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TRANSIENT CHLORIDE BINDING AS A CONTRIBUTORY FACTOR TO CORNEAL STROMAL SWELLING IN THE OX

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SUMMARY

1. Investigations were made of the cation exchange capacity of fresh isolated ox corneal stroma (Q, units: mequiv fixed stromal charge/kg stromal fluid) at pH 7·4 over a variety of stomal hydrations (H, units: kg stromal fluid/kg dry tissue) both above and below the physiological hydration of 3·2, whilst the stromas were immersed in a variety of sodium chloride solutions (range 5–1000 mM).

2. At any particular salt concentration, the product QH (dry tissue exchange capacity, units: mequiv/kg dry tissue) appeared constant, over all the hydrations investigated.

3. Dry tissue exchange capacity (QH) varied, however, when the bathing salt concentration was altered. It varied between 55 mequiv/kg dry tissue (e.g. Q = 17 mequiv at $H = 3{\cdot}2$) in 5 mM-NaCl to 240 mequiv/kg dry tissue (e.g. Q = 75 mequiv/l at $H = 3{\cdot}2$) in 1000 mM-NaCl.

4. The variation of stromal exchange capacity in NaCl solutions of different concentrations was similar when detected by three independent procedures: stromal gel pressure measurements, intrastromal sodium ion distributions, and intrastromal electrical potentials.

5. Intrastromal chloride ion distributions were anomalous. Total chloride (measured by radio-isotopes) was consistently higher than that predicted by Donnan theory.

6. The data were consistent with Elliott's hypothesis that a fraction of intrastromal chloride ions bind to the corneal stromal matrix and in so doing contribute to the fixed negative charge of the stroma.

7. Our observations may be explained by a model of the cation exchange capacity of ox cornea which has two types of components. On is (at constant pH) invariant, and has a dry tissue exchange capacity of about 50 mequiv/kg dry tissue, and is probably generated by the sulphonic and carboxylic acid groups of the glycosaminoglycans. The other is explained by supposing it to consist of a chloride binding ligand which exhibits first order binding, is half occupied at ambient chloride concentrations of 300 mM, and has a total capacity of 240 mequiv/kg dry tissue.

8. Partial stromal extraction with 4 M-guanidine HCl indicated that the chloride

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binding ligand is not associated with the collagen molecules in the corneal stromal fibrils.

9. It is suggested that such a stromal chloride ion binding ligand would help to stabilize the hydration and transparency of the living cornea when it is exposed to environments of varying tonicity (such as in river or sea bathing).

INTRODUCTION

It has been suggested that passive swelling of mammalian corneal stromas is driven by Donnan potentials (Hodson, 1971), and when the intrastromal electrical potential (Elliott, 1980), the rate at which the corneal stroma swells (Elliott, Goodfellow & Woolgar, 1989) and the stromal gel pressure (Hodson, O'Leary & Watkins, 1991) are measured in isotonic saline, the quantitative fit to the Hodson–Donnan theory is very good. In the theory, the fixed negative electrical charges of the stromal matrix molecules (i.e. their cation exchange capacity) generate electrical and osmotic imbalances and, because in the Hodson model there is supposed to be little or no significant chemical cross-linking between neighbouring collagen fibrils, the model is relatively simple to use and unambiguous in its predictions. In the most intensively studied stroma (that of the ox), it is found that all the associated phenomena (corneal gel pressure, intrastromal electrical potentials, stromal swelling rates) may be explained by a fixed negative charge (nominated Q) of 40 mequiv/kg stromal fluid at the physiological hydration (H, units: kg stromal fluid/kg dried tissue) of 3·2 when the stroma is immersed in physiological saline (154 mM-NaCl).

In initial treatments of the cation exchange capacity of corneal stroma (Otori, 1967; Hodson, 1971) it was assumed that the fixed negative changes arose from the sulphonic and carboxylic acid groups of the stromal glycosaminoglycans which would make the magnitude of Q a function of pH (depending on the pK_a of the acid groups). Otherwise, it was believed, there would be a (commonsense) dependence of Q only upon the hydration of the corneal stroma. It became clear, however, that Q was additionally dependent upon a third factor: the concentration of salt in the bathing medium. The simple observation was that free stromas swelled 'too slowly' in hypotonic saline (suggesting a reduced stromal fixed charge) and 'too quickly' in hypertonic saline (suggesting an enhanced stromal fixed charge). (For a review of the data see O'Leary, 1985). Elliott and his co-workers (Elliott, 1980; Elliott *et al.* 1980) proposed an explanation for the observation that the calculated cation exchange capacity, Q, depended upon the concentration of salt present. He suggested that the chloride ion is able to bind to some unidentified ligand in the corneal stromal matrix and this 'bound' chloride provided a significant contribution to the physiological value of Q.

We recently developed a simple osmotic method for measuring corneal gel pressure (Hodson *et al.* 1991) by immersion in saline solutions including (non-penetrating) polyethylene glycol – nominal molecular weight 10000 Da (acronym PEG10K). We have used this technique to check Elliott's (1980) chloride binding theory by an independent method.

METHODS

Ox eyes were transported over ice from the abattoir and used on the same day. Experiments were performed at room temperature (20–22 °C) on ox corneas dissected from the eye with (usually) an annulus of sclera about 3 mm wide.

