25.1 ± 1.3</td>
<td>20.3 ± 1.7</td>
<td>22.7 ± 1.5</td>
</tr>
</tbody>
</table>
CHAPTER 5

THE ESTIMATION OF STROMAL FIXED NEGATIVE CHARGE USING RADIO-ISOTOPES

INTRODUCTION

Having obtained a measure of the charge concentration within the rat corneal stroma using the swelling pressure apparatus described in the last chapter it was considered important to have an alternative method of estimating this charge concentration. Comparisons could then be made between both the magnitude of the values in the two cases and also the variation, if this applied in the second case, of the charge concentration with changing ionic strength.

The method chosen to obtain these comparisons was a modification of a technique first described by Maroudas and Thomas, 1970, for measuring the fixed charge concentration in connective tissues. Essentially the technique consists of measuring the concentration of permeant cations and anions within corneal stroma by loading the stroma with a radiolabelled cation and anion. As already described the presence of the negative charge groups within the stroma produces an asymmetrical distribution of permeant ions and the difference in stromal cation and anion concentrations should provide a measure of the negative charge concentration within the stroma.
METHODS

All the experimental corneas were bathed in solutions of sodium bicarbonate containing $^{22}\text{Na}$ and $^{14}\text{C}$ as radioactive tracers. Ideally solutions of sodium chloride with $^{22}\text{Na}$ and $^{36}\text{Cl}$ as tracers would have been used so as to afford a direct comparison with the results obtained in the last chapter. However because of the problems associated with the simultaneous counting of two tracers such as $^{22}\text{Na}$ and $^{36}\text{Cl}$ whose beta-particle spectra have a considerable overlap (maximum beta-particle energies of 0.54 and 0.71 Mev respectively) this study was done with $^{14}\text{C}$ and $^{22}\text{Na}$ (maximum beta-particle energies of 0.156 and 0.54 Mev respectively) using solutions of sodium bicarbonate. Then the scintillation counter used to measure the activities of $^{14}\text{C}$ and $^{22}\text{Na}$ in the solutions could be set up with one of its channels measuring purely the $^{22}\text{Na}$ activity, the sodium channel, whilst a second channel could be set up which measured a mixture of $^{14}\text{C}$ and $^{22}\text{Na}$ activity this being nominally the $^{14}\text{C}$ or bicarbonate channel. Then when measuring solutions containing both $^{22}\text{Na}$ and $^{14}\text{C}$ the overspill of $^{22}\text{Na}$ activity into the $^{14}\text{C}$ channel could be corrected for and a true measure of the $^{14}\text{C}$ activity and hence the bicarbonate ion concentration could be achieved.

As with the work described in Chapter 3, the scintillation counting was done on Packard Model 3380 Tri-carb scintillation spectrometer. The red channel of this spectrometer was used as the $^{14}\text{C}$ channel with the discriminators A and B set at 20 and
400 respectively and with a gain of 10%. The green channel was used as the $^{22}\text{Na}$ channel with its discriminators C and D set at 600 and 1000 respectively and with a gain of 10% also. Under these conditions the major part of the $^{14}\text{C}$ activity fell within the 20 - 400 window set by the discriminators on the red channel with no detectable $^{14}\text{C}$ in the 600 - 1000 window on the green channel. With these settings the red and green channels contained almost equal proportions of $^{22}\text{Na}$ activity. Before the double labelling technique could be applied to the determination of the charge concentration within the corneal stroma it had to be shown that both the sodium and the bicarbonate ions were freely exchangeable between the stroma and its bathing medium. To this end efflux experiments were carried out with corneas loaded with radiolabelled sodium and bicarbonate ions. The corneas were unloaded into 'cold' sodium bicarbonate and the time course of the efflux of the labelled material from the loaded corneas was used to indicate whether the sodium and bicarbonate ions were in fact freely exchangeable or whether there was binding or compartmentalisation of either or both ions.

