On the State of Hyaluronic Acid in Synovial Fluid

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Synovial fluid contains, besides proteins and salts, a polysaccharide of the type termed 'hyaluronic acid' (Meyer, Smyth & Dawson, 1939). This contains acetylglucosamine and glucuronic acid, and it has been claimed that these substances, in equimolar proportions, compose the whole of hyaluronic acid. In the past the isolation of hyaluronic acid from synovial fluid and from other sources has involved the use of relatively vigorous methods; our aim has been, by use of the mildest possible procedure, to find in what state hyaluronic acid occurs in native synovial fluid, that is, whether it is a separate component or is combined with other material.

It was found possible to separate the whole of the hyaluronic acid from synovial fluid by ultrafiltration, in the form of a complex which sedimented as a single component in the ultracentrifuge, giving a very sharp sedimentation boundary. It contained a constant amount of protein and its glucosamine content was possibly rather less than would correspond with an equimolar ratio of acetylglucosamine and glucuronic acid.

The high specific viscosity of solutions containing hyaluronic acid and the sharpness and rate of sedimentation of the boundary in the ultracentrifuge are properties likely to be affected by any degradative change which occurs during isolation. Synovial fluid, mucin, the material prepared by ultrafiltration and the material prepared by precipitation with ethanol have therefore been compared with respect to these properties. Samples of the ultrafilter residue, of mucin and of ethanol-precipitated material were obtained which contained undegraded hyaluronic acid, as judged by these criteria, though the formation of mucin tended to cause degradation. In every sample which was undegraded, the composition of the hyaluronic acid complex was the same, and any further reduction of the proportion of protein was always accompanied by signs of degradation. It is concluded, therefore, that hyaluronic acid occurs in synovial fluid as a definite complex with protein. The physico-chemical methods used have enabled certain physical characteristics of this complex to be measured.

METHODS

Collection and storage of synovial fluid. The fluid was withdrawn into a syringe through a lumbar puncture needle from the knee and ankle joints of the hindlimbs of cattle,

15–30 min. after slaughtering. The fluid from the ankle was withdrawn, after the skin had been removed, by introducing the needle into the sac-like dilatation of the joint cavity immediately anterior to the Achilles tendon. The knee-joint fluid was drawn from the supra-patellar pouch after the joint had been opened. Average volumes obtained were 20 ml. from the ankle joint and 5 ml. from the knee joint. Any fluid which was contaminated with a large amount of blood was discarded. The fluid was centrifuged to remove any blood or connective tissue, and then filtered through muslin. Preliminary experiments showed that fluid could be kept for several months at 1°, with short periods of exposure to room temperature, without any change of viscosity. At room temperature the viscosity fell markedly within 24 hr., owing to infection with bacteria; the addition of toluene considerably improved its stability at room temperature. All extended experiments were therefore done in the cold room and no special precautions were taken to ensure sterility.

Glucosamine. A modification of the method of Elson & Morgan (1933) was used, using the quantities of reagents and the hydrolysis procedure described by Palmer, Smyth & Meyer (1937). The procedure after hydrolysis differed somewhat from that of Palmer *et al.* (1937), because of the small quantities of glucosamine (0.1–0.5 mg.) which it was

Fig. 1. Variation of glucosamine colour with acetylation time. Extinction coefficients E measured at 535 $m\mu$.

required to estimate. In order to avoid diluting the hydrolysate more than necessary, it was made up in a standard flask to 10 ml., of which 4 ml. were taken for the preliminary titration with NaOH. A further 4 ml. were then neutralized and made up to 10 ml., of which 1 ml. was taken for the glucosamine estimation. The sensitivity of the method was further increased slightly by increasing the acetylation time from 15 to 30 min.; a preliminary experiment had shown that the maximum amount of glucosamine was acetylated after 30–45 min. and that longer times led to loss (Fig. 1). It was found that a variable amount of acetylacetone was lost by evaporation during the acetylation, and the technique of Palmer *et al.* (1937), of exposing the necks of the

flasks to a strong current of air, did not prevent this. Since this loss would be more serious with a longer acetylation time, a water condenser was made through which the necks

method of Palmer *et al.* (1937) was followed, the standard glucosamine solutions being introduced after the hydrolysis stage. Later, these standards were compared with standards

Fig. 2. Diagram of end-flow diffusion cell (the water jacket is not shown). The cell is of Perspex, except for the glass optical faces A, the glass outflow tube B and the pipette (not shown) which fits into the column D. The solution and buffer flow from the bottom of D through the channel C into the tunnel E and out through B, the boundary being established in D, C and E at the level of B.

of the flasks protruded, while their bulbs were immersed in a boiling water bath. This reduced evaporation to a small and constant amount, and markedly improved the reproducibility of the results. In most of the determinations, the

subjected to the hydrolysis procedure; the recovery after hydrolysis was rather variable, being from 72 to 96%, and averaging 85%. After this was discovered, hydrolysed standards were always used. The earlier values were corrected

on the assumption that the recovery of glucosamine from the unknown solutions also averaged 85%. Extinctions were measured at 535 m μ . in a Beckman spectrophotometer.

Total nitrogen. Total N was estimated by micro-Kjeldahl, using CuSO₄ and K₂SO₄ as catalyst, and 5–6 hr. digestion. Use of selenium catalyst and of 8 hr. digestion gave the same results.

Dry weight. Dry weights were determined by heating the material in a weighed crucible in an electric oven at 105° to constant weight.