Corneal gel pressure at various salt concentrations

Six preparations were individually immersed in 50 ml of lightly buffered (1 mM-HEPES, pH 7·4) solution containing a combination of 5, 10, 30, 60, 150, 300, 600 or 1000 mM-NaCl crossed with 2, 3, 4, 5, 6, 7 or 8% polyethylene glycol (BDH organics) nominal molecular weight 10000 Da (PEG10K) giving in all fifty-six combinations of solutions and 336 preparations. They were each gently agitated intermittently until they achieved equilibrium weight, which took less than 4 h. At this equilibrium, the osmotic pressure of the external non-penetrating PEG10K balanced the stromal gel pressure (with which the corneal stroma strives to swell). For details of the method please refer to Hodson *et al.* 1991. After 4 h, the equilibrated corneas together with their scleral rims were removed from their solutions, drained and gently blotted with tissues and an 8 mm diameter biopsy was trephined from the central cornea. The sharp trephine was attached to a clavulus to ensure a clean vertical cut through the preparation which lay anterior surface down on a moulded wax table. The biopsy, called here the 'corneal button', was gently released from the trephine and rapidly weighed to within 10 μg (the hydrated biopsies weighed in the range 30–50 mg). Each button was dried to constant weight under an infrared lamp for 2 h and the tissue dry weight (typically 11 mg) was determined also to within 10 μg. To check if tissue salt deposits were significant, the buttons exposed to high salts were rinsed in de-ionized water and then re-dried to constant weight. These corrections were very small. Hydration of the cornea (H) was calculated from tissue water weight, i.e. (total tissue weight − tissue dry weight): tissue dry weight ratio. During the experiment, the NaCl is free to penetrate into the corneal stroma to equilibrium, but PEG10K cannot and its osmotic pressure contribution to the external solution (calibrated after the method given in Hodson *et al.* 1991) is equal to the corneal stromal gel pressure. Consequently, this method gave a value for stromal hydration and stromal gel pressure for each preparation.

Intrastromal concentrations of total sodium and chloride

Ox corneas were dissected with their scleral rims and were conditioned by immersion in 20 ml of 1 mM-HEPES buffered at pH 7·4 including 30 mM-NaCl and 7·7% (w/w) PEG10K ($n = 8$), 60 mM-NaCl and 6·5% (w/w) PEG10K ($n = 8$), 154 mM-NaCl and 4% (w/w) PEG10K ($n = 22$), 300 mM-NaCl and 3·4% (w/w) PEG10K ($n = 8$) or 600 mM-NaCl and 2·4% (w/w) PEG10K ($n = 8$). The experimental solutions, described above were arranged (from data gathered from the previously described experiment) so that whilst the total NaCl in the bathing solution was altered, the average final hydration was always the same. After 30 min, the preparations were removed, drained, gently blotted and then transferred to 10 ml of a chemically identical solution including either 37 kBq ^{22}NaCl (for intrastromal total sodium determinations) or 32 kBq Na^{36}Cl (for intrastromal total chloride determinations) and allowed to equilibrate for 3 h with gentle agitation. Each preparation was then drained, gently blotted and then rapidly de-epithelialized with a motorized rotating bristle brush. (The reason for removing the epithelium was because we confirmed the observation of Otori (1976) that corneal epithelium has a very slowly exchanging sodium pool, which significantly interferes with the determination of stromal sodium concentrations. Although no such effect was noted with chloride determinations, we standardized our observations on de-epithelialized preparations.) A central 8 mm diameter was then taken from the central stroma, weighed and then the tissues were individually back-extracted into 2 ml of saline of the same tonicity as that of the loading solution. Preliminary experiments on the efflux rate of the isotopes indicated a single log linear efflux of isotope and effective extraction (> 99%) after 65 min. Corneal buttons were extracted, rinsed in distilled water, and dried to determine (a) the biopsy's hydration and (b) the biopsy's (small) volume contribution to the extraction fluid. From the extraction fluid, triplicate samples of 0·5 ml were each added to 5 ml Ecoscint in plastic vials and counted in a scintillation counter to 10000 sample counts. Their activity was compared to equivalent samples taken from the loading solution after the biopsies were removed. From these experiments it was possible to determine the total sodium and chloride concentrations within the corneal stroma at physiological hydrations whilst immersed in a variety of concentrations of NaCl solutions.

Ox corneal electrical potential

It proved to be difficult to achieve stable readings of electrical potentials within ox corneal stroma when they were stabilized by immersion in PEG10K. Penetration of glass microelectrodes through either the epithelium (covering the anterior surface) or Descemet's membrane (covering the posterior surface) led to unpredictable tip damage. An 8 mm diameter biopsy was taken from the central region of a de-epithelialized ox cornea and then bisected across its diameter. The D-shaped sample was then clamped between two glass slides separated (by a spacer) by a known distance so that its straight edge lay slightly recessed below the parallel edges of the glass slides. Stromal penetration by a borosilicate glass microelectrode filled with 154 mM-KCl (tip resistance 600–800 M) was then possible. Details of the method for recording the potentials may be found in Hodson & Wigham (1989). Average intrastromal potentials were measured after tissues had equilibrated against 150, 60, 30 or 10 mM-NaCl buffered at pH 7·4 with 1 mM-HEPES. Tissue hydration was determined as described above.

Guanidine extraction experiments

De-epithelialized ox cornea of diameter 8 mm were clamped between perforated Perspex plates to an average hydration of about 3–4 and immersed in 50 ml of 4 M-guanidine hydrochloride which extracts proteoglycans from corneal stroma (Gyi, 1988). The solution was vigorously stirred at ambient temperatures (20–24 °C) and after 3 days of extraction, the biopsies were unclamped, blotted, and allowed to swell in 154 mM-NaCl solutions. At 15 min intervals for the following 2 h the weight of the biopsy ($n = 8$) was measured. After 2 h, the biopsies were dried to constant weight and their weights were recorded. Control experiments (omitting the 3 day guanidine hydrochloride extraction) were performed on ox corneas taken from 3 day postmortem ox eyes. During the course of these investigations it was remarked that corneas taken from 3 day postmortem eyes showed remarkably similar swelling and intrastromal concentrations of sodium and chloride to those properties of fresh ox corneas. Dry weights of guanidine hydrochloride extracted were compared to the dry weights of both 3 day and fresh postmortem ox eyes.

