Investigative Ophthalmology & Visual Science, Vol. 29, No. 6, June 1988

Water Gradients Across Bovine Cornea

John A. Castoro, Adriel A. Bettelheim, and Frederick A. Bettelheim

Twenty-six bovine corneas, 30 μm thick, were sectioned perpendicular to the visual axis. Sections, weighing 10–20 mg, were analyzed for total water content by thermogravimetric analysis and for freezable water content by differential scanning calorimetry. The total water content as well as the free (freezable) water content increase, while the bound (nonfreezable) water content decreases in progressing from epithelium to endothelium. These results are correlated with the distribution of proteoglycans and their water sorptive and retentive capacity in bovine cornea. Invest Ophthalmol Vis Sci 29:963–968, 1988

There is much experimental evidence that the proteoglycan ground substance plays a significant role in corneal hydration. Especially, when a change of corneal hydration occurs (swelling), the locus of the change is in the ground substance.[1] Evidence for this statement is that one can account for the measured form birefringence of the cornea by assuming that during swelling, the hydration of the collagen fibrils remain constant.[2] Confirming this belief is the electron microscopic observation that shows no change in the diameter of collagen fibrils when the cornea swells.[3] On the other hand, when corneas are treated with hyaluronidase or cetylpyridinium chloride the stromal swelling is greatly reduced. This indicates the involvement of the ground substance in swelling.[4]

An increase in total water concentration across the cornea from epithelium to the center has been found by Maurice,[2] by measuring the change in refractive index of layers starting from epithelium and going deeper and deeper into the stroma. This was in contrast to the observations of Smelser[5] and Coulombre and Coulombre,[6] who found that during development the lamallae are laid down from within outwards and the posterior layers appeared to have higher concentrations of collagen and ground substances. However, it may be that this observation on the polymeric concentration gradient does not apply for mature corneas.

Maurice's observation on the refractive index gradient was substantiated by Kikkawa and Hirayama.[7] In their experiments, both in vivo and in vitro, the frontal third of the cornea absorbed water to a lesser extent than the rest. Turss et al[8] also found that the posterior layers of rabbit cornea are more hydrated (3.85 g H_2O/g dry weight) than the anterior (3.04 g H_2O/g dry weight). The same thing is true in bovine cornea.[9]

Our own observation[10] on the distribution of proteoglycans across the bovine cornea indicated that in the anterior part the keratan sulfate/chondroitin-4-sulfate ratio was lower than in the posterior part. This concentration gradient of the different proteoglycans bears significance if one takes into account that proteoglycans that predominantly contain chondroitin-4-sulfate have less swelling ability but greater water retentive power than the predominantly keratan sulfate bearing proteoglycans.[11–13] Thus, it is possible that not only the ground substance as a whole is responsible for the hydration of the cornea, but the specific distribution of the different proteoglycans which have different hydrating power may also play a role in the establishment of water gradients across the cornea.

Beside the uneven hydration across the cornea there is also a question of how much of the water is available to solvate metabolic products and how much is "bound" in the matrix and not available for solvation. Maurice[14] showed that by applying pressure up to 30 atm, all but 1 g water per gram dry weight can be squeezed out of the cornea. Observation on the distribution of labeled amino acids, sugars and proteins between bathing solution and confined pieces of stroma showed that only 0.25 g H_2O/g dry weight is not available to solvate small molecules. On the other hand, about 2 to 3 g H_2O/g dry weight is unavailable to solvate proteins.

Therefore, it is of interest to determine (1) what is the amount of bound (nonfreezable) water content in the cornea; and (2) if such bound water will show a distribution gradient across the cornea.

From the Chemistry Department, Adelphi University, Garden City, New York.

Supported by an NIH grant EY-02571, Bethesda, Maryland.

Submitted for publication: June 25, 1987; accepted December 15, 1987.

Reprint requests: Dr. F. A. Bettelheim, Chemistry Department, Adelphi University, Garden City, NY 11530.

