J. Physiol. (1980), **298**, *pp.* 453–470
With 11 *text-figures.*
Printed in Great Britain

SWELLING STUDIES OF BOVINE CORNEAL STROMA WITHOUT BOUNDING MEMBRANES

BY G. F. ELLIOTT, J. M. GOODFELLOW* AND A. E. WOOLGAR

From The Open University, Oxford Research Unit, Foxcombe Hall, Berkeley Road, Boars Hill, Oxford

(*Received 6 March 1979*)

SUMMARY

1. The swelling characteristics of demembranated bovine corneal stroma were studied as a function of time and of the pH and ionic strength of the bathing solution.

2. Compared with other pH values, the stroma swelled least near pH 4.

3. In the pH range 6–10, increasing the pH resulted in an increase both in the rate of swelling and in the hydration reached in a given time.

4. At pH 2 and 4, a final constant value of hydration was attained. At higher pH values no such equilibrium was attained when the hydration of the tissue was followed for at least 100 hr.

5. The swelling at high pH values was consistent with the hypothesis that the Donnan-osmotic contribution is the major component of the swelling pressure.

6. The ionic strength dependence was complex. There was a general decrease of swelling with increase in the ionic strength (μ) until around $\mu = 0{\cdot}1$. The swelling at $\mu = 0{\cdot}15$ was greater than at $\mu = 0{\cdot}1$ and $\mu = 0{\cdot}25$.

7. The results were interpreted on the assumption that the Donnan-osmotic effect is the major component of the swelling pressure.

INTRODUCTION

A major property of the corneal stroma is its ability to swell to many times its original weight when placed in aqueous solution *in vitro* (Kinsey & Cogan, 1942) and also *in vivo* when the cellular layers lining the cornea are damaged (Maurice & Giardini, 1951). The increase in water content is related to a decrease in the transparency of the tissue (Kinsey, 1948). This ability to swell and the transparency of fresh corneal stroma itself are both unusual properties for a connective tissue (Payrau, Pouliquen, Faure & Offret, 1967; Maurice, 1969).

The origin of the driving force for swelling, the swelling pressure, is a matter for discussion. It is accepted that the glycosaminoglycans present in the stroma are essential for swelling to take place to any extent (Hedbys, 1961). Theories of the origin of the swelling pressure differ in the structural relationship assumed between the glycosaminoglycans, glycoproteins and collagen fibrils which together make up the corneal stroma. These different assumptions result in differences in importance ascribed to various components of the total swelling pressure.

* Present address: Department of Crystallography, Birkbeck College, Malet Street, London.

0022-3751/80/6020-0079 $07.50

Farrell & Hart (1969) based their theory of the cornea on the premise that the glycosaminoglycan molecules act as cross-links between the collagen fibrils in the manner described by Matthews (1965) for cartilage. On this assumption the contributions to the swelling pressure include the Donnan-osmotic effect, stretching of the glycosaminoglycan chains, excluded-volume effects and the interaction of the glycosaminoglycans with the solvent. On the other hand, Hodson (1971) supposed that no effective cross-links exist in the cornea; he suggested that the glycosaminoglycan molecules surround the collagen fibrils (Hodson & Meenan, 1969; Myers, Highton & Rayns, 1973). In Hodson's model, the swelling pressure is held to be due entirely to the Donnan-osmotic pressure between the stroma and the external solution. The pressure represents the unequal distribution of small permeant ions between the polyelectrolyte phase and the bathing solution (Overbeek, 1956). In the cornea the distribution of ions results from the presence of a fixed charge (polyelectrolyte) concentration in the stroma, due in part to the glycosaminoglycan molecules. Contributions to this fixed charge are made in chondroitin sulphate by both carboxylic and sulphonic acid groups and in keratan sulphate by sulphonic acid groups alone.

The magnitude of the fixed charge concentration in corneal stroma has been estimated by various methods. Otori (1967) calculated that the fixed charge concentration is 36 mM in fresh tissue, from the data of Anseth & Laurent (1961). However, Hodson (1971) states that the fixed charge concentration is more likely to be 48 mM when all the charged groups, carboxyl as well as sulphonic acid groups, are included in the calculation. He obtained an experimental value of 47·4 mM using the Donnan exclusion techniques of Maroudas & Thomas (1970).

Friedman & Green (1971*a*), on the other hand, used experimental results from sodium binding in the stroma and calculated only 16 mM of charge in the stroma; no mention of the pH of the bathing solution was made.

In our experiments, the behaviour of the swelling has been studied in detail as a function of the pH and the ionic strength (μ) of the external bathing solution. Both these parameters are known to affect the magnitude of the swelling (Kinsey & Cogan, 1942; Loeven & van Walbeek, 1954; Pau, 1954; Smelser, 1962). We have interpreted these swelling results with a theory derived on the premise that the Donnan contribution is the major component of the swelling pressure (see Appendix). We used corneal stroma from which the epithelium and endothelium had been removed. At first sight this may appear to be a rather non-physiological system but it seems important to us to try to understand the detailed physical chemistry of the demembranated stroma because this must surely govern the ion-pumping effects of the metabolic systems within the limiting membranes.

METHODS

Tissues

Bovine eyes were obtained from slaughtered cattle within 4 hr of death. The corneas, without sclera, were dissected from the eyes with care and the cellular layers (the epithelium and endothelium) were gently removed by scraping a scalpel across the surfaces of the cornea. The stroma (cornea minus cellular layers) was dehydrated over silica gel at room temperature until it achieved constant dry weight. This technique has been used by many workers to preserve the

stroma for both experimental studies and lamellar keratoplasty (e.g. van Walbeek & Neumann, 1951; Loeven & van Walbeek, 1954; Hedbys, 1961; Payrau & Pouliquen, 1960).

The drying procedure ensures that all stroma are initially in the same condition at zero hydration. Drying agents other than silica gel have been shown to be unreliable and to lead to structural changes (Payrau *et al.* 1967). The swelling of dried tissue is thought to be similar to that of fresh tissue (Payrau *et al.* 1967; Goodfellow, 1975).

The pieces of dried stroma were kept small ($\approx$4 mm sq.) because supplies of cornea were limited, but they were heavy enough ($\approx$10 mg) to be weighed to a 1 % accuracy. We are aware that the size and shape of the specimen is a factor in the time course of swelling and we are investigating these effects separately.

Solutions

Both the pH and the ionic strength of the bathing solutions were varied. Six pH values were chosen and suitable buffers were used at each value; the details are given in Table 1. The required ionic strength was obtained by addition of NaCl where necessary; at all but the lowest ionic strength ($\mu = 0{\cdot}02$), the concentration of NaCl was in excess of the concentration of the buffer ions. Five values of ionic strength were used at each pH value. The pH was checked with a Pye Unicam pH meter.