Ox corneas extracted for 3 days in guanidine hydrochloride show a greatly diminished tendency to swell and advantage was taken of this observation to determine intrastromal sodium and chloride concentrations without the need for PEG10K in the bathing medium. Extracted corneas were washed for 1 h in 150 mM-NaCl in order to condition the preparation and then immersed in 10 ml 150 mM-NaCl including 74 kBq ^{22}NaCl (for intrastromal total sodium determinations, $n = 8$) or 64 kBq $Na^{36}Cl$ (for intrastromal total chloride determinations, $n = 7$). The extracted corneal stromas were loaded, with continuous stirrings, for 3 h, removed, blotted, weighed and back-extracted in 2 ml of 150 mM-NaCl solution for 90 min. Each corneal button was then removed from the unloading solution, blotted and dried to constant weight. Extracts (0·5 ml × 3) were added to 5 ml Ecoscint and counted as described above in a scintillation counter where the activity was compared to that in the original loading solution.

RESULTS

When corneal gel pressure ($\Delta\gamma$, which is the chemical potential of water in corneal stroma) is examined as a function of corneal hydration (H), a family of curves is obtained (Fig. 1). It is clear that the concentration of salt in the bathing medium has a significant effect upon the relationship. When the bathing salt concentration is 154 mM-NaCl, it has previously been established that the $\Delta\gamma$, H relationship may be adequately explained by the Donnan theory (Hodson *et al.* 1991) where the relationship between Q, cation exchange capacity of the stroma, C, the concentration of salt in the bathing medium and the gel pressure ($\Delta\gamma$) is predicted as:

$$Q^2 = \Delta\gamma^2 + 4C\Delta\gamma \quad (1)$$

From this relationship, and the measured values of tissue hydration (H), it was possible to calculate the dry tissue exchange capacity (QH) and when this was

plotted against hydration (H) the tissue exchange capacity appears constant over the range of hydrations tested (Fig. 2) for any particular salt concentration. It is clear, however, that on this type of analysis, the dry tissue cation exchange capacity varies with the concentration of salt in the bathing medium.

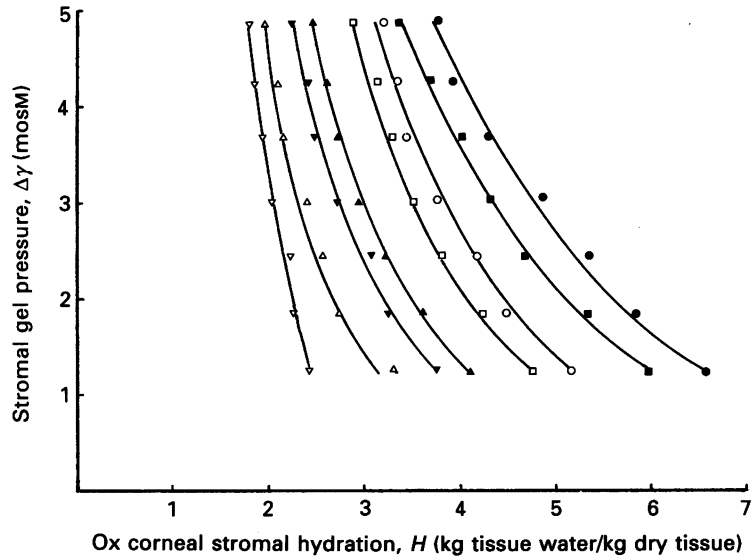

Fig. 1. The gel pressure ($\Delta\gamma$) of ox corneal stroma depends upon stromal hydration (H) and the concentration of NaCl in the bathing medium, whose various concentrations are (mM): ▽, 1000; △, 600; ▼, 300; ▲, 150; □, 60; ○, 30; ■, 10; ●, 5.

It is, for several purposes, convenient to use the value of Q_p, the cation exchange capacity of ox corneal stroma at the physiological hydration, H, of 3·2. These values of Q_p are calculated from Fig. 2 and plotted against salt concentration in the bathing medium, C (Fig. 3). It can be seen that Q_p increases with salt concentration to a saturating value and that the curve indicates a positive value for Q_p at 'zero salt'. The curve is a best fit to a model of corneal exchange capacity whose derivation will be explained later.

Intrastromal total sodium concentrations

When the stroma was maintained at physiological hydration, $H = 3{\cdot}2$, in 154 mM-NaCl, the total stroma sodium concentration was measured at $172{\cdot}4 \pm 3{\cdot}4$ mequiv/l (means ± S.E.M., $n = 22$). If the epithelium was retained on the preparation, whole corneal analysis gave a significantly lower average tissue sodium concentration of $167{\cdot}7 \pm 2{\cdot}2$ mequiv/l (mean ± S.E.M, $n = 8$). The existence of this epithelial effect has been noted previously in the rabbit (Otori, 1967). If we take the values of physiological stromal cation exchange capacity under these conditions of $39{\cdot}5 \pm 0{\cdot}8$ mequiv/l, (Hodson *et al.* 1991) then the Donnan relationship between the intrastromal sodium concentration (Na_i^+), the concentration of salt in the bathing medium (C) and Q is:

