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The distributions and diffusivities of small ions in chondroitin sulphate, hyaluronate and some proteoglycan solutions

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The distributions and diffusivities of Na^+, Ca^{2+} and Cl^- in chondroitin sulphate (CS), hyaluronate (HA) and proteoglycan solutions were measured using equilibrium dialysis and a capillary tube method. Measurements were made for a range of glycosaminoglycan (GAG) concentrations up to those normally found in dense connective tissue (10% CS, 2.5% HA), ionic strengths up to normal physiological concentrations (0.15 M) and for different combinations of monovalent and divalent cations. The partition coefficients, K_i, of the positive ions increased with increasing matrix concentration and with decreasing ionic strength but with one exception the selectivity coefficient $K_{Na}^{Ca} = \sqrt{K_{Ca}}/K_{Na}$ was close to unity, indicating nearly ideal Donnan distributions. The ionic diffusivities decreased very much like those of small neutral solutes with increasing matrix concentration and with one exception were relatively independent of ionic strength, The exception in both cases was low matrix concentrations and low ionic strengths for which the diffusivity of Ca^{2+} was an order of magnitude lower and selectivity coefficients were ≈ 2. We conclude that at physiological ionic strengths and GAG concentrations the distributions of small ions are determined by simple electrostatic interactions, without binding or condensation, and the diffusivities are not affected by the electrostatic field.

1. Introduction

The distribution and mobility of small ions in the extracellular matrix are important determinants of cellular activity and of the mechanical and physico-chemical properties of connective tissues [1]. In load-bearing connective tissues the polyionic fixed charge density is sufficiently high for the small counterions to generate an osmotic pressure which makes a major contribution to the mechanical properties of the tissue [2] and interest is developing in other electro-chemical effects [3]. The ionic environment influences the conformations and interactions of the connective tissue macromolecules themselves [4] and it is frequently cited as a factor controlling the precipitation and organisation of matrix material during tissue growth and remodelling [5]. It has been suggested that by virtue of their anionic nature, the glycosaminoglycans (GAGs) play a role in the control of the calcium content of tissues [6] and in calcification processes, both normal and pathological [7,8]. In pathological situations, the preferential deposition of certain crystals (e.g. sodium urate) in distinct locations of joint tissues has been linked with the distribution of the polyelectrolyte molecules in these tissues. Also, ions have been implicated as ligands promoting the deposition of lipids in atherosclerosis [9]. Moreover, inorganic ions are known to regulate a number of cellular processes [10] and the transport of ions from the extracellular spaces into cells must depend,

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amongst other factors, on the activity and diffusivity of these ions in the surrounding extracellular environment.

Corresponding to this diversity of functions is an extensive literature on ionic interactions in connective tissues. Experiments on intact tissues (e.g. refs 11, 12) have provided valuable information but the complexity of the tissue structure makes it imperative to establish the underlying mechanisms on isolated and well characterised tissue components. Since one of the clearer conclusions to emerge from the above work has been that proteoglycans play a dominant role in determining the behaviour of ions in the extracellular matrix, this has stimulated a number of studies on the behaviour of GAGs or proteoglycans in isolation.

At least two parameters are required to characterise the influence of a proteoglycan matrix on small ions; one is a measure of the equilibrium distribution volume for ions within the matrix and the other a measure of ionic mobility. Distribution volumes have been measured in a number of ways such as radioactive tracer techniques [13], titration [14], ion exchange [15], or equilibrium dialysis [4,16] and the results have been expressed variously in terms of ionic activities, affinities, binding constants and partition coefficients. The variety of terminology is an indication of the diversity of theoretical models of the interaction between polymer and ion which have been employed, and although the data often support the chosen model, the range of experimental conditions is generally rather limited and so the agreement is less than compelling. One particularly striking limitation is the absence of data on the behaviour of GAGs at concentrations approaching those occurring in cartilaginous tissues.

The problem of ionic mobility has received rather less consideration. The flux of ions across membranes containing GAGs or proteoglycans has been measured [17], but the calculation of diffusivity requires an independent measurement of the partition coefficient for the ion between membrane and solvent and the method has, in any case, been employed only at low GAG concentrations. Direct measurements of diffusivity are few [18,19] and these too have been made at concentrations much lower than those occurring in some of the tissues of major interest.

The principal objective of the present work was to obtain data on ionic distribution volumes and diffusivities at GAG concentrations within the physiological range. However, measurements were made over a sufficiently wide range of matrix concentrations and buffer compositions to allow comparisons to be made both with previous experimental work and with theoretical models. In the present paper an attempt is made to present results in terms which make no assumptions about underlying mechanisms; discussions of their theoretical implications are considered in a companion paper [40].

The GAGs we chose to investigate first were chondroitin sulphate (CS) and hyaluronic acid (HA), two of the most common GAGs in connective tissues. HA has a single negatively charged group (carboxyl) per disaccharide while CS has two (carboxyl and ester sulphate) resulting in a smaller intercharge distance. Since intercharge distance (or equivalently, linear charge density) is an important parameter in all polyelectrolyte theories, these two GAGs were expected to exhibit somewhat different behaviour. In addition, rat chondrosarcoma proteoglycans (PGs), were used in a small series of experiments to investigate how the PG structure affected the polyelectrolytic nature of its constituent GAGs.

Data are given on the partitioning of monovalent (Na^+) and divalent (Ca^{2+}) cations and of monovalent (Cl^-) anions between buffers of various ionic compositions and solutions of HA, CS and PG of various concentrations. The technique of equilibrium dialysis has been described elsewhere [20,21]. In order to extend the range of measurements to high matrix concentrations, dialysis was performed against buffer containing an iso-osmotic concentration of polyethylene glycol (PEG). Under these conditions, the effect of the PEG itself had to be taken into account and this was done by measuring the effects of externally applied PEG solutions on partition coefficients in cartilage.

Measurements of ionic diffusivity were made for the same range of ions, matrix concentrations and buffers using a capillary tube technique [22].

