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The Effects of the Hyaluronic Acid Complex on the Distribution of Ions

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Manery (1954) and Meyer & Rapport (1951) have suggested that hyaluronic acid, which is believed to be widely distributed in connective tissue, may have important effects on the distribution of ions within the body fluids. The present work was undertaken primarily to test this suggestion. Since there is evidence (Ogston & Stanier, 1950, 1952; Blumberg & Ogston, 1957a, b) that hyaluronic acid exists in synovial fluid and connective tissue as a complex with protein, it was decided to use this material, isolated from synovial fluid.

Although this preparation is chemically more complex than the polysaccharide isolated by Meyer & Palmer (1934) and others, the results obtained have also thrown light on its properties as a poly-electrolyte. Theoretical consideration of this aspect of the work leads to certain criticisms of the commonly used concept of 'ion-binding' (Klotz, 1953).

Ionic distributions have been commonly used for the estimation of extracellular volumes, and the general question of the steric exclusion of solutes from solutions of hyaluronic acid complex has been studied through the investigation of the distribution of the trisaccharide raffinose in solutions of the complex.

EXPERIMENTAL

Hyaluronic acid complex. This was prepared by the method of Ogston & Stanier (1950). The complex was washed on a sintered-glass ultrafilter of average pore diameter 1μ by redispersion in wash fluid followed by refiltration. Six washes were carried out with 0.2N-NaCl solution, and six with water. Wash fluids were not buffered, so that ions extraneous to the experiments might not be introduced. Curtin (1955) showed that this variation in wash fluids was permissible.

Dialysis. Dialysis bags containing about 3 ml. of a solution of hyaluronic acid complex in water were dialysed at 4° in glass conical flasks against solutions containing the ions under study or raffinose. All solutions were saturated with chloroform as antiseptic. After 5-7 days the phases in

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equilibrium were sampled for analysis. Forty-two dialyses of the complex against solutions of NaCl were performed. The concentration of NaCl was varied between 18 and 145 mM. Comparative experiments with KCl, KSCN and CaCl₂ were also performed.

Variation of pH. The unbuffered NaCl solutions showed a range of pH 5.6–6.3. A higher range, pH 7.8–8.1, was obtained by the addition to the NaCl solutions of NaHCO₃ in such concentration that $[\text{HCO}_3^- \text{ ion}] = 0.32 [\text{Cl}^- \text{ ion}]$, which follows approximately the relative concentration of the ions in serum (Gamble, 1954).

Determination of chloride. Chloride in the solutions was determined by potentiometric microtitration of weighed samples of solution containing about 300 μg . of chloride with standard AgNO₃ solution. The electrode system was Ag–unknown solution–0.1N-NH₄NO₃ bridge–sat. KCl/AgCl–Ag. Mixing was given by the stationary electrodes while the vessel which contained the solution was rotated on a pneumatic turntable. Care was needed to ensure that annular concentration gradients were not set up in the viscous solution.

Solutions were made up to 1 ml. with water before titration, unless NaHCO₃ was present, when 0.1N-HNO₃ was used to eliminate interference by HCO₃⁻ ions. Preliminary experiment showed that the hyaluronic acid complex did not interfere in the determination of chloride. The accuracy of this method was sufficiently high for the small differences in chloride concentration between the phases to be estimated with significance. The S.E.M. of four replicate determinations was about 0.1%.

Determination of thiocyanate. This ion was determined by the same method as that given above for chloride. The same order of accuracy was obtained.

Determination of bicarbonate. Bicarbonate was determined by volumetric measurement of CO₂ formed by acidification of weighed samples of solution with 0.1N-HCl in a Warburg constant-volume-manometer apparatus. The apparatus was used in the standard manner, with special attention to attaining the highest possible accuracy with it. The samples yielded 100–150 μl . of CO₂; when it was necessary, the volume in the flasks was made up to approx. 3 ml. with CO₂-free water. The S.E.M. of duplicate determinations was about 0.3%, which is rather better than is usually obtained with the Warburg apparatus.

Determination of sodium. Sodium was determined flame photometrically with a Beckman model DU spectrophotometer with flame attachment as modified by Exley & Sproat (1958). Replicates of solution containing about 5 μg . of sodium were weighed into polythene weighing bottles, and diluted to 2 ml. with water which had been passed through a column of ion-exchange resin Amberlite IR-120-H so that its sodium concentration was less than 20 μg ./l. Hyaluronidase (0.2 i.u.) was then added to each and the solutions were kept at 37° for 16 hr. in a water bath. The solutions were then diluted with the special 'low-sodium' water to about 5 g. by wt. Hyaluronic acid complex present was then so degraded and diluted that the viscosities of the solutions were not sensibly different from that of water, and sodium could be determined by direct comparison with standards in the flame photometer. The photoelectric signal of the instrument was directly amplified and measured with a pen recorder. With experience, only four replicates were necessary to obtain an S.E.M. of 0.1–0.2%.