Materials and Methods

Twenty-six bovine eyes were obtained from a slaughterhouse. The eyes were kept at 37°C. The cornea was excised as follows: The eyeball was inserted into a plastic bag and the bag was quickly immersed in a dry ice-acetone bath (−70°C). Some of the corneas were frozen in this manner, maintaining the natural curvature, while others were flattened against a plate and frozen to obtain flat corneas for sectioning. The thickness of each cornea was measured with a micrometer before attaching it to a planchet for sectioning. The thickness varied between 0.75 to 0.87 mm.

The frozen corneas were sectioned in a refrigerated microtome at −20°C. Some sectioning was done from anterior to posterior, others from posterior to anterior. The thickness of the sections were 30–40 μm. The sections weighed 10–20 mg. Adjacent sections were taken for the analysis of their water contents.

Each cornea sample was hermetically sealed into preweighed, coated aluminum sample pans and stored at −30°C until the actual measurements. For the measurement of the freezable water content, differential scanning calorimetry (DSC) was used.

A hermetically sealed, empty, coated aluminum pan served as a reference. The sample and reference pans were placed in a DSC (DuPont 990; Dupont, Wilmington, DE) cell and cooled to −30°C by an external dry ice-acetone bath. DSC curves were obtained by heating the sample at a programmed rate of 3°C/min. Most experiments were performed in N_2 atmosphere with a flow rate of 50 cm^3/min. The instrument was calibrated with a sapphire disc and the DSC cell calibration constant was obtained periodically. The DSC curves recorded the differential heat flow (Δq) as a function of time. The Δq was recorded simultaneously with two different sensitivities, for example, 0.5 mV/cm, high sensitivity, and 10 mV/cm, low sensitivity.

The area under the curve gives the number of joules of heat used to melt the measured mass of water. Since it was our intention to convert the area of an endotherm (in joules per gram sample) into a certain amount of freezable water per gram sample, we ran a calibration curve with distilled water and with aqueous NaCl solutions with different concentrations having different melting (freezing) points.[15]

After the DSC measurements, the pans were punctured, taking care not to disturb the samples. The pans were next placed in a thermogravimetric analyzer (DuPont 951) and the total water content of the samples was obtained from the weight loss which occurred during heating the pans to and maintaining them at 105°C. The nonfreezable water content was obtained as the difference between the total and freezable water content, and it was expressed as a percentage of the total water content. In some of our corneas a maximum hydration of 90% was found. Any sample containing a water content greater than this in any of its sections was discarded as swollen cornea, due perhaps to the low temperatures in the slaughterhouse.

The concentration gradient of the different water contents across the cornea was obtained by regression analysis using the Minitab II program in a Prime computer (Prime, Boston, MA).

Sections of separate bovine corneas were also used to measure the concentration of predominantly chondroitin-4-sulfate bearing proteoglycans (fraction Ia) and also predominantly keratansulfate bearing proteoglycans (fraction Ib) in each section. The extraction was done in 4.0 M guanidinium chloride at pH 7.5 and the separation involved ion exchange chromatography as described previously.[13] The concentration of each proteoglycan fraction in the cornea sections were obtained by analyzing the eluents from ion exchange chromatography, using the absorbance at 280 nm.

Since both proteoglycan fractions Ia and Ib have similar protein core content and similar amino acid composition,[13] we used the 280 nm absorbancy to calculate % of fraction Ia and Ib of the total extractable proteoglycan (% TEPG) in 1 g cornea as follows: we multiplied the 280 nm absorbancies (A 280) with the volume in each fractionating tube from V_1 to V_n containing a particular proteoglycan fraction.