Three efflux experiments were performed with loading times of 2, 5 and 20 minutes using one rat cornea for each experiment. The corneas were used as previously described and subsequently dehydrated over silica gel in an oven at 30°C for 24 hours. The loading solutions were volumes of 100 mM sodium bicarbonate in small glass vials to which were added $^{22}\text{Na}$ in the form of
sodium chloride having a specific activity of approximately 0.2 mCi.ml\(^{-1}\) and \(^{14}\)C in the form of sodium bicarbonate having a specific activity of approximately 0.7 mCi.ml\(^{-1}\).

Using a Hamilton syringe, 5 \(\mu\)l of \(^{14}\)C sodium bicarbonate was added to 1 ml of 'cold' sodium bicarbonate to make up one part of the loading solution. Similarly 50 \(\mu\)l of \(^{22}\)Na sodium chloride were added to 1 ml of 'cold' sodium bicarbonate to make up the second part of the loading solution. Both these solutions were then sampled by removing 3 x 10 \(\mu\)l from each and placing each 10 \(\mu\)l in a scintillation vial to which was added 990 \(\mu\)l of 'cold' sodium bicarbonate and an additional 9 ml of scintillation fluid as detailed in Chapter 3. The six scintillation vials were then shaken and placed in the scintillation spectrometer to determine the activity of the loading solutions and the amount of 'crossover' of \(^{22}\)Na activity into the bicarbonate channel.

The two single labelled loading solutions were then pooled to form the double labelled solution and 3 x 10 \(\mu\)l samples were taken from this as described above. Each dehydrated cornea was then placed in a glass vial containing approximately 2 ml of the loading solution and shaken on a flask shaker for 2, 5 and 20 minutes respectively. At the end of the loading time the cornea was removed from the loading solution, carefully, blotted to remove excess fluid, and then unloaded into a series of 'cold' 100 mM sodium bicarbonate solutions of 10 ml volume. The cornea was unloaded for 5s intervals up to 30s, then for 10s intervals up to 1 minute, then
for a further minute up to 2 minutes, a further 8 minutes up to
10 minutes and finally up to a total of 20 minutes in the 'cold'
bicarbonate solutions. A 1 ml sample was then taken from each
of the twelve unloading solutions and the activity in each
determined. With suitable blanks to measure the background 'noise'
from the spectrometer and applying corrections to the bicarbonate
channel figures for the 'cross-over' of $^{22}\text{Na}$ into the bicarbonate
channel the activity recovered in each of the unloading solutions
could be determined and a time course for the efflux of both
labels from the corneas could be constructed.

Using doubly radiolabelled sodium bicarbonate solutions a
series of experiments was then performed to measure the negative
charge concentration at different ionic strengths of the bathing
medium and at different hydrations of the cornea.

Labelled solutions of 20, 50, 100 and 150 mM sodium bicarbonate
were used, with three corneas loaded with the label at each ionic
strength.

The loading solutions were prepared as already described for
the efflux experiments with a suitable adjustment of the quantities
of both 'cold' and 'hot' portions of the mix. The first part of
the loading solution contained 3 ml of sodium bicarbonate to which
was added 40 $\mu$l of $^{14}\text{C}$ in the form of sodium bicarbonate solution
having a specific activity of approximately 0.7 mCi.ml$^{-1}$. The
second part of the loading solution had 3 ml of sodium bicarbonate
to which was added 60 $\mu$l of $^{22}\text{Na}$ in the form of sodium chloride with
a specific activity of approximately 0.2 mCi.ml\(^{-1}\). As before these components of the loading solution were sampled and counted, to check window settings, activity, and the degree of 'cross-over' of sodium signal into the bicarbonate channel. The two components were then pooled to form the final loading solution which was also sampled and counted.

