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Further Observations on the Preparation and Composition of the Hyaluronic Acid Complex of Ox Synovial Fluid

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Ogston & Stanier (1950) concluded that hyaluronic acid occurs in synovial fluid in combination with protein. The undegraded state of this complex in solution, prepared by ultrafiltration, was judged by viscosity measured in an Ostwald viscometer, and by sedimentation in the ultracentrifuge. It was not possible to account satisfactorily for the composition of the complex in terms of the analytical values for its components.

The main object of the present work was to make better and more extensive analyses. As a preliminary, methods of preparation were examined in the hope of finding one which would give undegraded material more conveniently than by ultrafiltration. In this search, a more sensitive test of degradation, based on the variation of viscosity with velocity gradient, was used. It is concluded that ultrafiltration remains the method of choice and improved analyses of material thus prepared are reported.

The results of this work generally confirm the previous conclusions, though altering some of them in detail. Nearly the whole weight of complex is now accounted for in terms of protein, acetylglucosamine, glucuronic acid and mineral content.

In the course of the work, the zero-concentration value of the sedimentation constant of the complex has been re-determined and the action of hyaluronidase has been briefly investigated. The values given by Ogston & Stanier (1951*a*) for the physical dimensions of the particle of complex have been adjusted in the light of the new results.

METHODS

The methods for collection and storage of synovial fluid, preparation of the mucin clot from 1 ml. of fluid and determination of dry weight described by Ogston & Stanier (1950) were used.

The ultrafilter residue. This was prepared by the method described by Ogston & Stanier (1950), using a sintered-glas

'5 on 3' filter of average pore diameter about 1 mμ. (made by Baird and Tatlock Ltd.). 100–200 ml. of synovial fluid were used on each filter and the loss of hyaluronic acid through the filter was small.

Glucosamine. This was estimated by the method of Johnston, Ogston & Stanier (1951).

Glucuronic acid. This was estimated by the method of Ogston & Stanier (1951*b*). It has been found that protein makes a very small contribution to the CO_2 evolved during the glucuronic acid determination (1 mg. protein ≡ 0·023 ml. 0·01 N-HCl) and the results have been corrected accordingly.

Total nitrogen. This was estimated by micro-Kjeldahl, using selenium catalyst and 7–8 hr. digestion; a Markham (1942) apparatus was used for distillation.

Ash. This was determined on samples of hyaluronic acid complex, first dried to constant weight at 105° and then ignited at 550°.

pH. This was determined using an electrode of Corning 015 glass and an AgCl reference electrode in 0·1 M-KCl, with a liquid junction, phthalate and borate buffers (Cecil, 1951) being used to calibrate.

Ions. Na and Ca were determined by flame photometry (Leyton, 1951) in material which had been ignited at 550–600°, dissolved in HCl and centrifuged.

Paper chromatography. For amino-acids this was carried out by the methods of Williams & Kirby (1948) and Consden, Gordon & Martin (1944). For carbohydrates irrigation with 40 % *n*-butanol-10 % ethanol-50 % water (v/v) on Whatman no. 1 filter paper and development with aniline hydrogen phthalate was employed.

Ultraviolet absorption. This was measured in 0·1 N-NaOH using a Beckman spectrophotometer or a Hilger 'Uvispek' spectrophotometer.

Refractive increment. This was measured in the refractometer described by Cecil & Ogston (1951).

Viscosity. This was measured at 25° either in an Ostwald or Couette viscometer, as described by Ogston & Stanier (1950).

Ultracentrifuge. A Svedberg oil-turbine ultracentrifuge with a Philpot (1938) optical system was used. The procedure of running, and methods of calculating sedimentation constants and estimating the quantities of components were as described by Cecil & Ogston (1948).

Electrophoresis. A Hilger Tiselius-type apparatus with a Philpot (1938) optical system was used.