$$Na_i^+ = \frac{Q + (Q^2 + 4C^2)^{\frac{1}{2}}}{2}, \qquad (2)$$

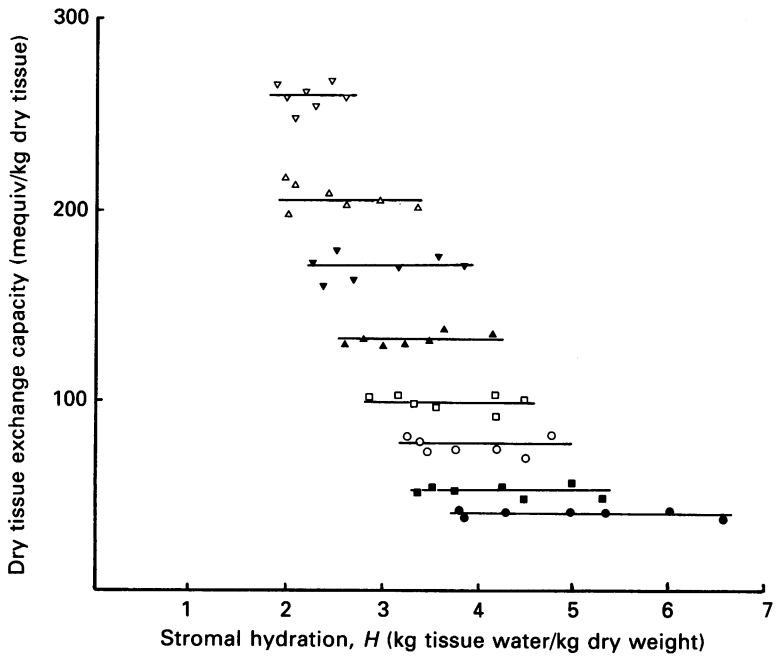

Fig. 2. The data represented in Fig. 1 is transformed (see text eqn (1)) from Donnan theory to give dry tissue fixed negative charge (QH) which appears nearly constant at any salt concentration. Symbols as in Fig. 1.

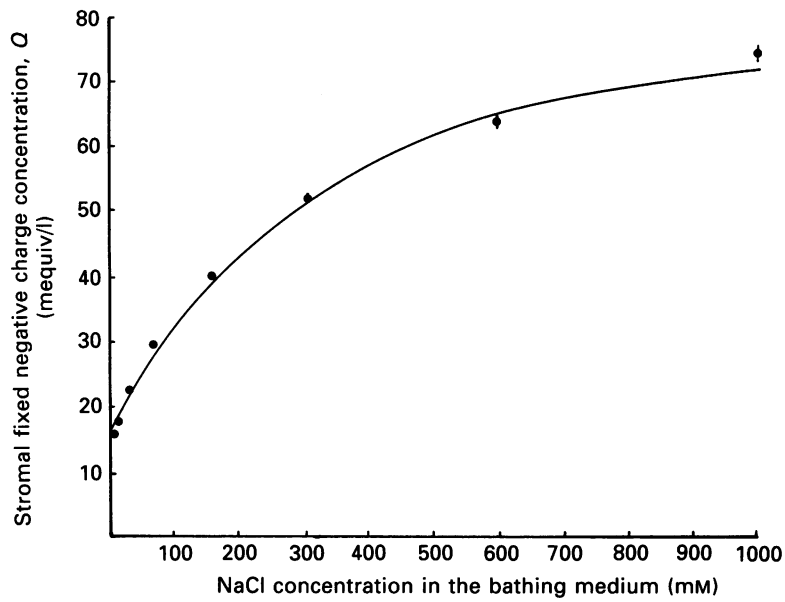

Fig. 3. The fixed negative charge (Q_p) of the cornea varies with salt concentration. The curve is a best fit for a model which assumes a chloride binding ligand (dissociation constant, $K_m = 300$ mM) to exist within the corneal stroma. Half-bars represent one standard error.

which predicts Na_i^+ to be $175{\cdot}0 \pm 0{\cdot}5$ mequiv/l. This is not significantly different from our measured value and Donnan theory is further supported by this correspondence which indicates that just about all the corneal stromal sodium is in free solution.

Intrastromal sodium concentrations were recorded (by radiotracer experiments) over a wide range of concentrations of NaCl in the bathing medium (C). In all cases

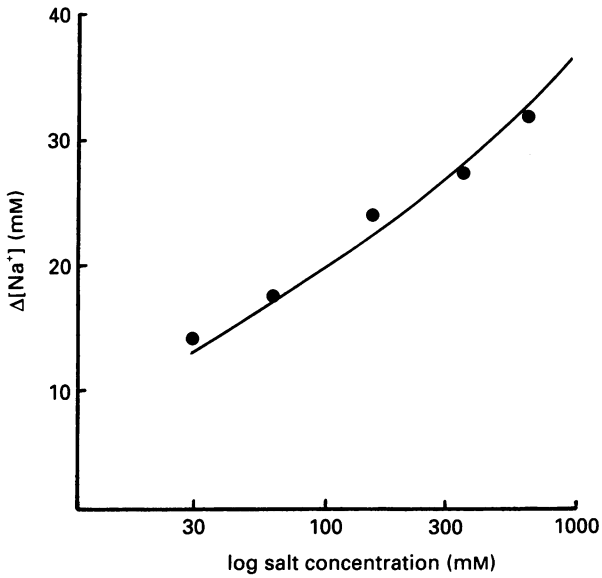

Fig. 4. The excess sodium concentration ($\Delta[Na^+]$) within ox corneal stroma above its concentration C (mM) in the bathing medium. Each point represents the mean of eight determinations. The curve is a best fit for a model which assumes a chloride binding ligand (K_m = 300 mM) to exist within the corneal stroma.

