Model Connective-Tissue Systems

A STUDY OF POLYION-MOBILE ION AND OF EXCLUDED-VOLUME INTERACTIONS OF PROTEOGLYCANS

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The osmotic pressure of solutions of sulphated proteoglycans isolated from the intervertebral discs of animals of various ages was determined. The behaviour of the solutions in salt-added systems was investigated in terms of the Donnan distribution of the mobile ions. It is evident that this effect is the dominating factor in explaining the observed non-idealities. Although marked variations in the compositions of the proteoglycan, with regard to their chondroitin sulphate and keratan sulphate content and hence charge content, occur with increasing age of parent tissue, the osmotic activities of the various preparations are very similar. This is explained by the 'fixation' of the counterions in such a way as to counteract any change in the charge content of the polyion; an 'osmotic buffering' effect. The swelling behaviour of gelatin gels containing the proteoglycan preparations has been measured. In all cases pressures in excess of the sum of the osmotic pressures of the individual components are observed. However, the magnitude of the excess decreases with increasing age of the parent tissue. It is suggested that the age changes, as reflected by a decrease in water content of the gel system, are not the result of changes in the osmotic properties of the individual components but rather reflect changes in the entropic interaction of the proteoglycan with the gelatin matrix. The relevance of this observation to the situation *in vivo* is discussed.

It is generally supposed that the efficient functioning of connective tissues may be dependent on their water content. Moreover, numerous investigators (Loeven & Van Walbeek, 1954; Hedbys, 1961; Meyer *et al.*, 1971) have suggested that the extent of hydration of the tissues is regulated primarily by the distribution and concentration of the glycosaminoglycans found in the extracellular matrix. Similarly it has been concluded (Hirsch *et al.*, 1953; Gower & Pedrini, 1969) that the age-related variations in the composition of the protein-glycosaminoglycan complex isolated from such tissues as the intervertebral disc may be responsible for the progressive desiccation and subsequent malfunction of the tissue with age.

The glycosaminoglycans of connective tissues, although of differing chemical structure, do possess certain common properties. They are all polyelectrolytes and carry carboxyl and *O*-sulphate ester groups which are dissociated at physiological pH. Studies of interactions occurring between the glycosaminoglycans and ions have been reported in which such techniques as electrophoresis (Bungenberg de Jong, 1949), ion-exchange behaviour (Dunstone, 1962) and

equilibrium-dialysis measurements (Farber *et al.*, 1957; Preston *et al.*, 1972) have been used. A further common feature of the glycosaminoglycans is that many of them exist in the form of high-molecular-weight molecules having a very diffuse molecular configuration which is capable of forming a continuous network of polymer chains in solution (Laurent, 1968; Ogston, 1970). For steric reasons the space available in such a network for an added macromolecule is considerably decreased. The net effect of this steric exclusion is to increase the activity or activity coefficient of the added macromolecules. This behaviour is an entropic effect and has a direct effect on the various physicochemical equilibria in the system. Such effects have been observed by a range of experimental techniques (Laurent & Ogston, 1963; Gerber & Schubert, 1964; Preston *et al.*, 1965; Ogston & Preston, 1966; Meyer *et al.*, 1971; for review see Laurent, 1972). It is evident that the glycosaminoglycan network may have a dual role in affecting the distribution of molecules between the circulation and the extracellular space of connective tissues. The charged character of the network will affect the behaviour of ionic species present and the steric exclusion properties of the matrix may regulate the behaviour of the soluble polymers known to exist in

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connective tissues (Gross, 1958; Fricke, 1961). Both these factors may be important in determining osmotic-pressure relationships *in vivo*.

