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The Nature of the Protein in the Hyaluronic Complex of Bovine Synovial Fluid

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Ogston & Stanier (1950, 1951, 1952) found that the hyaluronic acid of ox synovial fluid is always combined with 25–30% of protein when it is isolated by ultrafiltration under such conditions that its physicochemical properties are unchanged. Approximately the same amount of protein accompanies hyaluronic acid migrating by electrophoresis, sedimentation or diffusion. The protein complex is considered to be the form in which the hyaluronic acid occurs in the native fluid, since removal of the protein leads to changes in physicochemical properties of the material. Relatively little is known of the origin or composition of the protein, although Humphrey & Stanier (unpublished) found that the hyaluronic acid complex gave antisera in the rabbit which cross-reacted with some of the components of ox blood serum. Since, apart from its hyaluronic acid content, synovial fluid has the composition of a plasma exudate, similar to lymph and ascitic fluid (Hesslevik, 1940), it appears that at least some of the proteins in the hyaluronic acid complex could be derived from the free proteins of the synovial fluid. It was considered desirable to confirm this conclusion by isolation and characterization of the protein and this is described in this paper.

MATERIALS AND METHODS

Ox synovial fluid. This was collected and stored according to the methods of Ogston & Stanier (1950).

Hyaluronic acid complex. This was prepared as an ultrafiltrate residue using a sintered-glass '5 on 3' filter, average

pore diameter 1 μ . (Ogston & Stanier, 1952). The residue was taken up in phosphate buffer (pH 7.0, $I=0.1$) and the small amount of insoluble material was removed by centrifuging at 15 000 g for 20 min. Where studies were made on the ultrafiltrate this included the initial filtrate and the first five washings, concentrated by freeze-drying, if necessary.

Buffers. The following buffers were used, diluted to the desired concentrations:

pH 5.8	0.53M-KH ₂ PO ₄ , 0.056M-KOH
pH 7.0	0.50M-KH ₂ PO ₄ , 0.177M-KOH
pH 8.0	0.50M-KH ₂ PO ₄ , 0.47M-KOH
pH 10.0	0.62M glycine, 0.62M-KCl, 0.38M-KOH.

Physiological saline. This was 0.155M-NaCl. All solutions were saturated with chloroform as an antiseptic precaution.

Hyaluronidase. Benger hyaluronidase (Hyalase) was used. This preparation has been found to be free from proteolytic activity (Ogston & Stanier, 1952). Immunological analysis showed that it contained 0.8 mg. of substances reacting with antialbumin per mg. of N.

Ox serum fractions. These were prepared by an extension of the low-temperature ethanol precipitation method used by Hess & Deutsch (1948) to isolate γ -globulin from ox serum. The techniques of fractionation followed those established by Cohn (Edsall, 1947). Details of the fractionation are given in Fig. 1. Where no exact temperature is marked the value is in the range 0–1°. pH values were adjusted with 0.05N acetic acid or 0.05M-Na₂HPO₄. Ethanol-containing solutions were diluted 5 times with distilled water before their pH values were measured with the glass electrode. Each precipitate was washed twice with 500 ml. of fluid having the same pH and salt and alcohol concentration as its supernatant. All the fractions were dissolved in and dialysed against physiological saline and were stored as frozen 3% stock solutions. After one cycle of freezing and thawing it was found that some lipid material separated from F3. This was discarded. The fractions, with the exception of F5, gave single peaks on

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Fig. 1. Low-temperature ethanol-water fractionation of bovine serum.

Table 1. Mobilities of the ox serum fractions and their relationship to the electrophoretic components of unfractionated serum

pH 7.7, I = 0.2, phosphate.

Fraction no.	Appearance on electrophoresis	Mobility × 10 ⁻⁵ (cm. ² sec. ⁻¹ V ⁻¹)	Relation to electrophoretic components	Remarks
1	Single peak	2.57	β-Globulin	Acid-insoluble
2	Broad, single peak	0.95	γ-Globulin	γ ₁ and γ ₂ unresolved
3	Single peak	3.52	α-Globulin	Acid-insoluble
4	Single peak	3.31	α-Globulin	Acid-soluble
5	Two peaks (1) 75% (2) 25%	1.98 2.47	β-Globulin albumin	Acid-soluble
6	Single peak	4.91		

electrophoresis in pH 7.7, I = 0.2, phosphate buffer. Fraction 5 contained 25% of a slow and 75% of a fast component. The mobilities of the fractions and their relation to the electrophoretic components of unfractionated serum are given in Table 1.

It must be emphasized that this fractionation was not undertaken to obtain highly purified protein fractions as such, but in order to obtain reproducible and antigenically distinct fractions which could be used to prepare antibody for immunological analysis, following the principles of Kendall (1938).