This involved the injection of a tracer into the centre of a closed capillary tube containing the experimental solution and measuring its distribution along the capillary after an appropriate time. It was found that this method avoided end effects, permitted detection of non-Fickian diffusion and was generally more accurate than the more familiar method of measuring the concentration of tracer in a bath external to an open-ended capillary [18]. In addition, multiple isotope counting techniques were used either to compare ionic diffusivity with that of a low molecular weight reference material ([^{3}H]proline) or to compare the diffusivities of two ions directly in the same capillary.

2. Materials and methods

2.1. Measurement of partition coefficient

The partition coefficients of sodium and calcium between CS or HA solutions and salt solutions of different composition were measured using equilibrium dialysis and the radioactive tracers $^{22}Na^+$ and $^{45}Ca^{2+}$. The partition coefficients of $^{36}Cl^-$ were measured only for the physiological salt concentration. The GAG containing solutions were confined within small pore size dialysis tubing (Spectrapor membrane No. 3, cutoff 2000 Da, Spectrum Medical Industries, U.S.A.) and allowed to equilibrate against solutions of different composition. When concentrations of HA or CS greater than 4% were used, polyethylene glycol 20000 (PEG) (Fluka, F.R.G.) was added to the external solution so that the osmotic pressure of the GAG within the dialysis sac was matched by the osmotic pressure of the PEG in the outside solution. In this manner the GAG solution was prevented from diluting itself by imbibition of bathing solution. The amount of PEG to be added to the external solution in order to maintain a given concentration of GAG in the dialysis tubing was determined from the curves of osmotic pressure vs. concentration which had been previously obtained [20]. It was necessary, however, to determine the effect of PEG itself on solute partition and this will be described below.

A limited number of measurements were also carried out on proteoglycan (PG) preparations – both monomer and aggregate – kindly given to us by Dr. J. Kimura. They were prepared in his laboratory from rat chondrosarcoma, and were characterised by standard procedures [23].

The actual experimental procedure was as follows. Freeze-dried CS (Sigma, Grade III, whale or shark cartilage, 99%, mixed isomers) or HA (Sigma, Grade III, human umbilical cord) was exhaustively dialysed against distilled water and freeze dried. Samples of this preparation were then taken for uronic acid analysis. The freeze dried GAG was dissolved, in suitable amounts, in a NaCl-$CaCl_2$ solution of the desired ionic composition. The initial concentration of HA solutions had to be low enough to ensure ease of handling since concentrated HA solutions are extremely viscous. Once in the dialysis tubing, the solution could be concentrated against a PEG solution of suitable osmotic pressure. A small quantity (usually 2 ml) of the GAG solution was placed in small pore dialysis tubing which was sealed using special clips (Mediclips, Spectrum Medical Industries, U.S.A.) after all of the air had been expelled. The sacs were immersed in 100–200 ml of the chosen bathing solution, prepared with or without PEG as necessary. Tracer – $^{22}NaCl$ (~ 0.2 μCi/ml), $Na^{36}Cl$ (~ 0.4 μCi/ml) or $^{45}CaCl_2$ (0.1 μCi/ml) – was added to the bathing solution and the sacs were left on rollers at 4°C for an initial period of 48 h. Thereafter, samples were withdrawn from both the dialysis sacs and the external solution at regular time intervals until no further change in the radioactivity was observed. In most experiments equilibrium was attained after 2–3 days. However, in experiments where very high partition coefficients were measured, the equilibration period was of the order of 1–2 weeks.

During sampling, care was taken to remove by blotting any solution adhering to the sacs before taking 'inside' and 'outside' samples. These samples were immediately weighed (the weights were usually about 0.1–0.2 g) and then suitably diluted (10–100 times) with distilled water to ensure that there was no quenching during counting (where a scintillant was being added to the aqueous solution). After dilution, the samples were left on

shakers to make sure that the final solutions were uniform. This step was particularly important for HA since HA solutions take a long time to become uniformly dissolved. The final GAG concentrations in the sacs were determined by taking aliquots of the 'inside' solution, freeze drying them and measuring the uronic acid content. The automated procedure of Bitter and Muir [24] was employed. The fixed charge density of the GAG solutions at equilibrium was calculated using the formulae:

$$\begin{aligned} \mathrm{FCD(CS)} &= 2 \times (\mathrm{wt\ CS})/513 \\ \mathrm{FCD(HA)} &= (\mathrm{wt\ HA})/460 \end{aligned} \tag{1}$$

where the weight is expressed as g/l. These formulae are based on the known chemical compositions of the sodium salts of CS and HA.

In a number of cases a desorption (i.e. reverse equilibration) step was carried out at the end of the experiment. This consisted in placing the dialysis sac with its radioactively labelled GAG solution in a non-radioactive external solution of equivalent electrolyte concentration and allowing the two to come to equilibrium. At the new equilibrium the isotope ($^{22}Na^+$ or $^{45}Ca^{2+}$) distribution was determined. In some of the experiments (numbered 3b and 11b in the results), no unlabelled calcium was originally introduced into the GAG solution so that it was certain that the $^{45}Ca^{2+}$ taken up from the external solution was completely representative of the total calcium uptake. By comparing the results of these experiments with those in which some 'cold' $CaCl_2$ had been initially added to the GAG solution (experiments number 3a and 11a), information could be obtained as to whether there was any portion of the sites at which the calcium ions were more strongly bound.

The molal partition coefficients of Na^+, Ca^{2+}, and Cl^- were calculated from the following formula:

$$K_i = \frac{(\bar{N}_i/\bar{W}_w)}{(N_i/W_w)} \tag{2}$$

where K_i = molal partition coefficient of the ith species; $\bar{N}_i$ = radioactivity in the inside solution expressed as cpm/g of solution at equilibrium; N_i = radioactivity in the outside solution expressed as cpm/g of solution at equilibrium; $\bar{W}_w$ = weight fraction of water in the inside solution at equilibrium; W_w = weight fraction of water in the outside solution at equilibrium.