Determination of calcium. Calcium was determined flame photometrically in preparations of hyaluronic acid complex with the same method of degradation and dilution of the sample as was used for sodium. The final concentration of calcium in the solutions analysed was about 8 μg ./g. The accuracy was about 2%.

Determination of hyaluronic acid complex. The concentration of the complex was determined from the difference between the dry weight of a weighed sample of solution in water (after heating at 102–104° for 2 hr.) and the weight of the same sample after ignition.

Determination of raffinose. The sugar was determined colorimetrically by the method of Kulka (1956), after deproteinization of solutions with HPO₃. A small correction was necessary for the colour developed from the complex.

Direct determination of the equivalence of hyaluronic acid complex. The equivalence of the complex was directly determined by titration with cetylpyridinium chloride solution (Scott, 1956) of buffered solutions of the complex. Two buffers were used. The first, of pH 7.8–7.9, contained 0.1N-NaHCO₃ and 0.01N-Na₂SO₄. The second contained Na₂HPO₄ and NaH₂PO₄ at pH 7.0 and had ionic strength 0.175, and Na₂SO₄ was dissolved in it to 0.01N.

Hyaluronic acid complex solution (0.5 g.) was weighed out, and about 2 ml. of buffer solution added. An excess of cetylpyridinium chloride solution of known concentration was added slowly with mixing and the solution allowed to stand for 15 min. The SO₄²⁻ ion present assisted the formation of a compact precipitate, which was removed by centrifuging. The excess of reagent was then estimated spectrophotometrically at 260 m μ .

The cetylpyridinium chloride was twice recrystallized from water; the equivalence of its solution (3.85 m-equiv./l.) was determined by titrating its chloride content by the method described.

THEORETICAL

The interaction of ions with a polyelectrolyte may be treated in two ways: (i) by the description of the system as an equilibrium between the polyelectrolyte, the interacting ion and a polyelectrolyte-ion complex; or (ii) by the statement of the parameters of a Donnan equilibrium. The first method is potentially more powerful, in that it can describe other forms of interaction supplementing electrostatic interaction, but it suffers from the disadvantage that it implies that the interaction can be forced to completion by increasing the ionic concentration. This is not necessarily so, as the interacting ion can only be introduced into solution together with ions of opposite sign, which also enter the interacting system. The second method does not take into account forces of interaction other than electrostatic forces, but it is mathematically simpler, and has the advantage of describing the distributions of ions of both signs. Adamson (1952) has pointed out that the two treatments are thermodynamically consistent, differing only in their formulation of the standard states.

It is now recognized (Overbeek, 1956) that, for the univalent ions of the salt MA, the simple statement of the Donnan equilibrium

$$[\text{M}^+][\text{A}^-] = [\text{M}^+'][\text{A}^-'] \quad (1)$$

is not valid in dilute solutions of a polyelectrolyte with a high surface potential, and the true equilibria have been

described (i) by the introduction of empirical activity coefficients, (ii) by the description of deviations as 'ion-binding', (iii) by the recalculation of the Donnan equation without the use of the mathematical approximations of Debye and Hückel. Klaarenbeek (1946) has calculated and experimentally verified that the Donnan equilibrium in a solution of a colloid that bears charges only of negative sign can be described in terms of two parameters, which we may call E , the equivalence of the colloid in m-equiv./g., and α_- , the fraction of the colloidal equivalence which is neutralized by a deficiency in the solution of ions of the same sign as the colloid. α_- should be independent of ionic strength in a given system, and lies between 0 and 0.5. It will be apparent that these parameters can also be used to describe systems where other types of interactions occur. Then for a negative colloid

$$\left. \begin{aligned} \Delta[A^-] &= -\alpha_- E \\ \Delta[M^+] &= (1 - \alpha_-) E \end{aligned} \right\} \quad (2)$$

where $\Delta[A^-]$ and $\Delta[M^+]$ are the changes, in m-equiv./g. of colloid, in the concentrations of the anion and cation respectively in the colloid phase following from interactions of the ions with the colloid.

This treatment can be extended to solutions of colloids bearing charges of both signs. The proteins are an example of this type of colloid. The charge on an amphoteric colloid can be considered as occurring on a large number of equally charged zones of both signs. These charges either may interact with adjacent charges of opposite sign, so that the charge is in some part internally neutralized, or may induce local Donnan equilibria. Then a solution of 1 g. of a colloid can be considered to contain n_1 zones of unit negative charge and n_2 zones of unit positive charge, of which n_3 zones of each charge interact totally with each other, so that $(n_1 - n_3)$ negative zones and $(n_2 - n_3)$ positive zones induce local Donnan equilibria.