Standard buffer. This contained 0·2 M-NaCl, 0·0077 M-Na_2HPO_4 and 0·0023 M-KH_2PO_4 (pH 7·3), and was used for all measurements of refractive increment, viscosity, sedimentation and electrophoresis.

RESULTS

Anomalous viscosity as a criterion of degradation

Ogston & Stanier (1951*a*) showed that the variation of viscosity with velocity gradient and concentration can be represented by a plot of

$$\log\{(\eta_{rel.}-1)/\phi\}$$

against ϕ, where ϕ is the concentration expressed as volume fraction of unsolvated hyaluronic acid complex (Fig. 1). This method of plotting was suggested by Dr J. P. Johnston. An index of the degree of variation of viscosity with velocity gradient is given by the difference Δ between the values of $\log\{(\eta_{rel.}-1)/\phi\}$ extrapolated to zero and to infinite velocity gradient at a stated value of ϕ. Table 1 shows: (i) that values of Δ are reasonably constant for native fluids, (ii) that a marked fall of Δ of derived material may occur without a corresponding change of viscosity measured in an Ostwald viscometer or of intrinsic viscosity ($\phi=0$). Δ thus appears to be a more sensitive test of degradation than those previously used. Fig. 1 shows examples of the behaviour of an undegraded (*a*) and a degraded (*b*) material.

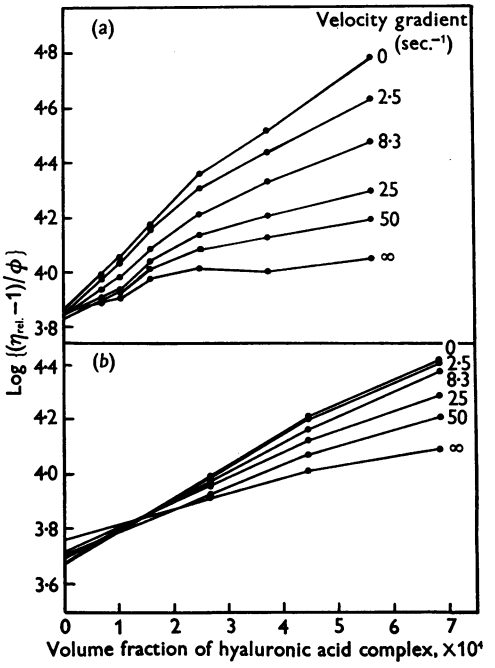

Fig. 1. Variation of $\log\{(\eta_{rel.}-1)/\phi\}$ with volume fraction ϕ at different velocity gradients. (*a*) synovial fluid; (*b*) hyaluronic acid complex precipitated by ethanol.

Methods of preparation

Mucin. Ogston & Stanier (1950) found that a single precipitation of hyaluronic acid as mucin might not greatly affect the Ostwald viscosity. It now appears (Table 1) that even a single precipitation produces a large lowering of Δ. Therefore no method of preparation which involves precipitation of mucin is satisfactory.

Precipitation with ethanol. Ropes, Robertson, Rossmeisl, Peabody & Bauer (1947) used precipitation with ethanol in alkaline solution at −20° to prepare hyaluronic acid free of protein after a preliminary precipitation of mucin; Ogston & Stanier (1950) concluded that this method would give an undegraded product, provided that the protein content was not reduced too much. Since

the mucin precipitation is undesirable, we have now used a preliminary concentration of synovial fluid, and partial removal of protein, on a glass filter; this starting material showed a value of Δ equal to that of the original fluid. Precipitation with ethanol at −20° was then carried out, both on the material maintain good hyaluronic acid boundaries in a small electrophoresis cell, though their behaviour in the 150 ml. cell was better. Experiments were carried out because of the claim by Blix (1940) to have separated from synovial mucin by this method hyaluronic acid containing only 10 % of protein.

Table 1. *Values of* $\log\{(\eta_{rel.}-1)/\phi\}$ *for all products*

(All measurements at 25° except Couette measurements on fluid 29*K* (19°.)