(Fig. 4) the intrastromal sodium concentration was higher than the sodium in the bathing medium. It is possible to calculate stromal fixed charge Q from these data by the Donnan relationship:

$$\frac{(Na_i^+)^2 - C^2}{Na_i^+} = Q, \tag{3}$$

and these values of Q_p (at the physiological hydration of 3·2) calculated from the measured intrastromal sodium concentrations are shown in Fig. 5. The errors in these determinations of Q_p are greater than those calculated from gel pressure determinations (Fig. 3) but for purposes of comparison, the same best-fit curve from Fig. 3 is reproduced in Fig. 5.

Intrastromal total chloride concentrations

Table 1 shows how the measured total intrastromal chloride concentration varies with NaCl concentration in the bathing medium at physiological hydration of 3·2. Determinations of intrastromal chloride were noticeably more reproducible than the

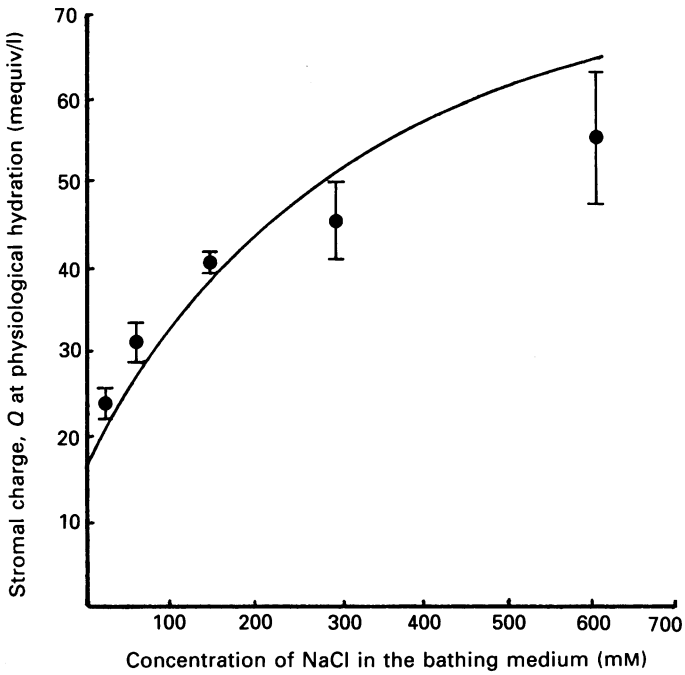

Fig. 5. The fixed negative charge (Q_p) of ox corneal stroma calculated from the sodium distributions in Fig. 4 for various concentrations of NaCl in the bathing medium. The curve is not fitted to the points but represents the values expected from chloride binding (see text for details). Half-bars represent one standard error.

TABLE 1. The relationship between the passively distributed total intrastromal chloride concentration (Cl_i^-) in ox corneal stroma at physiological hydration 3·2 and chloride in the bathing medium (Cl_o^-)

Cl_o^- (mM)	Cl_i^- (mM)	n
30	26·4 ± 0·4*	8
60	56·7 ± 0·8	8
150	150·4 ± 0·8	20
300	307·9 ± 4·2	8
600	605·5 ± 5·6	8
1000	1014 ± 8	8

* Means ± S.E.M.

determinations of intrastromal sodium (e.g. when eight pairs of ox corneas were bathed in 154 mM-NaCl, the intrastromal chloride was 150 ± 2·5 mM (mean ± S.D.), whereas the intrastromal sodium was 173 ± 12 mM (mean ± S.D.)). The evaluation of the data in Table 1 is the central consideration of this study and will be discussed later, but it is clear that total intrastromal chloride concentrations are significantly higher than Donnan theory would predict for a cation exchanger (such as the corneal stroma) where the intrastromal free chloride concentrations (Cl_i^-) would be consistently lower than the chloride in the bathing medium, Cl_o^-.

Intrastromal electrical potentials

Our results were similar in magnitude to those reported by Goodfellow (1975). We found a standard deviation associated with the determination of intrastromal electrical potentials under standard conditions of around 4 mV. It was not clear

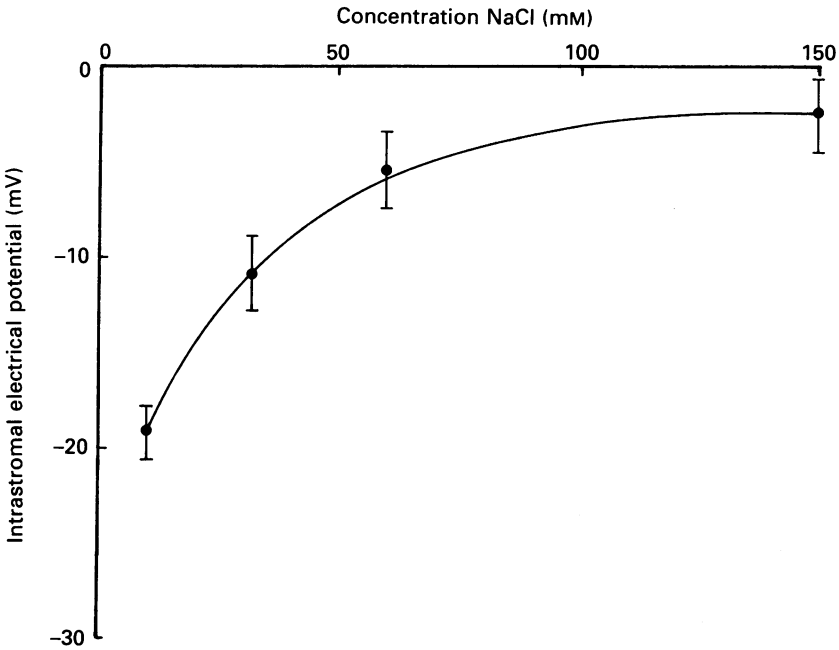

Fig. 6. The spontaneous intrastromal electrical potential, at physiological hydration 3·2, varies with the concentration of NaCl in the bathing medium. Half-bars represent one standard error.