At any one of the negative areas, j , which induces a Donnan equilibrium,

$$\left. \begin{aligned} j\Delta[A^-] &= -j\alpha_- e_- \\ j\Delta[M^+] &= (1 - j\alpha_-) j e_- \end{aligned} \right\} \quad (3)$$

where $j e_-$ is the equivalence of area j , which is equal to the equivalence of each of the other negative areas. The gross negative equivalence $E_- = \sum j e_- = n_1 e_-$. Of this gross negative equivalence, the quantity $E_i = n_3 e_-$ will interact internally with positive zones. Then the negatively charged areas will induce as a sum shifts in the concentrations of M^+ and A^- , as given by

$$\Delta[A^-]_1 = \sum_j (-j\alpha_- e_-) = -\alpha_- (E_- - E_i), \quad (4)$$

$$\text{and} \quad \Delta[M^+]_1 = (1 - \alpha_-) (E_- - E_i), \quad (5)$$

where α_- is defined by equation (4) and will lie between 0 and 0.5.

We may similarly write for the Donnan shifts due to the positive charges, of total equivalence $E_+ = n_2 e_+$,

$$\Delta[A^-]_2 = (1 - \alpha_+) (E_+ - E_i), \quad (6)$$

$$\Delta[M^+]_2 = -\alpha_+ (E_+ - E_i). \quad (7)$$

Equations (4) and (6) and equations (5) and (7) may be summed to give the following identities, which describe the observed total shifts in ionic concentrations:

$$\Delta[A^-] = E_+ (1 - \alpha_+) - E_- \alpha_- - E_i (1 - \alpha_+ - \alpha_-), \quad (8)$$

$$\text{and} \quad \Delta[M^+] = E_- (1 - \alpha_-) - E_+ \alpha_+ - E_i (1 - \alpha_+ - \alpha_-). \quad (9)$$

Several remarks can be made on these equations: (i) that, when $\alpha_- = \alpha_+ = 0.5$, $-\Delta[A^-] = \Delta[M^+] = 0.5(E_- - E_+)$, which is the classical statement of the Donnan equilibrium for negative colloid ($E_- > E_+$) due to its net charge ($E_- - E_+$). This would be expected to hold when the ionic strength is very low so that electrostatic shielding between charged areas on the colloid is negligible; (ii) that when the ionic strength is high, so that charged areas, shielded from each other, tend to induce Donnan equilibria independently of each other, then E_i becomes disappearingly small and $\Delta[A^-]$ will increase and may reach positive values.

This phenomenon, for example, of anion concentrations in solutions of proteins, showing progressively increasing deviations from the predictions of a Donnan function with increasing ionic strength, is well known and is termed 'ion-binding'. The deviations are commonly used as a measure of the number of positive 'sites' to which the anion is 'bound', i.e. as a method of investigation of the fine structure of colloids. It will be apparent from equations (8) and (9) that a knowledge of $\Delta[A^-]$ and $\Delta[M^+]$ from ionic-distribution experiments is not sufficient to permit the calculation of E_- and E_+ .

In the experiments described, apparent values of α_- have been computed from equation (2), and interpretation of the variation of α_- has been restricted to qualitative statements of the zwitterion character of the hyaluronic acid complex.

RESULTS

Distribution of sodium salts in solutions of hyaluronic acid complex

Competition between chloride and bicarbonate ions.

The statement of the parameters of a Donnan distribution will describe the changes in anion and cation concentrations in the colloidal phase, but will not distinguish between ions of the same sign and charge. An ion may preferentially displace another ion which is electrically identical from an ionic double layer because of differences other than charge. Two ions are said to behave ideally if the changes in their concentrations in the colloid phase are proportional to their concentrations in the diffusate. Table 1 shows the determined deficiencies in Cl^- and HCO_3^- ions in the colloid phase in two dialyses of hyaluronic acid complex solutions against solutions containing NaCl and NaHCO_3 . The concentration deficiencies of each ion are expressed as a percentage of their concentrations in the dialysing solution. In each dialysis, the percentage deficiency of HCO_3^- ion was the same, within the limits of experimental error, as that of Cl^- ion. It was therefore proper, knowing the ratio between the concentrations of the two ions in the dialysing solutions as prepared, to calculate the total deficiency of anion, $\Delta[A^-]$, for all dialyses where both Cl^- and HCO_3^- ions were present, as

$$\Delta[A^-] = \frac{[A^-]}{[\text{Cl}^-]} \Delta[\text{Cl}^-]. \quad (10)$$

Table 1. *Donnan expulsion of chloride and bicarbonate ions from hyaluronic acid complex solutions*

C is the concentration of an ion in the dialysing solution. Percentage deficiency is the concentration deficiency of an ion in the colloid phase, expressed as a percentage of its deficiency in the dialysing solution. S.E. is the standard error of the percentage deficiency.