Antisera. These were prepared by intravenous injection of the ox serum fractions into rabbits. Two injections, each containing 100 mg. of material, were given into an ear vein

at an interval of 6 days and the rabbits were bled through the heart 12 days after the second injection. The antibodies were purified and concentrated by the low-temperature ethanol precipitation method of Nichol & Deutsch (1948). Agar diffusion tests showed that all of the fractions were immunologically heterogeneous, each giving at least four rings. The results of precipitin tests for cross-reaction are given in Table 2.

It can be seen that there is significant cross-reaction between the antibodies to fractions 5 and 1 and 3 and 1. The cross-reacting components were removed by incubating the antibodies with a slight excess of the appropriate antigen at 37° for 60 min. The precipitates were removed by centrifuging at 2800 g for 10 min.

Table 2. *Immunological cross-reaction between the different fractions of ox serum*

Rabbit antiserum and approximately 0.1% solution of each antigen was used. The values are dilutions of antiserum which give a just-visible precipitate.

Antigen	Antiserum					
	F1	F2	F3	F4	F5	F6
F1	320	2	32	2	32	2
F2	2	280	8	2	10	2
F3	64	16	300	4	16	2
F4	16	2	8	160	8	2
F5	64	2	16	16	240	16
F6	8	2	2	2	2	320

Precipitin test. The ring test was used (Medical Research Council, 1931) with antiserum in constant dilution in the lower layer and falling dilutions of antigen forming the superficial layer. Readings were made in 100 min., after which no increase in titre was obtained.

Agar-diffusion test. The immunological heterogeneity of the antigen was examined by the Oudin (1949) agar-gel-diffusion technique.

Quantitative immunological analysis. This followed, in general, the procedures given by Kabat & Mayer (1948). Each antibody was calibrated by forming its specific precipitate on incubating a slight excess of the antibody for 60 min. at 37° with a series of antigen solutions of known concentration. The precipitates were centrifuged at 2800 *g* for 10 min. and washed twice with cold physiological saline. The N content of the washed precipitates was determined by the micro-Kjeldahl method and a calibration curve was constructed giving the concentration of the antigen against the N content of its specific precipitate. The concentration of a given fraction in a particular sample of protein was determined by preparing the specific precipitate under the same conditions as were used for the calibration (always ensuring that an excess of antibody remained after the precipitation) and comparing its N content with that of the standard curve.

Nitrogen determination. This was carried out by the micro-Kjeldahl method, using a selenium catalyst and 8 hr. digestion. A Markham (1942) apparatus was used for the distillation.

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

Ultraviolet absorption. This was measured in a Hilger Uvispek spectrophotometer.

Viscosity. This was measured using the Couette viscosimeter described by Ogston & Stanier (1953a).

Electrophoresis. This was carried out in a Tiselius (1948) apparatus with Philpot-Svensson optics. The following buffer was used: Na_2HPO_4 (0.003 M), KH_2PO_4 (0.00034 M), NaCl (0.18 M). (pH = 7.7, $I = 0.2$).

Refractive increment. This was measured in the refractometer described by Cecil & Ogston (1951). Protein refractive increments were taken to be 0.00182 for a 1% solution and hyaluronic acid 0.00144.

RESULTS

Separation of the protein from the ultrafilter residue

Attempts at separation using a synthetic resin. Attempts were made to separate the protein by adsorption of the hyaluronic acid complex on

columns of the cationic resin, Dowex 2, which was used in the bicarbonate form. Although it was hoped that the positively charged resin would displace the protein from the hyaluronic acid, it was found that the whole complex was adsorbed. Low yields of the protein (never more than 50%) could be eluted from the column with either 0.1 M-NaCl or 20% ethanol. In the latter case the ethanol solution and the column were chilled to -5°. Since there was no guarantee that the low yield would give a representative sample of the protein in the complex these experiments were discontinued.

Separation of the protein after hyaluronidase treatment. Since the resin experiments indicated that at least some of the protein was bound firmly to the hyaluronic acid, the effect of depolymerization of the latter by hyaluronidase was investigated. The ultrafilter residue was dissolved in phosphate (pH 7, $I = 0.1$) to give a 0.25% solution. This was incubated for 16 hr. at 37° with a minimal amount of Benger Hyalase (hyalase N = 0.0008 mg./ml. in the final solution). After dialysis against distilled water the glucosamine content of the treated material fell from 33 to 27% of the dry weight, indicating that some of the hyaluronic acid had been broken down to a diffusible size. Electrophoresis in phosphate buffer, pH 7.7, $I = 0.2$, showed the presence of two major components (Fig. 2a), one with a mobility of $9.1 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ v}^{-1}$ [corresponding to the values obtained for hyaluronic acid by Hesselvik (1940)] and the other with a mobility of $3.48 \times 10^{-5} \text{ cm}^2 \text{ sec}^{-1} \text{ v}^{-1}$. The proportions of protein and hyaluronic acid, 38 and 62% respectively, calculated from the areas of the peaks correspond closely with those obtained from chemical determination, indicating that the hyaluronidase treatment had dissociated all of the protein from the hyaluronic acid. Attempts were made to remove the degraded hyaluronic acid by adsorption on Dowex 2 resin, but more than 60% of the protein, as measured by ultraviolet absorption, was lost by this treatment.