The values of the selectivity coefficients of calcium in relation to sodium ions were calculated from the formula

$$K_{Na}^{Ca} = \sqrt{K_{Ca}}/K_{Na} \tag{3}$$

The selectivity coefficient, K_{Na}^{Ca} is a measure of the averaged activity of the calcium ion as compared with that of the sodium ion within the polyelectrolyte. Under conditions of ideal Donnan equilibrium $K_{Na} = 1/K_{Cl}$ and $K_{Na}^{Ca} = 1$.

At higher GAG concentrations (i.e. when PEG was present in the outside solution), the effective partition coefficients of the ionic species had to be corrected for the altered activity coefficients in the outside solution as a result of the presence of PEG. The procedure employed made use of the fact that intact articular cartilage which contains high concentrations of GAGs does not change volume when immersed in saline solution, even in the presence of PEG. It is thus possible to determine the partition coefficients of ions for cartilage immersed in 0.15 M NaCl with different PEG concentrations. In some of the tests 2.5 mM $CaCl_2$ was added to the solution. The method for the determination of the partition coefficients is based on the use of radioactive isotopes and has been previously described [21,25,26].

Consider the distribution of Na^+, Ca^{2+} and Cl^- between cartilage and an external solution in the absence and presence of PEG. Equating the activity of the diffusible salts in the two compartments in the absence of PEG requires that

$$\begin{aligned} \bar{a}_{Na}\bar{m}_{Na} \times \bar{a}_{Cl}\bar{m}_{Cl} &= a_{Na}m_{Na} \times a_{Cl}m_{Cl} \\ \bar{a}_{Ca}\bar{m}_{Ca} \times (\bar{a}_{Cl}\bar{m}_{Cl})^2 &= a_{Ca}m_{Ca} \times (a_{Cl}m_{Cl})^2 \end{aligned} \tag{4}$$

where m_i is the molal concentration and a_i is the activity coefficient of the ith ion and barred quantities refer to the cartilage. If PEG is added, the activity coefficients in the external solution will be different because of the excluded volume effects of the PEG. The activity coefficients in the carti-

lage, however, will not be affected since the tissue is surrounded by small pore size dialysis tubing to prevent penetration by PEG. Again the diffusible ions will distribute so that the activities in both compartments are equal

$$\begin{aligned} \bar{a}_{Na}\bar{m}'_{Na} \times \bar{a}_{Cl}\bar{m}'_{Cl} &= a'_{Na}m_{Na} \times a'_{Cl}m_{Cl} \\ \bar{a}_{Ca}\bar{m}'_{Ca} \times (\bar{a}_{Cl}\bar{m}'_{Cl})^2 &= a'_{Ca}m_{Ca} \times (a'_{Cl}m_{Cl})^2 \end{aligned} \quad (5)$$

where primes indicate values in the presence of PEG.

The ratios of activity coefficients necessary to correct the selectivity coefficients for the presence of PEG follow readily from eqs. 4 and 5

$$\begin{aligned} \frac{a'_{Na}a'_{Cl}}{a_{Na}a_{Cl}} &= \frac{\bar{m}'_{Na}\bar{m}'_{Cl}}{\bar{m}_{Na}\bar{m}_{Cl}} \\ \frac{a'_{Ca}/a'^2}{a_{Ca}/a^2} &= \frac{\bar{m}'_{Ca}/\bar{m}'^2_{Cl}}{\bar{m}_{Ca}/\bar{m}^2_{Cl}} \end{aligned} \quad (6)$$

The values of the mean activity coefficient ratios obtained for cartilage immersed in different concentrations of PEG are given in table 2 and are used as correction factors in table 3.

2.2. Measurement of diffusivity

Ionic diffusivity was measured over a range of buffer compositions and matrix concentrations similar to those in the equilibrium dialysis experiments, using GAGs and PGs from the same batches. The method of measuring tracer diffusivity has been previously described in detail [22]. In brief, a nylon capillary tube (i.d. 1 mm, length 6.5 cm, Portex) was filled with de-gassed GAG or PG solution (or, for measurements of free diffusivity, buffer alone) and sealed at both ends. A microinjection (≈ 0.2 μl) of the buffer used in preparing the solution containing the desired mixture of radioactive isotopes (generally, [^{3}H]proline together with either $^{45}CaCl_2$, $^{22}NaCl$, $Na^{36}Cl$, or $Na_2{}^{35}SO_4$, Amersham International) was made into the centre of the capillary tube and the tubes were kept at 4°C for between 18 and 48 h. The incubation time was generally chosen to allow the tracer to diffuse over a distance of 2 cm. The capillary tube was then frozen in liquid nitrogen and a length of 4 cm centred on the injection site was sectioned into 2 mm lengths using a specially constructed jig. The radioactivity in each section was assayed by either β or γ-counting, care being taken as outlined above that samples for β-counting were completely mixed and equilibrated with scintillant. When more than one isotope was employed, the 'crossover' between the isotope counting windows was measured and a microcomputer was used to correct the data for crossover and to calculate tracer diffusivity. For simple Fickian diffusion, a plot of ln C vs X^2 where C is concentration and X is distance from the injection site should be linear with slope $(-1/4DT)$ where T is the incubation time and D the solute diffusivity. Effective diffusivities were determined from the data by using a least squares method to determine the slope of the line of best fit.

3. Results

3.1. Partition coefficients

The experiments fall into three main groups. In the first, a buffer of 'physiological' ionic strength (0.15 M NaCl, 2.5 mM $CaCl_2$) was used and the effect of changing GAG concentrations on the partition coefficients was investigated. The GAG concentrations covered the medium to high range of those found in connective tissues. In the second set, a GAG concentration corresponding to the very top of the above range was used and the effect of changing ionic strength was investigated. The third group of experiments were conducted at low (0.05%) GAG concentration and low ionic strength. Both the second and the third groups were designed to clarify mechanisms of interaction between polyelectrolytes and small ions and do not apply to physiological conditions. The initial compositions of the 'inside' and 'outside' solutions are given in table 1.

