

PROTEIN ASSOCIATED WITH HYALURONIC ACID IN OX SYNOVIAL FLUID

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Summary

Protein which is associated with hyaluronic acid, prepared in a physico-chemically unchanged state from ox synovial fluid by ultrafiltration, has been separated by preparative equilibrium sedimentation in a caesium chloride density gradient and by chromatography on DEAE-Sephadex. Earlier work has been confirmed by showing that the protein consists of at least two chemically distinct lipoprotein components. It has also been confirmed that its removal from the hyaluronic acid causes marked changes of viscosity, without the occurrence of degradation of the hyaluronic acid. The separated proteins appeared in a number of differently aggregated forms, and this fact has prevented our drawing conclusions about their molecular sizes in synovial fluid.

I. INTRODUCTION

Ogston and Stanier (1950, 1952) and Ogston and Sherman (1958) showed that hyaluronic acid can be isolated from ox synovial fluid in a physicochemically unchanged state by ultrafiltration or by electrofiltration. Material prepared in this way contains 20–30% of residual protein. Ogston and Stanier (1952) claimed that this protein cannot be removed without causing changes in the physicochemical properties, particularly the non-Newtonian viscosity, of solutions of the hyaluronic acid. Balazs and Sundblad (1959) denied this claim, but it has recently been confirmed by Silpananta, Dunstone, and Ogston (1968).

Curtain (1955) separated this residual protein from ultrafilter residue (UFR) in good yield after enzymic degradation of the hyaluronic acid, and showed that it normally consisted of two proteins which resembled serum α - and β -lipoproteins. Preston, Davies, and Ogston (1965) separated the greater part of the protein by chromatography on DEAE-Sephadex, but reported only its amino acid composition. Silpananta, Dunstone, and Ogston (1967, 1968) showed that hyaluronic acid can be obtained almost free from protein by equilibrium sedimentation in a caesium chloride density gradient (hereafter called for brevity "gradient sedimentation"); the recovery of the protein that they obtained was low and they did not report on its properties.

This paper describes work on the protein, separated from UFR both by gradient sedimentation and by chromatography on DEAE-Sephadex. We have confirmed Curtain's (1955) claim that two lipoproteins are present, differing in amino acid

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composition though containing the same range of lipids. Their tendency to appear in different states of aggregation has prevented our reaching a conclusion about their molecular sizes in UFR. We have also shown, in confirmation of the work of Ogston and Stanier (1952) and of Silpananta, Dunstone, and Ogston (1968), that removal of the protein affects the viscous properties of the hyaluronic acid.

II. METHODS

Methods described by Silpananta, Dunstone, and Ogston (1967, 1968) were used, with some modifications described in Section III.

Lipid Analysis.—Dr. M. W. Simpson-Morgan did these analyses by a modification (unpublished data) of thin-layer chromatography on silicic acid (Korn 1959).

III. EXPERIMENTAL DETAILS AND RESULTS

(a) Recovery of Protein from UFR by Gradient Sedimentation, Initial Density 1.66 g/ml

The preparative experiment of Silpananta, Dunstone, and Ogston (1967) was repeated, care being taken to collect all the protein which was concentrated at the top of the gradient and which tended to stick to the tubes and their caps. 94% of the total protein of the UFR was recovered.

(b) Separation of Protein from UFR by Gradient Sedimentation at Lower Initial Density

In an analytical experiment (initial density 1.32 g/ml; density range 1.25–1.37 g/ml; 20 hr at 44,770 rev/min; 20°C), the protein formed two partly overlapping zones at mean densities 1.30 and 1.32 g/ml; the hyaluronic acid and chondroitin sulphate (Silpananta, Dunstone, and Ogston 1967) sedimented to the bottom of the gradient [Fig. 1(a)]. Opacity in the upper protein zone showed that partial precipitation of this material had occurred. From the zone widths, approximate molecular weights of 6×10^5 (upper zone) and 1×10^6 (lower zone) were calculated by the method of Meselson, Stahl, and Vinograd (1957).

This experiment was repeated on a preparative scale in the No. 50 rotor of a Beckman model L2 ultracentrifuge (initial density 1.35 g/ml; 96 hr at 42,000 rev/min; 20°C), the tubes being sliced at levels corresponding to densities 1.31 and 1.34 g/ml to separate the two protein zones. 84% of the total protein, only slightly contaminated by uronic-acid-containing material, was recovered. The upper zone (fraction 1) contained a considerable amount of precipitate; the lower zone (fraction 2) contained relatively little precipitate.

Each fraction, still in the caesium chloride solution in which it was separated and without removal of precipitate, was then examined by gradient sedimentation in the analytical ultracentrifuge under the same conditions as before. Fraction 1 showed only an opaque zone at mean density 1.30 g/ml, with no material of higher density [Fig. 1(b)]. Fraction 2 showed a main zone containing some precipitate, at mean density 1.32 g/ml, with none of the less dense material but with a minor zone of greater density [Fig. 1(c)]. This showed that the two protein fractions had been fairly well separated.

Both fractions were dialysed free from caesium chloride against changes of buffer (0.2M sodium chloride–0.12 M sodium hydrogen carbonate). The precipitated material did not redissolve. As nearly the whole of fraction 1 had precipitated the whole fraction was used for analysis, but fraction 2 was separated into soluble (fraction 2a) and insoluble (fraction 2b) parts.