The present investigation is an extension of our earlier studies (Meyer *et al.*, 1971; Preston & Meyer, 1971) of the osmotic behaviour of model connective-tissue systems. We report on the osmotic and the 'ion-binding' characteristics of the protein-glycosaminoglycan (proteoglycan) molecules isolated from the intervertebral disc of animals of various ages. The influence of the chemical and structural variations of the proteoglycan on its osmotic properties is evaluated. Further studies have been made on the effect of incorporating the proteoglycans into a gelatin gel on the swelling behaviour of these gel systems. We have applied a modified form of the osmotic-pressure relationships for quaternary systems to the swelling behaviour of the networks. An attempt is made to estimate the contributions to the total osmotic behaviour of the gels arising from the polyion-mobile ion interactions and from the polymer-polymer excluded-volume effects. The interpretation of the data in this way may have relevance to the understanding of the role that the proteoglycans play as hydration determinants in tissues.

Theory

In discussing osmotic relationships, macromolecular solutes (components 2 and 4) are considered as confined by a semipermeable membrane to a single phase denoted by ' ; this will contain solvent (component 1) and a uni-univalent salt (component 3). A phase containing only components 1 and 3 is unprimed. Osmotic pressures are expressed for example by ${}^2\pi_{1,3}$ which means that the membrane is impermeable to component 2 but permeable to components 1 and 3, which are at equilibrium across it. Any distribution of component 3 across the membrane as a result of steric interaction with any other component is taken as negligible. All physical quantities are expressed in c.g.s. units unless otherwise stated.

For a ternary system containing components 1, 2 and 3, assuming that component 2, a polyelectrolyte, and component 3 behave ideally as point charges, the osmotic pressure across the semipermeable membrane can be expressed as (Tanford, 1961):

$$\frac{{}^2\pi_{1,3}}{RT} = \frac{c'_2}{M_2} + \frac{10^3 V_1 z_2^2}{4M_1 m_3 (M_2)^2} (c'_2)^2 + \text{higher order terms in } c'_2 \quad (1)$$

where m_3 is the molarity of component 3, c_2 and z_2 are the concentration in g/ml and algebraic charge of component 2 respectively, M_1 and V_1 are the molecular weight and molar volume of component 1 respectively and M_2 is the molecular weight of component 2; R is the gas constant and T the absolute temperature.

However, the behaviour of a high-molecular-weight polyelectrolyte in solution does not conform to eqn. (1) owing to non-idealities arising from polyion-mobile ion interactions and from polymer-polymer (excluded-volume) effects. An attempt to allow for the polyion-mobile ion interactions has been given by Katchalsky *et al.* (1966) by the use of a coefficient $(\phi_P)_2$ which represents the fraction of counterions associated with the polyelectrolyte (component 2) that is osmotically active. We then have (ignoring higher terms in c'_2):

$$\frac{{}^2\pi_{1,3}}{RT} = \frac{c'_2}{M_2} + \frac{10^3 V_1 (\phi_P)_2^2 z_2^2}{4M_1 m_3 (M_2)^2} (c'_2)^2 \quad (2)$$

If it is assumed that contributions to the osmotic pressure arising from the polyion-mobile ion interactions and from the excluded-volume effects are additive, then further modification of eqn. (2) to allow for the latter effects is achieved by use of:

$$\frac{{}^2\pi_{1,3}}{RT} = \frac{c'_2}{M_2} + \left[(A_2^0)_2 + \frac{10^3 V_1 z_2^2 (\phi_P)_2^2}{4M_1 m_3 (M_2)^2} \right] (c'_2)^2 \quad (3)$$

where $(A_2^0)_2$ represents the non-ideality of component 2 arising from excluded-volume effects together with interactions between charges on different polyions.

The coefficient of $(c'_2)^2$ is a complex quantity, which is commonly expressed as the second virial coefficient of component 2, $(A_2)_2$, that is:

$$(A_2)_2 = (A_2^0)_2 + \frac{10^3 V_1 z_2^2 (\phi_P)_2^2}{4M_1 m_3 (M_2)^2} \quad (4)$$

For a quaternary system, which contains two polyionic components (components 2 and 4), we make the simplifying assumption that the Donnan distribution of component 3 is determined only by component 2, that is

$$\left[z_2 (\phi_P)_2 \frac{c_2}{M_2} \gg z_4 (\phi_P)_4 \frac{c_4}{M_4} \right]$$