The mean activity coefficient ratios measured in order to correct for the effect of PEG on selectivity coefficients are given in table 2. The values for NaCl are close to those calculated from the formula given by Wells [27] using the data of Edmond and Ogston [28]. The ratio $(a'^2_{Na}/a'_{Ca})/(a^2_{Na}/a_{Ca})$ was obtained only for one PEG con-

Table 1

Details of initial conditions in the partition experiments

No.	Inside solution			Outside solution				
	NaCl (M)	$CaCl_2$ (M)	Solute (%)	NaCl (M)	$CaCl_2$ (M)	^{45}Ca (μCi/ml)	^{22}Na	PEG (%)
1	0.15	0.0025	1% CS	0.15	0.0025	0.08	0.02	–
2	0.15	0.0025	4% CS	0.15	0.0025	0.08	0.02	8
3a	0.15	0.0025	5% CS	0.15	0.0025	0.08	0.02	9
3b	0.15	–	5% CS	0.15	0.0025	0.08	0.02	9
4	0.15	0.0025	10% CS	0.15	0.0025	0.08	0.02	15
5	0.15	0.0025	0.2% HA	0.15	0.0025	0.08	0.02	–
6	0.15	0.0025	2% HA	0.15	0.0025	0.08	0.02	9
7	0.15	0.0025	RCS (A1D1)	0.15	0.0025	0.08	0.02	9
8	0.15	0.0025	RCS (A1)	0.15	0.0025	0.08	0.02	9
9	0.002	–	7.6% CS	0.002	0.0000004	0.08	0.02	20
10	0.015	0.0025	7.6% CS	0.015	0.00025	0.08	0.02	20
11a	0.0004	0.0025	0.05% CS	0.0004	0.0000004	0.08	0.02	–
11b	0.0004	–	0.05% CS	0.0004	0.0000004	0.08	0.02	–
12	0.0004	–	0.05% HA	0.0004	0.0000004	0.08	0.02	–

centration, viz. 15%, and was extrapolated to other concentrations using the formula of Wells [27]. The measured selectivity coefficients were corrected as follows:

$$K_{Na}K_{Cl} = K'_{Na}K'_{Cl}\frac{a_{Na}a_{Cl}}{a'_{Na}a'_{Cl}} \tag{9}$$

$$K_{Na}^{Ca} - K_{Na}^{Ca\prime}\sqrt{\frac{a'^2_{Na}/a'_{Ca}}{a^2_{Na}/a_{Ca}}} \tag{10}$$

The single ion partition coefficients may be corrected for the effect of PEG with formulae derived from eqs. 4 and 5, and using the mean activity coefficient ratios in table 2, if it is assumed that the GAG solutions have no net charge at equilibrium, i.e. that

$$\bar{m}'_{Na} + 2\bar{m}'_{Ca} = FCD + \bar{m}'_{Cl} \tag{11}$$

$$\bar{m}_{Na} + 2\bar{m}_{Ca} = FCD + \bar{m}_{Cl} \tag{12}$$

Table 2

Ionic activity coefficients in solutions containing PEG

PEG (g PEG/g solvent)	$\frac{a'_{Na}a'_{Cl}}{a_{Na}a_{Cl}}$	$\frac{a'^2_{Na}/a'_{Cl}}{a^2_{Na}/a_{Cl}}$
9	1.10 ± 0.02	–
15	1.15 ± 0.03	1.16 ± 0.01
20	1.26 ± 0.03	–

The partition coefficients for the three groups of experiments proper are summarised in table 3a–c and the coefficients are also shown after correction for the presence of PEG in the external medium. As shown in table 3a, at physiological ionic strength, the partition coefficients for both Na^+ and Ca^{2+} increase approximately two-fold and four-fold respectively in response to a seven-fold increase in GAG concentration. The effect of changing ionic strength is much more marked, as shown in table 2b, particularly for Ca^{2+}, where a 75-fold change in ionic strength results in a 3000-fold change in K_{Ca}. Despite this range in absolute values, the selectivity coefficients of calcium with respect to sodium are in the range 1.06–1.18 for all concentrations of GAG tested (i.e. from 1–10%) in physiological saline and in the range 1.14–1.28 for a a high CS concentration (7%) in 0.015 NaCl and in de-ionised water.

For the four experiments where values of K'_{Cl} were obtained, it was possible to determine directly the effect of the GAG or PG solution on ionic activity coefficients, as

$$\sqrt{\frac{\bar{a}_{Na}\bar{a}_{Cl}}{a_{Na}a_{Cl}}} = \frac{1}{\sqrt{K'_{Na}K'_{Cl}}} \tag{13}$$

The mean activity coefficient, $\sqrt{\bar{a}_{Na}\bar{a}_{Cl}}$, for NaCl in the internal solutions was, on average, 89%

Table 3

Partition coefficients for Na^+, Ca^{2+} and Cl^- between polyelectrolytes (CS, HA, and PG) and pure electrolyte ($NaCl + CaCl_2$) solutions under different conditions

a) Medium to high polyelectrolyte concentrations at physiological ionic strengths (0.15 M NaCl + 2.5 mM $CaCl_2$)

No.	GAG	FCD (M)	K'_{Na}	K_{Na}	K'_{Ca}	K_{Ca}	K'_{Cl}	K_{Cl}	K_{Na}^{Ca}	m_{Na}/m_{Ca}	$K_{Na}K_{Cl}$
1	CS	0.05	1.33 –	1.33	2.14 –	2.14	–	–	1.10	37	–
2	CS	0.20	2.09 ± 0.08(22)	2.03	4.80 ± 0.21(10)	4.93	0.68 ± 0.03(11)	0.64	1.09	26	1.30
3a	CS	0.25	2.54 ± 0.09(10)	2.46	7.34 ± 0.23(10)	7.53	–	–	1.11	21	–
4	CS	0.36	3.00 ± 0.05 (8)	2.89	9.75 ± 0.16 (8)	10.47	–	–	1.12	18	–
5	HA	0.02	1.22 –	1.22	1.68 –	1.68	–	–	1.06	43	–
6	HA	0.10	1.47 ± 0.10(12)	1.42	2.43 ± 0.20 (4)	2.47	0.80 ± 0.06(12)	0.75	1.11	36	1.06
7	PG	0.23	2.26 ± 0.03 (2)	2.19	6.47 –	6.67	0.68 –	0.64	1.18	21	1.40
8	PG	0.24	2.24 ± 0.01 (2)	2.18	6.44 –	6.64	0.63 –	0.59	1.18	21	1.29