Refractive increment. A simple double prism refractometer (designed by Dr J. St L. Philpot) was used, with light of 546 m μ ; its accuracy was 10⁻⁶ of refractive index, corresponding to a concentration of about 0.0005 g./100 ml. The specific refractive increments of materials were determined and the refractive increments of dialysed solutions were subsequently used to estimate concentrations.

Ultraviolet absorption. Ultraviolet absorption was measured between 240 and 400 m μ . using a Beckman spectrophotometer.

Ultracentrifuge. A Svedberg oil-turbine ultracentrifuge with a Philpot (1938) optical system was used. The procedure of running and the methods of calculating the sedimentation constants and of estimating the quantities of components were as described by Cecil & Ogston (1948). All materials were dialysed against a standard buffer: NaCl, 0.2M; Na₂HPO₄, 0.0077M; KH₂PO₄, 0.0023M; pH 7.3.

Diffusion. Diffusion was measured with a Gouy diffusometer (Coulson, Cox, Ogston & Philpot, 1948), with a modified cell (Fig. 2). In this cell the flow of solution and solvent takes place along, instead of across, the optic axis, from a concentric reservoir at one end to a circular outflow tube at the other. The level of the boundary adjusts itself so that the cross-section of the outflow tube is available to solution and solvent in proportion to their viscosities; adjustment of the boundary stop of the optical system must be made when it has been seen at what level the boundary is formed by a given solution. This cell gave correct values for the diffusion constants of glycine and of lactoglobulin. In spite of the adaptation of the cell for viscous solutions, it was found that, in order to form a satisfactory boundary, the concentration of hyaluronic acid must not exceed 5 mg./ml.

and a considerable amount of free protein must also be present. For example, it was not possible to obtain a boundary with hyaluronic acid prepared by ethanol precipitation. Analysis of the records was made by the method of Ogston (1949).

Viscometry. Routine measurements of viscosity were made with an Ostwald viscometer with flow time for water of about 12 sec.; 2 ml. of fluid was required. All measurements were made at 25°. In order to study the anomalous viscosity of solutions containing hyaluronic acid, a Couette viscometer was designed and made in this laboratory, modified from that described by Lawrence, Needham & Shen (1944). The fluid (10 ml.) was contained in the annular gap (0.148 cm.) between concentric cylinders. The outer cylinder was rotated at speeds up to 150 rev./min., giving rates of shear numerically 0.5 times the speed in rev./min. The inner cylinder was suspended by a torsion wire and its deflexion was measured.

Estimation of the concentration of various materials. It was convenient to relate the properties of synovial fluid and its derivatives to their concentrations of hyaluronic acid glucosamine per ml. The glucosamine contents of ultrafilter residues and of ethanol-precipitated hyaluronic acid were measured directly. Since synovial fluid contains considerable amounts of non-hyaluronic acid glucosamine, it was necessary to estimate glucosamine in mucin derived from it. The mucin glucosamine represents all the hyaluronic acid glucosamine of synovial fluid, and was found to be constant; the weight of mucin, determined directly or refractometrically, could therefore be used to estimate hyaluronic acid glucosamine in synovial fluid.

EXPERIMENTS AND RESULTS

Chemical composition of synovial fluid. Preparation and chemical composition of its derivatives

(1) *Synovial fluid*

The viscosity of each sample of synovial fluid was measured in the Ostwald viscometer immediately after collection and the maintenance of this viscosity

Table 1. *Analysis of synovial fluid*

(Numbers of samples analysed are given in brackets.)

Source	Total N		Mucin N		Mucin		Mucin N (% of mucin)	
	Range	Mean	Range	Mean	Range	Mean	Range	Mean
Ankle	128–360	182 (10)	16–39	24 (6)	142–455	244 (11)	9.9–12.3	11.1 (6)
Knee	249–373	311 (11)	34–47	40 (8)	320–510	408 (14)	9.8–11.6	10.8 (8)

Source	Mucin		Total glucosamine		Total glucosamine (% of mucin)	
	Range	Mean	Range	Mean	Range	Mean
Ankle	180–205	193 (2)	36–42	39 (2)	20.2–20.6	20.4 (2)
Knee	405–446	426 (2)	86–87	86.5 (2)	19.3–21.5	20.4 (2)

(c) Mucin glucosamine in relation to mucin			
No. of precipitations of mucin	...	1	1
Mucin glucosamine (% of mucin)	...	7.3	8.4
Mean specific refractive increment of mucin = 0.00164			

was used as a criterion of its stability on storage, any sample which showed a fall of viscosity being rejected. The spontaneous precipitation of mucin during storage, described by Davies (1944), was never observed. The fluid was yellow and highly viscous, that from the knee joint being smaller in volume, of darker colour and more viscous than that from the ankle joint: the knee-joint fluid contained a higher percentage of hyaluronic acid and of protein. Some analyses of fluid are given in Table 1. The mucin clot contains all the hyaluronic acid of the fluid. Since, however, mucin contains about 8% of glucosamine, while the table shows that the total glucosamine of the fluid may be 20% (w/w) of the mucin, only about half the total glucosamine of synovial fluid is accounted for by hyaluronic acid. This was confirmed by precipitating and washing mucin from a sample of fluid; the mucin was found to contain 49% of the total glucosamine and precipitation of the residual solution with trichloroacetic acid removed a further 46% of the total glucosamine, leaving only 5% in solution. It appears, therefore, that most of the non-hyaluronic acid glucosamine is associated with protein, which would contain about 1.5% of glucosamine.