Serum albumin as a precipitating agent for the degraded hyaluronic acid. Ogston & Stanier (1950) and Blix (1940) have shown that serum albumin preferentially forms mucin with hyaluronic acid, even in the presence of other proteins. It has also been shown (Ogston & Stanier, 1953b) that the quantitative recovery of hyaluronic acid in mucin is not affected by its prior degradation with hyaluronidase. In the present work, however, it was found impossible to precipitate all of the degraded hyaluronic acid without introducing an excess of albumin into the solution, in some instances as much as 20% of the total protein. It was decided, therefore, to use ox serum albumin made insoluble by coupling with azobenzylcellulose, according to the method of Campbell, Luescher & Lerman (1951).

Table 3. *Isolation of protein from the ultrafilter residue and analytical data*

Unless otherwise indicated, values are given in mg./ml.

	Sample no.			
	4	6	7	12
Ultrafilter residue				
Dry weight	1.92	1.55	1.68	1.47
Glucosamine (Elson-Morgan)	0.421	0.384	0.429	0.368
Protein (total N - glucosamine N) $\times 6.45$	0.53	0.41	0.49	0.38
Protein content (% of dry weight)	28	26	29.5	26
Isolated protein				
Dry weight	0.61	0.47	0.58	0.43
Glucosamine (Elson-Morgan)	0.011	0.013	0.013	0.0082
Protein (total N - glucosamine N) $\times 6.45$	0.49	0.40	0.46	0.37
Ash	0.06	0.04	0.08	0.04
Recovery of protein (%)	92.6	97.5	93.9	97.4

Fig. 2. Electrophoresis diagrams. Right, ascending boundary migrating from left to right. Left, descending boundary migrating from right to left. *a*: hyaluronidase-treated ultrafilter residue. pH 7.7, $I=0.2$, phosphate buffer. 4800 sec. at 3.25 v cm.⁻¹. *b*: protein isolated from the ultrafilter residue. pH 7.7, $I=0.2$, phosphate buffer. 8800 sec. at 3.30 v cm.⁻¹. *c*: ultrafiltrate from preparation 6. pH 7.7, $I=0.2$, phosphate buffer. 10 400 sec. at 3.28 v cm.⁻¹. *d*: hyaluronic acid-free synovial fluid. pH 7.7, $I=0.2$, phosphate buffer. 6500 sec. at 3.25 v cm.⁻¹.

The material prepared contained approximately 15 mg. of albumin per g. of azobenzylcellulose, and it was found that this bound approximately 5 mg. of hyaluronic acid at pH 5.5, $I=0.1$.

Final method of separating the protein. This was carried out as follows: the ultrafilter residue from

100 ml. of synovial fluid was dissolved in 200 ml. of phosphate (pH 7.0, $I=0.1$), 0.1 ml. of Hyalase solution (containing 0.8 mg. N/ml.) was added, and the mixture was incubated at 37° for 16 hr. The incubated solution was dialysed for 24 hr. against two changes of excess of 0.1M-NaCl. A slight precipitate was removed by centrifuging for 20 min. at 2500 g and the supernatant was mixed with 50 g. of the cellulose-albumin, stirred vigorously and acidified to pH 5.5 with 10 % acetic acid. The adsorbent was removed by centrifuging, and after dialysis against distilled water the glucosamine, nitrogen, ash and dry weight of the supernatant were determined. In Table 3 are given the figures for the treatment of the different batches of the ultrafilter residue.

It can be seen that this method leads to the recovery of nearly all the protein present in the complex. A precipitin test showed no trace of albumin in this protein. The protein solution was concentrated by freeze-drying and examined by electrophoresis in phosphate (pH 7.7, $I=0.2$) (Fig. 2*b*). The major component was found to give a pattern and mobility, 3.44×10^{-5} cm.² sec.⁻¹ v⁻¹, similar to that of the slow component in Fig. 2*a*. The mobility is close to that of serum α -globulin (Svennson, 1946).