b) High CS concentration, varying ionic strength

No.	m_{Na} (M)	m_{Ca} (M)	FCD (M)	K'_{Na}	K_{Na}	K'_{Ca}	K_{Ca}	K_{Na}^{Ca}	m_{Na}/m_{Ca}
9	0.002	10^{-7}	0.30	146.0 ± 6.0 (3)	146.0	22600 ± 300 (3)	27523	1.14	200
10	0.015	0.00025	0.29	14.7 ± 0.8 (3)	13.6	288 ± 15 (4)	300.0	1.28	3.06
3a	0.15	0.0025	0.25	2.54 ± 0.09(3)	2.46	7.34 ± 0.23(10)	7.53	1.11	21
3b	0.15	0.0025	0.25	2.50 –	2.43	7.33 –	7.59	1.13	21

c) Low polyelectrolyte concentration, low ionic strength

No.	GAG	m_{Na} (M)	m_{Ca} (M)	FCD (M)	K_{Na}	K_{Ca}	K_{Na}^{Ca}	m_{Na}/m_{Ca}
11a	CS	0.001	10^{-5}	0.002	2.12 ± 0.04 (2)	22.5 ± 4.0 (2)	2.20	100
11b	CS	0.001	10^{-6}	0.002	2.20 ± 0.03 (2)	16.8 ± 0.5 (2)	1.94	5
12	HA	0.001	10^{-6}	0.001	1.6 –	9.03 –	1.88	100

(SD = 5%) of that in the external medium. This together with the fact that the selectivity coefficients of calcium to sodium are close to unity indicates that under a wide range of conditions departures from the ideal Donnan equilibrium are small. This is true even in the case of very high partition coefficients (i.e. of very low ionic strength) and for very low Ca^{2+} to Na^+ ratios although the latter conditions might have been thought to be particularly conducive to the 'condensation' of Ca^{2+}. The fact that the experiment at high ionic strength in which no Ca^{2+} was initially present in the GAG solution (experiment 3b) gave exactly the same result as that in which non-radioactive Ca^{2+} was initially added to the GAG solution (experiment 3a) shows that the partition coefficients obtained are representative of the total Ca^{2+} population and that there are no artifacts present due to the initial binding of 'cold' Ca^{2+}.

The only conditions which did show considerable departures from ideality for K_{Na}^{Ca} were those in which both the GAG concentration and the ionic strength were very low. In these experiments the selectivity coefficients lay in the range 1.9–2.2. The selectivity coefficient was slightly lower when 'cold' $CaCl_2$ was originally present in the CS solution. The reason for the latter effect is probably the increased Ca^{2+} concentration due to the added $CaCl_2$ although it may also indicate the existence of a small fraction of closely bound Ca^{2+} sites which, in the case of the cold Ca^{2+} being introduced first, do not subsequently participate in the tracer exchange within the time scale of the experiments and thus do not contribute to the measured calcium partition coefficients.

With or without cold Ca^{2+}, the partition coefficients for Ca^{2+} are high, and the selectivity coefficients are significantly higher than unity; hence there are definite deviations from the ideal Donnan equilibrium for these conditions.

3.2. *Diffusivities*

The principal variables in this investigation were GAG concentration, the ionic strength of the medium and the charge on the diffusing ion. A representative tracer distribution is shown in fig. 1a and in fig. 1b the same data are plotted in the reduced form ln C vs. X^2. Theory predicts that irreversible binding of tracer to the matrix would cause an accumulation of tracer at the site of injection and other departures from Fickian diffusion such as concentration dependent or multi-component diffusion would also give rise to non-linearities in this plot [22]. For the data shown any such effects are clearly very small and this was true of the whole range of experimental conditions and incubation times, even those of low matrix concentration and low ionic strength where interaction was strongest. It also held irrespective of whether the tracers were injected singly or in combinations of two or three. The ionic strength of the injected solution was always the same as that of the buffer used to prepare the GAG solution, except for the experiments using de-ionised water, where the ionic strength of the radioactive tracers themselves (typically 200 μM for $^{45}CaCl_2$ and 50 μM for $^{22}NaCl$) was not necessarily negligible. However, in this case some pilot experiments in which the injectate was 0.15 M NaCl and 2.5 mM $CaCl_2$ gave similar values of diffusivity and it was concluded that the effects of changing ionic strength along the diffusion tube in these experiments were negligible.

The diffusivity values were therefore calculated on the assumption of simple Fickian behaviour. The values quoted are means derived from several groups of experiments with different preparations of GAG solutions, different combinations of tracer ions and different incubation times. The accompanying standard deviations refer to the whole group of experiments. As is indicated by fig. 1b, the precision of a particular measurement is quite high, and the standard deviation within a particular batch of experiments was less than the variation between batches. These variations were at-