whether this variation represented tissue variation or technique variation, because the variation was as great in the same tissue (possible represented by local fixed charge variation) as in different samples of ox cornea. In high salt concentrations (greater than 150 mM-NaCl) it was not possible to establish any values of intrastromal electrical potentials significantly different from zero. Determination of potentials were considered to be acceptable only if two criteria were fulfilled: first, that microelectrode tip potential and resistances did not vary significantly pre- and post-penetration, and second, that tissue hydration values (determined after the electrical determinations) were within the range 3·0–3·4 (physiological hydration is $3{\cdot}2 \pm 0{\cdot}2$ (mean $\pm$ S.D.)). Eight preparations were made for each salt concentration on six different preparations. It was noted that stable stromal potentials were only recorded when the tip was at least 10 μm within the tissue's cut edge. The results of these determinations are shown in Fig. 6. It is possible, from these data, to calculate from Donnan theory what fixed stromal charge (Q) would generate these stromal potentials from the relationship between V, stromal potential (in mV), Q and C (bathing salt concentration):

$$Q = C(10^{-\frac{V}{57}} - 10^{\frac{V}{57}}). \quad (4)$$

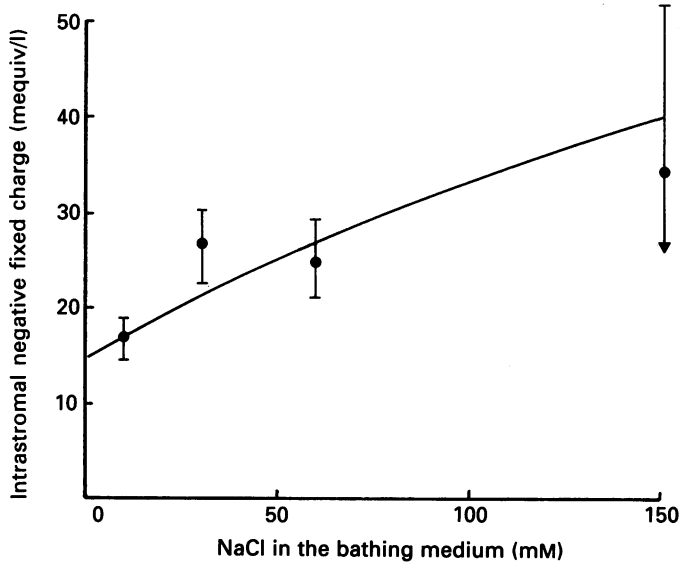

Fig. 7. Stromal cation exchange capacity calculated from text eqn (4) and the intrastromal electrical potentials (Fig. 6) as a function of NaCl concentration in the bathing medium. The curve is not fitted to the points but is derived (see text) from the chloride binding assumption. Half-bars represent one standard error.

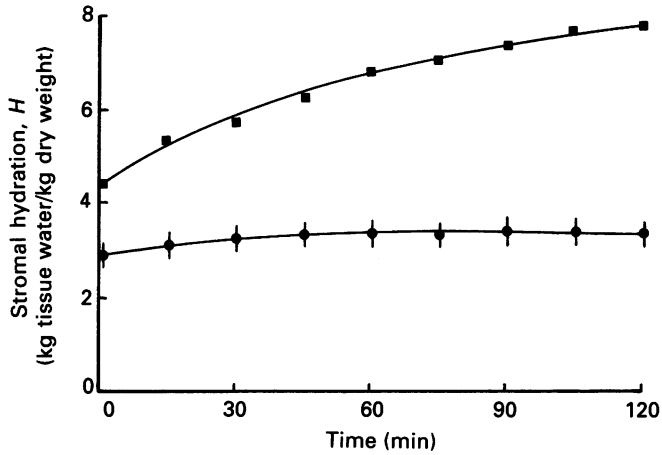

Fig. 8. Changes in hydration (H) with time of ox corneal stromas immersed in 150 mM-NaCl solution after 3 days storage in the excised eye (■) and after 3 days extraction in 4 M-guanidine hydrochloride (●) where the swelling rate is slowed about 20-fold. Half-bars represent one standard error.

The calculations are presented graphically in Fig. 7, where it will be seen that the errors associated with these calculations are so large as to render the significance of the data doubtful. Nevertheless it does seem that there is an increase of Q with increasing salt concentration and the data does not contradict the model curve (fitted to Figs 3 and 5 also) whose origin will be described later.