TABLE 1. The content and ionic strength of buffers used at pH 2, 4, 6, 7, 8 and 10. Sodium chloride was added to obtain the required final ionic strength

pH	Buffer	μ
2	25 ml. 0·2 M-KCl in 100 ml. + 6·5 ml. 0·2 M-HCl	0·06
4	18 ml. 0·2 M-CH_3COONa in 1000 ml. + 82ml. 0·2 M-CH_3COOH	0·02
6	12·3 ml. 0·1 M-Na_2HPO_4 in 1000 ml. + 87·7 ml. 0·1 M-NaH_2PO_4	0·01
7	61 ml. 0·1 M-Na_2HPO_4 in 1000 ml. + 39 ml. 0·1 M-NaH_2PO_4	0·02
8	94·7 ml. 0·1 M-Na_2HPO_4 in 1000 ml. + 8·3 ml. 0·1 M-NaH_2PO_4	0·03
10	50 ml. 0·05 M-$NaHCO_3$ in 100 ml. 10·7 ml. 0·1 M-NaOH	0·035

Swelling

Small pieces of dried corneal stroma (see above) were weighed on a sensitive balance to $\pm$0·1 mg and then transferred to jars containing at least 30 cm^3 of the bathing solution of known pH and ionic strength. At different time intervals the corneal pieces were removed from solution, carefully blotted to remove excess fluid, and then reweighed. At all times the solutions were covered to prevent evaporation.

The hydration, H, was calculated as the weight of water per unit dry weight. There will obviously be errors in blotting and weighing the stromal pieces, although this is a standard technique for this type of study. The hydrated weight measurement is accurate to 1 %. Blotting

probably causes errors at large hydrations where the tissue is only loosely held together so that a shear force could remove some tissue, especially from the surface. The method is not likely to produce significant errors at hydrations less than 10 if care is taken in blotting.

Each swelling curve (Figs. 1–9) represents data from at least five separate corneal specimens. In all cases the curves are fitted to the data by eye.

RESULTS

Swelling studies

The hydration of the pieces of corneal stroma was followed for approximately 100 hr in thirty different solutions. Initially swelling was fast, the rate of increase of hydration depending on the solution. The rate of hydration decreased continually with time. At low pH values (2 and 4), the rate of hydration became zero and a final constant value of hydration was attained. At higher pH values, the hydration increased continually within the period investigated and no constant value was obtained. Because of this difference in behaviour, the results are divided into two sections: those at low pH and those near to or above physiological pH (6–10).

Swelling at low pH

Fig. 1 shows that the corneal stroma approached a constant value of hydration within 10 hr when placed in solutions buffered at pH 4. The final value of the hydration depended on the ionic strength of the bathing solution. In general, the higher was the ionic strength the larger was the final value of the hydration; values are given in Table 2 from which it can be seen that the lowest final hydration was found at $\mu = 0{\cdot}06$ when $H = 2{\cdot}5$ and the highest value of $H = 4{\cdot}5$ was found when the ionic strength of the bathing solution was at $\mu = 0{\cdot}3$.

At pH 2, swelling occurred at a faster rate than at pH 4 (Fig. 2) but still reached a final constant value within 20 hr. The value of this final hydration depended on the ionic strength but in the inverse manner to the dependence at pH 4. Increasing the ionic strength at pH 2 resulted in a decrease in the final amount of swelling. Values for the final hydration for pH 2 are also shown in Table 2.

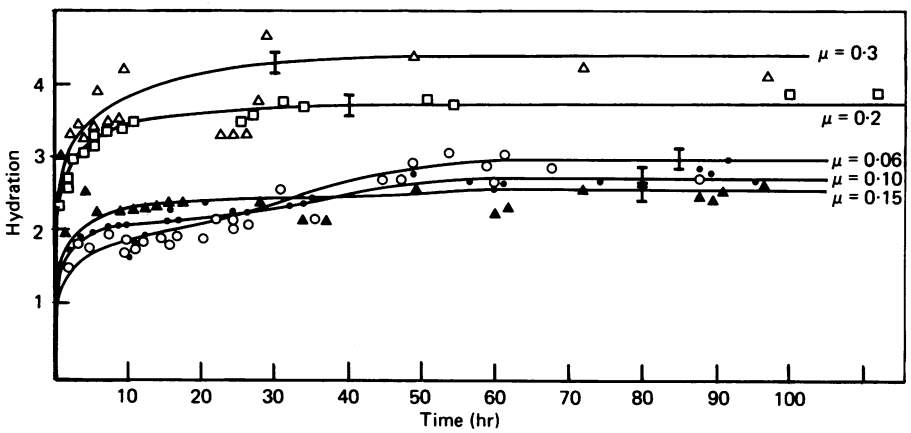

Fig. 1. The hydration as a function of time of swelling, in solutions buffered at pH 4. The final hydration is seen to increase as μ, the ionic strength of the bathing medium, increases.

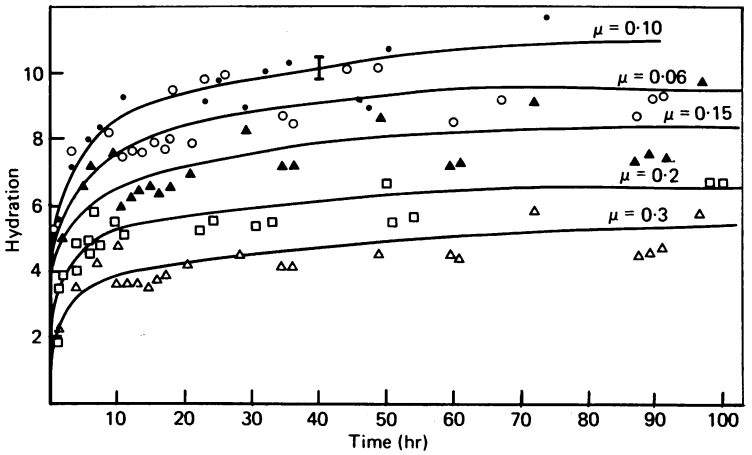

Fig. 2. The hydration as a function of time of swelling, in solutions buffered at pH 2. The final hydration is seen to decrease as μ increases.

TABLE 2. The final values of the hydration for corneal stroma swollen in solutions at pH 2 and 4 at five ionic strengths. Swelling at pH4 increases with increasing ionic strength while at pH 2 swelling decreases

Ionic strength (μ)	Final hydration at pH 4	Final hydration at pH 2
0·06	2·75–3·0	10·5–11·5
0·1	2·75–3·0	9·0–10·0
0·15	2·75–3·0	8·0–9·0
0·20	3·25–3·75	6·0–7·0
0·30	4·00–4·5	4·5–5·5

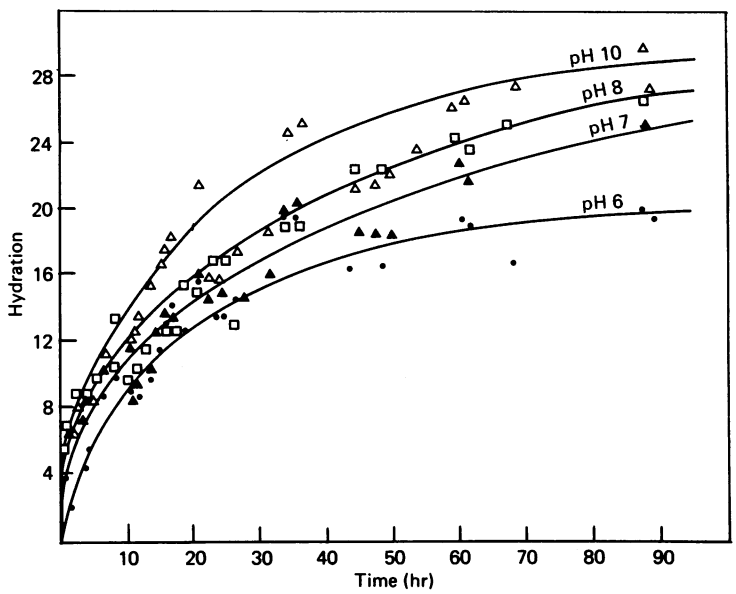

Fig. 3. The hydration as a function of time of swelling, for solutions at $\mu = 0{\cdot}02$, and pH 6, 7, 8 and 10. (. , pH 6; ▲, ph 7; □, pH 8; △, pH 10).

