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The Partition of Solutes between Buffer Solutions and Solutions Containing Hyaluronic Acid

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(*Received* 24 *August* 1960)

This paper describes experiments which show that hyaluronic acid may affect markedly the partition of diffusible solutes between solutions that contain it and buffer solutions; this effect can be described as the exclusion of the solute from a part of the volume of solution occupied by the hyaluronic acid. Johnston (1955) suggested the occurrence of a large degree of exclusion to explain the effect of hyaluronic acid on the ultracentrifugal sedimentation of serum albumin; however, Blumberg & Ogston (1956) were able to find only a small effect, by observing the effect of albumin on the sedimentation of hyaluronic acid. Aldrich (1958) observed directly a small degree of exclusion of raffinose by hyaluronic acid.

In spite of Blumberg & Ogston's result, steric considerations suggested that with large solute molecules quite large degrees of exclusion might be expected; such exclusions would be of considerable interest in relation to the partition of solutes between blood and tissue spaces believed to contain hyaluronic acid (reviewed by Rogers, 1961). Moreover, the occurrence of mutual exclusion is familiar in the physical chemistry of high polymers (Flory, 1943; Scott, 1949). Accordingly, we decided to investigate the effect by the direct method of dialysing solute to equilibrium between buffer solutions and solutions containing hyaluronic acid.

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EXPERIMENTAL

Methods of dialysis

β-Dextrin. As this solute passes easily through cellophan, a method like that of Aldrich (1958) was used. Since β-dextrin could not be estimated accurately in the presence of hyaluronic acid (because the latter reacts to some extent with periodate), only the buffer fluid was analysed after equilibration. The amount of β-dextrin in the hyaluronic acid solution was obtained by subtracting from the total β-dextrin that remaining in the buffer. Since the volume of the hyaluronic acid solution might change a little during dialysis, this was estimated by mopping buffer off the sac which contained it (at the end of dialysis), weighing the sac and subtracting the weight of the wet cellophan. A reasonably accurate estimate of the concentration of β-dextrin in the hyaluronic acid solution was thus made.

Other solutes. Instead of cellophan, Millipore HA membrane, of average pore diamter 0·45 μ, was used (Millipore Filter Corp., Watertown, Mass., U.S.A.). The membrane separated two compartments each of 5 ml. capacity, in the Perspex apparatus shown in Fig. 1. All the

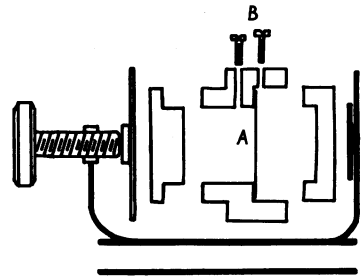

Fig. 1. Diagram of dialysis cell and clamp. *A*, Millipore membrane; *B*, filling plugs.

solutes used dialysed readily through this membrane, whereas hyaluronic acid was completely retained. When the compartments were filled, by syringe, a small air bubble was left in each, and stirring was effected by rotating the whole apparatus about its axis at 1 rev./min. The solute investigated was present initially in one or other compartment, usually in the buffer. Dialysis took up to 6 days at room temperature. At the end of equilibration, both solutions were withdrawn for analysis.

Light-scattering

Light-scattering measurements on a sample of hyaluronic acid complex were made by Dr B. N. Preston of this Department, using a Brice–Phoenix light-scattering apparatus. The hyaluronic acid was clarified by centrifuging it at a concentration of 0·14 g./100 ml. for 8 hr. at 30 000 rev./min. in the 30 head of a Spinco Model L ultracentrifuge. Measurements were made in the standard buffer over the concentration range 4–64 mg./100 ml. The results were calculated from the Zimm plot (Zimm, 1948).

Sedimentation

Sedimentation was observed in the Svedberg oil-turbine ultracentrifuge at 900 rev./sec. and 26°, by the method of Cecil & Ogston (1948) with the bar-schlieren optics of Baldwin (1953).

Materials

Cellophan tubing. This was Visking 1 cm. tubing. Before use it was thoroughly washed in water, to ensure that it contained no soluble material reacting with periodate.

Millipore membranes. It was found necessary to wash these before use only in experiments with inulin. Fresh membranes gave rise to soluble material which analysed as fructose and even exhaustive washing did not entirely remove this. However, in control experiments, it was established that the soluble material distributed itself equally between hyaluronic acid solutions and buffer. Correction could therefore be applied by assuming equipartition of any material in excess of the total inulin added.