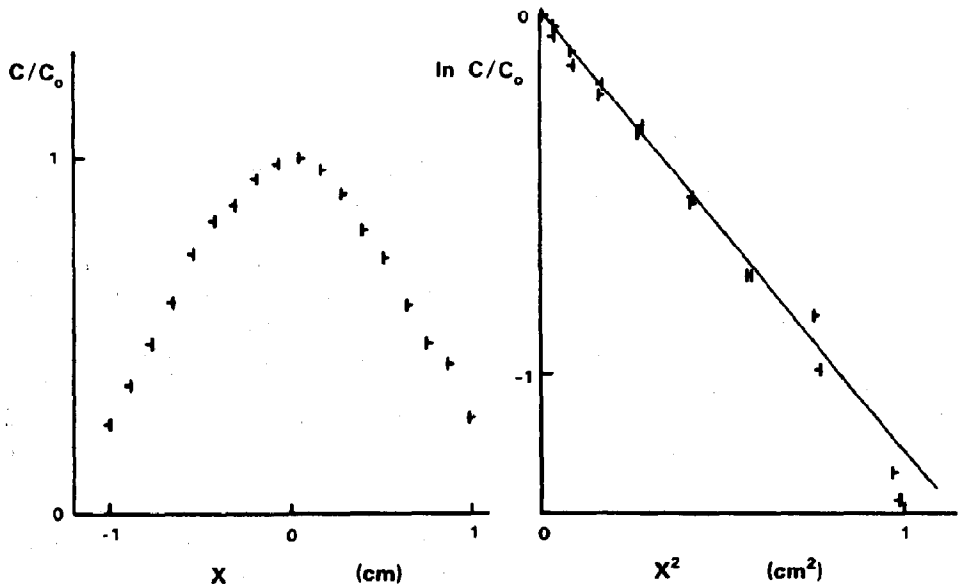

Fig. 1. The determination of the diffusivity of Na^+ in 10% CS ($T = 46$ h). (a) Measured distribution (⊢) rhs, (⊣) lhs; (b) (———) linear best fit, slope $= -1/4DT$.

tributed in particular to fluctuations in incubation temperature. They could be largely eliminated by calculating the ratio of ion diffusivity to that of the neutral reference material, proline, but as the ratio does not alter or add to the conclusions reached from examination of absolute values, it will not be referred to at length.

Although one of the initial motivations for this study was to measure the diffusivities of ions through GAG matrices at concentrations comparable to those occurring in dense connective tissues at physiological ionic strengths, it quickly became apparent that under these conditions there is little difference between the behaviour of ions and small neutral solutes. It therefore became of interest to establish the reasons for this and to try to define the conditions under which electrostatic interactions are important. Accordingly we will present our results in two groups. We will first investigate the effect of changing matrix concentration on ion diffusivity in the absence of added electrolytes, a condition chosen to maximise interactions and facilitate comparison with theory. We will then consider the effect of increasing ionic strength up to physiological levels at high and low matrix concentrations.

The measured diffusivities of Na^+, Ca^{2+}, Cl^-, SO_4^{2-} and [^{3}H]proline in CS solutions with no added electrolytes are shown in fig. 2. The free diffusion coefficients measured for these solutes were compared with those reported by Preston et al. [4] after correcting them to 4°C by means of the Stokes–Einstein equation. Considering the simplifications inherent in this correction, the agreement is reasonable. The diffusivity of proline decreases with increasing CS concentration. As discussed in the companion paper [40], this reduction in diffusivity may be accounted for by the tortuosity of the diffusion path around the polymer chains and the magnitude of the effect is estimated using the semi-empirical theory of diffusion of neutral particles in a polymer solution of Ogston et al. [29] which predicts that

$$\frac{D}{D_0} = \exp\frac{-r+a}{a}\sqrt{c_0 v} \qquad (14)$$

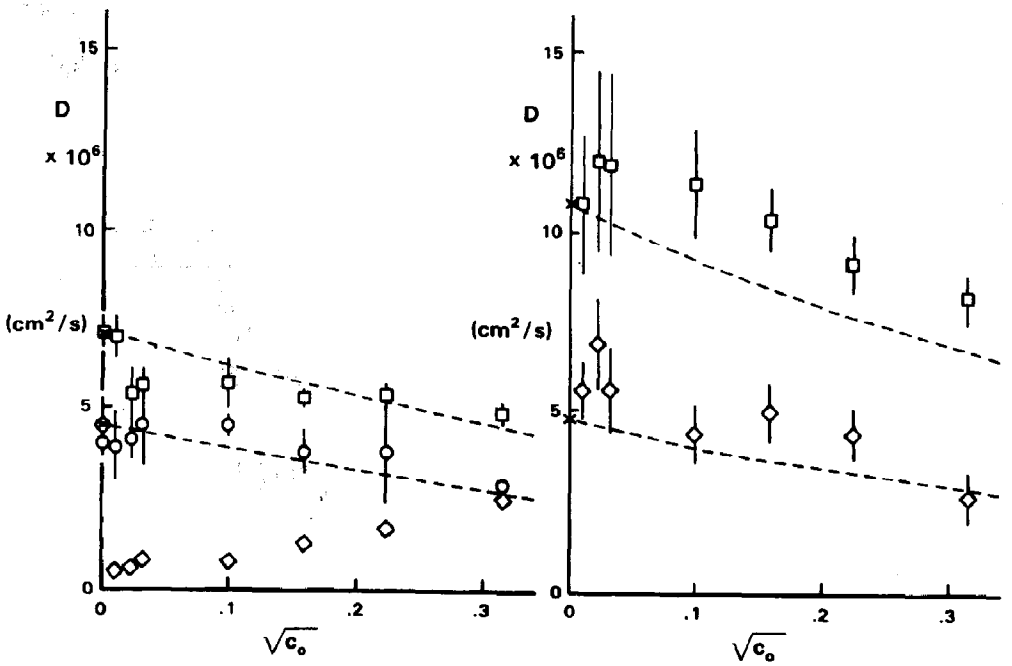

Fig. 2. The diffusivities of (a) (○) proline, (□) Na^+, (◇) Ca^{2+} and (b) (□) Cl^- and (◇) SO_4^{2-}. (×) Free diffusivities [4,18]; (- - - - -) theoretical effect of steric interaction [24].

where r is the radius of the diffusing particle, a is the radius, v the specific volume and c_0 the concentration of the polymer. The dotted lines in fig. 2 were calculated assuming $a = 0.5$ nm, $v = 0.54$. [29]. The Stokes' radius of the hydrated calcium ion was taken to be 0.3 nm [30] and the radii of the other tracers were calculated from the free diffusivities quoted in the literature [4,18] using the Stokes–Einstein relationship. The diffusivity of the neutral proline and of the two anions follows this behaviour closely over the range of concentrations tested. The diffusivity of Na^+ behaved similarly at the higher concentrations but was slightly lower than that predicted by purely steric effects at the lower concentrations. This suggests that electrostatic effects are not negligible at the lower concentrations. The diffusivity of Ca^{2+} was qualitatively different from the other solutes. At low matrix concentrations, its diffusivity was almost an order of magnitude lower than that in free solution. Behaviour of this type has been reported before and attributed to 'condensation' of ions onto the polymer [18]. As the matrix concentration increased, so did the calcium diffusivity, until at a concentration of 10% the reduction in diffusivity was comparable to that measured for proline; i.e. an amount which could be accounted for by steric interactions alone.