For each ionic strength three dehydrated corneas were placed in the loading solution contained in small glass vials and the vial shaken on a flask shaker for 5 minutes. After this period the corneas were successively removed from the loading solution, carefully blotted, and then rapidly weighed on an electronic microbalance (Beckmann, IM 500) to determine the wet weight of each cornea.

Individual corneas were then unloaded into 10 ml of 'cold' sodium bicarbonate of the same ionic strength as the loading medium for 1 hour. The unloading solutions were then sampled by removing 1 ml of solution for subsequent counting.

The experimental corneas were then dried down to constant weight in Petri dishes in a dessicator over silica gel for 3 weeks and after this time weighed again on the microbalance. The wet and dry weights enabled the hydration of each cornea to be determined.

Knowing the activity of \(^{22}\text{Na}\) and \(^{14}\text{C}\) recovered from each cornea and the activity of the original loading solution it was possible to calculate the stromal concentration of sodium and
bicarbonate ions, as described in the next section of this chapter.

One other experiment was performed using the double labelling technique described in this chapter. This was a comparison of the negative charge concentration in control corneas and those that had previously been 'rapidly' frozen in Freon 22 as described in Chapters 1 and 2.

As indicated earlier one of the original aims of this work was the application of the technique of ultracryotomy in conjunction with X-ray microanalysis to the study of the elemental composition of the stroma under various experimental conditions. As rapid freezing of the cornea in Freon 22 would be the first step in this protocol it was considered of interest to apply the double labelling technique to corneas so treated and obtain a measure of the negative charge concentration of the rapidly frozen corneas.

Six fresh rat corneas were obtained and three of them were rapidly frozen in melting Freon 22 as previously described and then allowed to melt by placing them on a glass slide at room temperature.

Both the unfrozen control corneas and the experimental frozen/melted corneas were then dehydrated over silica gel in an oven at 30°C for 24 hours.

Using doubly labelled 100 mM solutions of sodium bicarbonate the six corneas were then loaded with the label and subsequently unloaded into 'cold' 100 mM sodium bicarbonate. The activity in the unloading solution was counted and a measure of the negative charge concentration for each cornea was obtained as already described.
Calculation of Stromal Sodium and Bicarbonate Ion Concentrations

Consider as an example a cornea bathed in 20 mM doubly labelled sodium bicarbonate which upon unloading gave a total of 15,000 cpm of $^{22}\text{Na}$ activity and that the $^{22}\text{Na}$ activity of the loading solution was 22,000 cpm per 20 µl.

Then we might say that the equivalent volume of $^{22}\text{Na}$ or sodium ions is given by $N_a V$ where,

$$N_a V = \frac{15,000}{22,000} \times 20 \mu l$$

$$= 13.63 \mu l \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (1)$$

Knowing the wet weight and dry weight of the cornea the weight of water in the cornea immediately after it has removed from the loading solution can be determined. In this example let that value be 6.40 mgs. Then the concentration of sodium ions, $N_a C$ within the stroma is given by,

$$N_a C = \frac{N_a V}{\text{Volume of Water}} \times 20 \text{ mM} = \frac{13.63}{6.40} \times 20\text{mM}$$

$$= 42.59 \text{ m.equiv.} \cdot \text{l}^{-1} \quad \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots \ldots (2)$$
Similarly for the bicarbonate ion, if the $^{14}$C activity recovered was 90,000 cpm and the activity of $^{14}$C in the loading solution was 290,000 cpm per 20 µl then, the equivalent volume of $^{14}$C or bicarbonate ions generally is given by,

$$C_V = \frac{90,000}{290,000} \times 20$$

$$= 6.21 \text{ µl}$$

and the concentration of bicarbonate ions is given by,

$$C_C = \frac{C_V}{\text{Water Volume}} \times 20 = \frac{6.21}{6.40} \times 20$$

$$= 19.40 \text{ m.equiv.}^{-1}$$

And the net negative charge in this case is then, (2) - (5) i.e.