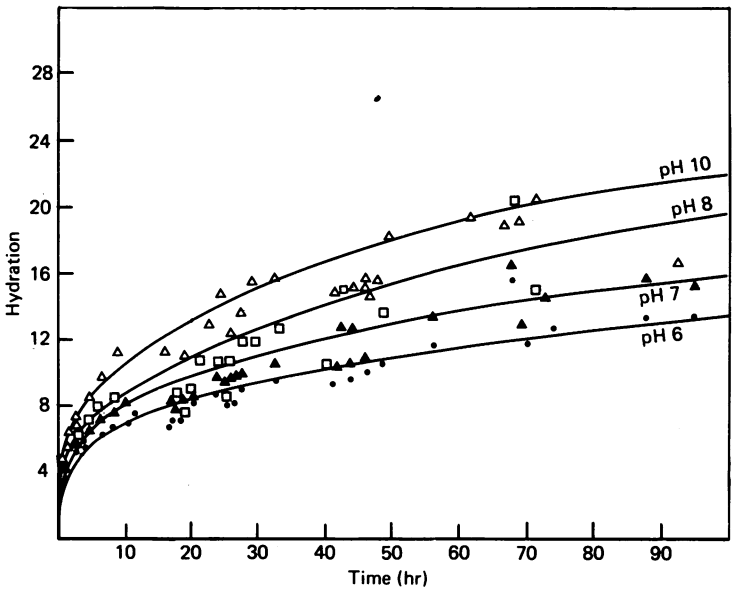

Fig. 4. The hydration as a function of time of swelling, for solutions at $\mu = 0{\cdot}05$ and pH 6, 7, 8 and 10. (pH key as in Fig. 3.)

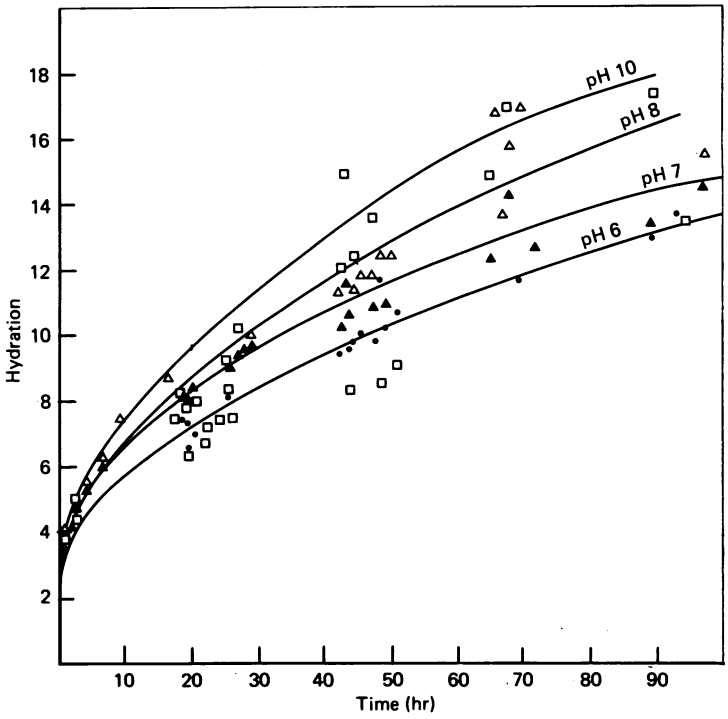

Fig. 5. The hydration as a function of time of swelling, for solutions at $\mu = 0{\cdot}1$ and pH 6, 7, 8 and 10. (pH key as in Fig. 3.)

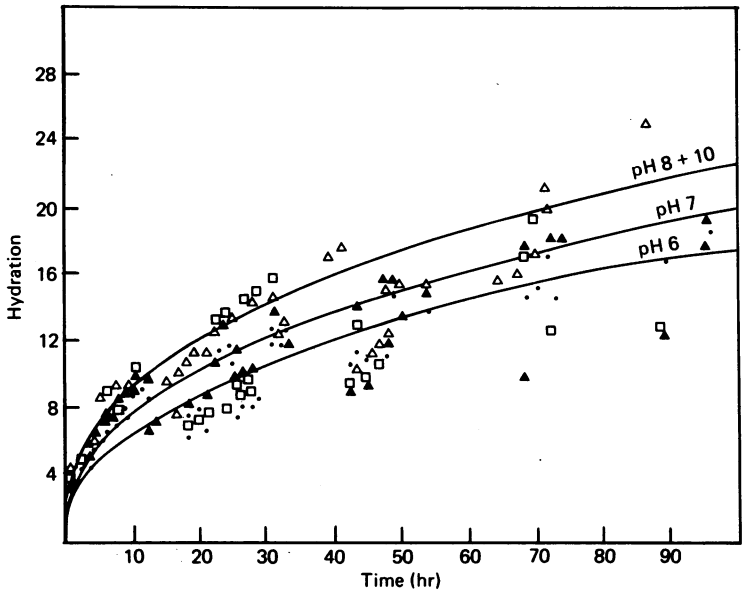

Fig. 6. The hydration as a function of time of swelling, for solutions at $\mu = 0{\cdot}15$ and pH 6, 7, 8 and 10. (pH key as in Fig. 3.)

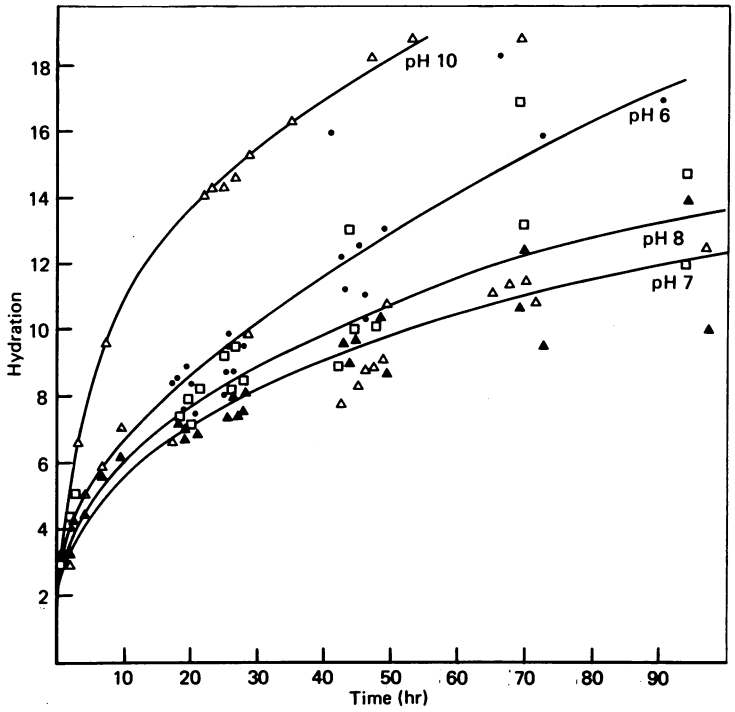

Fig. 7. The hydration as a function of time of swelling, for solutions at $\mu = 0{\cdot}25$ and pH 6, 7, 8 and 10. (pH key as in Fig. 3.)