			$\phi = 7\times10^{-4}$ ml./ml.				$\phi = 0$
			Couette: velocity gradient G sec.$^{-1}$				Couette: (all velocity
Material	No.	Treatment	0	∞	Δ	Ostwald	gradients)
Fluid	29*K*	—	4·67	3·87	0·80	3·74	3·94
	36*A*	—	4·97	4·21	0·76	3·85	3·89
	37*A*	—	4·97	4·06	0·91	3·90	4·00
	53	—	4·65	3·92	0·73	—	—
	54	—	4·86	4·00	0·86	—	—
	55	—	4·80	3·99	0·81	—	—
Mucin	26*K*	One pptn.	4·09	3·69	0·40	—	3·65
	28*A*	One pptn.	3·97	3·66	0·31	3·41	3·55
	35*K*	One pptn.	4·21	3·88	0·33	3·73	3·54
Hyaluronic acid complex ppted. by ethanol	38–40	Three pptn. from twice ppted. mucin	4·49	4·10	0·39	4·02	3·72
	50	One pptn. from alkaline conc. fluid	4·67	4·08	0·59	3·92	3·99
	51	One pptn. from neutral conc. fluid	4·34	3·83	0·51	3·60	3·65
Hyaluronic acid complex prepared by ultrafiltration	33	32 washings: no toluene	4·36	3·96	0·40	3·85	—
	35*K*	55 washings: no toluene	4·42	4·03	0·39	3·87	3·74
	55	Toluene: three washings	4·75	3·95	0·80	—	—
		Eight washings	4·87	3·98	0·89	—	—
		16 washings	4·88	4·03	0·85	—	—
		28 washings	4·76	4·03	0·73	—	—
		39 washings	4·57	4·01	0·56	—	—

dissolved in water, and after making it alkaline with NaOH as in the method of Ropes *et al.* (1947). The protein content was reduced by both procedures, though more so with alkali. Both products had a normal Ostwald viscosity, but a reduced Δ, showing some degree of degradation, though not so much as is produced by precipitation as mucin (Table 1). Ethanol precipitation after precipitation of mucin causes no further lowering of Δ (Table 1). The lowering of Δ is not caused merely by the removal of free protein in this method, since adding synovial fluid protein to a sample made by ethanol precipitation caused no increase in the value of Δ. It seems, therefore, that the process of precipitation with ethanol causes degradation.

Electrophoresis. The use of electrophoresis as a preparative method was not considered seriously, since it seemed likely to be more trouble and to give lower yields than ultrafiltration. The high viscosity of solutions of hyaluronic acid made it impossible to

A sample of mucin made on the larger scale (Ogston & Stanier, 1950) was run in the 10 ml. cell. The boundaries due to free protein were measured, and their contribution to the total refractive increment was compared with that of total protein, taken as 78 % of mucin. The free protein was less by 12 % than the total protein, and this means that 25 % of the hyaluronic acid complex is protein. The amount of albumin was estimated from the photographs to be 80–93 % of the total free protein. In the ultracentrifuge (Ogston & Stanier, 1950) only one free component was visible, sedimenting at the rate of serum albumin.

A second experiment was carried out in the 150 ml. cell with synovial fluid which had been concentrated on a glass filter. After running for a suitable time, four samples were removed by pipette from different levels of the upper section and two from the lower section of the anode limb. These were analysed for total nitrogen and glucosamine (Table 2). The

lowest value for the ratio of total nitrogen to glucosamine indicates a higher proportion of protein than is found in the best ultrafilter products (Table 3). All the samples showed normal Ostwald viscosity.