Hyaluronic acid. The material used throughout was the hyaluronic acid–protein complex, prepared from ox synovial fluid by the filtration method of Ogston & Stanier (1950). The washing process was repeated 10–20 times. The product contained no free protein.

Bovine-serum albumin. Bovine-serum albumin (The Armour Laboratories, Hampden Park, Eastbourne, Sussex) batch no. M 12007, was used.

Carbon monoxide–haemoglobin. This was prepared from washed erythrocytes of horse blood saturated with CO. The cells were lysed by dilution of the suspending fluid with water, and the stroma was removed by centrifuging at 60 000 *g* for 1 hr.

β_A-Lactoglobulin. This was prepared from identified milk essentially as described by Ogston & Tombs (1957).

Tropomyosin. This was prepared from rabbit muscle by the method of Bailey (1948).

β-Naphthol-3:6-disulphonic acid azodiphenylazo derivatives of proteins. (R-salt proteins). The preparation of the diazonium chloride from benzidine and β-naphthol-3:6-disulphonic acid (R-salt) and the coupling with proteins were carried out as described by Kabat & Heidelberger (1937). For 1 g. of serum albumin the equivalent of 0·11 g. of benzidine was used; for 1 g. of β_A-lactoglobulin the equivalent of 0·09 g. of benzidine was used. The resulting solutions of highly coloured proteins were exhaustively dialysed against buffer and any insoluble material was removed by centrifuging. Stock solutions contained 0·1 g. of coupled protein/100 ml.

Dinitrophenyl derivatives of protein. Preliminary experiments showed that the reaction of small quantities of 1-fluoro-2:4-dinitrobenzene was complete in 48 hr. at 2° in 0·067 M-Na_2HPO_4 containing 5% ethanol (v/v), about half the total fluorodinitrobenzene reacting with the protein. To 1 g. of protein in 40 ml. of 0·067 M-Na_2HPO_4 was added 10·6 mg. of fluorodinitrobenzene in 2 ml. of ethanol; this introduces 1–2 equiv. of dinitrophenyl/mole of serum albumin. After 100 hr. at 2°, the mixture was acidified with 3–4 m-equiv. of HCl (pH approx. 4) and extracted ten times with 45 ml. of light petroleum (b.p. 60–80°). The last traces of light petroleum were removed by suction, with the solution at 35°. The solutions were exhaustively dialysed against buffer and any insoluble material was removed by centrifuging. Dinitrophenyl derivatives of bovine-serum albumin, β_A-lactoglobulin and tropomyosin were made.

Polyglucose. Synthetic polyglucose was a present from Dr P. T. Mora (Mora, 1958). The sample had been precipitated by ethanol between 62·5 and 72·5% (v/v). It was stated to have a number-average molecular weight of 50 000 by osmotic pressure and of 21 000 by measurement of reducing end-groups. Light-scattering measurements made by Dr P. Johnson (personal communication) gave a weight-average molecular weight of 33 000 with an error of ±3000. The sample was labelled with ^{14}C by use of $K^{14}CN$ (The Radiochemical Centre, Amersham, Bucks.) by the procedure of Isbell (1953).

Inulin. Four preparations of inulin were used. Sample (A) was Kerfoot biochemical reagent inulin (Thomas Kerfoot Ltd., Vale of Bardsley, Lancs), twice recrystallized by dissolving it in dilute NH_3 soln. [0·1 ml. of NH_3 soln. (sp.gr. 0·88)/100 ml. of water], filtering off insoluble material and cooling to 1°. Sample (B) was reagent quality (British Drug Houses Ltd.), twice recrystallized, first after solution in hot 0·1 M-NaOH, then as above. Sample (C) was a present from Dr D. J. Bell, prepared by him and recrystallized as sample (A). Sample (D) was inulin from British Drug Houses Ltd. heated in glycerol for 20 min. and cooled; ethanol was then added to 30% (v/v) and the precipitate was discarded; then ethanol was added to 50% (v/v) and the precipitate was collected. Inulin was dried in air at 105° and finally exhaustively *in vacuo* over P_2O_5.

Synthetic amyloses of degrees of polymerization (D.P.) of 19 *and* 38. These were a present from Dr W. J. Whelan (Whelan & Bailey, 1954).