This assumption is valid in the present case where the pH conditions so chosen were close to the isoionic point of component 4 (gelatin) thereby allowing any charge effects on the behaviour of the gel matrix to be considered as negligible. The osmotic pressure is then given by:

$$\begin{aligned} \frac{{}^{2,4}\pi_{1,3}}{RT} = & \frac{c'_2}{M_2} + \left[(A_2^0)_2 + \frac{10^3 V_1 z_2^2 (\phi_P)_2^2}{4M_1 m_3 (M_2)^2} \right] (c'_2)^2 \\ & + \frac{c'_4}{M_4} + (A_2)_4 (c'_4)^2 + A^* c_2 c_4 \quad (5) \end{aligned}$$

where c_4 , z_4 , M_4 and $(A_2)_4$ are the concentration, algebraic charge, molecular weight and second virial coefficient of component 4 respectively. A^* is the interaction coefficient between components 2 and 4.

It has been shown (Meyer *et al.*, 1971; Preston & Meyer, 1971) that the osmotic or swelling behaviour of a gel cross-linked in the presence of another macromolecular component can be successfully understood in terms of the statistical mechanical properties of simple three-dimensional structures (Flory & Rehner, 1943). If we regard the added macromolecular component as component 2 and the gel-forming material as component 4, then following previous nomenclature (Meyer *et al.*, 1971) and by use of eqn. (5), we can express the swelling pressure of the gel, P , as:

$$P = \frac{c'_2}{M_2} + \left[(A_2)_2 + \frac{10^3 V_1 z_2^2 (\phi_P)_2^2}{4 M_1 m_3 (M_2)^2} \right] (c'_2)^2 + (A_2)_4 (c'_4)^2 - \frac{1}{\bar{v}_4 M_c} \cdot \left[(\bar{v}_4 c'_4)^{1/3} - \frac{\bar{v}_4 c'_4}{2} \right] + A^* c'_2 c'_4 \quad (6)$$

where v_4 is the partial specific volume of component 4 and M_c is the molecular weight per cross-linked unit in the network. Note that the third term on the right-hand side of eqn. (5) is omitted from eqn. (6), since it is assumed that the molecular weight of the network (component 4) is infinite. The first two terms on the right-hand side of eqn. (6) express the 'normal' osmotic pressure of a non-ideal charged solute, the third and fourth terms the swelling pressure of gel cross-linked in the absence of component 2 and the last term expresses the interaction contribution. Earlier work (Meyer *et al.*, 1971) has shown that M_c is unaffected by the presence of component 2.

Variation in the swelling pressure of the network can be achieved by immersing the gel into a phase denoted by double prime ("') containing components 1, 3 and a non-penetrating uncharged osmotically active solute (component 6). Changes in the volume of the gel will occur and an equilibrium state is reached where:

$$P = \left[\frac{\pi}{RT} \right]'' = \frac{c''_6}{M_6} + (A_2)_6 (c''_6)^2 + (A_3)_6 (c''_6)^3 \quad (7)$$

where c_6 , M_6 , $(A_2)_6$ and $(A_3)_6$ are the concentration, molecular weight, second and third virial coefficient of component 6 respectively.

In the swelling measurements of our model gel systems we can identify the components as water (1), proteoglycan (2), NaCl–NaHCO₃, (3), gelatin (4) and dextran (6). NaCl is the major salt and the assumption is made that the HCO₃[−] ion behaves similarly to the Cl[−] (Aldrich, 1958).

Experimental

Materials

The samples of gelatin and dextran were those described previously (Meyer *et al.*, 1971; Preston & Meyer, 1971). The buffer used was 0.15M–NaCl–

0.01M–NaHCO₃, pH 8.3, containing chloramphenicol, cycloheximide and penicillin (each at a concentration of 100 µg/ml) to prevent bacterial action. This buffer was chosen so that the pH conditions were close to the isoionic point of the gelatin thereby allowing any charge effects on the behaviour of the gel matrix to be considered as negligible.