Swelling at higher pH

At pH values 6, 7, 8 and 10, the initial fast rate of swelling decreased continually but did not become zero within 100 hr. The swelling depended on the pH and ionic strength of the bathing solutions as shown in Figs. 3–7. Notice that the data, particularly at higher hydrations, can be very scattered, and the curves in Figs. 3–7 are fitted by eye only. A better idea of the pH effect is given by the function $G(H)$ which we introduce in Section III of the Discussion.

In general, an increase in the pH of the bathing solution increased the hydration rate for a given time and a given ionic strength. Exceptions occurred at $\mu = 0{\cdot}25$ where the rate at pH 6 was larger than at pH 7 or 8 and at $\mu = 0{\cdot}15$ where the rates at pH 8 and pH 10 were similar.

Swelling in distilled water

Corneal stroma swelling in distilled water (Fig. 8) behaves differently to stroma swelling in solutions containing permeant ions (Na^+ and Cl^-). In distilled water the hydration of the corneal pieces increased very rapidly for about 6 hr; in the first hour the hydration increased from zero to 8 or 9. After this very rapid swelling the hydration rate became practically zero at a hydration of around 30; this final hydration was higher than any value obtained in salt solutions within 100 hr.

DISCUSSION

I. *Effects near to the isoelectric point*

We have found a minimum swelling occurring near to pH4, as reported previously by Kinsey & Cogan (1942), Loeven & van Walbeek (1954) and Hedbys (1961).

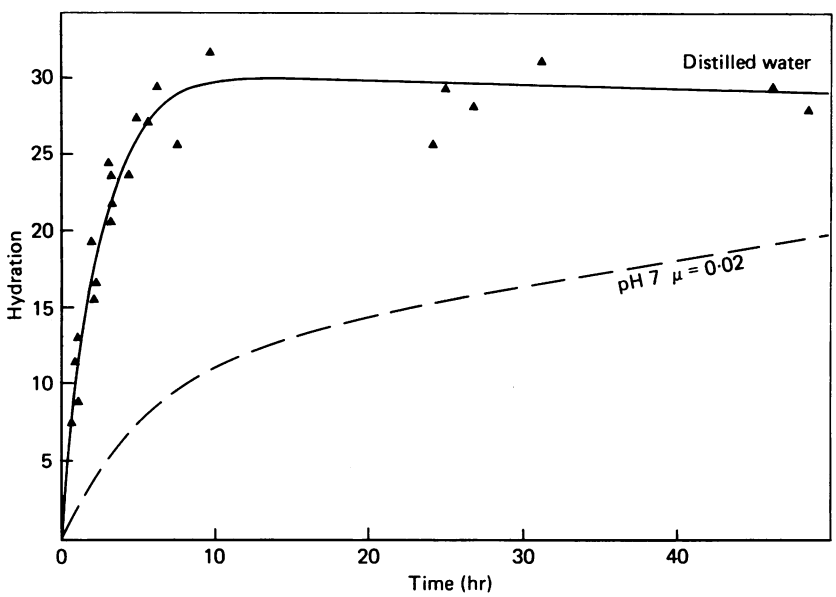

Fig. 8. The hydration is shown as a function of time for swelling of corneal stroma in distilled water. The swelling curve for a salt solution (pH 7, $\mu = 0{\cdot}02$) is shown in comparison.

Within the time course of our experiments, the swelling at pH 4 becomes zero, giving final constant values of the hydration which increase with increasing ionic strength, and are not very different from physiological hydration ($H \simeq 3{\cdot}5$–$4{\cdot}0$). The swelling also becomes close to zero at pH 2 but the final values of the hydration decrease with increasing ionic strength and are generally greater than corresponding values at pH 4. The most straightforward explanation of this effect is that the isoelectric point of the tissue is close to pH 4 and that the net fixed electric charge is zero at this point. The system can be considered as a polyelectrolyte gel and the behaviour close to the isoelectric point is governed by zwitterion pairs, which give attractive forces between the equal number of positive and negative charges (Katchalsky, 1954). Under these conditions the main effect of added salt is to screen the attractive forces and the system will swell more in higher salt concentration because the forces are less and the gel becomes looser. This is exactly the behaviour which we observed at pH 4.

II. *Effects away from the isoelectric point*

Moving the pH away from the isoelectric point will reduce the number of zwitterion pairs and will also produce a net fixed charge in the tissue. The net charge will be negative above the isolectric point and positive below it and will affect the swelling in two ways. First, the fixed charge will require more counterions in the gel to maintain the electrical neutrality. This accumulation of small permeant ions causes an excess internal osmotic pressure and will increase the amount of swelling. Secondly, the reduction in the number of attractive zwitterion pairs will cause a reduction in the attractive forces and thus will loosen the system, again causing increased swelling. This is the behaviour which we observe in the cornea. Swelling at pH 2 is larger than at pH 4, for a given ionic strength, and swelling at pH 6 is again larger than at pH 4. Increasing the pH above the isoelectric point from pH 6 to 10 produces a notable increase in the rate and amount of swelling. Above the isoelectric point, as the pH moves through the pK values of the various charged amino acids, an increase of pH will lead to an increase in the net fixed negative charge concentration in the stroma. This behaviour is also seen in other biological systems (e.g. muscle, Rome, 1968; Collins & Edwards, 1971; Elliott, Naylor & Woolgar, 1978).

Away from the isoelectric point the addition of salt has the opposite effect on the swelling than at the isoelectric point (Katchalsky, 1954). Screening reduces the repulsive forces between the negatively charged groups at pH values greater than 4. In this way the swelling capacity is reduced by increased salt as we observe for the swelling of the corneal stroma at pH 2 and in general at pH values above 4 (but see (IV) below).

III. *Donnan-osmotic theory of swelling rates*

Qualitatively, the pH dependence of the swelling in the range pH 6–10 is consistent with a Donnan-osmotic theory of swelling (Overbeek, 1956) so we have followed this concept further. The details of this analysis are given in the Appendix. We have assumed that the swelling pressure is totally Donnan-osmotic in origin, and that its value is as calculated by Hodson (1971). This swelling pressure causes water to flow into the stroma, and in calculating the flow we have used the experiments and calculations of Hedbys & Mishima (1962) and Friedman & Green (1971*b*). Although the

experimental fluid flow in their work was set up by hydrostatic pressure differences, we see no reason why the concepts should not be equally applicable to osmotic pressure differences.