Schardinger β-dextrin. This was a present from Dr P. W. Kent.

Buffers and measurement of pH. All measurements except those with the β-dextrin were made in 0·2 M-NaCl, 7·7 mM-Na_2HPO_4, 2·3 mM-KH_2PO_4, pH 7.3. Solutions of hyaluronic acid complex and of proteins were exhaustively dialysed against large volumes of this buffer. Measurements of pH were made with a Cambridge pH meter, standardized with 0·05 M-potassium hydrogen phthalate (pH 4·0) and 0·05 M-sodium borate (pH 9·24).

Methods of analysis

Hyaluronic acid complex. The concentration was determined by drying to constant weight at 105° after thorough

dialysis against distilled water. Closed Visking sacs were used for dialysis; any change of volume during dialysis was checked by direct weighing of the sacs after mopping them free from excess of liquid.

Coloured proteins. It was necessary to use these in the partition experiments to distinguish the partitioned protein from that present in the hyaluronic acid complex. The latter showed no absorption at the wavelengths at which the coloured proteins were estimated in a Hilger Uvispek spectrophotometer: carbon monoxide–haemoglobin at 570 mμ, R-salt proteins at 527 mμ, dinitrophenyl proteins at 360 mμ.

Polyglucose. This was estimated by radioactive counting. In order to allow for self-absorption due to hyaluronic acid, solutions from the buffer compartment were diluted with a solution of hyaluronic acid complex before estimation. Solution from the hyaluronic acid-complex compartment was similarly diluted with buffer, so that both final solutions contained the same concentration of salts and of hyaluronic acid complex. Samples (0·05 ml.) of solution were transferred to circles of lens tissue (diameter 16 mm.) on 19 mm. coverslips and counted with a mica end-window tube. The counting rate was about 3000 counts/min. A minimum of 10 000 counts were obtained.

Inulin. This was estimated by the resorcinol method essentially as described by Kulka (1956). Inulin (5–50 μg. in 2 ml.) was heated for 40 min. at 80° with 3 ml. of resorcinol in ethanol (0·05%, w/v) and 3 ml. of HCl (sp.gr. 1·18). Extinctions were measured at 480 mμ after cooling. Where hyaluronic acid complex was present deproteinization was necessary. Metaphosphoric acid (7%, w/v) was satisfactory for this; 1 ml. was added to 2·5 ml. of solution of inulin and hyaluronic acid, and the solution was centrifuged for 15 min. at 10 000 *g* before estimation. Control experiments showed recoveries of 99±1% of inulin. There was still a small error due to the hyaluronic acid, amounting to not more than 2% of the extinction due to inulin, and correction for this was made. It has been mentioned that the Millipore membranes gave rise to small amounts of material reacting as fructose, even after exhaustive washing. Control dialysis experiments, without inulin, showed that this material is equally partitioned between buffer and hyaluronic acid. Correction was therefore made by assuming equal partition of excess of apparent inulin over that known to be present (after allowing for the contribution of the hyaluronic acid) by subtracting half the apparent excess of inulin from the amount estimated in each compartment.

Amyloses. These were estimated by the method of Whelan & Bailey (1954) by use of 0·2% (w/v) I_2 in 2% (w/v) KI in water. D.P. 19 amylose was measured at 510 mμ and D.P. 38 amylose at 560 mμ, after dilution to 40–80 μg./ml. of final solution. Neither hyaluronic acid nor any material from the Millipore filters interfered.

Schardinger β-dextrin. The estimation was based upon the reaction with metaperiodate (Aspinall & Ferrier, 1957). The following conditions were most satisfactory: Dextrin (2 ml., 0·1 g./ml.) was added to freshly made $NaIO_4$ (5 ml., 0·015 M) with a crystal of KCl and incubated under N_2 in a Thunberg tube in the dark at 35° for 10 hr. Under these conditions the reaction was complete and there was little loss of periodate in the absence of dextrin. The presence of phosphate in the standard dialysis buffer led to erratic results, but reproducible results were obtained in the presence of NaCl alone: accordingly, these dialyses and estimations were performed in 0·2 M-NaCl. The extinctions of the original and remaining periodate, and of equimolar iodate, were read at 223 mμ. Hyaluronic acid complex was found to react with periodate over long periods to too great an extent to make correction for its reaction reliable. Small quantities of material reacting with periodate were found in the diffusate on prolonged dialysis of hyaluronic acid complex against buffer (without dextrin). Reliable correction could be made for this by use of a relatively high concentration of dextrin.