Dialysis no.	Ion	C (m-equiv./kg.)	Percentage deficiency	S.E.
43	Cl^-	10.55	2.2	0.4
	HCO_3^-	3.62	1.9	0.4
44	Cl^-	10.56	3.2	0.1
	HCO_3^-	10.65	2.9	0.4

Table 2. *Changes in ionic concentrations in solutions of hyaluronic acid complex and sodium salts, and calculation of coefficient α_-*

$[\text{NaA}]$ is the concentration of sodium salts. Experimental values of the changes in concentration of Cl^- and Na^+ ions, $\Delta[\text{Cl}^-]$ and $\Delta[\text{Na}^+]$, are in units of m-equiv./kg. of solution, and have a negative sign when the ion is in a deficiency in the colloid phase. The standard errors of $\Delta[\text{Cl}^-]$ and $\Delta[\text{Na}^+]$ are shown in parentheses. In the last 16 rows of results, HCO_3^- ion was present, and $\Delta[\text{A}^-] = 1.32 \Delta[\text{Cl}^-]$. α_- is calculated from equation (2), $\Delta[\text{A}^-] = -\alpha_- E$, where E is estimated from equation (11), and $\Delta[\text{A}^-]$ is in units of m-equiv./g. of hyaluronic acid complex (HAC).

pH	Concn. of HAC (%, w/w)	$[\text{NaA}]$ (m-moles/kg.)	$\Delta[\text{Cl}^-]$	$\Delta[\text{Na}^+]$	α_-
5.6	0.34	144	2.4 (0.55)	4.3 (0.59)	-1.42
5.8	0.31	143	2.2 (0.22)	3.9 (0.34)	-1.22
5.7	0.22	143	0.7 (0.24)	1.4 (0.40)	-0.59
5.6	0.14	144	0.7 (0.23)	1.4 (0.42)	-1.00
5.7	0.16	141	0.6 (0.15)	1.6 (0.48)	-0.70
5.7	0.12	142	0.7 (0.12)	1.1 (0.45)	-1.07
5.8	0.20	96	0.3 (0.14)	1.1 (0.36)	-0.26
5.6	0.22	96	0.7 (0.21)	1.7 (0.40)	-0.64
5.6	0.19	48	-0.3 (0.18)	1.0 (0.18)	0.32
5.8	0.21	49	-0.2 (0.08)	1.0 (0.21)	0.17
5.9	0.39	139	1.9 (0.15)	3.6 (0.48)	-1.02
5.7	0.17	138	0.7 (0.31)	—	-0.76
5.8	0.40	47	-0.67 (0.07)	1.9 (0.26)	0.29
5.8	0.14	46	-0.15 (0.04)	—	0.19
6.1	0.31	136	0.3 (0.15)	2.6 (0.24)	-0.14
6.0	0.32	136	-0.1 (0.14)	—	0.05
5.9	0.27	46	-0.69 (0.15)	—	0.43
6.1	0.28	46	-0.61 (0.06)	—	0.32
5.8	0.29	19	-0.88 (0.08)	—	0.52
6.3	0.28	20	-1.10 (0.10)	1.53 (0.13)	0.51
6.2	0.16	136	-0.2 (0.10)	—	0.18
6.1	0.24	136	-0.1 (0.07)	—	0.06
5.9	0.22	46	-0.30 (0.06)	—	0.23
6.1	0.17	46	-0.20 (0.03)	—	0.17
5.9	0.16	19	-0.33 (0.05)	—	0.34
5.9	0.16	19	-0.38 (0.06)	—	0.32
7.8	0.40	142	-1.6 (0.14)	—	0.40
7.8	0.40	140	-1.4 (0.13)	—	0.34
8.0	0.14	140	-0.2 (0.09)	—	0.15
8.0	0.13	139	-0.3 (0.10)	—	0.22
7.8	0.32	138	-1.9 (0.13)	2.0 (0.11)	0.59
8.0	0.30	136	-1.3 (0.14)	—	0.41
8.1	0.28	45	-0.51 (0.03)	—	0.17
8.0	0.29	46	-0.93 (0.07)	—	0.30
7.8	0.30	19	-0.87 (0.03)	—	0.29
7.8	0.30	18	-0.79 (0.04)	2.73 (0.07)	0.27
7.9	0.16	135	-0.4 (0.14)	—	0.23
7.9	0.15	135	-0.4 (0.17)	—	0.24
7.9	0.17	45	-0.45 (0.05)	—	0.26
8.0	0.15	45	-0.35 (0.04)	—	0.22
7.9	0.16	19	-0.36 (0.03)	—	0.22
7.8	0.16	18	-0.38 (0.06)	—	0.23