Synovial fluid, dialysed against the standard buffer (Fig. 7a), gave a sedimentation diagram identical with that of undialysed fluid. Three boundaries were present: a very sharp boundary due to the hyaluronic acid complex, whose rate of sedimentation varied rapidly with concentration, and two boundaries whose rates of sedimentation (S_{20} about 4 and 6×10^{-13}) were typical of the main albumin and globulin components of normal serum. In one case the relative proportions of these latter components were measured. The combined concentration was found to be 1.13 g./100 ml. and the albumin/globulin (A/G) ratio 4.4. Bauer, Ropes & Wayne (1940) found by salting-out the A/G ratio of synovial fluid to be 3.9.

(2) Mucin

Preparation. A compact clot of mucin is formed when synovial fluid is acidified with acetic acid to pH 4.5. On a small scale, 1 ml. of synovial fluid was washed from an

Ostwald pipette into 1 ml. of distilled water and about 0.06 ml. of 10% (w/v) acetic acid was added. The entire clot was collected on a glass rod and washed six times with 1% (w/v) acetic acid, and then twice with distilled water. It was carefully scraped off into a weighed crucible and dried at 105° to constant weight. The dried residue could then be used for determining total N. For preparation of mucin on a larger scale, the method of Ropes, Robertson, Rossmeisl, Peabody & Bauer (1947) was followed. After a suitable number of washings and reprecipitations, the mucin was dissolved in NaHCO_3 or Na_2HPO_4 and dialysed against the standard buffer.

Composition. Mucin collected and analysed without washing contained entrained protein up to 60–90% of the final weight of washed mucin; successive washings on the small scale removed this protein, and after six washings no further diminution in the weight of the clot occurred. Some analyses of mucin are given in Table 1. It can be seen that the glucosamine content of washed mucin tended to be higher if the mucin had been several times reprecipitated.

The sedimentation diagram (Fig. 7b) resembled that of synovial fluid except that relatively less protein was present and this consisted of a single component which sedimented at the rate of serum albumin. The amount of this component was estimated from the ultracentrifuge diagrams and was compared with the total refractive increment of the solution. The refractive increment due to hyaluronic acid was calculated from the glucosamine content and the specific refractive increment found from experiments on the ultrafilter residue (see below).

The combined refractive increments of the free albumin and of the hyaluronic acid were found to fall short of the total refractive increment (Table 2) by an amount considerably greater than could be accounted for by experimental errors. It was concluded, in view of later experiments, that this difference can be accounted for by protein which forms a complex with the hyaluronic acid. The total nitrogen also was in excess of that accounted for by free albumin plus glucosamine, and this excess corresponded with the amount of combined protein deduced from refractive measurements. The proportions of hyal-

Table 2. *Components of mucin*

Mucin sample	12K	18A	21
(a) Glucosamine (mg./ml.)	0.22	0.47	0.35
(b) Refractive increment of hyaluronic acid (=glucosamine $\times 100/32 \times 0.000144$)	0.000100	0.000213	0.000159
(c) Refractive increment of free protein	0.000279	0.000543	0.000426
(d) Refractive increment of hyaluronic acid + free protein	0.000379	0.000756	0.000585
(e) Total refractive increment	0.000420	0.000836	0.000670
(f) Refractive increment of protein in complex (=e-d)	0.000041	0.000080	0.000085
(g) Fraction of free protein (=c/(c+f))	0.87	0.87	0.83
(h) Proportion of complex in total (=b/(b+f))	0.34	0.35	0.36
(i) Proportion of hyaluronic acid in complex (=b/(b+f))	0.71	0.73	0.65
(j) Proportion of protein in complex (=f/(b+f))	0.29	0.27	0.35

uronic acid and protein which form this complex have been calculated and are given in terms of refractive increment in the last two rows of Table 2.

Selection of protein in the formation of mucin. Separated hyaluronic acid (Meyer & Palmer, 1936) will form mucin clots with a variety of proteins. It was confirmed that the ultrafilter residue will do so with serum albumin, serum globulin, haemoglobin and lactoglobulin. On the other hand, the mucin prepared from synovial fluid contains albumin exclusively. An experiment was performed in which lactoglobulin was added to a sample of synovial fluid in sufficient amount to form a mucin clot with the whole of the hyaluronic acid present. Mucin formed from this mixture was shown in the ultracentrifuge to contain only serum albumin.

(3) The ultrafilter residue

Preparation. Gradocol graded collodion membranes, obtained from St Mary's Hospital, were used for a few preparations. All the hyaluronic acid was retained by membranes of average pore diameter (A.P.D.) 0.19μ , and most of it by membranes of A.P.D. 0.52μ . The free protein passed through readily. In later preparations Jena '5 on 3' sintered glass filters, of A.P.D. about 0.6μ , were used, and almost completely retained the hyaluronic acid. These proved to be much faster than the collodion membranes and showed less tendency to become clogged. Their only disadvantage was that, when the residue on the filter was resuspended, a certain amount of glass powder was always scraped off, even by a rubber-tipped rod.

About 20 ml. of synovial fluid or redissolved mucin was placed in each glass filter; the filter was covered with a lid and connected to an evacuated reservoir. Filtration was complete after about 24 hr. The residue was then scraped off the filter and resuspended *in situ* in distilled water; when washing with saline, an equal volume of 0.4M -NaCl was then added. Usually six washings with 0.2M -NaCl were given and then twenty or more with distilled water. The whole process was carried out in the cold room. The removal of protein was followed by examining small samples of resuspended

material. Their concentrations were determined refractometrically, and the amount of protein was estimated by ultraviolet absorption in 0.1N -NaOH; the difference of extinctions at 285 and $330\text{ m}\mu$. (the latter to allow for haze: Holiday & Ogston, 1938) was taken as a measure of the protein. Correlation between total N and ultraviolet absorption of the final products was satisfactory (Table 3).