The effect of added electrolytes and the ratio of monovalent to divalent cations in 0.05% CS is shown in table 4. The first entry corresponds to experiments done under the conditions of fig. 2, i.e. the NaCS made up in de-ionised water. The calcium salt of CS was prepared by dialysing 0.05% NaCS first against 2.5 mM $CaCl_2$ and then against de-ionised water, a procedure which was shown in the equilibrium dialysis experiments described above to result in complete exchange of Na^+ and Ca^{2+}. In this case, the measured diffusivity was double that measured at the same ionic strength with the Na-salt predominating. This is in qualitative agreement with the expected behaviour of the electrostatic interaction and with the existence of a small 'tightly bound' calcium fraction.

Table 4

Effect of ionic strength and ionic composition on Ca^{2+} diffusivity in 0.05% CS ($D \times 10^6$ cm^2/s)

$m_{Na} + m_{Ca}$ (M)	m_{Na}/m_{Ca}	D_{Ca}
0.001	$\sim\infty$	0.6±0.1 (12)
0.001	~0	1.2±0.1 (6)
0.0014	2.5	1.5±0.6 (6)
0.003	0.5	2.4±0.3 (6)
0.1525	60.0	3.2±0.7 (5)

Table 5

Measured diffusivities in different solutions at different ionic strengths ($D \times 10^6$ cm^2/s)

Matrix	m_{Na} (M)	m_{Ca} (M)	$D_{Proline}$	D_{Na}	D_{Ca}
Free	0	0	4.0±0.5 (12)	7.2±0.4 (5)	4.5±0.8 (5)
	0.015	0.00025	4.6±0.4 (12)	6.8±0.8 (6)	4.0±0.8 (6)
	0.15	0.0025	5.4±0.6 (18)	7.9±1.3 (12)	4.9±1.1 (12)
0.05% CS	0	0	4.1±0.5 (12)	6.7±1.3 (6)	0.6±0.1 (12)
	0.015	0.00025	–	–	–
	0.15	0.0025	6.0±0.4	–	3.2±0.7 (5)
10% CS	0	0	2.9±0.1 (18)	5.0±0.7 (12)	2.5±0.3 (6)
	0.015	0.00025	2.7±0.2 (12)	4.4±0.3 (6)	2.3±0.1 (6)
	0.15	0.0025	2.9±0.3 (48)	5.9±0.6 (24)	2.1±0.5 (36)
0.05% HA	0	0	4.8±0.7 (6)	–	1.8±0.2 (6)
	0.015	0.00025	–	–	–
	0.15	0.0025	4.8±0.8 (6)	–	3.9±0.6 (6)
2.5% HA	0	0	3.8±0.3 (18)	6.2±0.5 (12)	3.5±0.6 (6)
	0.015	0.00025	3.8±0.2 (12)	5.8±0.8 (6)	2.8±0.5 (6)
	0.15	0.0025	3.8±0.3 (18)	6.6±0.4 (6)	3.7±0.4 (12)

In addition, it is known that the Ca-salt is more compact than the Na-salt [4] and steric considerations may therefore contribute slightly to the effect. As more electrolytes were added to the solution, the diffusivity of calcium increased until at physiological levels (0.15 M NaCl + 0.0025 M $CaCl_2$) it was not significantly different from its free diffusivity.

The effects of ionic strength on diffusivity at higher concentrations of CS and in HA are shown in table 5. The decrease in diffusivity of Ca^{2+} seen at low concentrations of CS is also seen in 0.05% HA, but the effect is smaller as might be expected because of the smaller charge density of HA. Perhaps the most significant feature of these data, however, is that at the high GAG concentrations (10% CS and 2.5% HA) the diffusivities are unaffected by the ionic strength of the solution.

A few measurements of diffusivity in both aggregated and disaggregated proteoglycan solutions (RCS, A1 and A1D1) at physiological ionic strength were made. None of the results were significantly different from those measured in CS solutions of the same concentration (weight percent).

4. Discussion

Since a companion paper [40] is devoted to development of the theory underlying these experiments, this discussion will be limited to a comparison of the present data with previous experiments and to consideration of their implications to connective tissue physiology. Considering first the measurement of partition coefficients, the data at low matrix concentrations can be compared with previous in vitro experiments and the data for higher concentrations can be compared with measurements on intact tissue. An impediment to the former comparison is the diversity of indices used to characterise glycosaminoglycan-ion interactions. The earlier literature was reviewed in some detail by Dunstone [12,15]. There is actually very little overlap between the experimental conditions he employed and those used in the present work, although we have both considered the effect of changing ionic strength on ionic partition. His observation of large variations of the parameter κ with ionic strength seems to contradict our finding of little effect of ionic strength. However, this disagreement is illusory because his parameter κ contains an additional factor of total ionic strength, which when removed results in a nearly constant partition of ions. Of the more recent experiments, those of Cleland [31,32] using a titration method and those of Preston et al. [4] using equilibrium dialysis covered only the lowest of our range of concentrations and were for monovalent ions only. The partitions for Na^+ in CS were comparable but were somewhat different for HA. In view of the large effect of protein content of the HA preparation demonstrated by Preston et al. the difference may well be attributable to differences in HA composition. The choice of model used by these authors to analyse the interaction will be considered in the following paper.

Woodward and Davidson [33] using a titration technique found no evidence of Ca^{2+} 'binding' in CS (in contrast to CS-proteoglycan) but they worked at very low concentrations where effects may be small and their criterion of 'binding' (departure from linearity in the plot of Ca^{2+}-electrode potential vs. added Ca^{2+}) is somewhat unspecific. We did not find differences between CS and PG and this is supported by another equilibrium dialysis study [34] again at low glycosaminoglycan concentrations. This paper viewed the interaction between matrix and ions in terms of a simple binding reaction and the model was found to break down outside a limited range of ionic strengths. The conclusion that this breakdown represents a departure from Gibbs–Donnan behaviour and demonstrates the need to invoke other than electrostatic interactions does not, however, seem justified.