Equivalence of the hyaluronic acid complex. The analytical data describing the dialyses performed are given in columns 1-5 in Table 2. After transforming $\Delta[\text{Cl}^-]$ and $\Delta[\text{Na}^+]$ to units of m-equiv./g. of complex, and calculating $\Delta[\text{A}^-] = 1.32 \Delta[\text{Cl}^-]$ for the dialyses where HCO_3^- ion was present (pH 7.8-8.1), values were calculated of the equivalence of the complex/g., $E = \Delta[\text{Na}^+] - \Delta[\text{A}^-]$, for the 16 dialyses where both $\Delta[\text{Na}^+]$ and $\Delta[\text{A}^-]$ were determined. These values of E were linearly correlated with pH and [NaA], the millimolar concentration of sodium salts. There was found to be no significant correlation between E and [NaA], but the correlation between E and pH was highly significant, the relationship being

$$E = 0.37 \text{ pH} - 1.57, \quad (11)$$

where E is in units of m-equiv./g. of complex. It is realized that the true form of this relationship is likely to be that of a titration curve, and not linear. However, the results were not sufficiently accurate to permit the fitting of any particular titration curve, and the linear form was sufficient to demonstrate a correlation between E and pH and to allow estimates of E to be made for the calculation of α_- . The approximation is good enough to demonstrate the polyelectrolyte character of the complex, and the fitting of a more plausible form of function would not affect the conclusions drawn.

Fig. 1. Scatter diagram of values of the coefficient α_- calculated from experimental results (see text) and plotted against concentration of salt. Some experimental values have been omitted to avoid crowding of the diagram. O, pH 5.5-5.8; +, pH 5.9-6.3; ●, pH 7.8-8.1.

Experimental values of α_- , and its correlation with pH and salt concentration. From values of $\Delta[\text{A}^-]$ in the consistent units of m-equiv./g. of complex, and estimates of E from equation (11), values of α_- could be calculated with equation (2). These values are shown in the last column of Table 2, and are plotted against [NaA] in Fig. 1. In this figure different symbols are used to indicate pH ranges. At low pH values, and higher values of [NaA], negative values of α_- occur. Statistical correlation of these values showed that, in the range pH 7.8-8.1, α_- was independent of pH and ionic strength, being constant at 0.3. In the range pH 5.5-6.3, the data can be fitted to the regression equation

$$\alpha_- = -1.39 \times 10^{-1} [\text{NaA}] - 9.46 \times 10^{-1} (\text{pH}) + 2.22 \times 10^{-2} (\text{pH}) [\text{NaA}] + 6.19. \quad (12)$$

The coefficients of equation (12) are significant at the 0.1% level. The partial correlations are probably not linear, as this regression equation implies, but the experimental scatter does not permit more accurate curve fitting. The high significance of the coefficients in equation (12) indicates, however, that it can be stated qualitatively that, in this pH range, α_- does not conform to the Klaarenbeek prediction that it will lie between 0 and 0.5, and will be independent of ionic strength. Rather, in this range, pH 5.5-6.3, it decreases and reaches negative values with increasing ionic strength, the effect being more pronounced with decreasing pH. In the range pH 7.8-8.1, $\alpha_- = 0.3$, and is independent of ionic strength, and therefore conforms with the predictions of Klaarenbeek.

Distribution of other electrolytes. The results of comparative experiments which examined the independent distributions in solutions of hyaluronic acid complex of the ions of NaCl, KCl, KSCN and CaCl_2 are shown in Table 3. Comparison of the results for NaCl, KCl and KSCN shows that these salts are all distributed with a value of α_- of 0.3. The value of α_- in CaCl_2 solutions, at a mean of 0.06, is, as would be expected for a salt of Cl^- ion with a bivalent ion, lower than 0.3.

Equivalence of hyaluronic acid complex; titration with cetylpyridinium chloride. The titration of samples of the complex with a standard solution of cetylpyridinium chloride provided an independent estimate of the equivalence of the complex. The results are shown in Table 4. At both pH 7.0 and 7.8, four samples of the complex were found to have equivalence values which were constant at 3.2 m-equiv./g. One of the samples was prepared by Mr T. F. Sherman by the electro dialysis of synovial fluid (Ogston & Sherman, 1958). Another had been extracted with the chelating agent ethylenediaminetetra-acetic acid (EDTA) to remove metals which might be masking acidic groups. This

Table 3. *Changes in ionic concentrations in solutions of hyaluronic acid complex and various electrolytes, and calculation of α_-*

For explanation see Table 2. s.e. of $\Delta[A^-] = 0.03-0.06$.

Salt	pH	Concn. of HAC (%, w/w)	Concn. of salt (m-moles/kg.)	[A ⁻] (m-equiv./kg. of soln.)	α_-
NaCl	5.6	0.20	21	0.36	0.36
	5.7	0.18	21	0.25	0.26
KCl	5.7	0.19	22	0.29	0.28
	5.7	0.19	22	0.32	0.31
KSCN	5.6	0.19	20	0.35	0.36
	5.8	0.19	20	0.32	0.29
CaCl ₂	5.7	0.18	17	0.04	0.04
	5.6	0.21	17	0.08	0.08

Table 4. *Equivalence of samples of complex by cetylpyridinium chloride titration*

Duplicate results are shown for each sample. Samples 7 and 8 were prepared by ultrafiltration of synovial fluid. Sample ED was prepared by electro dialysis of synovial fluid. Sample 7V is sample 7, extracted with ethylene-diaminetetra-acetic acid by dialysis and then dialysed against distilled water.