In developing the analysis in the Appendix we have used the equilibrium Donnan theory in a non-equilibrium system, and it must be asked whether this is legitimate. Helfferich (1962) states that if the interface offers no resistance to the flow of solutes then the equations derived by Donnan are valid; this is likely to be the case with cornea, especially at high hydrations when there will be little resistance from the glycosaminoglycans. We have also ignored any inhomogeneity which may exist within the swelling cornea, and have assumed that any internal gradients are unimportant compared with the ionic gradient across the surface of the stroma (caused by the fixed charges on the stromal matrix, whose effective reflexion coefficient is unity at that surface).

Having made these assumptions we arrive at the conclusion (Appendix) that the time of swelling should be directly related to the function $G(H)$, where $G(H) = H^2/2 + 0{\cdot}67H$. The time course of this function is shown for $\mu = 0{\cdot}05$ and pH 7 in Fig. 9. The linear regression fit is represented by a continuous line through the data points. The details of the regressions (the slope $\pm$ standard error, intercept and number of data points) are given in Table 3 together with the f ratios for the regression. (The f ratio is the ratio of the variance of the regression data to the deviation of the regression data from the calculated values; see Weatherburn, 1968.)

All the values are significant at the 1 % level of f ratio tables, suggesting that the Donnan-osmotic assumption is correct at least as a first approximation. The slopes of

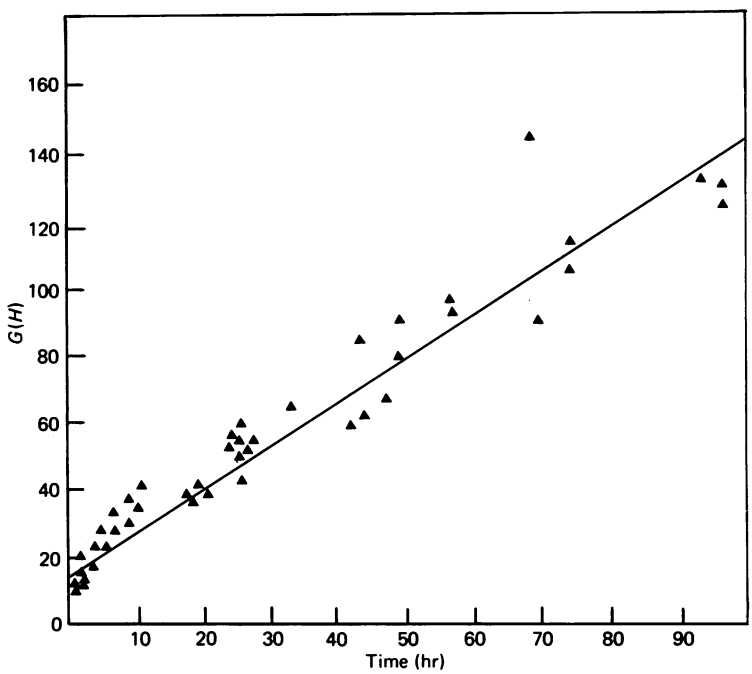

Fig. 9. The function $G(H)$ ($= H^2/2 + 0{\cdot}67H$, see Appendix) is plotted as a function of swelling for solution of $\mu = 0{\cdot}05$ and pH 7.

TABLE 3. The details of the computed linear regression fits of the swelling data for four pH values each at five ionic strengths. The function $G(H)$ ($=H^2/2+0{\cdot}67H$, see Appendix) gives a significant linear fit with the time of swelling. The slope, intercept and number of data points N^1 are given for each solution as well as the f ratios for the slopes which are all significant at the 1 % level

Solution					
μ	pH	Slope	Intercept	N^1	f ratio
0·02	6	2·58 ± 0·3	39	56	76
	7	3·60 ± 0·23	33	63	241
	8	3·92 ± 0·19	33	67	436
	10	4·94 ± 0·39	65	69	163
0·05	6	1·01 ± 0·05	15	45	381
	7	1·27 ± 0·05	17	45	643
	8	1·86 ± 0·15	14·6	35	147
	10	2·1 ± 0·13	36	66	254
0·1	6	1·02 ± 0·04	10·9	30	555
	7	1·06 ± 0·04	16·8	35	533
	8	1·28 ± 0·11	8·7	54	125
	10	1·41 ± 0·10	16·1	26	181
0·15	6	1·55 ± 0·06	15·5	83	729
	7	1·68 ± 0·08	18·9	93	402
	8	2·08 ± 0·20	27	93	106
	10	1·99 ± 0·14	28	90	196
0·25	6	1·62 ± 0·13	8·8	32	153
	7	0·76 ± 0·06	12·1	35	142
	8	1·1 ± 0·11	12·8	35	108
	10	1·48 ± 0·26	32	76	45

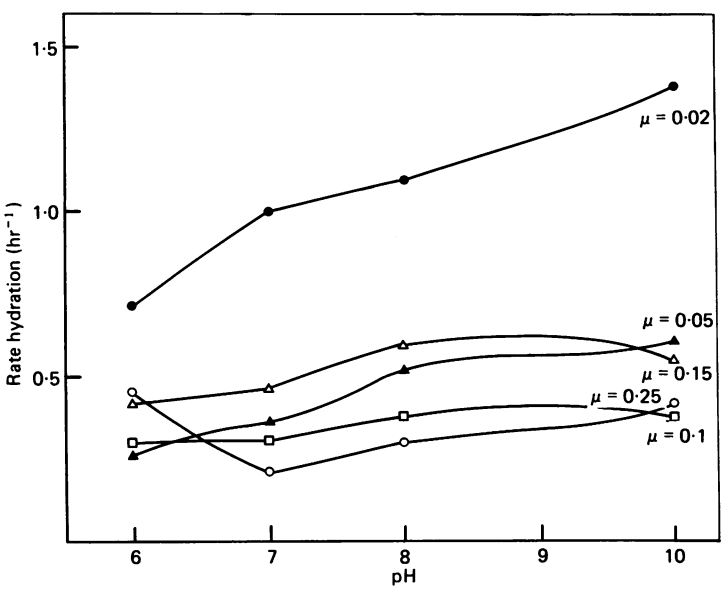

Fig. 10. The rate of hydration, estimated from the slope of the linear regression of $G(H)$ against time, as a function of the pH of the bathing solution at $H=3{\cdot}5$.

the regression lines can be used to calculate the rate of hydration, dH/dt, for any value of the hydration (see Appendix). This rate is shown in Figs 10 and 11 as a function of pH and ionic strength, respectively, at $H = 3{\cdot}5$.

It is of interest that the swelling rate at $\mu = 0{\cdot}1$ is less than that at $\mu = 0{\cdot}05$ and $\mu = 0{\cdot}15$ (all at pH 7). It is also of interest that the pH dependence of the swelling rate at $\mu = 0{\cdot}1$ is very small. Presumably the stroma is specialized to give a swelling pressure which while finite is small and relatively independent of μ and pH near to physiological conditions, so that the ion pumping mechanism which opposes the swelling (Hodson, 1971) works in a stable environment. Other features of Figs 10 and 11 probably relate to ion-binding to the protein which we will consider in section IV below.