Guanidine hydrochloride-extracted corneas

When ox corneas were clamped for 3 days in 4 M-guanidine hydrochloride, washed in 154 mM-NaCl and then released, their swelling rates were considerably diminished (Fig. 8). Determination of the intrastromal total sodium and chloride concentrations of 3-day guanidine-extracted corneas equilibrated against 154 mM-NaCl gave intrastromal sodium values of $159{\cdot}6 \pm 1{\cdot}4$ mM ($n = 14$) and intrastromal chloride values of $151{\cdot}9 \pm 1{\cdot}6$ mM ($n = 14$). These twenty-eight corneas had a mean hydration of $4{\cdot}1 \pm 0{\cdot}1$ (mean $\pm$ S.E.M.). The dry weights of the guanidine-extracted corneas were significantly lower $9{\cdot}52 \pm 0{\cdot}11$ mg (mean $\pm$ S.E.M.) than fresh biopsies whose weight was $11{\cdot}4 \pm 0{\cdot}02$ mg which represents a 16% loss of dry weight after 3 days extraction in 4 M-guanidine HCl.

DISCUSSION

Ox corneal stroma has swelling properties in isotonic saline (Elliott, 1980; Elliott *et al.* 1980; Hodson *et al.* 1991) which can be quantitatively explained by the Donnan theory of corneal swelling (Hodson, 1971). The single variable which regulates all the phenomena associated with the Hodson–Donnan theory of corneal swelling is the cation exchange capacity, Q, of the matrix molecules. Part of the cation exchange capacity of the cornea is donated by the carboxylic and sulphonic acid groups of the glycosaminoglycans (Hodson, 1971) which are associated with the collagen fibrils (Scott, Orford & Hughes, 1981; Wall, Elliott, Gyi, Meek & Branford-White, 1988). Elliott and his co-workers (Elliott, 1980; Elliott *et al.* 1980) proposed that a substantial contribution to the cation exchange capacity Q arose by chloride ions binding to some unidentified ligand in the stromal matrix. It was the purpose of this investigation to examine Elliott's suggestion of 'chloride binding'. As the amount of chloride bound to any such stomal ligand would depend upon the concentration of free chloride ions, we examined the changing properties of the corneal stroma as a function of changing the bathing salt concentrations. As the free intrastromal chloride increased then any such chloride binding to the ligand should also increase.

This proposal we found to be the simplest explanation of our data: a chloride binding ligand exists in ox corneal stroma which exhibits first order kinetics, a K_m (dissociation constant) of 300 mM and a total capacity of 75 mequiv/l at physiological hydration, 3·2. When the chloride ion is complexed, the cation exchange capacity of the stroma correspondingly increases.

The reaction is between the free ligand (L, units mM) of single valency which binds reversibly with free intrastromal chloride ions (Cl_i^-, units: mequiv/l stromal fluid) and consequently produces a ligand: chloride ion complex (LCl^-, units: mequiv/l stromal fluid). The reaction is:

$$L + Cl_i^- \rightleftharpoons LCl^-, \tag{5}$$

and the dissociation constant K_m (mM) may be written:

$$\frac{[L][Cl_i^-]}{[LCl^-]} = K_m, \tag{6}$$

where square brackets represent concentrations.

Total ligand (nominated L_T) is the sum of the free ligand (L) and complexed ligand (LCl^-) and so eqn (6) may have L substituted out:

$$\frac{([L_T]-[LCl^-])[Cl_i^-]}{[LCl^-]} = K_m, \tag{7}$$

and be re-arranged to give:

$$\frac{[L_T]}{[LCl^-]} = \frac{K_m}{[Cl_i^-]} + 1. \tag{8}$$

Consequently, when $[Cl_i^-] = K_m$, half the total ligand is free and the other half is complexed to a chloride ion and at high values of Cl_i^- ($[Cl_i^-] \to \infty$ all the ligand is complexed ($LCl^- \to L_T$).

$[LCl]_i$ is the contribution to stromal fixed charge Q from the coupled chloride and may be renominated Q_{Cl}. The other contribution to fixed charge will be from the acidic groups of the corneal glycosaminoglycans and will be invariant and is nominated Q_i for their final concentration depends only upon the hydration of the stroma but not the concentration of salt.

$$Q = Q_{Cl} + Q_i, \tag{9}$$

$$\frac{[L_T]}{Q-Q_i} = \frac{K_m}{[Cl_i^-]} + 1. \tag{10}$$

Although it is not possible to measure $[Cl_i^-]$ by any of the techniques described in this paper (radioactive labelling gives free chloride plus chloride complexed to the ligand) it is readily possible to calculate $[Cl_i^-]$ from the Donnan relationships and knowledge of corneal gel pressure ($\Delta\gamma$), or intrastromal potentials or intrastromal sodium concentrations. This simple theory is the source of the best-fit curve of the weighted mean of these three independent determinations which is shown in Figs 3, 5 and 7 where $K_m = 300$ mM and, at the physiological hydration of 3·2, $L_T =$ 75 mequiv/l and $Q_i = 15$ mequiv/l.

Total intrastromal chloride figures then provide a crucial check of Elliott's hypothesis of chloride binding. Chloride values should include free chloride and complexed chloride. Inspection of the chloride data in conjunction with the intrastromal electrical potentials indicates very clearly that there is some extra factor in play. For example at stromal hydration 3·2 and bathing salt concentration of 30 mM, electrical potential is −10·8 mV which predicts a free chloride concentration within the stroma (from the Nernst potential) of 20·6 mM which is considerably different from that measured (26·8 ± 1·3 mM). In contrast to chloride, the intrastromal sodium values are determined (46·0 ± 2·8) mM close to the values expected from the Nernst potential (43·7 mM).