Samples of sulphated proteoglycans were prepared from bovine and equine intervertebral discs obtained from animals ranging in age from foetal development to 24 years old. There was no apparent species differentiation as evidenced by chemical composition, molecular weight and electrophoretic studies (Comper & Preston, 1971). Extraction of the proteoglycans from pooled discs of a number (two to eight) of animals of each age-group was carried out by using mild non-dissociative techniques (Hascall & Sajdera, 1969; Meyer *et al.*, 1971). Subsequent purification of the samples was by CsCl-density-gradient centrifugation (Meyer *et al.*, 1971).

A proteoglycan sample prepared from 3-year-old steers was digested with chondroitinase (*Proteus vulgaris*) in Tris–HCl buffer ($I = 0.01$, pH 8.0) for 24h at room temperature (22°C). The product, which contained less than 5% of the chondroitin sulphate chains, was purified on a CsCl density gradient at an initial density of 1.55 g/ml. After 36h at 2°C and 100000g in the no. 50 rotor of the Beckman-Spinco model L ultracentrifuge, the degraded material was isolated from the bottom half of the tube and the CsCl removed by repeated dialysis against the standard buffer. The chemical composition of the various proteoglycans was determined by using the methodology as described previously (Meyer *et al.*, 1971). Assuming that all the hexosamine carried -N-acetyl and -O-sulphate groups in a 1:1 molar ratio and allowing for a cation equivalence (as Na⁺) of all the negatively charged groups, the analytical values account for a recovery of 90% of the dried material.

Physicochemical methods

Viscosities were measured over concentration range 1–5 mg/ml at 20°C in a Zimm rotating-cylinder viscometer (Beckman Instruments, Palo Alto, Calif., U.S.A.) at a shear stress of 9.8×10^{-3} dyn·cm^{−2}. These viscosities were taken as equal to that at zero shear. The reduced viscosities were calculated by comparison of the rotation times obtained with solvent.

The molecular weights of the proteoglycan preparations were determined by sedimentation equilibrium at low concentrations (0.2 mg/ml). The experiments were performed at 20°C in a Spinco model E ultracentrifuge in the J rotor as described by Chervenka (1970). Calculations of the various average molecular weights, both whole-cell and point values,

were performed on a CDC 3200 digital computer. Ultracentrifugation carried out at different rotor speeds indicated a molecular-weight heterogeneity of the preparations; an absolute variation in the whole-cell averages of about $\pm 10\%$ was observed. The molecular-weight values reported correspond to about 80–90% of the material in the cell.

The osmotic activities of solutions of dextran and gelatin were calculated by sedimentation-equilibrium techniques as described previously (Meyer *et al.*, 1971). Estimates of the osmotic pressure of solutions of proteoglycans and of the swelling pressure of the gel systems were made by equilibrium dialysis at 5°C against solutions of known concentration of dextran as described previously (Meyer *et al.*, 1971; Preston & Meyer, 1971).

Proteoglycan–mobile ion interactions were studied by measurement of distribution of ions at equilibrium by use of isotopic species as described previously (Preston *et al.*, 1972). The concentration of component 3 (in this case NaCl) was varied from 0.005 to 0.05 M.

Results

The chemical compositions of the proteoglycan preparations are shown in Table 1. In agreement with other investigators, it is seen that as the age of the species increases, the ratio of chondroitin sulphate content to that of keratan sulphate decreases

(Gower & Pedrini, 1969; Hallen, 1962) and the degree of sulphation of the *N*-acetylhexosamine residue increases (Cifonelli *et al.*, 1967). The charge content of the proteoglycan calculated on the basis of the chemical assay, assuming that the carboxyl and sulphate ester groups carry unit negative charge, appears to decrease with age of tissue.

Some physical properties of the different preparations are summarized in Table 2. It is evident that molecular size of the proteoglycan decreases with aging of the animal source. This is in agreement with studies made on material isolated from human intervertebral discs (Lyons *et al.*, 1966).