In no case was it found possible to remove all the protein by this method. In two cases (16K and 23) washing was continued until no further fall in the ultraviolet absorption could be produced; in other cases (19K(1) and 22K) washing was stopped short of this point.

The material finally obtained was quite colourless and most of it went into solution. The undissolved solid, including suspended glass, could be removed by centrifuging at 4500 g. The removal of suspended solid was found not to have changed the composition significantly with respect to total N and glucosamine; subsequently all analyses were made on the cleared solution.

Composition. Exhaustively washed samples showed a single boundary in the ultracentrifuge (Fig. 7d). Nitrogen and glucosamine values for a number of ultrafilter residues are given in Table 3, together with a summary of their physico-chemical properties. 19K(1), 22K and 23 were samples in which both the viscosity and sedimentation boundary were normal, and can therefore be regarded as containing undegraded hyaluronic acid. Of these, 22K contained some free protein which sedimented separately in the ultracentrifuge, and this is confirmed by the high values for nitrogen and ultraviolet absorption.

By partition chromatography (see below) it appeared that the non-hyaluronic acid component of the ultrafilter residue was a protein of normal composition. This was therefore assumed to have a normal nitrogen content of 15.5%, and the non-glucosamine nitrogen values were multiplied by 6.45 to give values for protein. The amount of hyaluronic acid was taken to be the total weight less protein,

Table 3. *Composition of ultrafilter residues and of ethanol-precipitated hyaluronic acid*

Sample no.	Ultrafilter residues						Precipitated hyaluronic acid	
	16K	19K(c)	19K(1)	19K(2)	22K	23	23	23
Filter	Glass	Collodion	Glass	Glass	Glass	Glass	—	—
No. of washings	38	27	19	39	22	25	—	—
No. of precipitations	—	—	—	—	—	—	3	5
Total N (%)	5.4	3.7	6.9	5.4	10.0	7.0	7.0	4.8
$E_{285}-E_{330}$ at 10 mg./ml.	0.265	0.32	0.54	0.39	0.79	0.44	0.54	0.20
Glucosamine (%)	26	25	24	20	14	18	32	32.5
Non-glucosamine N (%)	3.4	1.8	4.9	3.8	8.9	5.6	4.5	2.1
Protein (% non-glucosamine N $\times 6.45$)	22	11	32	24	57	36	29	14
Non-protein (%)	78	89	68	76	43	64	71	86
Glucosamine (% of non-protein fraction)	33	29	36	27	33	27	45	38
Total specific refractive increment $\times 10^5$	151	151	152	156	167	156	155	146
Specific refractive increment of non-protein fraction	143	147	139	148	149	143	145	140
Sedimentation constant	—	Normal	Normal	Normal	Normal	Normal	Normal	—
Non-protein sedimentation boundary	—	Diffuse	Sharp	Diffuse	Sharp	Sharp	Sharp	—
Viscosity	Normal	Low	Normal	Low	Normal	Normal	Normal	Low

and the glucosamine was finally expressed as a percentage of hyaluronic acid.

It can be seen that the glucosamine constituted 27–36% of the hyaluronic acid. In the two normal samples known to contain no free protein, 19K(1) and 23, the combined protein constituted 32 and 36% of the ultrafilter residue. Degradation in samples 19K(c) and 19K(2), shown by the low viscosity and diffuse sedimentation boundary, was associated with an abnormally low percentage of non-hyaluronic acid nitrogen. 16K, however, was physico-chemically normal, although its non-hyaluronic acid nitrogen was low. In 22K the free protein was found to have the sedimentation constant of serum globulin. This was to be expected, since albumin passes through a fine filter more rapidly (Fig. 7c). Measurement of this free protein boundary in the ultracentrifuge diagram and calculation of its contribution to the total nitrogen showed that the nitrogen and glucosamine contents of the hyaluronic acid complex were 6.8 and 22% respectively.

A paper chromatogram of hydrolysate of 16K was made for us by Mr D. S. Parsons. This showed the presence of the following amino-acids: aspartic and glutamic acids, glycine, alanine, tyrosine, tryptophan and phenyl alanine, and possibly traces of serine and proline. The presence of tyrosine and tryptophan was also indicated by the ultraviolet absorption of the ultrafilter residue.

Mucin formation by the ultrafilter residue. Ultrafilter residues containing 7% of nitrogen gave only slight cloudiness when the solution was acidified. After addition to the ultrafilter residue of mixed protein from synovial fluid, or of albumin, globulin, haemoglobin or lactoglobulin, good clots were formed on acidification. The dry weight and nitrogen content of these clots were the same as those of clots formed from synovial fluid containing a corresponding amount of hyaluronic acid. This showed that the hyaluronic acid had not lost its ability to form a normal mucin clot.

(4) *Ethanol-precipitated hyaluronic acid*

Preparation. The method of Ropes *et al.* (1947) was followed exactly. Initially the hyaluronic acid was precipitated three times; a part of the product was then precipitated twice more. The product was quite colourless; it dissolved easily to form a somewhat cloudy, viscous solution which became clearer on adding alkali.

Composition. The analytical results for the two samples are given in Table 3. The thrice-precipitated material closely resembled the undegraded ultrafilter residues in its composition, except that its glucosamine content was higher, as was that of the five-times precipitated sample; it gave a single boundary in the ultracentrifuge (Fig. 7f). Both samples contained protein, as is shown by the absorption at

285 m μ . and by the high values of total nitrogen; the five-times precipitated material contained less protein than the other and had a correspondingly low specific refractive increment.