RESULTS

The first partitioned solute to be investigated was inulin, chosen because of its use in the measurement of extracellular volumes *in vivo* and *in vitro*. Preliminary experiments showed a surprisingly unequal partition, and this effect was fairly thoroughly investigated. Once it had been established that the effect is real and reproducible, other solutes required less extensive investigation. The results are described more or less in the order in which they were obtained.

Partition of inulin

Time course and direction of dialysis. Fig. 2 shows that the partition of inulin approached a stable end-point, and that this was the same whether the inulin was initially added to the buffer or to the hyaluronic acid solution.

Effect of different samples of inulin. The partitions of different samples under identical conditions are shown in Table 1. The sample found otherwise to contain material of relatively low molecular weight showed less unequal partition (see below): the 'good' samples show satisfactory agreement.

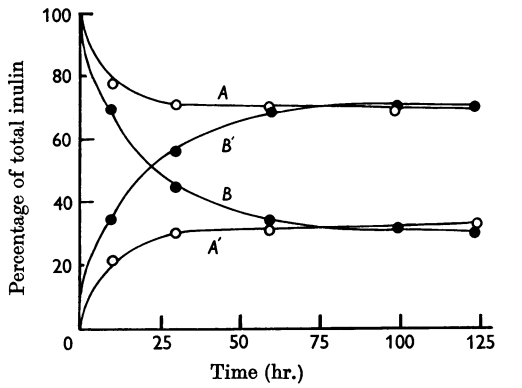

Fig. 2. Time courses of equilibration of inulin between buffer solution and a 0·18% solution of hyaluronic acid complex. Curves *A* and *A'* were with the inulin initially in the buffer: *A*, loss of inulin from the buffer; *A'*, gain of inulin by the hyaluronic acid solution. Curves *B* and *B'* were with the inulin initially in the hyaluronic acid solution: *B*, loss of inulin from the hyaluronic acid solution; *B'*, gain of inulin by the buffer.

Effect of varying the ionic strength and pH. Closely concordant results were obtained with 0·18% hyaluronic acid complex and inulin sample (A) (50 μg./ml.) in the standard buffer but containing NaCl up to 2 M, and in 0·2 M-NaCl with pH varied from 6 to 9 by phosphate and 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffers.

Table 1. *Partition of different samples of inulin between buffer and solution of hyaluronic acid complex*

Concn. of hyaluronic acid complex was 0·18%; concn. of inulin initially was 50 μg./ml. in standard buffer; dialysis was for 100 hr.

Sample of inulin	Percentage of total inulin in: Buffer	Hyaluronic acid
From T. Kerfoot Ltd., unpurified	60	40
From T. Kerfoot Ltd., recrystallized (A)	69	31
From British Drug Houses Ltd., recrystallized (B)	68	32
From D. J. Bell, recrystallized (C)	70	30
Ethanol-fractionated (D)	68	32

Effect of varying the concentration of inulin. With hyaluronic acid complex (0·18%) and standard buffer, the same result was obtained with inulin sample (A) concentrations from 25 to 250 μg./ml.

Effect of varying the concentration of hyaluronic acid. The partition of sample (A) varied with the concentration of hyaluronic acid complex as shown in Table 2.

Properties of samples of inulin

Comparison of crude inulin (Kerfoot) with recrystallized samples showed that it appeared to contain significant amounts of material of lower molecular weight. (i) The crude material passed through Visking tubing much more rapidly than the recrystallized material. (ii) Descending chromatography on Whatman no. 1 paper in butan-1-ol–pyridine–water (6:4:3, by vol.) showed the crude material to be markedly heterogeneous. (iii) The crude material showed a higher apparent solubility in water at 25°. (iv) The crude material showed a considerable fraction not precipitated by less than 50% (v/v) ethanol. It is possible that the

Table 2. *Partition of solutes between buffer and solutions of hyaluronic acid complex*

All dialyses were in standard buffer except for β-dextrin (see text). K = (concn. in buffer)/(concn. in hyaluronic acid).