Table 2. *Composition of fractions taken from anode side of Tiselius apparatus*

	Fraction	Total N (mg./ml.)	Glucosamine (mg./ml.)	Total N/glucosamine
Top section	1	0·113	0·226	0·50
	2	0·055	0·123	0·45
	3	0·077	0·210	0·37
	4	0·045	0·159	0·28
Second section	5	0·596	0·225	2·65
	6	0·704	0·216	3·26

Ultrafiltration. Although it had been shown by the earlier criteria that ultrafiltration produces undegraded material, it was now necessary to find out whether this material has the full degree of anomalous viscosity possessed by synovial fluid. Two samples, which had been judged to be undegraded

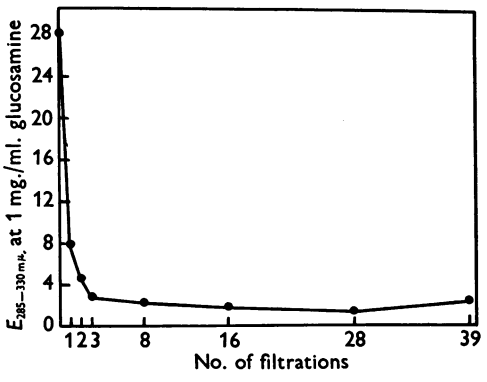

Fig. 2. Course of removal of free protein from synovial fluid by successive filtrations and washings.

by the earlier criteria, were found to have a lowered value of Δ (Table 1). This question was therefore investigated. Pooled fluid 55 was washed as usual, except that the saline and water used in washing were saturated with toluene. Samples taken at different stages of washing were dialysed and their viscosities were measured in the Couette viscometer. Their concentrations were estimated after dialysis on the basis of their glucosamine contents, these being converted to volume fraction of hyaluronic acid (see below). The results (Table 1) show that there was no degradation after 16 filtrations, possibly slight degradation after 28 filtrations and definite degradation after 39.

An approximate measure of the removal of the free protein by filtration was obtained from the values for the differences in extinction measured at 285 and at 330 mμ. (Fig. 2). This showed that the first three filtrations had removed some 90 % of the total protein of synovial fluid. Fig. 2 and the values of the ratio of total N to glucosamine (Table 3) show that further filtration removes protein at a very slow rate. However, a rise of the N/glucosamine ratio has occurred after 39 filtrations.

It seems, therefore, that ultrafiltration brings the protein content down to a fairly stable low value and that between 16 and 28 filtrations can be performed without any sign of degradation appearing by any of the criteria available.

Composition of the hyaluronic acid complex made by ultrafiltration

The material prepared by 16 filtrations (samples 16*W* and 16*D*) was subjected to more complete analysis, with a view to resolving the discrepancies noted by Ogston & Stanier (1950). Dialysed samples made by 28 and 39 filtrations (28*D* and 39*D*) were less completely analysed (Table 4).

Sample 16*W* was in solution in water; it was centrifuged at 5000 *g* to remove a small amount of solid matter. 16*D* was made by dialysing a sample of 16*W* against buffer. During this dialysis, a further small amount of solid separated and was removed by centrifuging. The N/glucosamine and dry weight/glucosamine ratios (Table 4) of 16*D* are less than those of 16*W*. These changes would be accounted for if the solid which separates on dialysis were protein. This was found to be the case when the N and glucosamine contents of another sample of hyaluronic acid complex were measured before and after dialysing against buffer and centrifuging: in this case, the N content showed a similar lowering while the glucosamine content was unchanged.

The molar ratio of glucuronic acid to glucosamine of 16*W* is 1·34. This ratio was determined on several other samples (Table 5), the ratio being in all cases well above 1·0. The ash content of 16*W* agreed well with a determination on another sample.