Physical properties of synovial fluid and derived preparations

Viscosity, measured in the Ostwald viscometer

It was realized that the measured viscosity of material of this sort varies rapidly with the rate of shear and that the rate of shear in a capillary viscometer is non-uniform and varies with the flow time.

Fig. 3. Variation of relative viscosity (Ostwald) of synovial fluid with concentration of sodium chloride.

Nevertheless, reproducible results are obtainable when the conditions (viscometer, temperature, concentration of solute and composition of solvent) are constant, and are adequate for the comparison of different materials. The variation of the viscosity of a given solution with the concentration of salt (Fig. 3) agreed with the results of Ropes *et al.* (1947). For all subsequent measurements the standard buffer was used.

Synovial fluid. The logarithm of the relative viscosity varied linearly with concentration, values for different samples of fluid falling on the same line. This is shown in Fig. 4, in which relative viscosity values for mucin, ultrafilter residues and the ethanol-precipitated hyaluronic acid are also given for comparison.

Mucin. The viscosities of solutions of mucin tended to be lower than those of synovial fluid at corresponding concentrations of hyaluronic acid glucosamine, though in one case a value was obtained which agreed well. Repeated precipitation appeared to lower the viscosity further. The viscosity seemed to be a more sensitive index of degradation than the rate of sedimentation.

Ultrafilter residue. The viscosity of two samples of ultrafilter residue fell close to the values obtained for synovial fluid at similar concentrations. Degradation in samples 19K(c) and 19K(2) was accompanied by a marked loss in viscosity.

Fig. 4. Variation of the log of the relative viscosity (Ostwald) with concentration of hyaluronic acid glucosamine: ●, synovial fluid; ○, mucins (number of precipitations are shown in brackets); ×, ultrafilter residues (19K(2) and 19K(c) are marked); □, ethanol-precipitated hyaluronic acid (three precipitations). All preparations dialysed against standard buffer.

Fig. 5. Variation of relative viscosity (Couette) with rate of shear for different concentrations of synovial fluid, dialysed against buffer. Curve A, 21 mg. of hyaluronic acid glucosamine/100 ml.; curve B, 14 mg./100 ml., curve C, 7 mg./100 ml.

Ethanol-precipitated hyaluronic acid. The viscosity of the thrice-precipitated material was normal while that of the five-times precipitated material was somewhat low compared with synovial fluid and the ultrafilter residue at similar concentrations.

Viscosity, measured in the Couette viscometer

Synovial fluid. The relation of viscosity to rate of shear was studied in the Couette viscometer at different concentrations of synovial fluid. The curves (Fig. 5) of $\eta_{rel.}$ against rate of shear (r) were found to be approximately hyperbolic and to be expressed by

$$(r + a)(\eta_{rel.} - \eta_{rel.(\infty)}) = \text{constant},$$

where a is a constant and $\eta_{rel.(\infty)}$ is the relative viscosity at infinite rate of shear. This relationship was used to obtain the values of $\eta_{rel.(\infty)}$, which were found to agree approximately with the values obtained in the Ostwald viscometer; the values of $\eta_{rel.}$

Fig. 6. Variation of the $\log [1/c (\eta_{rel.(0)} - 1)]$ (Couette) of synovial fluid with concentration. Curve A, dialysed against water; curve B, dialysed against buffer.

at zero rate of shear, $\eta_{rel.(0)}$, were obtained from the intercepts at $r=0$ of plots of $1/\eta_{rel.}$ against r . The values thus obtained of $\eta_{rel.(0)}$ were plotted against concentration as $\log [1/c (\eta_{rel.(0)} - 1)]$, and extrapolated to zero concentration (Fig. 6). Although the intrinsic viscosity, determined in this way, includes the contribution by serum protein, this is negligible.

Rate of sedimentation in the ultracentrifuge

Synovial fluid. The rate of sedimentation of the hyaluronic acid boundary, as a function of concentration, was studied in some detail. The results were plotted as $1/S_{20}(\text{corr.})$ against concentration of hyaluronic acid glucosamine, and were found to be fairly reproducible from one sample of fluid to another. In Fig. 8 are shown values for the same sample of fluid as was used in the Couette viscometer, at various concentrations. This plot was approximately linear in the more dilute range, but was curved at higher concentrations. Extrapolation to zero concentration could be made with fair accuracy, the limiting value of $S_{20}(\text{corr.})$ being 13×10^{-13} . Fig. 8 also shows values of the sedimentation constant for different samples of mucin, ultrafilter residue and ethanol-precipitated hyaluronic acid.

At higher concentrations, the hyaluronic acid boundary in synovial fluid was extremely sharp (Fig. 7a) and remained so throughout the sedimentation run. At lower concentrations, however, it became more diffuse with time.

Fig. 7. Ultracentrifuge diagrams: (a) synovial fluid; (b) mucin; (c) ultrafilter residue with free globulin; (d) ultrafilter residue with no free protein; (e) ultrafilter residue, diffuse boundary due to degradation; (f) ethanol-precipitated hyaluronic acid.

Fig. 8. Variation of sedimentation constant with concentration of hyaluronic acid glucosamine; ●, synovial fluid; ○, mucins (number of precipitations are shown in brackets); ×, ultrafilter residues; □, ethanol-precipitated hyaluronic acid (three precipitations).

Mucin. The sedimentation constant of the hyaluronic acid component of once-precipitated mucin appeared to be normal. Repeated precipitation, however, led to high values for the sedimentation constant (Fig. 8). Also, the sharpness of the boundary was impaired. The time taken for the boundary to become noticeably diffuse, as a function

of concentration, could in fact be used as an accessory criterion of the state of the hyaluronic acid. This is shown in Fig. 9, in which results for synovial fluid, ultrafilter residue and ethanol-precipitated hyaluronic acid are also given. It is evident that at a given concentration a much longer time is taken for the boundary to become diffuse in synovial fluid or in once-precipitated mucin than in mucin precipitated several times.