Solute	Concn. of hyaluronic acid complex (%)	Dialysis time (hr.)	Percentage of total solute in: Buffer	Hyaluronic acid	K
Inulin (A)	0·18	100	69	31	2·22
	0·09	100	61	39	1·56
	0·045	100	56	44	1·27
	0·023	100	53	47	1·13
Polyglucose	0·18	163	61	39	1·56
	0·09	163	55	45	1·22
	0·045	163	52	48	1·08
Amylose D.P. 19	0·18	160	59	41	1·44
Amylose D.P. 38	0·18	160	65	35	1·85
β-Dextrin	0·32	192	52	48*	1·08
Raffinose†	0·28	192	50·5	49·5	1·02
Serum albumin (R-salt deriv.)	0·18	140	72	33	2·18
	0·09	140	63	36	1·75
Dinitrophenyl serum albumin	0·18	160	72	28	2·57
	0·09	160	64	36	1·77
	0·045	160	58	42	1·38
	0·023	160	54	46	1·17
β-Lactoglobulin (R-salt deriv.)	0·18	140	66	34	1·94
	0·09	140	58	42	1·38
	0·045	140	53	47	1·13
Dinitrophenyl β-lactoglobulin	0·18	160	67	33	2·03
Dinitrophenyl tropomyosin	0·18	120	73	27	2·70
	0·09	120	62	38	1·63
	0·045	120	57	43	1·33
Carbon monoxide–haemoglobin	0·18	160	67	33	2·03
	0·09	160	59	41	1·44
	0·045	160	52	48	1·08

* Not determined directly, see text.

† From Aldrich (1958).

purified samples are not identical, since cursory examination showed that their apparent solubilities and rates of passage through Visking cellophan differ; the latter may, however, have been due to variations of Visking cellophan, even when pieces were taken from the same roll (Dr W. J. Whelan, personal communication).

Partition of serum albumins

With the R-salt protein, it was established that dialysis for 140 hr. was sufficient to establish equilibrium. Subsequent experiments with the dinitrophenyl albumin showed that the partition was not affected by the direction of dialysis, by increase of salt concentration up to 2M-NaCl, by variation of pH between 6 and 9 or by the concentration of protein between 0·1 and 2 g./100 ml. The effect of changing the concentration of hyaluronic acid was similar to that on inulin, and is shown in Table 2.

Partition of other solutes

The results are summarized in Table 2. In each case it was established that the time of dialysis was sufficient to attain equilibrium. In all but one of these experiments the solute was initially in the buffer. One experiment with polyglucose initially in the hyaluronic acid compartment gave a result close to that in which it was initially in the buffer.

Sedimentation of hyaluronic acid in the presence of β_A-lactoglobulin

These experiments were done because the partition experiments appear to contradict the conclusion of Blumberg & Ogston (1956) that there is only a small exclusion of protein by hyaluronic acid. β_A-Lactoglobulin was chosen as the protein because its sedimentation coefficient (2·8s) is much less than that of hyaluronic acid at low concentration of the latter. The sedimentation rate of hyaluronic acid (at a concentration of 0·016 %) was measured in the presence of various concentrations of protein. The results are shown in Table 3.

Table 3. *Sedimentation of hyaluronic acid complex in the presence of β_A-lactoglobulin*

Concn. of hyaluronic acid complex was 0·16 %. Speed was 900 rev./sec.; temp. 26°; sedimentation coefficients are corrected to 20° in the standard buffer.

Concn. of β_A-lactoglobulin (g./100 ml.)	Sedimentation coefficient of hyaluronic acid complex (s)
0	5·86
0·065	5·84
0·13	5·95
0·33	5·96

Light-scattering by the hyaluronic acid complex

A consistent Zimm plot was obtained, indicating a molecular weight of 13×10^6, a radius of rotation of 3750 Å and a second virial coefficient of $3{\cdot}7 \times 10^5$ c.g.s. units. These values are in fair agreement with those obtained on hyaluronic acid complex by dynamic methods (Blumberg & Ogston, 1958) and on suitable preparations of protein-free hyaluronic acid from ox synovial fluid (Balázs, 1958).