The equilibrium distribution of Na^+ and Cl^- ions brought about by the proteoglycan samples in solutions containing various amounts of added NaCl is shown in Table 3. Estimates of the osmotically active fraction of counterions associated with the macroion, ϕ_p , are made by the use of eqn. (5a) of Preston *et al.* (1972) here expressed as:

$$(\phi_p)_2 = \frac{(c_3)^2}{c'_3 z_2 c'_2} - \frac{c'_3}{z_2 c'_2} \quad (8)$$

where activity coefficients of the ions are assumed to be unity. It is seen that the values of $(\phi_p)_2$ within the range of c_3 studied are approximately constant for each preparation. The values of $(\phi_p)_2$ increase with increasing keratan sulphate content which parallels a decrease in the charge content of the polyion (Tables 1 and 4). A theoretical examination by Manning

Table 1. *Chemical composition and characteristics of the proteoglycans*

Chondroitin sulphate was identified by uronic acid content and keratan sulphate by hexose content. For further details see the text.

Proteoglycan sample ...	Amount of substance (g/100 g dry wt.)			
	Foetal	3-year-old	24-year-old	Chondroitinase digest
Hexosamine (free base)	28.4	27.9	22.3	23.2
Uronic acid (free acid)	23.0	13.1	11.1	<1.0
Hexose	7.9	14.5	21.3	25.4
Sulphate	12.8	15.4	14.1	14.5
Sialic acid (free acid)	1.9	2.7	3.6	4.9
Protein	8.5	13.8	15.8	22.7
Weight ratio of chondroitin sulphate/keratan sulphate	2.9	0.9	0.58	<0.05
Mol ratio of sulphate/hexosamine	0.85	1.03	1.18	1.16

Table 2. *Physical characteristics of proteoglycans isolated from intervertebral discs of animals of different ages*

For details see the text. Values are given as means $\pm$ S.E.M.

Proteoglycan sample ...	Foetal	3-year-old	24-year-old	Chondroitinase digest
Number-average molecular weight	7.5×10^5	6.0×10^5	3.1×10^5	3.0×10^5
Weight-average molecular weight	8.2×10^5	6.6×10^5	3.4×10^5	3.2×10^5
$10^{-1} \times [\eta]$	2.25 ± 0.15	2.20 ± 0.15	1.2 ± 0.1	—
Charge content (mequiv./g)	2.42	2.26	2.02	1.68

Table 3. *Distribution of Na⁺ and Cl⁻ between solutions of sulphated proteoglycan and solutions of NaCl, and other data*

Proteoglycan sample	For details see the text.				
	Concn. of NaCl, c_3 (mM)	$\frac{c_{Na^+}}{c_{Na^+}}$	$\frac{c_{Cl^-}}{c_{Cl^-}}$	$\frac{10^2 \times c'_2 z_2}{(\text{equiv./litre})}$	$(\phi_P)_2$
Foetal	78.4	1.19	0.94	1.93	0.52
	38.7	1.36	0.90	1.79	0.48
	20.7	1.42	0.87	1.15	0.53
	10.5	1.77	0.77	1.05	0.53
	8.0	1.74	0.76	0.78	0.58
	7.6	2.22	0.66	1.18	0.55
3-year-old	14.8	1.80	0.72	1.60	0.61
	10.5	1.84	0.74	1.16	0.58
	8.0	2.17	0.70	1.17	0.51
	5.4	2.50	0.65	1.00	0.58
	5.1	3.65	0.45	1.63	0.55
24-year-old	78.4	1.17	0.93	1.91	0.60
	57.7	1.21	0.91	1.72	0.67
	38.7	1.42	0.81	2.34	0.68
	20.0	1.70	0.76	1.94	0.59
Chondroitinase digest	78.4	1.10	0.95	1.16	0.72
	57.7	1.12	0.93	1.09	0.79
	38.7	1.24	0.87	1.42	0.78
	17.6	1.73	0.71	1.80	0.70
	14.3	2.01	0.64	1.96	0.74
	7.6	2.94	0.42	1.92	0.76
	5.1	3.46	0.38	1.57	0.75

Table 4. *Na⁺ fixation by various proteoglycan preparations: analysis of Donnan equilibrium measurements by use of eqn. (8) and other data*For details see the text. Values of $(\phi_P)_2^*$ are given $\pm$ S.E.M.