$$Na_C - C_C = 42.59 - 19.40$$

$$= 23.19 \text{ m.equiv.}^{-1}$$

**Calculation of $F_p$, the Negative Charge Concentration at Physiological Hydration**

If in this case the dry weight and the wet weight of the cornea was 0.6 mgs and 7.0 mgs respectively then the hydration $H_x$ of the cornea immediately after being removed from the loading solution was,

$$H_x = \frac{7.0 - 0.6}{0.6}$$

$$= 10.67$$
and using

\[ F \times H = F_p \times p \] .............................. (6)

with \( H = 3.24 \) then,

\[ F_p = \frac{23.19 \times 10.67}{3.24} \]

\[ = 76.37 \text{ m.equiv.}^{-1} \]

RESULTS

The rat cornea seemed to be fully radiolabelled whether the tissue was exposed for 2, 5 or 20 minutes to the loading solution.

Table 1 gives the activities of \(^{14}\text{C} \) and \(^{22}\text{Na} \), corrected for 'cross-over' and background, for the efflux experiment with a 5 minute loading time.

Table 2 gives the cumulative activities of \(^{14}\text{C} \) and \(^{22}\text{Na} \) for the time course of the efflux i.e. 20 minutes.

Figure 1 shows the corresponding efflux plot derived from Table 2 and illustrates the essentially linear nature of these plots indicating that both the sodium and bicarbonate ions are freely exchangeable between the stroma and its bathing medium and that the diffusional permeability of both ions, as defined in chapter 3, is about the same.

Table 3 summarises the results of the experiments to determine negative charge concentrations in 20, 50, 100 and 150 mM doubly labelled sodium bicarbonate solutions. It shows the activity in
the bicarbonate and sodium channels due to the loading solution and each of the unloaded corneas. The activity is given in cpm and has been corrected for 'cross-over' of sodium signal into the bicarbonate channel and also for background.

Table 4 summarises the calculated values of $F_x$ the negative charge concentration at hydration $H_x$ for each cornea. The calculation of $F_x$ being performed as detailed in the theory section of this chapter. Also included in Table 4 is the measured hydration $H_x$ of each cornea, the negative charge concentration $F_p$ at physiological hydration calculated using $F_x H_x$ and a value of $H_p = 3.24$ and equation (6), and finally the square root of the product of the sodium and bicarbonate concentrations within the stroma. The salt concentration in the stroma was always significantly higher than in the bathing medium. Student's t test showed no significant differences between the values of $F_p$ under the various experimental conditions tested.

Table 5 details the results of the experiment with the frozen/melted corneas together with the unfrozen corneas which served as controls. It shows the calculated values of negative charge concentrations $F_x$ together with $H_x$, $F_p$ and as in Table 4 the square root of the product of the sodium and bicarbonate ion concentration in the stroma.

It is interesting to note from Tables 4 and 6 that the effective concentration of sodium and bicarbonate ions within the stroma, given by the square root of the product of the calculated concentrations of these ions, is in every case greater than the...
corresponding values for the bathing medium i.e. 20, 50, 100
and 150 mM.

A Student's t test applied to the $F_p$ results for frozen/
melted and control corneas (Table 5) indicated a significant
difference between the measured values for $F_p$. Freezing a cornea
diminishes the estimated fixed negative charge in rat corneal
stroma.
Table 1. Efflux of $^{14}C_3$ and $^{22}Na$ from rat corneas loaded for 5 min