DISCUSSION

Exclusion of solutes by hyaluronic acid

It seems to be established by these experiments that solutes are unequally partitioned between buffer solutions and solutions containing hyaluronic acid complex, by what amounts to a partial exclusion of solute by the hyaluronic acid from the solution occupied by the latter. One may inquire first what is the general nature of this exclusion. The facts that the partition is quantitatively independent of ionic strength and pH of the solution, that it is not obviously affected (as between different proteins) by the isoelectric point, that there is a general relationship between partition coefficients (K) and the molecular sizes irrespective of the chemical nature of the solute, and that it is strongly affected by the concentration of hyaluronic acid, all point to the essentially steric nature of the exclusion. Hyaluronic acid is a chain polymer of very large molecular weight and of random chain configuration (Blumberg & Ogston, 1958; Balázs, 1958). The radius of its molecular domain is of the order of 3500–4000 Å, which means that each molecule occupies a volume of 10–15 l./g. At concentrations down to 0·01 % there will therefore be an extensive interpenetration of neighbouring chains, and at concentrations of the order of 0·1 % and upward a solution of it will consist of an almost uniform network of polysaccharide chain. It is clear, qualitatively, that a compact solute

Table 4. *Values calculated for R_B of hyaluronic acid from values of R_S, β and equation 2: see text*

Solute	β	R_S (Å)	R_B (Å)
Raffinose	3	4·9	4·1
β-Dextrin	11	6·5	2·8
Polyglucose	98	20*	3·3
β_A-Lactoglobulin	160	22	3·1
Carbon monoxide–haemoglobin	170	27	3·6
Serum albumin	250	27	3·1
Amylose, D.P. 19	88	9·0 (19)	1·6
Amylose, D.P. 38	150	11·4 (23)	1·6
Inulin	204	10·6 (26)	1·4
Tropomyosin	270	30 (29)	3·4

* Average mol.wt. taken as 33 000.

particle will be able to fit into only limited sites in this network, the fewer sites the larger the particle.

Ogston (1958) has given a geometrical treatment of the distribution of 'holes' in a random suspension of straight fibres, calculating the probability $P_{D>r}$ that an object of radius r can be placed at any point within the network. Since the partition coefficient $K = 1/P_{D>r}$ this theory could be applied to the present case by making the assumptions (i) that the hyaluronic acid chains consist of randomly-kinked straight segments, (ii) that the number of 'ends' is equal to the number of kinks. Knowing approximately the effective radius of a solute particle, the average length of straight segments can then be calculated from the partition coefficient and equation (13) from Ogston (1958). Unfortunately, this leads consistently to segment lengths of the order of 1 Å, which is absurd.

This result does, however, suggest another similar approach to the problem, which is to treat the hyaluronic acid chain as a string of beads which can be regarded (approximately) as randomly distributed in solution: this is equivalent to a modification of Ogston's treatment of fibres, by treating them as all 'ends' and leads directly to

$$1/P_{D>r} = K = \exp[4\pi\nu r^3/3], \qquad (1)$$

when ν is now the number of beads/ml. and r is the effective radius of the solute particle. This radius is the sum of the particle radius R_S and that of the bead R_B. If $\bar{v}$ is the partial specific volume of the beads, equation (1) becomes

$$\log K = \frac{\bar{v}}{2{\cdot}303}\left\{\frac{R_B+R_S}{R_B}\right\}^3 w = \beta w, \qquad (2)$$

where w is the weight of hyaluronic acid/ml. and β is a constant for a given solute. Fig. 3 shows that this relationship is approximately obeyed.

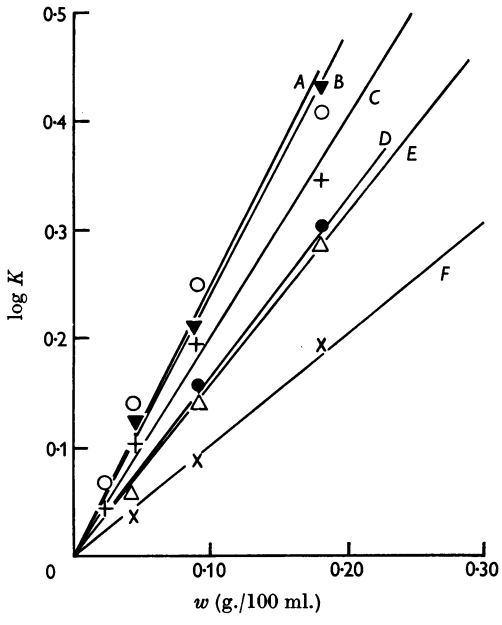

Fig. 3. Plots of log K against w for various solutes (see text). A (▼), Tropomyosin; B (○), serum albumin; C (+), inulin; D (●), carbon monoxide–haemoglobin; E (△), β_A-lactoglobulin; F (×), polyglucose.