Proteoglycan sample	Concn. range of NaCl, c_3 (mM)	No. of expts.	$(\phi_P)_2^*$	$(\phi_P)_2^* z_2 / M_2$ (mequiv./g)
Foetal	5-60	6	0.44 ± 0.05	1.06
3-year-old	5-60	5	0.46 ± 0.05	1.05
24-year-old	20-80	4	0.53 ± 0.04	1.07
Chondroitinase digest	5-60	7	0.62 ± 0.05	1.04

(1969) of counterion fixation has suggested that the measurement of the Donnan distribution as carried out in the present study would yield a high value of $(\phi_P)_2$ in error by a factor of 1.21. The average $(\phi_P)_2$ values corrected by this factor are shown in Table 4 and it is these values denoted by $(\phi_P)_2^*$ which will be used in subsequent calculations of osmotic pressure. The osmotic pressures of the various proteoglycan preparations as measured by equilibrium dialysis against dextran solutions are shown as a function of polymer concentration in Fig. 1. It is evident that the osmotic behaviour of all the preparations is very similar. The results have been analysed into the general form of the osmotic-pressure equation, eqn. (7), and values of $(A_2)_2$ calculated by least squares by using the method of Fletcher & Powell (1963) programmed for a CDC 3200 digital computer to per-

form the minimization. For all samples studied the value of $(A_2)_2$ was found to be $1.90 \times 10^{-3} \pm 0.2 \times 10^{-3}$.

The details of the deswelling experiments on the gel systems are shown in Table 5. In all cases the observed swelling pressures exceed the sum of the individual pressures of the two macromolecular components, in agreement with our previous results (Meyer *et al.*, 1971). By use of eqn. (6) and of the osmotic-pressure relationships shown in Fig. 1, which includes the effect of concentration on the swelling pressure of a 1% (w/w) gelatin gel, estimates of the interaction coefficient A^* are made (Table 5). The values of A^* for material from 24-year-old source are lower, a decrease paralleled by that of the molecular weight (Table 2). Although the comparison of A^* values is being made between systems having a different ratio

of gelatin to proteoglycan, it has been shown (Meyer *et al.*, 1971) that the value of A^* for a given system is independent of this ratio.

Discussion

It is well established that the major glycosaminoglycans of the intervertebral disc are chondroitin sulphate and keratan sulphate (Hallen, 1962; Rosenberg *et al.*, 1967; Gower & Pedrini, 1969). Investigations have shown (Lyons *et al.*, 1966; Rosenberg *et al.*, 1967; Comper & Preston, 1971) that these polysaccharides are linked together with a protein moiety to form high-molecular-weight hybrid species displaying a large compositional heterogeneity. By using discs from animals of various ages as source material, we have observed the characteristic changes in the chemical (Hallen, 1962; Cifonelli *et al.*, 1967; Gower & Pedrini, 1969) and physical properties (Lyons *et al.*, 1966) of the isolated proteoglycan preparations. Following the observation that both the chondroitin sulphate content of the proteoglycan decreased with age as did the degree of hydration of the parent tissue, it was tempting to suggest that these two phenomena were linked through the agency of the osmotic properties of the macroion. On the basis that the contribution of the Donnan equilibrium to the osmotic activity of the polyion far exceeds all other effects, it may have been reasonable to expect that the more heavily charged chondroitin sulphate-rich proteoglycan would have a greater osmotic activity than the keratan sulphate-rich material. It is evident from this study that the osmotic pressures of the various proteoglycan preparations are very similar in spite of an apparent 22% decrease in the charge content for

chondroitin sulphate-poor material (Table 2). Although a small part of this expected decrease in osmotic activity is compensated by an increase arising from the smaller molecular weight of the preparation,

Fig. 1. Effect of concentration on the swelling pressure of a 1% (w/w) gelatin gel (Meyer *et al.*, 1971) (—) and on the osmotic pressure of solutions of proteoglycans isolated from foetal tissue (●), from 3-year-old tissue (■) and from 24-year-old tissue (○)

For details see the text.

Table 5. Details of deswelling experiments on gels and values of the osmotic-pressure excesses and corresponding interaction coefficients, A^*

For details see the text.