Although the composition of sample 28*D* (dialysed and centrifuged) differs little from that of

Table 3. *Progress of removal of protein* N *by ultrafiltration*

Sample	3*D*	8*D*	16*D*	28*D*	39*D*
No. of filtrations	3	8	16	28	39
Glucosamine (mg./ml.)	0·255	0·248	0·256	0·282	0·249
Total N (mg./ml.)	0·086	0·077	0·067	0·065	0·076
Total N/glucosamine	0·34	0·31	0·26	0·23	0·30
Mucin (mg./ml.)	2·26	2·26	—	—	—

Table 4. *Analysis of hyaluronic acid complex made by ultrafiltration*

Sample	16*W*	16*D*	28*D*	39*D*
Dry wt. (mg./ml.)	1·28	1·09*	1·11*	1·04*
Glucosamine (mg./ml.)	0·280	0·256	0·282	0·249
Glucuronic acid (mg./ml.)	0·405	—	—	—
Ash (mg./ml.)	0·077	—	—	—
Na^+ (μequiv./ml.)	0·70	—	—	—
Ca^{++} (μequiv./ml.)	0·24	—	—	—
pH	6·1	—	—	—
Refractive increment $\times 10^5$	19·2	16·4	16·6	15·6
Total N (mg./ml.)	0·090	0·067	0·065	0·076
Protein N (mg./ml.) (Total N – glucosamine N)	0·068	0·047	0·043	0·057
Protein (mg./ml.) (protein N × 6·45)	0·44	0·30	0·28	0·37
Hyaluronic acid (mg./ml.) (dry wt.-protein)	0·84	0·79	0·83	0·67
Total N/glucosamine	0·32	0·26	0·23	0·30
Dry wt./glucosamine	4·6	4·2	3·9	4·2
Glucosamine % of hyaluronic acid	33	32	34	37
Protein % of dry wt.	34	28	25	36

* From refractive increment.

Table 5. *Glucuronic acid : glucosamine ratios in hyaluronic acid complex produced by ethanol precipitation and by ultrafiltration*

Sample	Protein (%)	Glucosamine (%)	Glucuronic acid (%)	Molar ratio glucuronic acid : glucosamine
One precipitation from ethanol	69	5·5	7·2	1·20
Several precipitations from ethanol	18	26	35	1·24
Ultrafiltration residue	34	23	32	1·28
Ultrafiltration residue (16*W*)	34	22	32	1·34

16*D*, sample 39*D* shows distinct changes of the N/glucosamine ratio and N content on a dry weight basis (Table 4).

Table 6. *Composition of hyaluronic acid complex*

Component	g./100 g. of complex
Glucosamine	(24·8)
Acetylglucosamine	30·6
Glucuronic acid	35·9
Protein	25·7
Ash	6·8
	99·0
Less 1 mole water/mole glucosamine	2·5
Less 1 mole water/mole glucuronic acid	3·3
	93·2

Since all the physical measurements reported by Ogston & Stanier (1950, 1951*a*) were made on dialysed and centrifuged material, it is the composition of this material (i.e. of 16*D* rather than 16*W*) which is of chief interest. However, since the dry weight determined directly is probably more accurate than that determined from the refractive increment, and since only the latter is available for dialysed samples, the composition on a dry weight basis given in Table 6 is for 16*W*, after subtraction of the weight of protein (assumed 15·5% N) removed following dialysis. This gives the most accurate estimate of the composition of dialysed material.

Composition of mucin

Mucin clots were made from 1 ml. of solution of ultrafilter residue to which synovial fluid protein had been added, by the method used for synovial fluid. Their dry weights were determined in duplicate and agreed within 2%. It was confirmed that further washing of the clots produced no loss of weight.

According to these results (Table 3), glucosamine forms 11·2% of mucin; from this and from the proportion of glucosamine in hyaluronic acid complex (Table 6), hyaluronic acid complex is found to form 45% of mucin. These values are higher than those given by Ogston & Stanier (1950), which were based on analysis of mucin made on a larger scale. Since Ogston & Stanier (1951*a*) assumed that hyaluronic acid complex forms only 32% of mucin (based on their previous work) their values for the physical constants of the complex require emendation (see below).

Chromatographic analysis

A two-dimensional chromatographic analysis for amino-acids was performed on a sample of hyaluronic acid and, in a parallel experiment, on a sample of serum albumin. No marked difference

between the chromatograms could be observed. This suggests that the protein in the complex has the usual N content (15·5 %).