Table 4 shows values for R_B calculated from the values of β, $\bar{v}$ (taken as 0·69, Ogston & Stanier, 1950) and of R_S. The last are calculated on the assumption that the solute molecules are spherical, compact and unsolvated (the partial specific volumes are taken as 0·75 for proteins and 0·60 for carbohydrates). In Table 4 the resulting values of R_B are collected into two sets, the first those for which the last assumption is likely to be approximately correct, and the second for solutes believed not to be of spherical or compact configuration. With the exception of tropomyosin in the second half, the values of R_B divide well into high and low. The values in the first half give an average for R_B of 3·34 Å, corresponding to a bead weight of 158. The approximate correspondence of this with that of a hexose unit must be fortuitous, but the value is at least a reasonable one for the 'bead model', and gives some sort of picture of steric exclusion.

The value for R_B of 3·34 Å for the compact particles may now be used to calculate R_S for the others: this gives the values in parentheses in the second half of Table 4. The three linear polysaccharides are seen to have effective radii much larger than those of the corresponding compact spheres. This is not unexpected, since such molecules are likely to be elongated, with a considerable degree of stiffness, and the size of the 'hole' required will be more closely related to the volume of rotation than to the molecular volume. By contrast polyglucose has a highly branched and relatively compact structure. The behaviour of tropomyosin seems to be anomalous since all the evidence (Bailey, 1954) suggests that it is a stiff molecule of large axial ratio.

Equations (1) and (2) show that the 'excluded volume' is not a constant quantity per weight of polymer (for a given solute) but varies with the concentration of polymer:

$$\text{Excluded vol./wt. of polymer} = \frac{1}{w}\left\{1-\frac{1}{K}\right\} = \frac{1}{w}\{1-10^{-\beta w}\}. \qquad (3)$$

Effect of protein on the sedimentation of hyaluronic acid

Blumberg & Ogston (1956) pointed out that exclusion of solute by hyaluronic acid would amount to selective solvation of the hyaluronic acid by

solvent, so that a large exclusion should markedly affect the sedimentation of the hyaluronic acid. From their observations on an ascending boundary of hyaluronic acid, at a concentration of 0·28 %, in the presence of serum albumin, they concluded that the exclusion could not under those conditions be more than 12 ml./g. of hyaluronic acid. By contrast, the present partition results, with use of equation (3), would predict an exclusion of nearly 300 ml./g. under their conditions. Our observations on the downward-moving boundary of hyaluronic acid in the presence of β_A-lactoglobulin qualitatively confirm those of Blumberg & Ogston in showing that the protein has far less effect than would be expected from the partition experiments.

The only possible explanation of this discrepancy seems to be that an equilibrium partition of protein in relation to hyaluronic acid is not nearly attained under the conditions of sedimentation, but that protein moves with the hyaluronic acid in excess of expectation.

Distribution of substances in tissue spaces and the estimation of extracellular volumes

A review of these questions would be out of place here. It is enough to point out that our experiments make it highly probable that macromolecular substances, in particular proteins and polysaccharides of the type of inulin, will be very unequally distributed between blood or incubation fluids and tissue spaces wherever the latter contain significant concentrations of hyaluronic acid or similar material. The effect of this will be to cause extracellular water to be underestimated. The normal distribution of protein *in vivo*, between blood or lymph and tissue spaces, will be similarly affected. It cannot be assumed, for example, that the concentration of serum protein in lymph is the same as that in extracellular water from which the lymph was derived; in general, it will tend to be greater. The situation here is likely to be complicated also by resistance offered by a polysaccharide network to the passage of solutes from blood to lymph through the tissue spaces.

SUMMARY

1. The partition of a number of diffusible solutes, including inulin and proteins, between buffer solutions and solutions of hyaluronic acid has been examined.

2. All substances examined are partitioned in favour of buffer, increasingly with increase of effective molecular volume.

3. The importance of these findings to the distribution of natural and artificial macromolecular substances between fluids and tissue spaces, *in vivo* and *in vitro*, and to the use of such substances for measuring extracellular volumes, is pointed out.

4. A simple steric theory is put forward to explain this effect.

We are grateful to the Medical Research Council for a Training Studentship held by one of us (C.F.P.), to Dr D. J. Bell, Dr P. W. Kent, Dr P. T. Mora and Dr W. J. Whelan for gifts of materials, and to Dr W. Bartley for advice.

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