The sum of these products was divided by net weight of each corneal section (grams).

$$\text{Ia/g} = \frac{\sum_{i=1}^{n} A_i\ 280 \times V_i}{g} \quad (1)$$

$$\text{Ia \% TEPG} = \frac{\text{Ia} \times 100}{\text{Ia} + \text{Ib} + \text{Ic} \cdots} \quad (2)$$

Results

The total water content of bovine corneal sections increases as one progresses from epithelium to endothelium. The positive total water gradient is evident when the sectioning was done from epithelium toward the endothelium (Figs. 1–3) and the negative gradient is seen when the sectioning was done from endothelium toward epithelium. The regression equation and the standard deviation, correlation coefficient of the data of Figures 1–5 are presented in Table 1.

The opposite trend exists in the nonfreezable water content as expressed as percent of the total water content. When the sectioning was done from epithe-

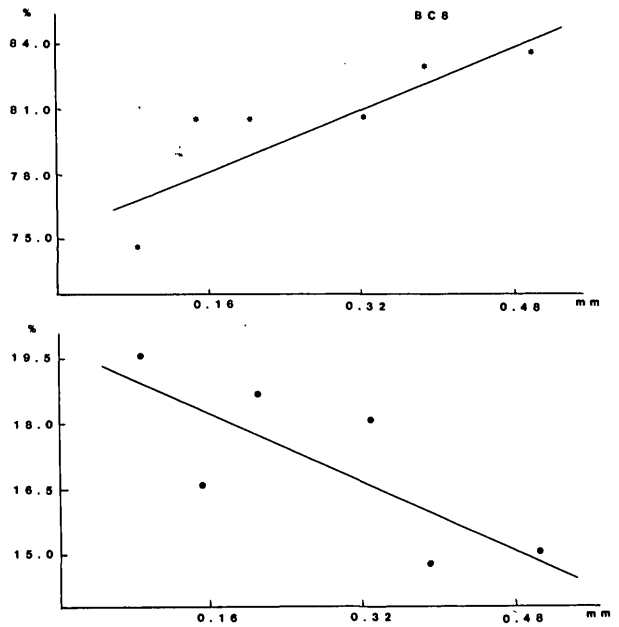

Fig. 1. Total water content (* —— *) and nonfreezable water content (as % of the total water) (● —— ●), across bovine cornea BC8 as a function of distance (in mm) from the epithelium.

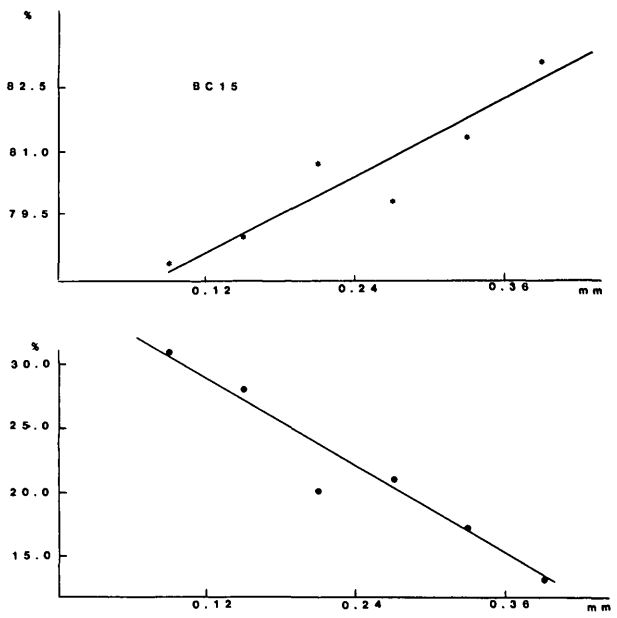

Fig. 2. Total water content (* —— *) and nonfreezable water content (as % of the total water) (● —— ●), across bovine cornea BC15 as a function of distance (in mm) from the epithelium.

lium to endothelium, the nonfreezable water content expressed as percent of the total water content had a negative gradient (Figs. 1–3). As expected the nonfreezable (bound) water increases when one progresses from endothelium to epithelium (Figs. 4, 5). The regression equations and standard deviations and correlation coefficients of the data for the nonfreezable water content are also summarized in Table 1.