Donnan-osmotic concepts can also explain the observation that in distilled water the volume first swells very rapidly but that the swelling rate decreases to zero after about 10 hr (Fig. 8). If the dried stroma (with its associated counter- and co-ions) is put into distilled water the swelling pressure is initially very large because the osmotic pressure of distilled water is zero and because there is a large internal concentration of diffusible ions. The diffusible counter- and co-ions will experience large concentration gradients so that they will begin to diffuse from the stroma, but the concentrations which they achieve in the bathing medium will always remain trivial supposing that there is a large excess of the (initially) distilled water. This implies that the external Donnan product will always be effectively zero so that the internal Donnan product must become so for equilibrium. The only way in which this can happen is for counter-ions to bind to the fixed charge groups so as to neutralize that fixed charge. When this has been achieved there will be no remaining net fixed charge and thus no further Donnan-osmotic swelling pressure. This interpretation is sup-

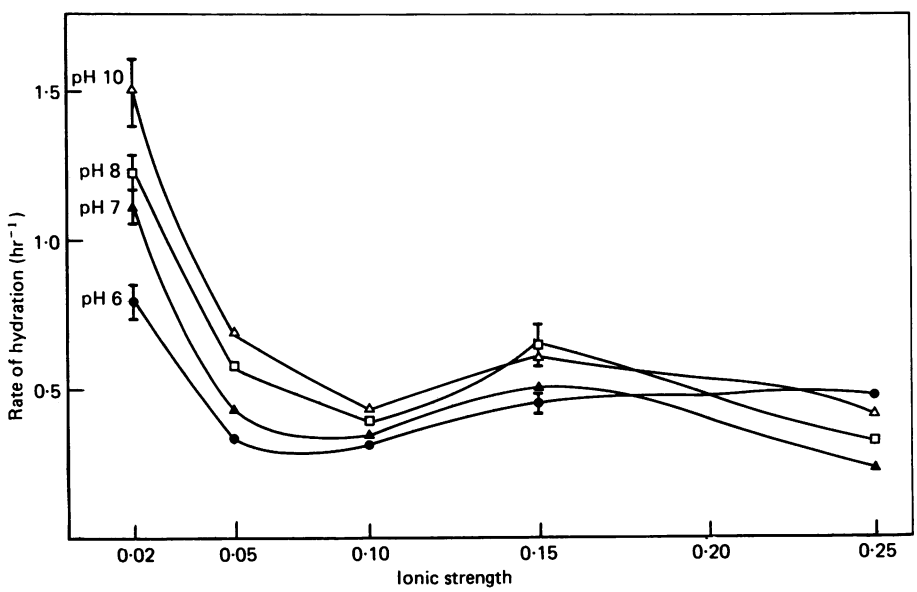

Fig. 11. The rate of hydration, estimated as in Fig. 10, as a function of the ionic strength of the bathing solution at $H = 3{\cdot}5$.

ported by the measurements of Green, Hastings & Friedman (1971), whose Table 1 shows that the fixed charge concentration in distilled water falls swiftly as a function of hydration, from about 43 mM at $H = 3{\cdot}6$ to about 13 mM at $H = 10{\cdot}6$ (expressing their values as mM fixed charge in the wet tissue).

Green *et al.* (1971), and several other authors, express the hydration as the percentage water content. Yet others (e.g. Farrell & Hart, 1969) express the hydration in terms of the ratio of the corneal thickness to its normal value. We have adopted the structurally convenient definition given under *Swelling* in the Methods.

IV. *The values of the fixed charge concentrations*

The regression fits of $G(H)$ can also be used to calculate the fixed charge concentration C_f (see Appendix). Values of this charge concentration, calculated at $H = 3{\cdot}5$, are shown in Table 4. Notice that the earlier points in the plots of $G(H)$ against t could often fit lines of higher gradient, sometimes by as much as a factor approaching 3 (e.g. Fig. 9). Since the gradient is proportional to the square of the charge concentration, the values shown in Table 4 might have to be increased by a factor which could approach $\sqrt{3}$.

The values shown in Table 4 are generally greater than the 12–16 mM reported by Friedman & Green (1971*a*) but are smaller than the 48 mM reported by Hodson (1971). Table 4 also shows that the (negative) fixed charge concentration increases with pH above the isoelectric point, as is expected, and also increases with the ionic strength of the bathing solution. This latter fact was previously observed by Green *et al.*

TABLE 4. The values of the fixed-charge concentration, C_f, estimated from the linear regression fits of the swelling data

Solution	pH	Fixed charge concentration (mM)
$\mu = 0{\cdot}02$	6	15
	7	18
	8	19
	10	21
$\mu = 0{\cdot}05$	6	15
	7	17
	8	20
	10	22
$\mu = 0{\cdot}1$	6	21
	7	22
	8	24
	10	25
$\mu = 0{\cdot}15$	6	32
	7	33
	8	37
	10	36
$\mu = 0{\cdot}25$	6	42
	7	29
	8	35
	10	41

(1971), who interpreted it as a *decrease* in the binding of Na^+ ions with an *increase* in the Na concentration, having made the assumption that there was no Cl^- binding. We are not convinced by this interpretation, and believe that a more logical approach is to assume the binding of negative ions (Cl^- in the work of Green *et al.* 1971, and Cl^- or phosphate in our own experiments). Moreover in the work of Green *et al.* (1971) and Friedman & Green (1971*a*) no pH buffers were used in the external salt solutions and the equilibration times for the radioisotopes were comparatively short (2 hr). We feel that these reasons, taken in all, may account for the numerical differences between the values of fixed charge derived from our experiments and those derived from the earlier work. The phenomenon of increasing negative charge with increasing strength of the bathing salt solution has also been observed for the myosin filaments in striated muscle in experiments where the measurements were made by a combination of micro-electrode and X-ray diffraction techniques (Elliott *et al.* 1978). We are continuing to measure the fixed charge concentration in stroma, particularly using the micro-electrode techniques which we have developed for muscle work.

General conclusions

From these experiments and from our X-ray work (Goodfellow, Elliott & Woolgar, 1978) it appears that the corneal stroma belongs to the group of systems which are formed from arrays (usually ordered) of cylindrical charged filaments or fibrils (Elliott, 1968). In muscle these charged filaments are proteins (mostly myosin and actin). The corneal stroma differs in detail, the protein collagen forms the cylindrical fibrils but the glycosaminoglycans certainly provide some of the fixed negative charge, though not all of it since some is probably due to the protein components, and also to negative-ion binding to the proteins. Our results could indicate that the glycosaminoglycans surround the collagen fibrils forming charged cylinders (Hodson & Meenan, 1969; Myers *et al.* 1973). Farrell & Hart (1969) have proposed an alternative model for the stroma in which the glycosaminoglycans form cross-links as suggested by Matthews (1965) for cartilage. This model is not supported by the electron microscopy of Myers *et al.* (1973) or that of Smith & Frame (1969) and we find it difficult to interpret our swelling results on any model which involves mechanical cross-links (at pH values away from pH 4).

In other systems, an equilibrium is obtained within 30 min between the osmotic pressure causing swelling and the attractive forces (e.g. in glycerinated muscle; see Rome, 1967, 1968). In muscle the attractive forces may be due to elastic cross-links or to van der Waals forces between neighbouring fibrils (Elliott, 1968; Rome, 1968; Miller & Woodhead-Galloway, 1971). As no equilibrium state is found in the corneal stroma at physiological pH and ionic strength, there presumably can be no effective force balancing the osmotic swelling pressure and thus no cross-links or osmotic constraint once the epithelium and endothelium have been removed.