Sample	pH	Equivalence (m-equiv./g. of complex)
7	7.0	3.11
		3.23
8	7.8	3.23
		3.26
ED	7.8	3.29
		3.16
7V	7.0	3.19
		3.29

Table 5. *Distribution data for raffinose in solutions of hyaluronic acid complex*

'Outside' and 'inside' refer to the diffusate outside the dialysis bag and the colloid phase inside the bag.

Concn. of raffinose		Concn. of complex (g./kg.)	Excluded vol. (ml./g. of complex)	r_E (Å)
Outside (g./l.)	Inside (% of outside concn.)			
5.0	98.2	2.8	6.6	13.2
5.0	98.4	2.8	5.7	12.3
5.0	98.4	2.7	5.9	12.5
5.0	98.3	2.7	6.3	12.9
2.4	98.7	2.8	4.6	11.1
2.4	97.9	2.8	7.5	14.1
2.4	98.5	2.7	5.6	12.1
2.4	98.1	2.7	7.0	13.7
Mean			6.2	12.7

value of 3.2 m-equiv./g. does not agree with the value of 1.3 m-equiv./g. at pH 7.8, which is given by the equivalence of sodium salts interacting with the complex (equation 11). This suggests that some other cation is present in the ultrafiltered prepara-

tions of the complex which can be displaced by the cetylpyridinium ion, but masks acidic radicals so that they do not interact with univalent ions.

Determination of calcium in samples of complex prepared by ultrafiltration. Calcium has been reported in samples of hyaluronic acid complex prepared by ultrafiltration (Ogston & Stanier, 1952). It therefore appeared possible that its presence might account for the disparity found between estimates of the equivalence by the two independent methods. Analysis of two preparations of the complex gave values of 1.86 and 1.78 m-equiv. of calcium/g. of complex. These figures lead to a total equivalence of 3.1 m-equiv./g. estimated to be interacting with ions, which shows good agreement with the equivalence determined directly to be 3.2 m-equiv./g.

Distribution of raffinose in solutions of hyaluronic acid complex. Blumberg & Ogston (1956) showed, using sedimentation techniques, that the domain of the hyaluronic acid complex random coil was permeable to solutes. In these experiments the observed distributions of raffinose were checked against the expected steric exclusion of solutes due to the threads of the random coil of the complex. The length of the disaccharide repeating unit of the complex (Meyer, 1953) may be taken to be about the same as that of cellulose, 10.25 Å (Sponsler & Dore, 1926). The length of polysaccharide chain in 1 g. of complex may then be calculated to be 1.23×10^{14} cm., and taking a cylindrical thread model for the complex, the specific volume of the complex at 0.69 (Ogston & Stanier, 1950) can be used to derive a mean radius of the complex thread of 4.2 Å. A solute of molecular radius r Å will therefore be sterically excluded by 1 g. of complex from a volume of

$$(r + 4.2)^2 \times 10^{-16} \times 1.23 \times 10^{14} \text{ cm.}^3.$$

$(r + 4.2)$, which we may call r_E , the radius of exclusion, can therefore be estimated from distribution data. The experimental results are shown in Table 5. The mean excluded volume to raffinose

was 6.2 ml./g. of complex, and $r_g = 12.7 \text{ \AA}$. This gives $8.5 \text{ \AA}$ as an estimate of the effective radius of raffinose; its molecular dimensions would be expected to be about $15 \text{ \AA} \times 5 \text{ \AA}$.

DISCUSSION

Equivalence of the hyaluronic acid complex. The equivalence of the complex has been determined by direct titration to be 3.2 m-equiv./g., and a balance has been struck with cations and anions in solution which supports this figure. It is, however, surprisingly high. The equivalence of the polysaccharide carboxyls on the complex, assuming one glucuronic acid residue to every *N*-acetylglucosamine residue, and 25 % of protein in the complex, is 2.0 m-equiv./g., and if the unconfirmed analysis of Ogston & Stanier (1952) is taken, it may be as high as 2.2 m-equiv./g. This determination therefore demands that the equivalence of the protein in the complex is about 4.0 m-equiv./g., which seems too high. It seems that the chemical constitution of the complex requires further examination before light can be shed on this question.