As for the distribution of keratan sulfate and chondroitin-4-sulfate chains across the bovine cornea, one can use the glucosamine/galactosamine ratio to represent the keratan sulfate/chondroitin-4-sulfate ratio. This ratio increases from epithelium to endothelium, as seen from the histograms reproduced from Bettelheim and Goetz[10] in Figure 6. This means that there is more chondroitin-4-sulfate near the epithelium than near the endothelium; keratan sulfate is more concentrated near the endothelium. A similar trend was obtained when fractionated proteoglycans were studied. The predominantly chondroitin-4-sulfate-bearing proteoglycans (Ia) have a negative concentration gradient from epithelium to endothelium. On the other hand, the predominantly keratan sulfate-bearing proteoglycans (Ib) have a positive concentration gradient from epithelium to endothelium. This can be seen in Figure 7, where the concentration of each fraction is expressed as a percent of the total extractable proteoglycan as a function of distance from epithelium.

Finally, to show the water sorbing and retentive capacity of these two kinds of proteoglycans, the water vapor sorption and desorption isotherms of these proteoglycans are reproduced in Figure 8 from our previous studies (reproduced with permission from Elsevier Science Publishers, Amsterdam, The Netherlands).[13]

Discussion

In the previous sections we referred to the proteoglycans bearing predominantly galactosaminoglycan

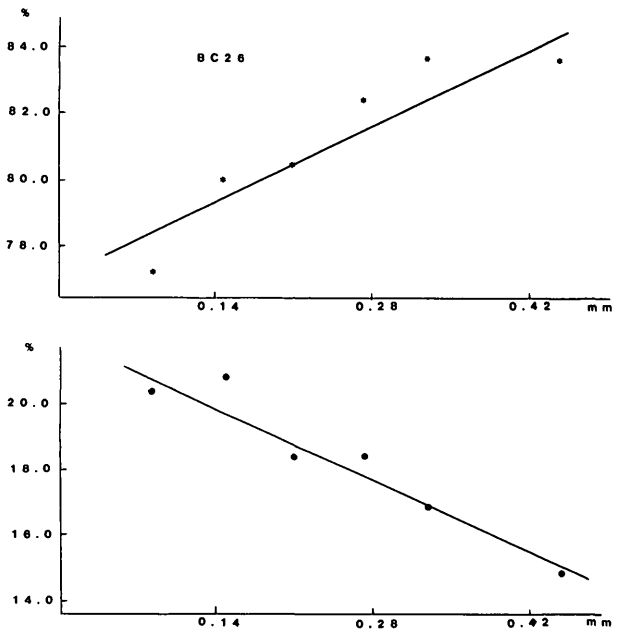

Fig. 3. Total water content (* —— *) and nonfreezable water content (as % of the total water) (● —— ●), across bovine cornea BC26 as a function of distance (in mm) from the epithelium.

Table 1. Regression parameters of the water contents of five individual bovine corneas

Sample	*Intercept*	*Slope*	*SD*	r^2%
Total water				
BC 8 (epi)	75.5	16.9	2.0	68.7
BC 15 (epi)	76.8	14.7	0.8	84.8
BC 26 (epi)	76.9	17.4	0.9	87.7
BC 7 (endo)	84.0	−11.1	0.4	92.5
BC 14 (endo)	85.5	−19.7	1.2	76.5
Nonfreezable water				
BC 8 (epi)	19.8	−9.7	1.5	57.7
BC 15 (epi)	35.0	−55.3	2.1	91.4
BC 26 (epi)	22.2	−16.1	0.6	93.9
BC 7 (endo)	19.8	16.3	1.5	63.3
BC14 (endo)	11.2	49.4	2.3	83.1

side chains as chrondroitin-4-sulfate proteoglycans. We did this because the isolation of these materials was done according to references in which such nomenclature is used. More recently, however, it was discovered that the galactosaminoglycans in the cornea have iduronic acids rather than glucuronic acids and, therefore, the proper classification of these galactosaminoglycans should be dermatan sulfate.[16,17] Thus, in the discussion, we shall refer to the galactosaminoproteoglycans as dermatan sulfate (DS) rather than chondroitin-4-sulfate (CS), and the glucosaminoproteoglycans as keratan sulfate (KS).