Fig. 9. Relation between concentration of hyaluronic acid glucosamine and time to appearance of diffuse boundary in ultracentrifuge. (The lower line represents materials in which degradation had taken place.) ●, synovial fluid; ○, mucins (number of precipitations are shown in brackets); ×, ultrafilter residues (19K(2) and 19K(c) are marked); □, ethanol-precipitated hyaluronic acid (three precipitations).

Ultrafilter residue. All samples of ultrafilter residue containing 7% of nitrogen or less showed only the typical hyaluronic acid component (Fig. 7d), which sedimented at the normal rate (Fig. 8). The sedimentation constants of those containing low proportions of nitrogen (19K(c) and 19K(2)) showed little departure from normal, but the boundaries of these samples showed a marked decrease in sharpness compared with normal (Figs. 7e, 9). The same abnormality was shown by an ultrafilter residue which had been prepared from a thrice-precipitated mucin.

Ethanol-precipitated hyaluronic acid. Only the thrice-precipitated material was used. The single sedimentation boundary was sharp, and showed only the presence of the hyaluronic acid component (Fig. 7f). Its sedimentation constant was a little higher than normal.

Diffusion

Mucin. Measurements were made on mucin 21 over a range of concentration and are given in Table 4 in terms of total refractive increment. Since mucin contains only two components, the hyaluronic acid complex and albumin, the diffusion record can be analysed for the diffusion constants and proportions of these components by the method of Ogston (1949). Even with the special cell, described above, difficulty

was too low for the diffusion record to be analysed in terms of two components. However, the proportion of protein, in terms of refractive increment, was known to be 40 %, from the sedimentation data; the diffusion constant of this protein was found, in a separate experiment, to be 4.0×10^{-7} , consistent with its being globulin. Accordingly, the diffusion constant of the hyaluronic acid complex could be obtained from the measured diffusion constant. The results are given in Table 4 and Fig. 10.

Table 4. *Diffusion constants of mucin and ultrafilter residue*

Material	Refractive fraction of hyaluronic acid complex	Refractive increment of hyaluronic acid complex $\times 10^6$	$D_{20} \times 10^7$	
			Hyaluronic acid complex	Protein
Mucin 21	0.40*	52	1.17	4.9
	0.43*	36	0.87	5.2
	0.40*	27	1.12	5.9
	0.33*	22	0.97	4.4
Ultrafilter residue 22K	0.605†	11.6	0.92	—
	0.605†	8.7	1.07	—
	0.605†	6.7	1.34	—
	0.605†	3.9	1.67	—

* Estimated from the diffusion record.

† Estimated from the ultracentrifuge diagram.

was experienced in forming satisfactory boundaries, so that the results are not likely to be very accurate. Nevertheless, they are reasonably consistent (Table 4 and Fig. 10); the proportion of components agrees

Fig. 10. Diffusion constant of hyaluronic acid complex for different concentrations of mucin and ultrafilter residue: ●, mucin; ○, ultrafilter residue.

with that estimated from the sedimentation diagram of this material (Table 2 and Fig. 7b) and the diffusion constant of the protein component is of the order of magnitude expected for albumin (D_{20} in this buffer should be about 5.7×10^{-7} ; Kekwick, 1938). The diffusion constant of the hyaluronic acid complex is evidently of the order of 1×10^{-7} and does not vary considerably with concentration.

Ultrafilter residue. Measurements were made on sample 22K. The highest concentration at which this material would form a boundary in the diffusion cell

Ultraviolet absorption

Ultrafilter residue and ethanol-precipitated hyaluronic acid. The absorption curves of several samples of ultrafilter residue and ethanol-precipitated hyaluronic acid were determined between 240 and 400 m μ . (Fig. 11). All show a hump or maximum in the region of 275 m μ . in neutral or acid solution, with a shift in the position of the maximum to about 285 m μ . in 0.1N-NaOH. This type of absorption curve is typical of proteins and is due, mainly, to the tyrosine and tryptophan which they contain (Holiday, 1936; Sizer & Peacock, 1947). An accurate analysis of the absorption curves in terms of tyrosine and tryptophan could not be made because of their low extinction compared with the non-specific scattering due, presumably, to the polysaccharide; the solutions were never quite clear even in alkali. Analysis by the method of Holiday & Ogston (1938) gave values for tyrosine of 1.3–1.7 % and for tryptophan of 0.4–1.0 % of total protein.

DISCUSSION

Methods of separating undegraded hyaluronic acid from synovial fluid

Definite relations were found to hold in synovial fluid between the concentration of hyaluronic acid glucosamine and the relative viscosity, rate of sedimentation and sharpness of the sedimentation boundary. The occurrence of similar relations in its derivatives was taken as evidence that they had been

prepared in an undegraded state. It was found possible to obtain undegraded preparations containing hyaluronic acid by ultrafiltration, by precipitation as mucin and by precipitation with ethanol.

over 7% was always accompanied by the presence of free protein, which sedimented as a separate component. Those preparations which contained 7% of total nitrogen or less showed the presence of only

Fig. 11. Ultraviolet absorption of ultrafilter residues and of ethanol-precipitated hyaluronic acid in 0.1N-NaOH: x, ultrafilter residue 19K(1); □, ultrafilter residue 19K(2); ●, ultrafilter residue 19K(c); ○, ultrafilter residue 16K; ▲ three-times precipitated hyaluronic acid; - - -, five-times precipitated hyaluronic acid.