There is a formal equivalence between Hodson's (1971) Donnan-osmotic swelling theory and the approach of Elliott (1968) which deals with the electrical double-layer repulsion between charged cylinders in an ionic solution. In both cases the swelling forces are due to the fixed charges and are modified by the nature of the surrounding salt solution. The detailed equivalence between these viewpoints remains a matter for theoretical investigation.

APPENDIX

The relation between rate of hydration and time

To compare the experimental swelling data with the theory of Donnan swelling of the corneal stroma, a relationship between the hydration of the stroma and the time of swelling was deduced from assumptions based on this theory of swelling. The basic assumption is that a Donnan distribution of ions exists between the stroma and the bathing solution. This distribution of small permeant ions depends on the fixed charge concentration in the stroma due to the charged groups of the glycosaminoglycans, etc. The value of the fixed charge concentration will vary with the hydration of the tissue as well as with the ionic strength and pH of the bathing solution.

The osmotic pressure difference, $\Delta\pi$, between the stroma and the external solution, which is the result of the distribution of permeant ions, can be expressed as a function of the fixed charge concentration, C_f, and the concentration of cations in the external solution, C_+, assuming that these are monovalent. (All concentrations are in molar units.)

$$\Delta\pi = RT((C_f^2 + 4\,C_+^2)^{\frac{1}{2}} - 2\,C_+).$$

This is equivalent to eq. (10) in Hodson, 1971. R is the gas constant (8·314 $J.K^{-1}$ mol^{-1}) and T is the absolute temperature, assumed to be 293 K. The expression under the square root in this equation can be expanded using the binomial theorem to give a simpler relationship between the osmotic pressure difference and the fixed charge concentration. Thus:

$$(1 + C_f^2/4C_+^2)^{\frac{1}{2}} = 1 + C_f^2/8C_+^2 - C_f^4/32\,C_+^4$$

The binomial expansion is convergent as long as C_f is less than $2C_+$; using the first two terms of the expansion gives:

$$\Delta\pi = RT\,C_f^2/4\,C_+. \tag{1}$$

The fixed-charge concentration will decrease as the hydration of the tissue increases, assuming that the total fixed charge remains constant throughout the experiment. Hence,

$$C_f \times H = Q, \tag{2}$$

where Q is a constant whose value depends on the quantity of glycosaminoglycans present, the pH and the ionic strength of the bathing solutions. H is the hydration of the tissue.

Substituting eqn. (2) into eqn. (1), the unknown value C_f can be eliminated, giving,

$$\Delta\pi = RTQ^2/4\,C_+H^2. \tag{3}$$

Thus for a given bathing solution the osmotic pressure difference is inversely proportional to the square of the hydration of the stroma. The rate of flow of fluid, J, per unit cross-section per unit time into the cornea can now be related to the osmotic pressure difference, using the arguments of irreversible thermodynamics (e.g. Katchalsky & Curran, 1967).

Hodson (1971) gives the rate of flow of fluid, J, as

$$J = \sigma\,L \times \Delta\pi, \tag{4}$$

where σ is the reflexion coefficient of the interface and L is the hydraulic conductivity of the stroma so that σL is an Onsager coefficient. (Note that Hodson (1971) is referring to the cornea with endothelium intact and in our experiments it is the stroma alone which is under discussion.) Assuming again that the swelling pressure is totally due to the osmotic pressure, substitution of eq. (4) into eqn. (3) gives

$$J = \sigma L\, RTQ^2/4\, C_+ H^2. \tag{5}$$

It is probable that the reflexion coefficient and the hydraulic conductivity will both depend on the hydration of the stroma, thus it might be expected that the more hydrated the stroma the less the resistance to flow of fluid. Experimentally, σ and L have been found only for the endothelium, which is not present in these experiments (Mishima & Hedbys, 1967).

Hedbys & Mishima (1962) measured the flow conductivity of the stroma which can be related to the reflexion coefficient and the hydraulic conductivity. Moreover, Friedman & Green (1971*b*) calculated the relationship between the flow conductivity and the hydration of the tissue based on the data of Hedbys & Mishima (1962). Following the nomenclature of Friedman & Green (1971*b*) and denoting the flow conductivity by K/η, then

$$J = -(K/\eta)\mathrm{d}P/\mathrm{d}x, \tag{6}$$

where J is the rate of flow of fluid and $\mathrm{d}P/\mathrm{d}x$ is the pressure gradient across the stroma. Following Fatt & Goldstick (1965), eq. (6) can be expanded as

$$J = -(K/\eta)(\mathrm{d}P/\mathrm{d}H)(\mathrm{d}H/\mathrm{d}x), \tag{7}$$

where $\mathrm{d}x/\mathrm{d}H$ is a constant, a, defined as the increase in thickness per unit increase in hydration.

J can be eliminated between eqns. (5) and (7), giving a relation between K and L

$$-K/\eta = \sigma L\, Pa/(\mathrm{d}P/\mathrm{d}H). \tag{8}$$

Once more we assume that the pressure in eqn. (7) is totally osmotic in origin. The value of the differential $\mathrm{d}P/\mathrm{d}H$ can be found by differentiating eqn. (3) (see eqn. 7), the expression for P in terms of hydration, to give

$$\mathrm{d}P/\mathrm{d}H = -2P/H. \tag{9}$$

Substituting eqn. (9) into eqn. (8) gives

$$\sigma L = (K/\eta)2/Ha. \tag{10}$$

Friedman (1971) and Friedman & Green (1971*b*) have shown that the data of Hedbys & Mishima (1962) are consistent with an empirical relation between the flow conductivity and hydration of the form

$$K/\eta = Q_1 H^3/(H+\epsilon), \tag{11}$$

where Q_1 and ϵ are constants and are equal to 31×10^{-9} cm^2 min^{-1} and 0·67 respectively. This expression (eqn. (11)) for the flow conductivity can be substituted into eqn. (10) so that the dependence of the hydraulic conductivity on hydration is given by

$$\sigma L = 2Q_1 H^2/a(H+\epsilon). \tag{12}$$

Substitution of eqn. (12) into eqn. (5) then gives

$$J = 2Q_1RTQ^2/a(H+\epsilon)4C_+. \tag{13}$$

The final step in the argument relates the flow of fluid into the cornea to the rate of increase of hydration of the tissue. Because the stroma increases in thickness in only one direction on swelling, it can be seen that

$$J = a\mathrm{d}H/\mathrm{d}t, \tag{14}$$

where a is the increase in thickness per unit increase in hydration ($a = \mathrm{d}x/\mathrm{d}H$, see above).

Combining eqns. (13) and (14) leads to

$$\mathrm{d}H/\mathrm{d}t = M/(H+\epsilon),$$

where

$$M = RTQ^2\,Q_1/2a^2C_+.$$

Rearranging this expression and integrating,

$$H^2/2 + H \times \epsilon = G(H) = Mt + D, \tag{15}$$

where D is a constant of integration.

Thus this theory, on the assumption that the swelling pressure of the corneal stroma is due to a Donnan-osmotic pressure difference, predicts that the time of swelling should be linearly related to $G(H)$, a function of the hydration

$$(G(H) = H^2/2 + 0{\cdot}67H).$$

One of the authors (J.M.G.) acknowledges the support of a Science Research Council post-graduate studentship. Our corneal research programme has also been supported by the Medical Research Council.