Elliott's 'chloride binding' requires that the stromal chloride is in two forms, free and complexed. The concentrations of complexed chloride may be calculated by subtracting the free chloride (calculated from the Nernst potential) from the measured total stromal chloride. If Elliott's 'chloride binding' is true then these calculated figures of complexed chloride should equal Q_{Cl}, which is that part of the fixed negative charge of the stroma which is a function of salt concentration. When

these two values, Q_{Cl} and complexed chloride are compared, it is seen that they are quite close in value to each other throughout the range of salt concentrations tested on the present study (Fig. 9). Although the stromal fixed negative charge, which is dependent on sodium chloride concentration (Q_{Cl}, ordinate) seems systematically

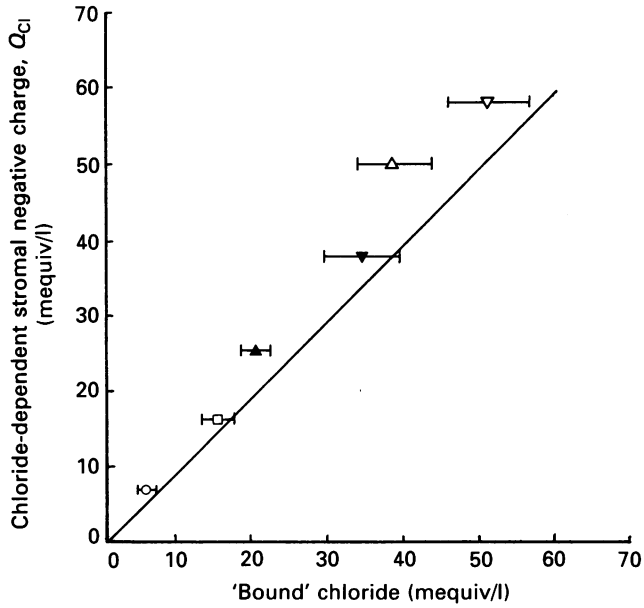

Fig. 9. The fraction of chloride ions in ox corneal stroma which is not in tissue water ('Bound' chloride; mequiv/l) is nearly equal to the additional fixed negative stromal charge (Q_{Cl}; mequiv/l) which arises in the presence of chloride for all NaCl concentrations (mM) tested: ▽, 1000; △, 600; ▼, 300; ▲, 150; □, 60; ○, 30. Half-bars represent one standard error. The line is the line of identity.

greater than the 'missing' chloride ion concentration by a few percentage points, the correspondence is remarkable and sufficiently close to provide positive support, in our view, for Elliott's chloride binding hypothesis: the 'missing' chloride is liganded to the stromal matrix and provides the sodium chloride-dependent increase in fixed negative charge.

Chloride binding would have an obvious physiological role in stabilizing corneal transparency when the cornea was immersed in solutions of different tonicity (such as when bathing underwater with open eyes). The first line of defence in maintaining corneal homeostasis (the dynamic balance between the stromal gel pressure and the regulating endothelial bicarbonate pump: Hodson & Miller, 1976) would be the 'tight' corneal epithelium (Maurice, 1951; Green, 1965; Marshall & Klyce, 1983) covering the apical surface of the cornea which would prevent any sudden change in the ionic concentrations of the underlying stroma. Nevertheless, there would be a slow but progressive decrease (in hypotonic solutions such as river water) or increase (in hypertonic solutions such as the sea) in both sodium and chloride ion concentrations in the stroma which if the fixed stromal charge, Q, were invariant (i.e. generated from the acidic groups of the glycosaminoglycans alone) would alter the

stromal gel pressure, $\Delta\gamma$. To a good approximation at or near isotonic saline the relationship between, Q, $\Delta\gamma$ and C_i, the concentration of salt within the stromal fluid is (Hodson, 1971):

$$\Delta\gamma = \frac{Q^2}{4C_i} \tag{11}$$

Consequently if C_i were to vary and Q were invariant, $\Delta\gamma$ would vary inversely with C_i. Let us call this gain in gel pressure $(\frac{d\Delta\gamma}{dC_i})$ G'. Then it can be calculated that the change in gel pressure, under the same circumstances, with unit change of C_i, nominated G, will be six times smaller than G' because of the tendency of Q to increase as C_i increases and vice versa. The attenuation of gel pressure change (G'/G) as a consequence of chloride binding increasing the stromal fixed negative charge would be 6-fold. The practical consequences are considerable. If one immerses one's eyes in tap water for 2 min the corneal stroma swells by about 15 μm (from 520 to 535 μm) and as a consequence of this, haloes may be observed around point light sources. If Elliott's 'chloride binding' hypothesis is correct, as the present study proposes, this effect would be nearly six times greater without the attenuation effect of the stromal chloride binding. If chloride binding did not exist, then the cornea would be sufficiently hydrated to impede vision, and our perception of water as a 'friendly' medium in which to immerse our face would be influenced adversely.

The guanidine hydrochloride extraction studies indicate that both the intrastromal sodium and chloride concentrations are nearly consistent with a residual fixed negative charge of around 8 mequiv/l with no strong evidence for chloride binding *per se*. Correcting to physiological hydration (3·2) this would correspond to a fixed charge of 10 mequiv/l and little or none of this would be contributed by a chloride binding ligand. It is possible from these figures to calculate that whilst about only 40% of the fixed negative charge is extracted by 4 M-guanidine hydrochloride exposure for 3 days, nearly all the chloride binding ligand is extracted by this procedure. As the dry weight of the preparation diminishes to 86% of the original dry weight and as collagen contributes about 90% of the dry weight of corneal stroma, it is clear that most of the collagen – probably all of it – remains in the tissue after guanidine extraction. Consequently, the chloride binding ligand of ox corneal stroma, which this study suggests plays a crucial role in the maintenance of corneal homeostasis and transparency, is not associated with the collagen molecules.

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