Relation of the isolated protein to the various serum fractions and the free proteins of the synovial fluid

Immunological analysis of the isolated protein. The isolated protein reacted in the precipitin test with the antibodies to γ -globulin, acid-insoluble α -globulin, and both β -globulins, but not to anti-albumin or acid-soluble α -globulin. The results of quantitative immunological analysis are given in Table 4.

These results show that the hyaluronic acid complex contains a large proportion of acid-insoluble α -globulin. This was confirmed when it was found that 75 %, as measured by ultraviolet

absorption, of the protein was precipitated by dilution with distilled water and acidification with 10 % acetic acid to pH 5.2. It must be noted, also, that 96 % of the protein can be accounted for in the fractions which react with the antisera, suggesting that all of the isolated protein is identical antigenically with the fractions present in bovine serum.

Table 4. *Quantitative immunological analysis of two samples of protein isolated from the ultrafilter residue*

Values given as mg./ml.			
Antigen		Sample 'A'	Sample 'B'
Fraction no.	Nature of component		
1	β -Globulin (acid-insol.)	0.75	0.63
2	γ -Globulin	0.16	0.31
3	α -Globulin (acid-insol.)	3.39	3.27
4	α -Globulin (acid-sol.)	0.03	0.04
5	β -Globulin (acid-sol.)	0.19	0.23
6	Albumin	0.02	0.05
Total material reacting with antibody		4.54	4.53
Dry weight of material in each sample		4.73	4.81

Table 5. *Composition as determined by electrophoresis of ultrafiltrates from three different preparations of the hyaluronic acid-protein complex*

The values given are percentages of total proteins.

Component	Sample no.		
	4	6	12
Albumin	64	66	65
α -Globulin	4	5	4
β -Globulin	14	12	16
γ -Globulin	18	17	15

Electrophoresis of the ultrafiltrates. The ultrafiltrates from three preparations (nos. 4, 6 and 12) were submitted to electrophoresis in phosphate buffer, pH 7.7, $I=0.2$. All three gave a 'serum type' pattern. The proportions of the components are given in Table 5. The electrophoresis pattern of preparation 6 is given in Fig. 2c. The ultrafiltrates contain a very high proportion of albumin, agreeing with the results obtained by Hesselvik (1940) and Ogston & Stanier (1951) for horse, human and ox synovial fluids.

Electrophoresis of hyaluronic acid-free synovial fluid. It was found possible to remove nearly all the hyaluronic acid from synovial fluid by treating it with hyaluronidase and adsorbing the degraded hyaluronic acid on cellulose-coupled albumin as described previously. Electrophoresis was carried out in phosphate buffer, pH 7.7, $I=0.2$, on three samples of synovial fluid treated in this manner. The proportions of components and the protein and

glucosamine contents of each sample are given in Table 6. The electrophoretic pattern of sample 12 is given in Fig. 2d.

Table 6. *Electrophoretic, immunological and chemical analysis of hyaluronic acid-free synovial fluids*

The results of the electrophoretic analysis are given in terms of percentage composition; all other values are in mg./ml.

	Sample no.		
	4	6	12
Electrophoretic analysis			
Albumin	60	59	62
α -Globulin	10	11	9
β -Globulin	12	13	14
γ -Globulin	18	17	15
Immunological analysis			
Acid-insoluble β -globulin	1.03	0.87	0.89
γ -Globulin	1.69	1.54	1.59
Acid-insoluble α -globulin	0.61	0.73	0.69
Acid-soluble α -globulin	0.22	0.18	0.19
Acid-soluble β -globulin	0.17	0.21	0.16
Albumin	4.31	4.49	4.22
Total material reacting with antibody	8.03	8.02	7.74
Dry weight of non-dialysable material in each sample	8.51	8.62	8.21
Chemical analysis			
Glucosamine (Elson-Morgan)	0.13	0.24	0.16
Protein (total N-glucosamine N) $\times 6.45$	8.33	8.25	7.85
Ash	0.04	0.08	0.09

Immunological analysis showed that 98 % of the protein could be accounted for in the precipitates to the antibodies to the six serum fractions. As expected, the difference between the concentrations, as determined electrophoretically, of the various components of the ultrafiltrate and the hyaluronic acid-free synovial fluid corresponded closely with the composition of the protein of the ultrafilter residue.