We are indebted to Dr David Maurice, Dr Ian Freeman and Dr Stuart Hodson for much encouragement and useful discussion of our results and to A. J. Bron, F.R.C.S. and the staff of the Nuffield Laboratory for Ophthalmology for their interest and advice.

REFERENCES

ANSETH, A. & LAURENT, T. C. (1961). Studies on the corneal polysaccharides: 1, separation. *Expl Eye Res.* **1**, 25–38.

COLLINS, E. W. & EDWARDS, C. (1971). Role of Donnan equilibrium in the resting potentials in glycerol-extracted muscle. *Am. J. Physiol.* **221**, 1130–1132.

ELLIOTT, G. F. (1968). Force – balances and stability in hexagonally packed polyelectrolyte systems. *J. theor. Biol.* **21**, 71–87.

ELLIOTT, G. F., NAYLOR, G. R. S. & WOOLGAR, A. E. (1978). Measurements of the electric charge on the contractile proteins in glycerinated rabbit psoas using microelectrode and diffraction effects. In *Ions in Macromolecular and biological systems*, Colston Papers No. 29, ed. EVERETT, D. H. & VINCENT B. Bristol: Scientechnica Press.

FARRELL, R. A. & HART, R. (1969). On the theory of spatial organisation of macromolecules in connective tissue. *Bull. math. Biophys.* **31**, 727–759.

FATT, I. & GOLDSTICK, T. K. (1965). Dynamics of water transport in swelling membranes. *Colloid Sci.* **20**, 962–987.

FRIEDMAN, M. H. (1971). General theory of tissue swelling with application to corneal stroma. *J. theor. Biol.* **30**, 93–109.

FRIEDMAN, M. H. & GREEN, K. (1971*a*). Ion binding and Donnan equilibria in rabbit corneal stroma. *Am. J. Physiol.* **221**, 356–362.

FRIEDMAN, M. H. & GREEN, K. (1971*b*). Swelling rate of the corneal stroma. *Expl Eye Res.* **12**, 239–250.

GOODFELLOW, J. M. (1975). Structural studies of the corneal stroma. Ph.D. Thesis, The Open University.

GOODFELLOW, J. M., ELLIOTT, G. F. & WOOLGAR, A. E. (1978). X-ray diffraction studies of the corneal stroma. *J. molec. Biol.* **119**, 237–252.

GREEN, K., HASTINGS, B. & FRIEDMAN, M. H. (1971). Sodium ion binding in isolated corneal stroma. *Am. J. Physiol.* **220**, 520–525.

HEDBYS, B. O. (1961). The role of polysaccharides in corneal swelling. *Expl. Eye Res.* **1**, 81–91.

HEDBYS, B. O. & MISHIMA, S. (1962). The flow of water in the corneal stroma. *Expl Eye Res.* **1**, 262–275.

HELFFERICH, F. (1962). *Ion Exchange*. New York: McGraw-Hill.

HODSON, S. (1971). Why the cornea swells. *J. theor. Biol.* **33**, 419–427.

HODSON, S. & MEENAN, A. (1969). The distribution of acidic mucopolysaccharides in corneal stroma. *Separatum Experimentia* **25**, 1305.

KATCHALSKY, A. (1954). Polyelectrolyte gels. *Prog. Biophys. biophys. Chem.* **4**, 1–59.

KATCHALSKY, A. & CURRAN, P. F. (1967). *Nonequilibrium Thermodynamics*. Cambridge, Mass.: Harvard University Press.

KINSEY, V. E. (1948). Spectral transmission of the eye for ultraviolet radiation. *Archs. Ophthal N.Y.* **39**, 508–513.

KINSEY, V. E. & COGAN, D. G. (1942). The cornea. III. hydration properties of excised corneal pieces. *Archs. Ophthal., N.Y.* **28**, 272–284.

LOEVEN, W. A. & VAN WALBEEK, K. (1954). Swelling and transparency of cornea and sclera as compared with a model system of pigskin gelatin and mucoitin sulphate. *Biochim. biophys. Acta* **14**, 471–481.

MAROUDAS, A. & THOMAS, H. (1970). A simple physiochemical micromethod for determining fixed anionic groups in connective tissue. *Biochim. biophys. Acta* **215**, 214–216.

MATTHEWS, M. B. (1965). The interaction of collagen and acid mucopolysaccharide. *Biochem. J.* **96**, 710–716.

MAURICE, D. M. (1969). In *The Eye*, 2nd ed., ed. DAVSON, H. Chapter 7. London: Academic Press.

MAURICE, D. M. & GIARDINI, A. A. (1951). Swelling of the cornea *in vivo* after the destruction of the limiting layers. *Br. J. Ophthal.* **35**, 791–797.

MILLER, A. & WRAY, J. S. (1971). Molecular packing in collagen. *Nature, Lond.* **230**, 437–439.

MILLER, A. & WOODHEAD-GALLOWAY, J. (1971). Long range forces in muscle. *Nature, Lond.* **229**, 470–473.

MISHIMA, S. & HEDBYS, B. O. (1967). The permeability of the corneal epithelium and endothelium to water. *Expl Eye Res.* **6**, 10–32.

MYERS, D. B., HIGHTON, T. C. & RAYNS, D. G. (1973). Ruthenium-red positive filaments interconnecting collagen fibrils. *J. Ultrastruct.* Res. **42**, 87–92.

OTORI, I. (1967). Electrolyte content of rabbit corneal stroma. *Expl Eye Res.* **6**, 356–367.

OVERBEEK, J. Th. G. (1956). The Donnan equilibrium. *Prog. Biophys. biophys. Chem.* **6**, 58–84.

PAU, H. (1954). Beitrag zur Physiologie and Pathologie der Hornhaut. *Albrecht v. Graefes Arch. Ophthal.* **154**, 579–602.

PAYRAU, P. & POULIQUEN, Y. (1960). Conservation des cornées et des sclères par silico-dessiccation; homogreffes et heterogreffes. *Annls Oculist.* **193**, 309–345.

PAYRAU, P., POULIQUEN, Y., FAURE, J. P. & OFFRET, G. (1967). *Le Transparence de la Cornée, les Mechanismes de ses Alterations*. Paris: Masson & Cie.

ROME, E. (1967). Light and X-ray diffraction studies of filament lattice of glycerol extracted rabbit psoas muscle. *J. molec. Biol.* **27**, 591–602.

ROME, E. (1968). X-ray diffraction studies of the filament lattice of striated muscle in various bathing media. *J. molec. Biol.* **37**, 331–344.

SMELSER, G. K. (1962). Corneal hydration; comparative physiology of fish and mammals. *Invest. Ophthal.* **1**, 11–32.

SMITH, J. W. & FRAME, J. (1969). Observations on the collagen and protein polysaccharide complex of rabbit corneal stroma. *J. cell. Sci.* **4**, 421–436.

VAN WALBEEK, K. & NEUMANN, H. (1951). Studies of corneal transparency under various experimental conditions. *Archs. Ophthal., N.Y.* **46**, 482–487.

WEATHERBURN, C. E. (1968). *A First Course in Mathematical Statistics* pp. 196–200 and 223–226. Cambridge: Cambridge University Press.