Attempts were made to detect the presence of any carbohydrate component other than glucosamine and glucuronic acid, following treatment with aqueous or methanolic hydrochloric acid, and paper chromatography; the results of these experiments were entirely negative.

Treatment with hyaluronidase

A few experiments were made on the action of a crude preparation of rat-testis hyaluronidase, in the hope that this might lead to a method for isolating the protein part of the hyaluronic acid complex following depolymerization of the hyaluronic acid. The action of the enzyme was followed by measuring the viscosity of a portion of the enzyme-containing solution, which contained about 1 mg./ml. of hyaluronic acid. The relative viscosity fell from 20·6 to 1·75 after 24 hr. and to 1·51 after 48 hr. At these times samples were examined in the ultracentrifuge. In each case a single diffuse boundary was observed. These boundaries had sedimentation constants 2·07 and $1·97 \times 10^{-13}$ (confirming Hahn, 1947) and represented respectively 83 and 58 % of the original refracting material. Since the protein in the original samples represented only 30 % of the refracting material, it is clear that the boundaries must still have contained a large amount of carbohydrate material, and that the hyaluronic acid was only partially depolymerized (see Discussion). The attempt to separate the protein in this way was abandoned.

During the treatment with hyaluronidase, the separation of a small amount of a granular precipitate was noticed. Treatment of a sample of hyaluronic acid complex with Benger's 'Hyalase' also led to the precipitation of about 7 % of the weight of dissolved material. This caused no detectable change of the N/glucosamine ratio. The 'Hyalase' was found to be without detectable proteolytic activity.

Sedimentation of purified hyaluronic acid complex

The values of the sedimentation constant at zero concentration and of its variation with concentration used by Ogston & Stanier (1951*a*) were obtained with dilutions of synovial fluid dialysed against buffer. Dr J. P. Johnston (personal communication) has observed that free protein may modify the rate of sedimentation of the complex. The opportunity was, therefore, taken to study the sedimentation of sample 16*D* over a wide range of concentration (Fig. 3). No sign of free protein could be observed. The boundary of hyaluronic acid remained as sharp as in synovial fluid at the same concentrations. The rates of sedimentation at lower concentrations were close to those observed with synovial fluid; at higher concentrations, the sedimentation constants in synovial fluid were somewhat lower (Fig. 3) and this agrees with the findings of Johnston.

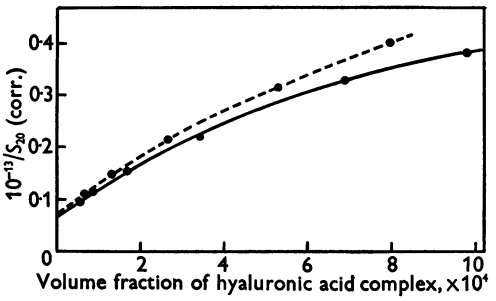

Fig. 3. Variation of sedimentation constant with volume fraction of hyaluronic acid complex. - - -●- - -, synovial fluid (Ogston & Stanier, 1950); —●—, ultrafiltration residue 16*D*.

DISCUSSION

The use of the variation of viscosity with velocity gradient as a criterion has confirmed the conclusion of Ogston & Stanier (1950) that ultrafiltration does not lead to degradation of the hyaluronic acid complex, whereas its precipitation by acid (as mucin) or by ethanol does cause degradation. The persistent presence of a proportion of protein in the complex is confirmed, though the amount of this protein is less than was estimated by Ogston & Stanier (1950). This difference was due to their analysing the solution in water, produced by ultrafiltration, whereas on dialysis against buffer a constant fraction of the protein separates as a precipitate. It is likely that this is denatured protein held in suspension, which separates when the viscosity is lowered by buffer. We have found that electrophoresis does not remove more protein than does ultrafiltration, whether the starting material is synovial fluid or mucin; this is contrary to the finding of Blix (1940).