The total water content has a positive gradient across the cornea, that is, it increases proceeding from

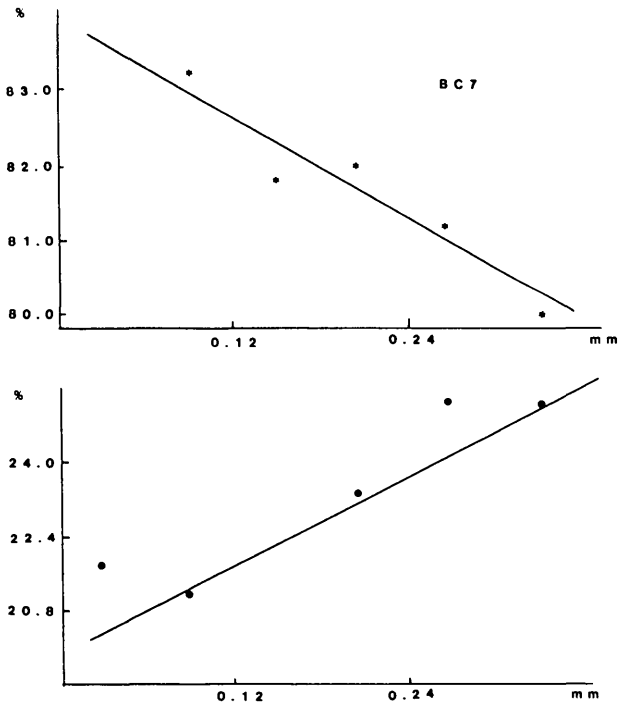

Fig. 4. Total water content (* —— *) and nonfreezable water content (as % of the total water) (● —— ●), across bovine cornea BC7 as a function of distance (in mm) from the endothelium.

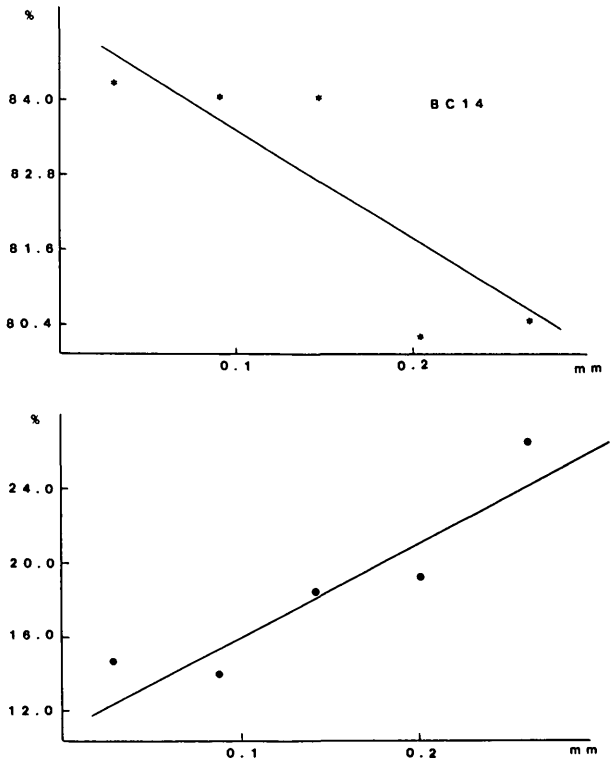

Fig. 5. Total water content (* —— *) and nonfreezable water content (as % of the total water) (● —— ●), across bovine cornea BC14 as a function of distance (in mm) from the endothelium.

epithelium to endothelium in each of the corneas studied. Most of the total water contents were between 75–85%, which corresponds roughly to the values found by Turss et al[8] (3.04 g water/g dry wt = 75% total water content and 3.85 g water/g dry wt = 79.3% total water content). The upper limit, 85%, is equivalent to 5.67 g water/g dry wt.