<table>
<thead>
<tr>
<th>Period of collection</th>
<th>$^{14}C_3$ counts/m</th>
<th>$^{22}Na$ counts/m</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-5 s</td>
<td>6169</td>
<td>1149</td>
</tr>
<tr>
<td>5-10s</td>
<td>4662</td>
<td>915</td>
</tr>
<tr>
<td>10-15s</td>
<td>3871</td>
<td>628</td>
</tr>
<tr>
<td>15-20s</td>
<td>3386</td>
<td>592</td>
</tr>
<tr>
<td>20-25s</td>
<td>3240</td>
<td>625</td>
</tr>
<tr>
<td>25-30s</td>
<td>3368</td>
<td>800</td>
</tr>
<tr>
<td>30-40s</td>
<td>4697</td>
<td>907</td>
</tr>
<tr>
<td>40-50s</td>
<td>3822</td>
<td>730</td>
</tr>
<tr>
<td>50-60s</td>
<td>2195</td>
<td>416</td>
</tr>
<tr>
<td>1-2m</td>
<td>5483</td>
<td>975</td>
</tr>
<tr>
<td>2-10m</td>
<td>1075</td>
<td>261</td>
</tr>
<tr>
<td>10-20m</td>
<td>-</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2. Cumulative activity of $^{14}C_3$ and $^{22}Na$ in cpm in rat corneas after loading for 20 min and then effluxing for time T

<table>
<thead>
<tr>
<th>T</th>
<th>Corneal $^{14}C_3$ activity</th>
<th>$^{22}Na$ activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>41968</td>
<td>8004</td>
</tr>
<tr>
<td>5s</td>
<td>35799</td>
<td>6955</td>
</tr>
<tr>
<td>10s</td>
<td>31337</td>
<td>5940</td>
</tr>
<tr>
<td>15s</td>
<td>27266</td>
<td>5312</td>
</tr>
<tr>
<td>20s</td>
<td>23888</td>
<td>4720</td>
</tr>
<tr>
<td>25s</td>
<td>20648</td>
<td>4095</td>
</tr>
<tr>
<td>30s</td>
<td>17272</td>
<td>3205</td>
</tr>
<tr>
<td>40s</td>
<td>12575</td>
<td>2388</td>
</tr>
<tr>
<td>50s</td>
<td>8753</td>
<td>1658</td>
</tr>
<tr>
<td>1m</td>
<td>6558</td>
<td>1242</td>
</tr>
<tr>
<td>2m</td>
<td>1075</td>
<td>267</td>
</tr>
<tr>
<td>10m</td>
<td>-</td>
<td>9</td>
</tr>
</tbody>
</table>
Table 3. Total recoveries of $^{14}$CO$_3^-$ and $^{22}$Na from rat corneas loaded originally in solutions whose activities are given in parentheses (cpm.ml$^{-1}$).

<table>
<thead>
<tr>
<th>Molarity of NaHCO$_3$ in loading soln</th>
<th>Exp. No</th>
<th>Recovered $^{14}$CO$_3^-$ activity (cpm)</th>
<th>Recovered $^{22}$Na activity (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM</td>
<td>1</td>
<td>12848 (266072)</td>
<td>1193 (14555)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>11570 (247872)</td>
<td>1227 (15744)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>16142 (275743)</td>
<td>1317 (13808)</td>
</tr>
<tr>
<td>50 mM</td>
<td>1</td>
<td>6091 (291204)</td>
<td>1172 (22579)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>9292 &quot;</td>
<td>1504 &quot;</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>5486 &quot;</td>
<td>1148 &quot;</td>
</tr>
<tr>
<td>100 mM</td>
<td>1</td>
<td>4510 (229149)</td>
<td>685 (23994)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4464 &quot;</td>
<td>758 &quot;</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6031 &quot;</td>
<td>834 &quot;</td>
</tr>
<tr>
<td>150 mM</td>
<td>1</td>
<td>3607 (94412)</td>
<td>508 (10648)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>4862 &quot;</td>
<td>642 &quot;</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3697 &quot;</td>
<td>560 &quot;</td>
</tr>
</tbody>
</table>
Table 4. The measured hydration, $H_x$, the value of fixed negative charge in the stroma, $F_x$ (meq.l$^{-1}$), and the calculated fixed negative charge at hydration 3.24, which is physiological, $F_P$ (meq.l$^{-1}$). $C$ is the root of the product of the stromal concentrations of bicarbonate and sodium.