The literature on ionic diffusivity is less extensive. Most directly comparable is the work of Magdelanat et al. [18] who used an open-ended capillary technique to investigate the effects of changes in solvent ionic strength on ion diffusion in a 0.05% CS gel. Using the Einstein model of diffusion to make temperature corrections, our results in this weak solution are very comparable to theirs. Dorabialska and Plonka [19] had earlier reported reduced diffusivity for sodium in heparin

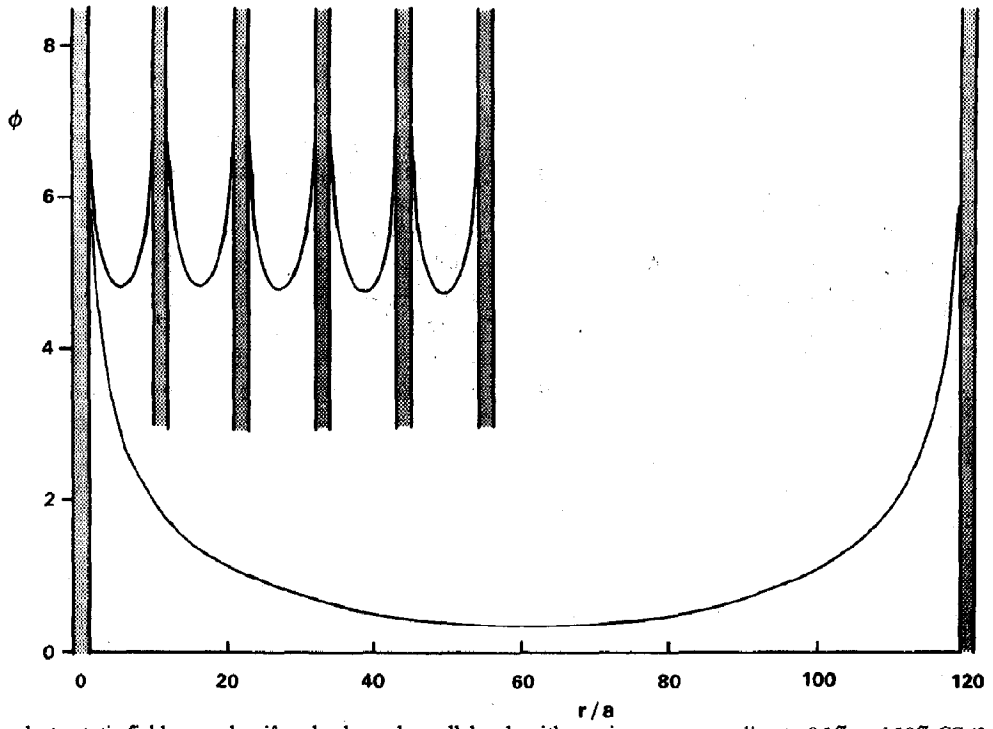

Fig. 3. The electrostatic field around uniformly charged parallel rods with spacings corresponding to 0.1% and 10% CS (for details of the calculation see ref. 40).

but not in HA but the experimental conditions were not fully described and we infer the concentrations to be $\approx 0.01\%$ for HA where we also would expect no effect. The membrane technique of Meyer et al. [17] has, as described above, been employed only at low concentrations and changes in permeability largely reflect changes in partition coefficient under those conditions.

The demonstration that, even in solutions of low ionic strength, cationic diffusivity is reduced at high glycosaminoglycan concentrations by steric, rather than electrostatic interactions, does not appear to have been previously reported. In the companion paper [40] we discuss this behaviour theoretically in terms of numerical solutions of the Poisson-Boltzmann equation. Qualitatively, however, it may be understood with the aid of fig. 3 which shows the electrostatic fields in 0.1% and 10% CS, modelled as regular arrays of parallel, appropriately spaced rods. The closer spacing of the rods at the higher concentrations reduces the potential gradient in the region between the rods and the effect on the diffusing ions of this 'smoothed' potential is expected to be reduced.

The main conclusions from our experimental results are that at physiological ionic strengths the partition and diffusion not only of monovalent ions such as Na^+ and Cl^- but also the divalent Ca^{2+} show very limited departures from the predictions of the ideal Donnan equilibria and can be approximately described in terms of this effect and the exclusion properties of the GAG. On the other hand, at low ionic strengths and low polyelectrolyte concentrations, the behaviour of the calcium ion becomes very different from that of the monovalent ions and exhibits low diffusivities and high selectivity coefficients. This difference in the behaviour of Ca^{2+} has never to our knowledge been clearly described and is probably one of the main reasons for the apparently contradictory reports present in the literature. It should be stressed

that we found no evidence of any calcium 'binding' under physiological conditions in either CS or HA. The high concentration of ionic calcium in the vicinity of the GAG moieties is merely a consequence of the electrostatic forces.

Although CS and HA are the major components of connective tissue proteoglycans, it seemed possible that the more complex configuration of the latter may affect the interaction between the negatively charged groups of the GAG and the ions in solution. Within our limited data using proteoglycans extracted from rat chondrosarcoma, this does not appear to be the case. Moreover, previous studies on articular cartilage [35], the intervertebral disc [16,36] and bovine nasal cartilage [37] have also led us to similar conclusions regarding partition and diffusion coefficients of small ions. One possible exception was a somewhat high value of K_{Na}^{Ca} obtained in human femoral head cartilage [38,39]. The reason for this may simply be that the results were based on total hydration, without taking into account the existence of an extra-fibrillar and an intra-fibrillar compartment with different fixed charged densities. In order to clarify such questions conclusively, a study similar to that described in the present paper but involving proteoglycans of different size, structure and composition rather than simple GAGs is being undertaken. It should be emphasised that the knowledge of the activity and diffusivity coefficients of the calcium ion in connective tissue matrices is important if attempts are to be made to describe quantitatively the conditions of precipitation of calcium salts in different physiological and pathological conditions.

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