Polyelectrolyte character of hyaluronic acid complex. The theoretical treatment which has been presented above of the Donnan distributions around amphoteric polyelectrolytes can be used to derive qualitative statements of the polyelectrolyte character of the complex. The experimental knowledge of the variables

$$\Delta[\text{Na}^+] - \Delta[\text{A}^-] = E_- - E_+$$

is not sufficient to solve equation (8) or (9) for any parameters of the molecule without unwarranted assumptions. Qualitatively it can be stated that in the range pH 7.8–8.1 the independence of α_- from ionic strength means that, in this range of pH, the complex acts as a colloidal particle bearing charges of only one sign. With decreasing pH the charge on the complex interacting with the ions of sodium salts decreases as might be expected for the complex of a polyacid and a protein above its isoelectric point. In the range pH 5.5–6.3 the anion is not always in a deficiency in the colloidal phase, and apparent 'ion-binding' has been observed, more particularly at low pH and high ionic strength. This effect is reflected in negative values of α_- under these circumstances. The comparison of the results for sodium chloride with those for other univalent ions provides no evidence of specific interactions. The variations in α_- for sodium chloride are explained under the theory given as indicating zwitterion character of the complex at low pH values. At low ionic strengths the effect is obscured through the interaction of the variously charged areas on the complex which results from incomplete electrostatic screening. The partial

equivalences of the zwitterion under any one set of conditions necessarily cannot be calculated from the data. The zwitterion character of the complex at low pH values is to be expected from its constitution. The statistical correlation of α_- against pH and ionic strength (equation 12) suggests that the amphoteric nature of the complex will not be important at physiological pH values. This is supported by the observation that the total equivalence as determined by direct titration is the same at pH 7.0 as at pH 7.8.

Use and abuse of the concept of 'ion-binding'. It has been common in describing the interactions of ions with polyelectrolytes for deviations from the predictions of the simple Donnan function (equation 1) to be denoted as being a result of the 'binding' of ions to the polyelectrolyte, and distributions are described in terms of a sum of the 'Donnan equilibrium' and the quantity of one of the ions which is 'bound' to the polyelectrolyte. The distinction is drawn between 'specific' and 'non-specific' ion-binding, where the first term is applied to cases where one type of ion shows highly individual effects. In these cases, where precipitation or stoichiometric interaction is observed, the chemical reaction which the term 'binding' connotes is obviously occurring, and these cases will not be discussed further.

However, in cases of non-specific ion-binding, where it is recognized (Klotz, 1953) that the effects are due to electrostatic 'binding' of ions to charged 'sites' on the polyelectrolyte, the use of the terminology of ion-binding in the description of ionic distributions is more open to criticism. In some cases its use can be criticized as being no more than clumsy, as in such cases where 'binding' is small, and constant with ionic strength (e.g. Farber & Schubert, 1957), where the statement of its occurrence is a result of the inadequacy of equation (1) as a Donnan function. Similarly, in describing the interactions of ions with amphoteric polyelectrolytes, such as the proteins, the statement of a degree of 'ion-binding' is defensible, if clumsy, but only if the term 'ion-binding' is recognized as having been defined to mean the empirically determined deviations from the predictions of an arbitrary law. However, if a pictorial meaning is given to the measured amounts of 'binding', and the chemical connotation of the word is transferred to it, as often has been done, fallacious interpretations of the results easily follow.

Equations (8) and (9) show that in the general case fine parameters of a colloid cannot be deduced from distribution experiments, and it will be apparent that the deduction of the 'number of binding sites' on a colloid must imply two assumptions: (i) that $E = E_- - E_+$, and (ii) that $\alpha_- = 0.5$ and $\alpha_+ = 0.0$. Nor must it be thought that alter-

native methods of measuring 'binding', such as the electrometric method of Scatchard, Sheinberg & Armstrong (1950) can circumvent this difficulty. These methods implicitly measure the deviations from the predictions of equation (1) in that they employ the same assumption on which equation (1) is based, that the ionic activity coefficients are the same in the two equilibrium phases, and define 'free' ion by this criterion. It is suggested that, in recognition of the general identity of Donnan equilibria and 'non-specific ion-binding' effects, the latter term might be conveniently discarded as introducing connotations which are improper and have proved dangerous, that 'non-specific ion-binding' is a chemical rather than an electrostatic phenomenon.

Interactions of calcium. The occurrence of calcium in preparations of hyaluronic acid complex which have been washed 12 times with sodium chloride solutions and with water shows that calcium is tightly associated with the complex, and cannot be easily displaced from that association by high concentrations of salts of univalent ions. This tight association is confirmed by the value $\alpha_- = 0.06$, measured for the distribution of calcium chloride in solutions of the complex. (There is no evidence on whether this association is strengthened by forces of interaction other than the electrostatic; it can be qualitatively attributed to the higher place in the Hofmeister series which is normally held by bivalent ions.) It would therefore be expected that hyaluronic acid in physiological systems would be largely associated with Ca^{2+} ions, and that little residual charge would remain to interact with other ions, and to influence their distributions. The presence of hyaluronic acid in tissues would not therefore be expected to introduce any sensible error into the measurement of extracellular space from the distributions of such ions as Na^+ and Cl^- . This conclusion is supported by the work of Ropes, Bennett & Bauer (1939), on synovial fluid, who showed that the distribution only of Ca^{2+} ion was influenced by the presence of 'mucin'. The balance obtained between the equivalence of the complex as determined by titration with cetylpyridinium chloride and that accounted for interactions of ions, including Ca^{2+} , suggests that the calcium found in the preparations of the complex is not chemically integral with it, in that the calcium is displaceable by the cetylpyridinium ion.