Sample and gelling conditions	$10(\pi/RT)$ external (mosm)	$10^2 \times$ Final concn. (g/g)		$10(\pi/RT)$ excess (mosm)	$10^3 A^*$
		Gelatin	Proteoglycan		
Foetal proteoglycan; 1% gelatin–0.53% proteoglycan	67.0	5.79	3.07	41.9	1.41
	79.0	6.17	3.32	49.5	1.42
	100.0	6.88	3.65	59.9	1.59
	117.0	7.41	3.93	69.7	1.62
Proteoglycan from 3-year-old; 1% gelatin–0.56% proteoglycan	21.0	3.35	1.87	8.5	1.37
	31.7	4.09	2.29	13.5	1.45
	51.7	5.13	2.86	22.5	1.52
	71.2	5.95	3.32	30.0	1.52
	91.0	6.65	3.72	37.0	1.57
Proteoglycan from 24-year-old; 1% gelatin–0.28% proteoglycan	10.6	3.44	0.97	2.3	0.69
	26.5	6.46	1.83	6.2	0.53
	63.7	7.85	2.23	22.0	1.25
	76.1	8.34	2.37	19.1	0.97
	91.6	9.13	2.59	26.9	1.14

Table 6. *Second virial coefficient of proteoglycans*

For details see the text.

Proteoglycan sample	$10^3 \times (A_2)_2$ experimental	From polyion-mobile ion interactions	From excluded- volume effects
		$10^3 \times \left[\frac{10^3 V_1 z_2^2 (\phi_p)_2^{*2}}{4M_1 m_3 (M_2)^2} \right]$	$10^3 \times \left[\frac{1.6 [\eta]_2}{M_2} \right]$
Foetal	1.9	1.75	0.48
3-year-old	1.9	1.72	0.60
24-year-old	1.9	1.79	0.62

the major compensating factor arises from the 'ion-binding' characteristics of the polyion. The Donnan distribution studies clearly indicate that a large fraction of the counterions associated with the proteoglycans is in some way 'bound' to the polyelectrolyte. Tables 1 and 4 show that with a decreasing chondroitin sulphate content and hence a decreased charge content, the value of $(\phi_p)_2^*$ steadily increases. However, we observe that the 'effective' charge content, i.e. $(\phi_p)_2^* z_2/M_2$, remains constant. This is further emphasized by the studies on the chondroitinase-digested material for which the charge content is decreased by 35% relative to foetal material but for which the value of $(\phi_p)_2^* z_2/M_2$ remains comparable with those for other preparations. These results indicate that in spite of the marked variation in charge content of the proteoglycan, whether it is derived from carboxyl or sulphate ester groups, the osmotic contribution arising from the counterions remains constant. This behaviour is predicted by a modified form of Manning's theory (Wells, 1973) and has been discussed as a general phenomenon by Katchalsky *et al.* (1966) in terms of an 'osmotic buffer'.

In Table 6 we have calculated the contribution to $(A_2)_2$ of the Donnan equilibrium as suggested by eqn. (4). It is seen that the polyion-mobile ion interactions account for the major part of the non-ideality of the polyion. Further support for this suggestion is provided from an approximate calculation of the excluded-volume effects.

As has been discussed (Nagasawa & Takahashi, 1972) there is no established method for evaluating the coefficient A_2^0 for a polyion. However, we have assumed that an approximate value may be obtained by using the 'dilute solution' treatment of non-ionic polymers as given by Flory (1953). The simplified view is taken that the polyion is considered as uncharged and behaves as a non-penetrating sphere. In this case:

$$(A_2^0)_2 = \frac{16\pi(r_2)^3 N}{3M_2^2} \quad (9)$$

where r_2 is the equivalent radius of component 2. An independent estimate of r_2 can be obtained from vis-

cosity data, if the same model is again assumed, by the use of the expression (Tanford, 1961):

$$[\eta] = \frac{\pi r^3 N}{0.3M} \quad (10)$$

where $[\eta]$ is the intrinsic viscosity.