Effect of variations in the method of preparation on the composition of the ultrafilter residue

Although the composition of the protein isolated from different samples of ultrafilter residue was fairly constant, the question was raised whether the composition could be varied by factors such as changes in the pH and ionic strength of filtration and washing. The method of Ogston & Stanier involves the use of distilled water and unbuffered salt solutions as washing fluids. To investigate the effect of pH and ionic strength, 60 ml. lots of synovial fluid were dialysed against the following: (a) phosphate buffers; pH 5.8, $I=0.1$; pH 7.0, $I=0.1$; pH 7.0, $I=0.5$; pH 7.0, $I=1.0$; pH 8.0, $I=0.1$, and (b) glycine buffer, pH 10.0, $I=0.1$. The samples were then submitted to ultrafiltra-

tion and each residue was washed 20 times with the appropriate buffer solution. Care was taken to ensure that all the ultrafilter residue was redissolved at each washing. In an attempt to reproduce 'physiological conditions' a sample of synovial fluid was dialysed against, and filtered in the presence of, the following solution: NaCl, 0.103M; K₂SO₄, 0.0056M; CaCl₂, 0.0056M; NaH₂PO₄, 0.002M; sodium lactate, 0.0055M; MgCl₂, 0.0018M; NaHCO₃, 0.027M. The pH of solution was 7.4. These are similar to those given by Bauer, Ropes & Wayne (1940) for the values of pH and electrolyte composition of ox synovial fluid. It was found that the solution maintained its pH satisfactorily if kept cold in a stoppered Pyrex bottle. In order to prevent the pH from rising, owing to diffusion of CO₂ from the mixture into the vacuum under the filter, the filtration flask was filled with CO₂ and evacuated to a pressure of 20 mm. Hg at 0°; this is equal to the pressure of the free CO₂ in the mixture and the vapour pressure of water at 0°. Frequent pH measurements made on the ultrafiltrate showed that the pH remained within the range of pH 7.3–7.5.

decreases, indicating a rough correlation between the isoelectric point of the protein bound and the pH of filtration. At neutrality increasing *I* leads to the binding of larger amounts of protein, including some albumin, which is not otherwise bound. The material prepared under 'physiological conditions' is not very different from that prepared at pH 7.0, *I* = 0.1, and both contain approx. 75% α-globulin; but it differs from that prepared by the method of Ogston & Stanier (1952). The latter contains 82% α-globulin and is closer in composition to the sample prepared at pH 5.8, *I* = 0.1.

Maximum amount of protein bound under various conditions. Since synovial fluid contains only a relatively limited concentration of each protein fraction it is possible that the amount of a given protein bound under a particular set of conditions would not necessarily be the maximum amount which could be bound, if a greater concentration of the protein were present in the fluid. Electrophoresis of the ultrafiltrate from the pH 8.0 preparation showed that this contained no γ-globulin, in contrast to ultrafiltrates prepared at

Table 7. *Protein content of the ultrafilter residue prepared under different conditions of pH and ionic strength and the electrophoretic composition of the isolated proteins*

	General conditions of preparation							
	Ogston & Stanier	'Physiological'	pH 5.8 <i>I</i> = 0.1	pH 7.0 <i>I</i> = 0.1	pH 7.0 <i>I</i> = 0.5	pH 7.0 <i>I</i> = 1.0	pH 8.0 <i>I</i> = 0.1	pH 10.0 <i>I</i> = 0.1
Dry weight (mg./ml.)	1.30	1.48	1.50	1.46	1.37	1.21	1.22	1.44
Protein (mg./ml.)*	0.36	0.40	0.41	0.38	0.43	0.53	0.23	0.19
Protein (% of dry weight)	27	28	27	26	32	44	22	13
Electrophoretic analysis of isolated protein (%):								
γ-Globulin	2	6	2	11	11	5	65	78
β-Globulin	16	18	11	15	16	14	30	22
α-Globulin	82	76	87	74	70	60	5	0
Albumin	0	0	0	0	3	21	0	0

* Calculated from (total N – glucosamine N) × 6.45.

The final ultrafilter residue from each preparation was taken up in 60 ml. of phosphate buffer, pH 7.0, *I* = 0.1, and treated with hyaluronidase and the hyaluronic acid was removed as described previously. The protein from each preparation was submitted to electrophoresis in phosphate buffer, pH 7.7, *I* = 0.1. The electrophoretic analysis of the isolated proteins is given in Table 7. The electrophoretic analysis of the protein isolated from an ultrafilter residue prepared by the method of Ogston & Stanier (1952) is included for comparison.

It can be seen that there is considerable variation between the different preparations, in both the type and amount of protein bound. As the pH increases, less protein is bound and its mobility

lower pH values. In order to see whether hyaluronic acid would bind more γ-globulin at pH 8.0, purified γ-globulin was added to synovial fluid until this represented 30% of the protein present. The ultrafilter residue prepared from this at pH 8.0 contained 28% protein, 80% of which reacted with the antibody to γ-globulin; this is in contrast to the normal pH 8.0 preparation which contains 22% protein, 65% of which is γ-globulin. Attempts were made to duplicate this phenomenon at pH 5.8 and 7.0 by dissolving ultrafilter residue prepared by the method of Ogston & Stanier (1952) in undiluted ox serum and refiltering and washing in the presence of the appropriate buffers. The ultrafilter residues did not differ significantly in either protein content

or composition, as determined by electrophoresis of the isolated protein, from the normal residues prepared at pH 5.8 and 7.0. This experiment was repeated using a 5% solution of albumin instead of undiluted serum. Electrophoresis and a precipitin test carried out on the isolated protein showed that no albumin was bound at either pH 5.8 or 7.0.