A single precipitation of mucin often caused degradation and repeated precipitation always did so; repeated precipitation of mucin with ethanol could cause degradation; in some cases, the product obtained by ultrafiltration had become degraded. Evidently precipitation of mucin is not a satisfactory first stage in the separation.

All the undegraded preparations which we made, except ultrafilter residue 16K, contained 7% of total nitrogen or more; any excess of the total nitrogen

a single component in the ultracentrifuge. All preparations which contained less than 7% of total nitrogen, except ultrafilter residue 16K, showed signs of degradation. We therefore conclude that the combination of hyaluronic acid with other nitrogenous material is essential to the maintenance of the physico-chemical state in which it occurs in synovial fluid.

The methods which have been used by previous workers for the separation of hyaluronic acid have

been, for the most part, relatively vigorous. Most of them have involved an initial precipitation of mucin, followed by removal of protein by shaking with chloroform and amyl alcohol (Blix & Snellman, 1945; Meyer *et al.* 1939), by use of zinc acetate (Meyer *et al.* 1939), or by digestion with trypsin (Ropes *et al.* 1947). Final isolation of the hyaluronic acid has usually been by precipitation with ethanol. Ropes *et al.* (1947) eliminated protein and precipitated hyaluronic acid in one operation by precipitating from an alkaline solution of mucin with ethanol at -20° . Hadidian & Pirie (1948) shook with pyridine in the presence of ammonium sulphate.

Many workers have measured the viscosities of hyaluronic acid solutions, but at various concentrations and in various buffers (reviewed by Hadidian & Pirie, 1948). This fact, together with the variety of the sources from which the hyaluronic acid was prepared, makes comparison difficult. However, our best samples of ultrafilter residue have a viscosity equal to that of synovial fluid at corresponding concentration. This corresponds to a relative viscosity of 39 at a concentration of 1 g. of hyaluronic acid per litre (0.4 mg. of hyaluronic acid glucosamine per ml.) in 0.2M-NaCl, and this value is considerably greater than any published value for isolated hyaluronic acid. This suggests that by the methods previously used hyaluronic acid was isolated only with some degree of degradation.

Composition of the hyaluronic acid complex

(a) *Protein.* It has been pointed out above that the undegraded hyaluronic acid complex, separated from synovial fluid by ultrafiltration, contains 7% of total nitrogen. Of the total nitrogen, 5% is unaccounted for by glucosamine present in the complex; the presence of amino-acids in the complex, shown by its ultraviolet absorption and by paper chromatography, suggests that this excess of nitrogen is contributed by protein. The specific refractive increments of protein, hyaluronic acid and complex are also consistent with this view. Analysis of the

sedimentation diagram of mucin and of its diffusion record have also shown that part of the protein present is bound to hyaluronic acid, to form a complex of the same composition as ultrafilter residue (Table 5). Other workers quoted above have shown that hyaluronic acid can be obtained free from protein, but only by use of relatively severe methods. Blix (1940) and Hesselvik (1940) did obtain protein-free hyaluronic acid by electrophoresis, but they applied no physico-chemical tests to their products.

(b) *Glucosamine.* Equimolar proportions of acetylglucosamine and glucuronic acid in hyaluronic acid require a glucosamine percentage of 42.6. However, a wide range of values has been reported, of which most have been lower than this. For example, Meyer *et al.* (1939) give values between 34.5 and 43.7 for synovial hyaluronic acid. Ropes *et al.* (1947) give a value of 40%. Hadidian & Pirie (1948) give values of 34–38% for viscous preparations from umbilical cord and 20–25% for non-viscous preparations. Blix & Snellman (1945) give a mean value of 33%.

Our analysis of hyaluronic acid, prepared by repeated ethanol precipitation, gave values near the theoretical. On the other hand, the proportion of glucosamine found in the non-protein fraction of the ultrafilter residue was low (Table 3). The estimation of glucosamine has not been entirely satisfactory, owing to the possibility of its destruction during hydrolysis; but a mean allowance has been made for this destruction in calculating the proportion of glucosamine and allowance for the maximum destruction found would still not, in most cases, give the theoretical proportion of glucosamine.

If a value of 25% for the proportion of glucosamine in the whole complex is accepted (Table 3), and if it is assumed that glucosamine constitutes 42.6% of hyaluronic acid, two alternative assumptions may be made about the composition of the non-hyaluronic acid part of the complex, which contributes 5 g. of nitrogen/100 g. of complex (Table 6). These are that (a) the protein contains 15.5% of nitrogen, in which case some third component free from nitrogen and

Table 5. *Proportion of hyaluronic acid complex in mucin, ultrafilter residue and ethanol precipitate, and its content of hyaluronic acid*

	Mucin			Ultrafilter residue			Ethanol-precipitate (3 precipitations) 23
	12 <i>K</i>	18 <i>A</i>	21	19 <i>K</i> (1)	22 <i>K</i>	23	
Proportion of complex in total material							
Refractive fraction*	0.34	0.35	0.36	Approx. 1.00	0.605	1.00	1.00
Weight fraction†	0.38	0.39	0.40	Approx. 1.00	0.64	1.00	1.00
Proportion of hyaluronic acid in complex							
Refractive fraction*	0.71	0.73	0.65	0.63	0.64	0.58	0.66
Weight fraction†	0.76	0.775	0.705	0.68	0.68	0.63	0.71

* I.e. fraction contributed to the total refractive increment.