Rearranging eqns. (9) and (10) we find that:

$$(A_2^0)_2 = \frac{1.6[\eta]_2}{M_2} \quad (11)$$

The values for $(A_2^0)_2$ based on the mechanistic approach of an excluded volume and calculated by use of eqn. (11) are considerably smaller than the experimental values of $(A_2)_2$ (Table 6). The absence of any dependency of $(A_2)_2$ on molecular weight of the proteoglycan is supportive evidence that the excluded-volume effects may contribute only a minor role in the non-ideal behaviour of the polyion.

The incorporation of the proteoglycans into the gelatin gels gives rise to marked excesses in the osmotic pressures. In the absence of any knowledge of the salt distribution arising from interactions within the complex gel system, a precise quantitative explanation of this behaviour is not possible. However, the decrease in the excess pressure with a parallel decrease in the molecular size of the proteoglycan and our previous studies (Meyer *et al.*, 1971) do suggest that the excess may arise from the mutual steric exclusion of the gelatin chains and the proteoglycan molecules. Further use of the treatment of Flory (1953) relates the interaction coefficient, A^* , to excluded-volume effects by the expression:

$$U_{2,4} = A^* M_2 M_4 \quad (12)$$

where $U_{2,4}$ is the molar excluded volume of pairs of molecules 2-4. This may also be expressed as $\epsilon_{2,4}$, the excluded volume of component 4 per g of component 2, by:

$$\epsilon_{2,4} = \frac{U_{2,4}}{M_2} = A^* M_4 \quad (13a)$$

or, conversely, as the excluded volume of component 2 per g of component 4:

Table 7. Values of the excluded volume ($\epsilon_{4,2}$) for proteoglycans per g of globular protein

For details see the text.

Protein	Proteoglycan sample	$10^{-3} \times \epsilon$ (ml/g)
Gelatin	Foetal	1.13
	3-year-old	0.90
	24-year-old	0.28
	Hyaluronate*	25.7
Bovine serum albumin	Hyaluronate†	13.6
	Hyaluronate‡	0.7
	Nasal septum§	1.23

* From Meyer *et al.* (1971).

† From Ogston & Preston (1966).

‡ From Laurent & Ogston (1963).

§ From Gerber & Schubert (1964) with $(M_n)_2 = 1 \times 10^6$.

$$\epsilon_{4,2} = \frac{U_{2,4}}{M_4} = A^* M_2 \quad (13b)$$

Expression of the excluded-volume effects per g of proteoglycan, that is by an evaluation of $\epsilon_{2,4}$, is precluded in these studies as the molecular weight of the gelatin gel (M_4) is regarded as infinite. Table 7 shows the values of $\epsilon_{4,2}$ calculated by eqn. (13b). Estimates of excluded volumes have been made for related systems and are included in Table 7 for comparative purposes. The similarity of these values of $\epsilon_{4,2}$ suggests that our present interpretation of the osmotic-pressure excesses may be a reasonably satisfactory one.

The concept that a gel state exists in the nucleus pulposus of the intervertebral disc has been suggested by Sylven *et al.* (1951), who postulated a loose entanglement of the collagen and proteoglycan constituents. More recent studies (for review see Szirmai, 1970) on the morphological and physicochemical characteristics of the tissue confirm this view. From the studies made on our model system, we suggest that the age changes as reflected in the dehydration of this tissue are not the result of changes in the osmotic properties of the constituent proteoglycan but rather reflect changes in the interactions of the proteoglycan with either the matrix material or other polymeric components. Soluble non-collagenous proteins exist in the nucleus pulposus of embryonic and young species (Fricke, 1961): we envisage that the mutual exclusion of these materials with the proteoglycan will be an important factor in the hydration of the tissue. Such a mechanism would become less effective with increasing age as the collagen becomes fibrillated and cross-linked (Sylven *et al.*, 1951) and the non-collagenous proteins crystallize and denature (Happley *et al.*, 1969). As Laurent (1968) pointed out, it is difficult to assess the magnitude of these inter-

actions *in vivo*, principally owing to the lack of knowledge of the amounts of constituents in the tissue and what factors maintain them.

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