Measurement of the anomalous viscosity of ultrafilter residues prepared under varying conditions

Because of the effect of varying pH and ionic strength on the protein content it was considered of interest to study the effect of these different conditions of preparation on the physicochemical properties of the complex. For this study the changes in anomalous viscosity investigated by Ogston & Stanier were used. Ogston & Stanier (1951) showed that the difference, Δ , between the respective values of $\log(\eta_{rel} - 1)/\phi$ (where η_{rel} is the relative viscosity and ϕ the hydrodynamic volume fraction occupied by the solute) extrapolated to zero and infinite velocity gradient at a given value of ϕ provides a sensitive test for hyaluronic acid degradation. The less the difference between the two values, the more degraded is the sample. In Table 8 are given some values of Δ for synovial fluid dialysed against the pH 5.8, 7.0, 8.0 and 10.0 buffers, for ultrafilter residue prepared in the presence of these buffers and then dissolved in phosphate buffer, pH 7.0, $I=0.1$, and for ultrafilter residue prepared at pH 7.0, $I=0.1$, and then dissolved in undiluted ox serum.

Table 8. *Values of $\log(\eta_{rel} - 1)/\phi$ for synovial fluid and ultrafilter residue prepared and dialysed under different conditions*

The ultrafilter residue was prepared in the presence of buffers, the pH of which is given in the second column. The residues were then dissolved in phosphate pH 7.0, $I=0.1$ and dialysed against buffers, the pH of which is given in the second column. The synovial fluid specimens were directly dialysed against buffers. In the last experiment the residue was prepared in phosphate pH 7.0, $I=0.1$ and dissolved in serum. I for all buffers was 0.1. Volume fraction $\phi = 4 \times 10^{-4}$ ml./ml.

Material	pH of buffer	Couette velocity gradient		Δ
		0	∞	
Synovial fluid	5.8	4.68	4.03	0.65
	7.0	4.66	3.99	0.67
	8.0	4.66	4.05	0.61
	10.0	4.20	3.97	0.23
Ultrafilter residue dissolved in buffer	5.8	4.58	3.96	0.62
	7.0	4.75	4.06	0.69
	8.0	4.53	3.95	0.58
	10.0	4.25	3.97	0.28
Ultrafilter residue dissolved in serum	—	4.63	3.96	0.67

It can be seen that Δ remains remarkably constant for all the products, except that prepared at pH 10 which shows signs of degradation, both as dialysed synovial fluid and as ultrafilter residue. In order to see whether the degradation was irreversible, some of the pH 10 ultrafilter residue was dissolved in its own ultrafiltrate which had been neutralized by dialysis against phosphate buffer, pH 7.0, $I=0.1$. The solution was filtered and the ultrafilter residue washed in the usual manner with the buffer of pH 7.0, $I=0.1$. The residue contained 18% protein (amount in original pH 10 preparation, 13%) and gave a value of Δ (0.26) only slightly higher than that given by the original pH 10 preparation. The protein was isolated and examined by electrophoresis in phosphate pH 7.7, $I=0.2$. This showed that most of the increase in protein concentration was due to the binding of α -globulin, with some reduction in the γ -globulin content—a composition differing markedly from that found for ultrafilter residue prepared from synovial fluid which had been dialysed against phosphate pH 7.0, $I=0.1$. It appears, therefore, that filtration at pH 10 impairs the ability of hyaluronic acid to combine reversibly with protein.

DISCUSSION

The immunological and electrophoretic results indicate that the protein content of the hyaluronic acid complex is wholly derived from the serum and is composed largely of α -globulin, with smaller amounts of β - and γ -globulins (Table 4, Fig. 2b). This composition varies with the pH and ionic strength of ultrafiltration. The results (Table 7) suggest that the hyaluronic acid complex of Ogston & Stanier (1952), prepared by washing the ultrafilter residue with unbuffered salt solutions and distilled water, may not be the same with respect to the type of protein as the complex present in the native synovial fluid—represented by the ultrafilter residue filtered and washed under 'physiological conditions' of pH and salt concentration.