<table>
<thead>
<tr>
<th>Molarity of NaHCO$_3$ in loading soln.</th>
<th>Exp. No.</th>
<th>$H_x$</th>
<th>$F_x$</th>
<th>$F_P$</th>
<th>$C$</th>
</tr>
</thead>
<tbody>
<tr>
<td>20mM</td>
<td>1</td>
<td>9.72</td>
<td>22.0</td>
<td>66.0</td>
<td>23.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>10.44</td>
<td>21.6</td>
<td>69.6</td>
<td>28.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>8.12</td>
<td>23.8</td>
<td>59.7</td>
<td>23.0</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td>65 ± 4</td>
<td>25 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50mM</td>
<td>1</td>
<td>7.50</td>
<td>27.8</td>
<td>64.3</td>
<td>56.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.75</td>
<td>32.3</td>
<td>57.5</td>
<td>60.3</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.39</td>
<td>28.4</td>
<td>56.0</td>
<td>55.1</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td>59 ± 3</td>
<td>57 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>100mM</td>
<td>1</td>
<td>5.14</td>
<td>41.1</td>
<td>65.2</td>
<td>109.9</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.73</td>
<td>51.5</td>
<td>91.1</td>
<td>105.6</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>6.72</td>
<td>30.4</td>
<td>63.1</td>
<td>108.7</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td>73 ± 11</td>
<td>108 ± 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>150mM</td>
<td>1</td>
<td>7.19</td>
<td>34.9</td>
<td>77.3</td>
<td>156.6</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>8.24</td>
<td>24.9</td>
<td>63.3</td>
<td>157.5</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>7.20</td>
<td>47.4</td>
<td>105.3</td>
<td>160.6</td>
</tr>
<tr>
<td>Mean ± S.E.</td>
<td></td>
<td>82 ± 12</td>
<td>158 ± 2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 5. The effect of freezing and then thawing of a cornea upon the estimation of fixed negative charge in rats. Determinations were made in 100mM NaHCO₃.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hydration</th>
<th>Fixed negative charge</th>
<th>Fixed charge at hydration 3.24</th>
<th>Stromal salt conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>7.27</td>
<td>27.2</td>
<td>61.0</td>
<td>115.1</td>
</tr>
<tr>
<td>None</td>
<td>7.24</td>
<td>26.0</td>
<td>58.0</td>
<td>106.7</td>
</tr>
<tr>
<td>None</td>
<td>5.67</td>
<td>33.6</td>
<td>58.8</td>
<td>112.0</td>
</tr>
<tr>
<td></td>
<td>Mean ± S.E.</td>
<td>59 ± 1</td>
<td></td>
<td>111 ± 3</td>
</tr>
<tr>
<td>Frozen/Thawed</td>
<td>6.42</td>
<td>22.5</td>
<td>44.6</td>
<td>110.6</td>
</tr>
<tr>
<td>Frozen/Thawed</td>
<td>6.15</td>
<td>17.0</td>
<td>32.3</td>
<td>114.1</td>
</tr>
<tr>
<td>Frozen/Thawed</td>
<td>5.98</td>
<td>19.8</td>
<td>35.4</td>
<td>116.8</td>
</tr>
<tr>
<td></td>
<td>Mean ± S.E.</td>
<td>37 ± 4</td>
<td></td>
<td>114 ± 2</td>
</tr>
</tbody>
</table>
Fig. 1. The loss of $^{22}\text{Na}$ and $^{14}\text{CO}_3$ from pre-equilibrated rat corneal stroma.
Ultracryotomy

The results from the work with the ultracryotome revealed two limitations to the technique.