† Calculated from the refractive fraction by use of the appropriate specific refractive increments.

glucosamine is also present, or (b) the protein contains only about 12 % of nitrogen and no third component is present. On the other hand, a higher glucosamine content (29 %) would make it unnecessary to assume the presence of either a third component or protein of an abnormally low nitrogen content. In any case, the presence of protein constituting at least 30 % of the complex is required.

Table 6. Possible compositions of hyaluronic acid complex, containing 25 % glucosamine and 5 % non-glucosamine nitrogen

Components as % of total	Assuming protein contains 15.5 % N	Assuming protein contains 12.2 % N
Hyaluronic acid	59	59
Protein	32	41
Third component	9	0

Physico-chemical properties

All our results suggest that the particles of the hyaluronic acid complex are very large and asymmetric. The filtration experiments indicate a particle of length between 0.2 and 0.5 μ .

Sedimentation and diffusion. The value of S_{20} (corr.) at zero concentration was found to be 13×10^{-13} . The diffusion results obtained with mucin and with ultrafilter residue (Table 4 and Fig. 10) agree moderately well, considering the technical difficulty of these measurements. Extrapolation to zero concentration is made uncertain by the apparent steep rise of the diffusion constant at the lowest concentrations. We have ascribed this rise to experimental error and have taken the limiting value of D_{20} (corr.) to be 1.0×10^{-7} , on the grounds (Singer, 1947) that the diffusion constants of asymmetric particles vary little with concentration in other cases and that this is in accordance with theoretical expectation.

Using these limiting values of S_{20} (corr.) and of D_{20} (corr.) and assuming a value of 0.69 for the partial specific volume, $\bar{V}$ (taking the complex to consist of 0.35 of protein and 0.65 of hyaluronic acid, having partial specific volumes 0.75 and 0.65 respectively) we obtain (Svedberg & Pedersen, 1940):

$$M = \frac{RTS}{D(1 - \bar{V}\rho)} = 1.0 \times 10^6,$$

$$\text{and } f/f_0 = 10^{-8} \left(\frac{1 - \bar{V}\rho}{D^2 S \bar{V}} \right)^{\frac{1}{2}} = 3.25.$$

From the relationship of Perrin (1936), this value of f/f_0 would be satisfied if the particles were elongated ellipsoids (unsolvated) having an axial ratio of 62. From this axial ratio, the particle weight and the partial specific volume, the dimensions of the particles would be: length 0.2 μ ; thickness 3.3 $m\mu$., which agree with those indicated by ultrafiltration.

Viscosity. The value of the intrinsic viscosity at zero rate of shear, derived from Fig. 6 and expressed as

$$V_0 \equiv \frac{1}{\phi} \left(\frac{\eta}{\eta_0} - 1 \right) \quad \phi \rightarrow 0,$$

where ϕ is the volume fraction of the hyaluronic acid complex, is 1.36×10^4 . This quantity can be used to determine the asymmetry of the particles by means of the relationship of Simha (1940), computed by Mehl, Oncley & Simha (1940). The axial ratio obtained, assuming that the particles are elongated and unsolvated, is 590.

This discrepancy between the indications of sedimentation and diffusion and of viscosity cannot be resolved by any assumption about the solvation of the particles and is more serious if the particles are assumed to be disk-shaped rather than rod-shaped. As a tentative explanation, we may suppose that the particles are interconnected to form a loose, sponge-like structure; the processes of sedimentation and diffusion involve only compression and expansion of this structure, which would not necessarily cause any widespread breaking of interparticle links; on the other hand, these links might contribute largely to the shearing force required to cause viscous flow and would thus increase the viscosity and the apparent asymmetry of the particles derived from it.

It is seen from Fig. 6 that the relative lowering of the viscosity by salt is nearly independent of the concentration of hyaluronic acid. The effect of salt should be to reduce the thickness of the electrical double layer surrounding the particles and so to reduce their interaction both with solvent and with each other. The effect on viscosity of interaction with solvent should not vary much with the concentration of hyaluronic acid, whereas the interparticle interaction should vary rapidly with concentration. The results suggest, therefore, that salt affects the former rather than the latter, and hence that the interparticle interaction is not mainly ionic in character.

The fact that treatment of the hyaluronic acid complex affected the viscosity more markedly than it affected the rate of sedimentation suggests that the first degradative changes involve a reduction of interparticle linking.

CONCLUSION

Evidence has been produced to show that hyaluronic acid occurs in synovial fluid in the form of a definite complex with protein and, perhaps, with another material which contains neither glucosamine nor nitrogen. Isolation of this complex by ultrafiltration gives an undegraded product: it appears, however, that other methods of separation are liable to cause degradation and, in particular, any removal of protein from the complex causes significant changes in its physico-chemical properties. The particle weight

of the complex is about 10^6 ; the particles are probably rod-shaped with a length of 200 m μ . and a thickness of 3.3 m μ .

SUMMARY

1. A hyaluronic acid complex was prepared from synovial fluid by ultrafiltration, and judged to be undegraded by viscosity and ultracentrifuge measurements.

2. Its composition and physico-chemical properties were compared with those of the native synovial fluid, mucin, and hyaluronic acid prepared from mucin by ethanol precipitation at -20° .

3. Hyaluronic acid occurs in synovial fluid in close association with protein, which constitutes about 30% of the complex; removal of this protein leads to degradation.

4. The particle weight of this complex, estimated by sedimentation and diffusion, is about 10^6 , with an axial ratio of about 60.

5. The viscosity at low rates of shear indicates considerable interaction between these 'particles', even at high dilution.

6. The hyaluronic acid was in most cases found to contain somewhat less than the theoretical percentage of glucosamine.

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