The sedimentation constants of protein samples isolated from complexes prepared by the method of Ogston & Stanier (1952), at pH 5.8, 7.0, 8.0, 10.0 and 'physiological conditions' all fall within the range previously reported for serum globulins (see Addendum).

The absence from the complex of proteins unrelated to those of serum is shown by the complete precipitation of the isolated protein by the antibodies to the bovine serum fractions. This complements the finding of Humphrey & Stanier (to be published) that ox serum absorbed all of the antibodies from a rabbit antiserum to the hyaluronic acid complex. The quantitative immunological tests carried out on synovial fluid from which the

hyaluronic acid had been removed by degradation and absorption showed that all of the proteins were derived from the serum (Table 6). This was confirmed by electrophoresis (Fig. 2*d* and Table 7) and agrees with the suggestion by Hesselvik (1940) that, apart from hyaluronic acid, synovial fluid is essentially a plasma exudate. It appears that the hyaluronic acid, secreted by the cells of the synovial epithelium, forms the complex with some of the serum proteins which diffuses through the walls of the joint capillaries.

The nature of the combination between the hyaluronic acid and the protein is obscure. The increase in the amount of protein in the complex as the pH of ultrafiltration is decreased suggests that the binding forces might be ionic, tending to become stronger with decreasing pH, when the proteins tend to acquire positive charges at a greater rate than the strongly charged hyaluronic acid would lose its negative charges. However, at pH 5.8 α -globulin predominates in the complex, whereas the β - and γ -globulins with much stronger positive charges are only bound in significant amounts as the pH is raised to 7 or 8, when these charges are considerably reduced. This suggests that the hyaluronic acid will combine with proteins whose isoelectric points are in the region of the pH of ultrafiltration. Since many of the serum proteins are least soluble in the neighbourhood of their isoelectric points it is possible that they might be adsorbed to the micelles of the randomly coiled hyaluronic acid (Ogston & Stanier, 1951) by a process akin to the 'salting-out adsorption' of proteins on solids observed by Tiselius (1948). This effect might explain the increase in the amount of protein in the complex, when ultrafiltration is carried out at high ionic strength. Under these conditions combination occurs even with albumin, although at ionic strength 0.1 this does not occur even when albumin is added to a concentration of 5% prior to ultrafiltration. It must be noted that no mucin formation was observed with the albumin at high ionic strengths, suggesting that mucin clot formation below pH 5.6 is dissimilar to the complex formation at higher pH.

Using the anomalous viscosity criterion of Ogston & Stanier (1951) it was found that the physicochemical structure of the complex remains constant under all of the conditions used for preparation, except pH 10 (Table 8), where the protein concentration is considerably reduced (Table 7). The properties of the complex prepared at this pH cannot be restored by re-filtering at pH 7 in the presence of synovial fluid proteins, indicating that the loss of protein and accompanying change in physicochemical structure are irreversible. Some evidence of the reversibility of the combination with protein of the material prepared by the

method of Ogston & Stanier (1952) is given by the experiments involving the re-filtration of this in the presence of whole serum at pH 5.8 and 7.0, where the complex assumes the composition of that prepared from synovial fluid dialysed and filtered at these pH values.

It is of interest to note that Blumberg & Oster (1954) have claimed that the protein is not essential to the structure of the hyaluronic acid, since they found by light-scattering studies that the protein-free hyaluronic acid prepared by the method of Weissman, Rapport, Linker & Meyer (1953) has essentially the same molecular structure as the hyaluronic acid complex of Ogston & Stanier (1952).

However, since they used a value of 1.8×10^{-3} for the specific refractive increment of protein-free hyaluronic acid, their conclusions may be doubtful. This value is certainly too high; Ogston & Stanier (1950) obtained a mean value of 1.44×10^{-3} for (protein-free) hyaluronic acid from ox synovial fluid and Ogston (private communication) has observed 1.40×10^{-3} for protein-free bacterial hyaluronic acid.

SUMMARY

1. The protein has been isolated from the hyaluronic acid-protein complex of Ogston & Stanier. Evidence was obtained showing by physical and immunological tests that the protein is entirely derived from the serum.

2. The protein composition of the hyaluronic acid complex varies with pH and ionic strength. At pH 5.8 α -globulins predominate, at pH 7-8 α - and β -globulins participate, and at pH 10 the protein content is reduced considerably, the protein combining being mostly γ -globulin. At neutrality increasing ionic strength led to a marked increase in the amount of protein in the complex.

3. It is suggested that the protein associates with the hyaluronic acid through a process akin to salting-out adsorption.