Composition of the hyaluronic acid complex. Of the weight of the complex 93 % can now be accounted for in terms of acetylglucosamine, glucuronic acid, protein and ash (Table 6). The following points deserve mention: (i) the molar ratio of glucuronic acid to glucosamine in the complex is markedly greater than the value of 1·0 claimed by Meyer, Smyth & Dawson (1939); (ii) it is assumed that the carbohydrate components are joined by anhydride links, so that the weight of 1 mole of water has been subtracted for each mole of glucosamine and of glucuronic acid; (iii) the assumption that the protein contains near to 15·5 % N is confirmed by chromatographic comparison of its amino-acid composition with serum albumin; (iv) the complex yields some 7 % of ash: Na^+ and

Ca^{++} together account for 1·8 % of the dry weight or 0·83 m-equiv./g. dry weight compared with 1·85 m-equiv. of glucuronic acid.

The change of composition which is associated with the onset of degradation observed in sample 39*D* is not in line with the observation of Ogston & Stanier (1950), who concluded that lowered N content is associated with degradation. Here the proportion of N to glucosamine, and of N to dry weight, have increased. Such changes could be due to microbial action.

Ionic composition and pH. Comparison of the amount of metallic ions with that of carboxyl groups suggests that less than half of the carboxyl groups are ionized. Since the pK of glucuronic acid is about 3·5 (using the value found by Karrer & Schwarzenbach, 1934, for galacturonic acid), and since the pH of the solution is 6·1, nearly complete ionization might be expected. However, Katchalsky (1951) has shown that the pH of a solution of half-ionized polymethacrylic acid may be several units higher than the pK of the monomer, owing to the interaction of the charged groups. In accordance with the observations of Katchalsky & Spitnik (1947) on polymethacrylic acid, the pH of a solution of hyaluronic acid complex was found to fall markedly when neutral salt was added.

The composition of mucin. This is not in itself of any importance since no such complex appears to exist in synovial fluid and since its separation is not a desirable stage in the preparation of undegraded hyaluronic acid. Nevertheless, the estimation of mucin is a useful method for measuring the amount of hyaluronic acid in the presence of protein and has been much used for this purpose. It is important to realize, therefore, that its composition may depend on the method of preparation. Small clots made on glass rods and thoroughly washed appear to have a constant composition: this differs from the composition of mucin made on a larger scale, probably through the presence in the latter of entrained protein.

The action of hyaluronidase. Treatment, even for a long period, leaves a considerable proportion of the hyaluronic acid in a state in which its sedimentation is observable in the ultracentrifuge. Hahn (1947) has investigated the sedimentation of such material over a range of concentrations. The sedimentation constant at higher concentration differs little from that of undegraded hyaluronic acid complex; the variation with concentration is much less. The data available (S_{20} at zero concentration, taken as $2{\cdot}0 \times 10^{-13}$ and the variation of $1/S$ with concentration (Fessler & Ogston, 1951), taken as $0{\cdot}49 \times 10^{13}$, both from Hahn's data; and the intrinsic viscosity $(\eta_{rel.} - 1)\phi = 5{\cdot}9 \times 10^2$ from our results) enable estimates to be made of the molecular dimensions of the degraded material, using the procedure of Ogston & Stanier (1951*a*). The results are: $M = 107\,000$; effective volume = 5·5 ml./g.; axial ratio = 30. These results will apply to the average of the more highly polymerized part of a polydisperse mixture, since this part will make the greatest contribution to the viscosity and to the observed average sedimentation. They are consistent with the production, by hyaluronidase, of shorter chain units from a very large chain-polymer: the average unit has about 1/100 of the weight of the original polymer and would be expected to have a much lower degree of solvation (or entrainment of solvent) and a larger axial ratio.