The individual bovine corneas had different gradients (Figs. 1–5) but each of them showed the same trend. Even more important, there was a negative

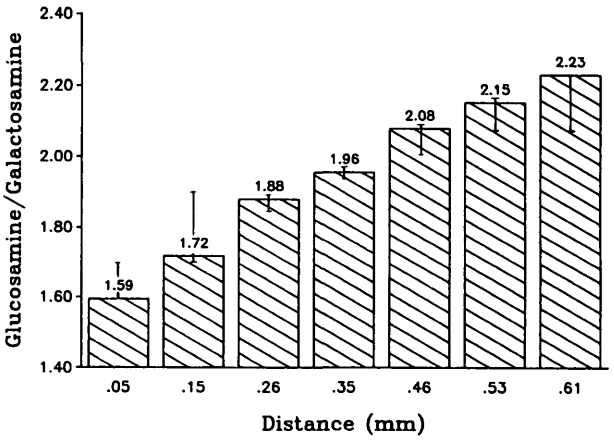

Fig. 6. Glucosamine/glactosamine ratio across the bovine cornea as a function of distance (in mm) from epithelium.

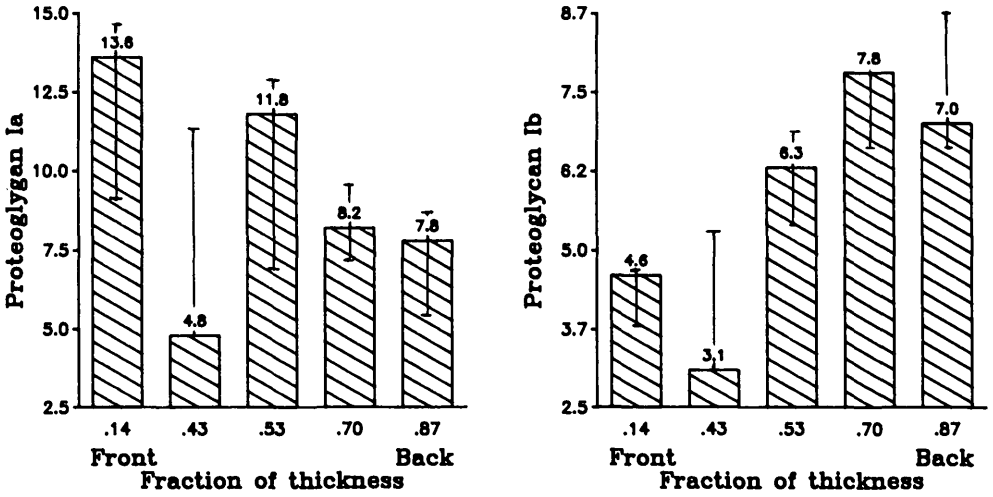

Fig. 7. Proteoglycan concentration (as % of the total extractable proteoglycans per gram cornea) as a function of fractional thickness of bovine cornea.

gradient of the bound (nonfreezable) water (expressed as % of the total water content) across the cornea. This bound water decreases from epithelium to endothelium. Again, the gradients of the bound water of the individual corneas vary but the trend is uniform.

If the hydration properties of the different proteoglycans, as studied by water vapor sorption,[13] also reflect their behavior in the corneal stroma, then their distribution may be directly responsible for the anisotropic hydration of the cornea. Keratan sulfate[11] and predominantly keratan sulfate-bearing proteoglycans have great water sorptive capacity, but meager water retentive capacity.[13] There are more of these keratan sulfate-bearing proteoglycans near the endothelium of the cornea (Fig. 6) than near the epithelium.[10] Thus, these proteoglycans may be able to sorb and transfer water in the cornea to solvate the lamellae near the endothelium, once such water has passed the cellular barrier separating it from the aqueous humor. On the other hand, dermatan sulfate[12] and dermatan sulfate-bearing proteoglycans[13] have much less water sorbing capacity than the predominantly keratan sulfate-containing proteoglycans, but their water retention power is greater.