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ADDENDUM

Examination in the Ultracentrifuge

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A number of samples of protein, isolated from the hyaluronic acid complex of ox synovial fluid as described in the preceding paper, were examined in the Svedberg oil-turbine ultracentrifuge, using the methods of Cecil & Ogston (1948) and the optical system of Baldwin (1953). This technique shows a much lower resolving power for the components of plasma than does electrophoresis: the aim was therefore merely to provide a check on the characterization of the proteins by the other techniques. Measurements were also made on a sample of α -globulin prepared (as described in the preceding paper) from ox serum. In two cases measurements

were made at different salt concentrations (Pedersen, 1945) in order to determine the apparent partial specific volumes.

RESULTS

The results are summarized in Table 1; Fig. 1 shows some typical sedimentation diagrams; Fig. 2 shows the corrected sedimentation coefficients plotted against concentration. In addition to the main component, there was always a small amount of heterogeneous material with a sedimentation coefficient $15\text{--}20 \times 10^{-13}$ sec.

Table 1. *Sedimentation coefficients of protein fractions prepared from the hyaluronic acid complex of ox synovial fluid*

For conditions of filtration, see preceding paper. All samples were separate preparations, except those bracketed together. The column 'buffer' gives the solution in which each sedimentation measurement was made. In the correction of the sedimentation coefficient to $S_{20,w}$ the values of $\bar{V}$ used were: complex protein pH 8 and below, 0.78; pH 10, 0.75; serum α -globulin 0.86 (see text).

Conditions of filtration	Buffer	Concentration (g./100 ml.)	$10^{13} \times S_{20}$ sec.	$10^{13} \times S_{20,w}$ sec.
NaCl-H ₂ O	0.2M-NaCl	1.60	5.57	5.87
{ NaCl-H ₂ O	0.2M-NaCl	1.14	6.07	6.40
{ NaCl-H ₂ O	0.385M-NaCl	1.14	5.79	6.36
{ NaCl-H ₂ O	1.03M-NaCl	1.14	4.98	6.39
'Physiological'	0.2M-NaCl	0.72	6.16	6.49
pH 5.8	0.2M-NaCl	1.56	5.71	6.00
pH 8	0.2M-NaCl	1.32	5.75	6.06
pH 10	0.2M-NaCl	1.19	5.88	6.19
pH 10	0.2M-NaCl	0.41	6.40	6.73
{ α -Globulin	0.2M-NaCl	0.47	6.47	6.89
{ α -Globulin	1.04M-NaCl	0.47	4.76	6.89

DISCUSSION

The present results on the sedimentation of ox serum α -globulin in 0.2M-NaCl and those of Johnston & Ogston (1946) on bovine γ -globulin are

Fig. 1. Sedimentation boundary diagrams of proteins isolated from the hyaluronic acid complex of ox synovial fluid; for conditions of isolation, see the preceding paper.

Measurements were made in 0.2M-NaCl at 900 rev./sec. and at approx. 25°.

Method of isolation	Concn. (g./100 ml.)	Time after full rotor speed (min.)
A NaCl-H ₂ O	1.14	22
B pH 5.8	1.56	57
C pH 8	1.32	47
D pH 10	1.19	34

closely alike. This makes it unlikely that the ultracentrifuge would reveal the change of the hyaluronic acid complex protein, with pH of filtration, from α - to γ -globulin, which is shown by other techniques. Fig. 2 shows that all the samples appear as a single family in sedimentation, which is consistent with the protein being α - or γ -globulin.

However, changing the concentration of buffer salt has different effects on the sedimentation of the complex protein (α -globulin) and on that of serum α -globulin (Table 1); a given change of salt concentration causes a larger change in the sedimentation of the serum α -globulin. If one may use this change to obtain the effective partial specific volume of the sedimenting material (cf. Ogston, 1954) the results are: complex protein α -globulin $\bar{V}' = 0.78$; serum α -globulin $\bar{V}' = 0.86$. The latter is quite close to the directly determined value of

$\bar{V}' = 0.84$ for human serum α_1 -globulin given by Oncley, Scatchard & Brown (1947), though they found a sedimentation coefficient of only 5×10^{-13} for this material. If the high value of $\bar{V}'$ of serum α -globulin were due to the association with the protein of lipid material of density about 1, then the hyaluronic acid complex protein could consist of the same protein with much less lipid.

Fig. 2. Sedimentation coefficients of proteins isolated from the hyaluronic acid complex of ox synovial fluid; for conditions of isolation, see preceding paper. The filled circle gives a value obtained for α -globulin isolated from ox serum.

SUMMARY

1. Sedimentation measurements in the ultracentrifuge have been made on proteins isolated under various conditions from the hyaluronic acid complex of ox synovial fluid.

2. The results are consistent with the protein resembling a serum globulin; however, the change of sedimentation rate with concentration of buffer salt differs from that shown by serum α -globulin.

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