(a) Rapid freezing of biological tissue almost always produced ice crystal damage to the ultrastructure of the tissue. Freezing rates are too slow. It is possible to achieve fast enough freezing rates so that ice crystal size is either below the resolvable limits of the electron microscope or even to vitrify the tissue water but only in a few cases which include isolated sub-cellular particles or individual cells (Bachmann and Schmitt, 1971; Ververgaert, 1973) or the outer few microns of tissue blocks (Dempsey and Bullivant, 1976a). A promising approach to obtaining practical volumes of rapidly frozen tissue with no or minimal ice crystals would seem to me to involve a hybrid specimen sampling/freezing device. In this method, some means of quickly biopsying tissue (100 μm thick slices, for example) could be combined with almost immediate freezing in (say) a propane jet (Moor et al, 1976; Müller et al, 1979). Then there would be the possibility of obtaining reproducible, working volumes of tissue relatively free of ice crystals. The beginnings of this kind of approach have been made already (Bernard and Krigman, 1974; Van Harreveld and Fifkova, 1975; Sitte et al, 1977).
(b) The second limitation of ultracryotomy is the inability consistently to obtain ultrathin (<100 nm) sections of frozen material. Personal communication with other workers in this field confirms this to be a problem and the usual published statement 'and then the frozen tissue was sectioned' describes a process which nearly always involves many difficulties. There is considerable differences in the physical properties of plastic embedded material, for which ultramicrotomes and knives were developed and, the physical properties of frozen tissue. It seems probable that developments in this field might increase the success rate of sectioning frozen tissue.

At the present moment the most practical approach to the preparation of biological specimens for subsequent X-ray microanalysis in the transmission electron microscope would seem to be the use of either freeze-substitution or freeze-drying with the important proviso that sectioning of the material be carried out on a dry knife or on a liquid which does not disturb the elemental composition of the section. Sections prepared in this way could then be examined in the now widely available scanning transmission electron microscope where the innate contrast in the section can be enhanced electronically.

**Swelling Pressure Measurements and Charge Concentration**

The results from the beam/strain gauge apparatus indicated that as the ionic strength of the bathing medium was increased so the calculated value of the charge concentration increased.
This variation in charge concentration with ionic strength has been noted by other workers (Green et al, 1971; Goodfellow, 1975; Elliot et al, 1980). The values of charge concentration calculated from the beam/strain gauge data ranged from 20 – 35 mequiv.1^-1 which compares with a range of 12 – 16^-1 mequiv.1 (Friedman and Green, 1971) and 15 – 42 mequiv.1^-1 (Elliot et al, 1980).

The absence of pH buffers in the stromal bathing media in both the work described in this thesis and that of Friedman and Green (1971) when compared to Elliot et al (1980), who used buffered solutions, might explain the differences in numerical values of the charge concentration. This charge arises from the dissociation of acidic groups and the degree of dissociation is dependent upon the pH within the stroma. Thus a clearer picture of charge concentration with varying ionic strength of the bathing medium is seen if the pH of the stroma is buffered by adding buffers to the bathing medium.

Green et al (1971) explain the increase in charge concentration with increasing ionic strength of the bathing medium in terms of binding of sodium ions within the stroma. The possibility of cation binding was suggested by Otori (1967). He measured the concentration of Na^+ and Cl^- ions in rabbit corneal stroma with various extraction techniques and found the sodium concentration higher in the stroma than in the aqueous humor but also that the stromal chloride concentration was comparable to that in the aqueous humor. One possible factor explaining this relative elevation of the chloride ion concentration above which would be expected from a simple Donnan equilibrium could, he suggested, be the binding of sodium ions in the stroma.
Green et al (1971) expanded this possibility and proposed a decrease in the binding of Na\(^+\) ions with increasing Na\(^+\) concentration to explain the apparent increase in charge concentration with increasing ionic strength of the bathing medium.

Alternatively Elliot et al (1980) think it more likely that binding of negative ions is occurring within the stroma with the binding fraction increasing with increasing concentration of these ions in the bathing medium.

Another possibility is that the basic equations from which values of the charge concentration are calculated need to be modified to account for changes in the activity coefficients of both the cation and anion as the ionic strength of the bathing medium is changed.