The overall glucosamine/galactosamine gradient is one good indicator of the uneven distribution of keratan sulfate- and dermatan sulfate-bearing proteoglycans across the cornea. Beyond that one can see that proteoglycans Ia and Ib, extracted from each corneal section, also show gradients across the cornea (Fig. 7). Proteoglycan Ia, on the average, contains five dermatan sulfate side chains for each keratan sulfate on the protein core. This is a predominantly dermatan sulfate proteoglycan. Proteoglycan Ib, on the average, has five keratan sulfate side chains for each dermatan sulfate side chain on the protein core.[13] This is a predominantly keratan sulfate-carrying proteoglycan. The histogram in Figure 7 shows the same kind of distribution of the two proteoglycans across the cornea as the overall glucosamine/galactosamine ratio indicated (Fig. 6). As the numbers on Figure 7 show, proteoglycans Ia and Ib make up only about 18% of the total extractable proteoglycans under the extraction conditions described above.[13] However, the fact that the individual (Ia and Ib) proteoglycan distributions parallel the overall glucosamine/galactosamine gradient gives significance to this assymmetric proteoglycan distribution.

We must emphasize that the water contents and the proteoglycan distribution analysis were done on separate bovine corneas. Therefore, one must be contented to point out only the trends (gradients) in each case *without* assuming that certain numerical relationships exist among them (ie, × mole ratio of keratan sulfate/dermatan sulfate proteoglycan represents y% total or z% nonfreezable water content).

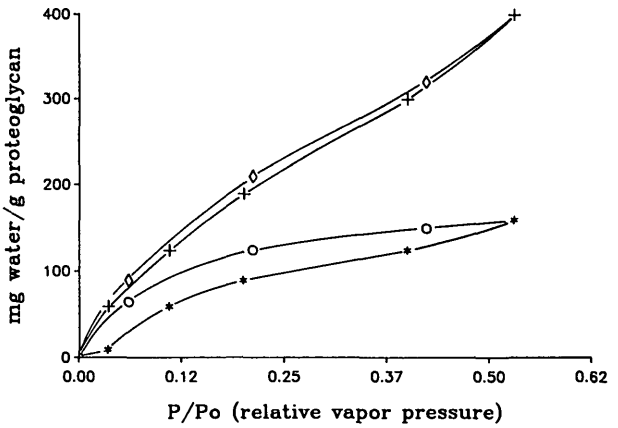

Fig. 8. Water sorption isotherm of (* —— *) and desorption isotherm (O —— O) of proteoglycan Ia; water sorption isotherm (× —— ×) and desorption isotherms (◇ —— ◇) of proteoglycan Ib at 29.6°C. The reproducibility of the sorption isotherms is ±1 mg water/grams proteoglycans.

The water sorption and retention properties of proteoglycans Ia and Ib (Fig. 8) parallel the water sorption and retention properties of the keratan sulfate[11] and dermatan sulfate[12] glycosaminoglycans.

In summary, across the cornea one finds asymmetric distributions of total water content, nonfreezable water content and of proteoglycans bearing predominantly keratan sulfate and predominantly dermatan sulfate side chains.

Thus it is plausable that the keratan sulfate-bearing proteoglycan gradient which has the highest concentration near the endothelium helps to set up the total water content gradient because of its great sorptive capacity.

On the other hand, the dermatan sulfate-bearing proteoglycans with their great water retentive capacity can help to set up the bound (nonfreezable) water gradient that is at maximum near the epithelium, where the dermatan sulfate-bearing proteoglycan concentration is also the greatest. Such a gradient would then serve the purpose of diminishing the dehydration of the front of the cornea that is exposed to the atmosphere.

Key words: bovine cornea, concentration gradient, nonfreezable water, proteoglycans, water

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