Physical properties. Ogston & Stanier (1951*a*) assumed that hyaluronic acid complex constitutes 32 % of the weight of mucin, in calculating the intrinsic viscosity of the complex. The analyses reported here show that hyaluronic acid forms 45 % of mucin. It appears from Table 1 that the value of $\log\{(\eta_{rel.} - 1)/\phi\}$ at $\phi = 0$ should be between 3·9 and 4·0, instead of the value of 3·8 taken by Ogston & Stanier (1951*a*); this would have the effect of increasing the values of V' given in their Table 3 by a factor of 1·4. The value of the sedimentation constant, extrapolated to zero concentration is 14·7 instead of $13{\cdot}2 \times 10^{-13}$. This change, together with that of the values of V', will increase the values of M by a factor of 1·45, the values of a by 1·26 and of a^3 by 2·0. The calculated values of χ° and of Δ° are little affected. The new experimental value of $d(1/S)/dc$ is 3·3, instead of $3{\cdot}9 \times 10^{-3}$. None of these changes significantly affects the conclusions already drawn.

General conclusions. We briefly summarize our conclusions about the composition and properties of the hyaluronic acid of ox synovial fluid: (i) The hyaluronic acid occurs in the form of a complex containing 25 % of protein; the carbohydrate part of the complex consists mainly, perhaps entirely, of acetyl hexosamine and uronic acid (glucosamine and glucuronic acid, according to Meyer *et al.* (1939)). The molar ratio of hexuronic acid to hexosamine is 1·25–1·35. (ii) The complex is very easily degraded and only the mildest methods of separation (ultrafiltration and probably electrophoresis) preserve its native properties. (iii) The particle weight is about 10^7: the dynamic properties of solutions of the complex show that the particles are nearly spherical and are very highly hydrated. These properties are consistent with the view that the complex is, essentially, a flexible chain-polymer.

SUMMARY

1. Methods of separating hyaluronic acid from ox synovial fluid have been re-investigated, using the variation of viscosity with velocity gradient as a criterion of degradation. Precipitation as mucin

and precipitation by ethanol cause degradation, while ultrafiltration does not.

2. The course of purification by ultrafiltration has been followed. It has been confirmed that the protein content of the complex is reduced to a limiting value of 25%. Electrophoresis is shown not to give products of lower protein content.

3. Analysis of the product of ultrafiltration shows that 93% of its weight is accounted for by acetyl hexosamine, hexuronic acid, protein and ash. The protein content of the complex is found to be lower than was previously reported.

4. A revised value is given for the composition of mucin, prepared on a small scale.

5. Degradation by hyaluronidase has been shown to leave a considerable fraction of the material in a polymerized state (molecular weight of the order of 10^5).

6. The ultracentrifugal sedimentation of purified hyaluronic acid complex has been re-investigated.

7. Slight modifications of the values of physical quantities are given, based on the revised analytical values and sedimentation constant.

8. Our conclusions about the composition and molecular configuration of the hyaluronic acid complex are summarized.

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Note added in proof. Since this paper was sent in, some of our preparations have been examined by Dr R. Consden, who finds that small amounts of neutral sugars are consistently present.

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Separation of Adenosine Phosphates by Paper Chromatography and the Equilibrium Constant of the Myokinase System

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This paper describes a procedure for the quantitative separation by paper chromatography of adenosinetriphosphate (ATP), adenosinediphosphate (ADP), adenosinemonophosphate (AMP) and inorganic orthophosphate. The technique is a development of those of Hanes & Isherwood (1949) which are satisfactory for many purposes, but are inadequate for the complete separation of ADP and inorganic phosphate. The formic acid *iso*propyl ether solvent of Hanes & Isherwood separates satisfactorily the three adenosine phosphates from inorganic phosphate and the *iso*propanol ammonia solvent separates ATP from ADP, AMP and inorganic phosphate. The imperfect separation of ADP and inorganic phosphate is especially evident when the mixture of phosphates contains $KH_2{}^{32}PO_4$. The