The bathing solutions both in the work described in Chapter 4 and in Elliot et al (1980) were assumed to act ideally i.e. the activity coefficients of the Na\(^+\) and Cl\(^-\) ions were unity. It is known that when the ionic strength of a colloidal electrolyte is changed the distribution of ions about the charged colloidal particles also changes (Feynman, et al 1964; Brenner and McQuarrie, 1973). As the ionic strength of the electrolyte increases the thickness of the ion sheath about a charged particle in the electrolyte decreases. One measure of this thickness is the Debye length \( L \) where at some temperature \( T \),

\[
L = \frac{A}{I^2} \quad \text{(1)}
\]

where \( A \) is some constant and \( I \) is the ionic strength of the electrolyte.
At low concentrations of the electrolyte, \( L \) is large and the distribution of ions and counterions is diffuse, whereas at high concentrations \( L \) is small and the distribution of ions is much more ordered.

The corneal stroma, if it consists of charged collagen fibrils, would be a good approximation to the simple colloid described above and the variation of the ionic strength of the stromal bathing medium would be likely to produce similar variations in the cation and anion distributions about the collagen fibrils. It is possible that in an ordered arrangement of cations and anions at high ionic strength the activity coefficients of these ions would have values of less than unity and be different for each ion, whereas at low concentrations with a more diffuse or disordered arrangement of ions the activity coefficients could more nearly approach unity. The value of \( F \) the charge concentration is then given by,

\[
F = \alpha_1 [\text{Na}]_1 - \alpha_2 [\text{Cl}]_1
\]

where \( \alpha_1 \) and \( \alpha_2 \) are the activity coefficients of the sodium and chloride ions respectively and both are a function of ionic strength. For this reasoning to produce an increase in \( F \) with increasing ionic strength there would have to be differences between \( \alpha_1 \) and \( \alpha_2 \) at high concentrations with \( \alpha_1 > \alpha_2 \).
Charge Concentration in the Double-labelling Experiments

The measurement of the charge concentration at physiological hydration, $F_p$, spanned a range from 65 - 82 mequiv. L$^{-1}$, some 2.5 times the magnitude of the results obtained with the beam strain/gauge apparatus. Although there is a suggestion of increasing $F_p$ with increasing ionic strength no statistically significant differences were found between any of the values of $F_p$.

The square root of the product of the stromal sodium and bicarbonate ion concentrations $C$ should, if we are dealing with a simple Donnan equilibrium between the stroma and its bathing medium, be equal to the concentration of the sodium bicarbonate in the bathing medium. In every case in these experiments this factor $C$ was significantly greater than the corresponding value of bathing medium concentration.

Why should there be such a large difference in the calculated values of $F_p$ between the two sets of experiments? Two possible explanations suggest themselves. One involves the radically different methods by which data were obtained for the calculation of $F_p$. The other explanation is that the equations set up to transform the swelling pressure measurements into values of $F_p$ do not properly describe the situation within the stroma and need to be modified to account for the binding of anions and possibly cations as well as the existence of non-ideal solutions within the stroma. In other words there is not a simple Donnan equilibrium between the stroma and its bathing medium. Further evidence for this is
provided by the values of $C$ which are all in excess of those predicted on the basis of Donnan equilibrium.

Interestingly the variation of $F_p$ with ionic strength showed no significant differences over the range of ionic strengths used. If, in fact, there is anion binding one wonders whether this result indicates an affinity of the stroma for the chloride ion.

Finally the double-labelling experiment with frozen and unfrozen corneas indicates a significant difference between the values of $F_p$ in the two cases. This is instructive but it does indicate that as far as the corneal stroma is concerned, rapid freezing may alter the physical chemistry and the chemical integrity of the tissue.
Appendix 1

Parts of the work described in this thesis have already been published, the relevant publications are listed below.


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