Distribution of raffinose. The approximate concordance between the distributions of raffinose observed and the requirements of steric exclusion by a geometrical model of the complex as a cylindrical thread supports the conclusion of Blumberg & Ogston (1956) that the domain of the random coil of the complex is permeable to solutes. Further support is forthcoming from the

ionic-distribution experiments, where there was no evidence that the steric exclusion was higher than that due to the specific volume of the complex, which was negligible in these experiments. The error in determinations of extracellular space which use raffinose distributions should be no greater than 1-2%.

SUMMARY

1. The distribution of sodium chloride in solutions of the hyaluronic acid complex have been examined. At low pH values and at ionic strengths approaching the physiological, anomalous distributions have been observed which could be characterized as following 'binding' of Cl^- ion.

2. The theory of the distributions of univalent ions around an amphoteric polyelectrolyte has been briefly treated, and it has been shown that distribution experiments cannot provide sufficient information to permit the derivation of parameters of the polyelectrolyte other than its net equivalence. On the basis of this theory, the use of the concept of 'ion-binding' is discussed and criticized.

3. Sodium chloride-distribution experiments have been qualitatively interpreted as indicating that hyaluronic acid complex exhibits zwitterion characteristics at values below about pH 6.1.

4. Brief studies of the distributions of potassium chloride and potassium thiocyanate in solutions of the complex did not show any differences from the distributions of sodium chloride.

5. The distribution of calcium chloride in solutions of the complex was briefly examined. With the observation of the occurrence of calcium in washed preparations of the complex, this showed that Ca^{2+} ion was tightly associated with it, and that it can be expected that hyaluronic acid would be expected to be physiologically associated with Ca^{2+} ion. The complex should not introduce any error into determinations of 'sodium space' and 'chloride space'.

6. The direct titration of the complex with solutions of cetylpyridinium chloride consistently gave estimates of the equivalence of the complex at 3.2 m-equiv./g., which is surprisingly high.

7. The distribution of raffinose in solutions of the complex was approximately consistent with the steric requirements of a thread model for the complex. The domain of the random coil of the acid is thus confirmed as being permeable to solutes. The error in determinations of 'raffinose space' due to this effect should not exceed 2%.

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The Isolation of 16 β -Hydroxyoestrone and 16-Oxo-oestradiol-17 β from the Urine of Pregnant Women

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While attempting to follow up the suggestion of Marrian & Bauld (1955) that 16-oxo-oestradiol-17 β (3:17 β -dihydroxyoestra-1:3:5-trien-16-one) might be the common metabolic precursor of oestriol (oestra-1:3:5-triene-3:16 α :17 β -triol) and 16-epioestriol (oestra-1:3:5-triene-3:16 β :17 β -triol) Watson & Marrian (1955) reported the detection of a ketonic Kober chromogen in extracts of the urine of pregnant women which they provisionally identified as the sought-for 16-oxo-oestradiol-17 β solely on partition-chromatographic and countercurrent-distribution evidence. Shortly afterwards Levitz, Spitzer & Twombly (1956) reported the detection by reverse isotope dilution of radioactive 16-oxo-oestradiol-17 β in the urine of subjects after the administration of [16-¹⁴C]oestradiol-17 β ([16-¹⁴C]-oestra-1:3:5-triene-3:17 β -diol).

The following year the ketonic Kober chromogen detected in pregnancy urine by Watson & Marrian (1955) was isolated from the same source as a crystalline solid by Marrian, Watson & Panattoni (1957), and evidence was presented that the isolated product probably consisted of 16 α -hydroxyoestrone (3:16 α -dihydroxyoestra-1:3:5-triene-17-one) and a small proportion of 16-oxo-oestradiol-17 β . It was furthermore suggested that the latter

might have been an artifact produced from the former by rearrangement during the extraction of the phenolic fraction of the urinary extract by aqueous alkali from ether. The major component of the isolated product was subsequently identified with certainty as 16 α -hydroxyoestrone by Marrian, Loke, Watson & Panattoni (1957), who also obtained evidence which apparently confirmed the earlier suggestion that the minor component in the product had been artifactually formed by rearrangement of 16 α -hydroxyoestrone in alkaline solution. This evidence led the authors to conclude that pregnancy-urine extracts contain no 16-oxo-oestradiol-17 β other than that which is formed artifactually in this way, and, because of this conclusion and of the difficulty in separating mixtures of 16 α -hydroxyoestrone and 16-oxo-oestradiol-17 β by crystallization they queried the validity of the claim of Levitz *et al.* (1956) to have detected 16-oxo-oestradiol-17 β in urine extracts, although the method of extraction used by the latter authors seemed to exclude the possibility of artifactual formation of 16-oxo-oestradiol-17 β .

Marrian, Watson & Panattoni (1957) and Marrian, Loke, Watson & Panattoni (1957) suggested that 16 α -hydroxyoestrone formed from