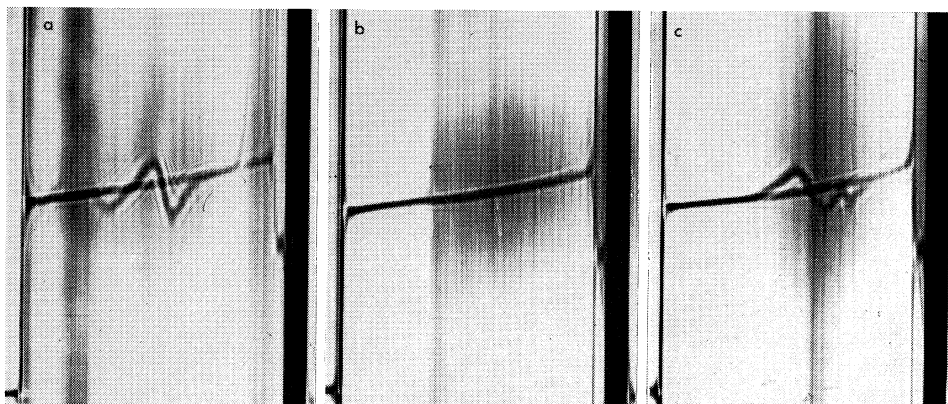


Fig. 1.—Schlieren diagrams of the gradient sedimentation of protein fractions. (a) Whole UFR at mean density 1.32 g/ml. Two zones of protein appear, the upper (as shown by the opacity) partly precipitated. (b) Upper protein zone (fraction 1; see text) separated by preparative gradient sedimentation, mean density 1.30 g/ml. Nearly all of this fraction has precipitated. (c) Lower protein zone (fraction 2; see text) separated by preparative gradient sedimentation, mean density 1.32 g/ml. The main zone, which contains some precipitate, is at density 1.32 g/ml, with a smaller amount of material of slightly greater density.

(c) Separation of Protein from UFR by Column Chromatography

An attempt to separate the protein on a Sepharose 2B column with caesium chloride (density 1.6 g/ml) as solvent throughout, was first made. This attempt failed because of clogging of the column, probably due to precipitation of protein. A column of DEAE-Sephadex A-50 (medium grade) as described by Preston, Davies, and Ogston (1965) was therefore used. The eluted protein appeared in three peaks (fractions 3, 4, and 5). However, contrary to the findings of Preston, Davies and Ogston (1965), only 50–60% of the total protein had come off the column by the time that small amounts of material containing uronic acid had begun to appear. The hyaluronic acid, which was recovered from the column by reversed flow (Preston, Davies, and Ogston 1965), still contained 13% of protein; Preston, Davies, and Ogston (1965) reported 6% of protein in hyaluronic acid recovered in this way.

(d) Amino Acid Analysis

Table 1 shows the amino acid compositions of fractions 1, 2a (soluble), 2b (insoluble), and 3, 4, and 5. Fractions 2a and 2b showed no marked differences from each other and resembled the protein analysed by Preston, Davies, and Ogston (1965). Fraction 1 differed significantly from them, particularly with respect to its

contents of arginine, glutamic acid, glycine, and valine. Fractions 3, 4, and 5 resembled each other fairly closely, except for the low proline contents of fractions 3 and 4; but these values may be unreliable, since only 50- μ g samples were analysed. Fractions 3, 4, and 5 all differed from the whole protein of Preston, Davies, and Ogston (1965) in containing more alanine and perhaps more glycine. It follows that the amino acid composition of the peptide material not recovered from the DEAE-Sephadex column must also differ from that of the whole protein in order to maintain the amino acid balance.

(e) Analysis of Lipids

All fractions gave similar qualitative patterns of identified and unidentified lipids. Figure 2 shows a typical example.

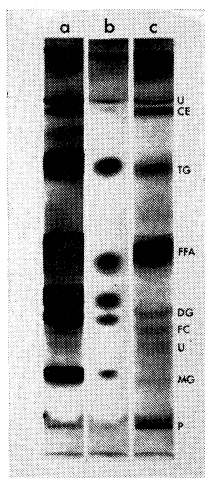


Fig. 2.—Typical thin-layer chromatogram on silica gel of protein obtained from UFR. (a), (b) Standard mixtures, run at different concentrations. (c) Lipid extracted from UFR protein. *U*, Unknowns; *CE*, cholesterol esters; *TG*, triglycerides; *FFA*, free fatty acids; *DG*, diglycerides; *FC*, free cholesterol; *MG*, monoglycerides; *P*, phospholipids.

(f) Sedimentation Velocity

The sedimentation velocity of fraction 2a and of whole protein separated by chromatography on DEAE-Sephadex was measured in 0.2M sodium chloride–0.12 M sodium hydrogen carbonate at 59,780 rev/min, 20°C. Fraction 2a was examined after treatment with sulphite (0.036M sodium sulphite–0.021M potassium dihydrogen phosphate–0.048M disodium hydrogen phosphate). The protein obtained from chromatography on DEAE-Sephadex was also treated with sulphite, its sedimentation velocity measured, and the measurement repeated in sodium chloride buffer, after removal of the sulphite and exposure to oxygen. Table 2 gives experimental details, and the sedimentation coefficients of the various components.

(g) Viscosity Experiments

When caesium chloride was dissolved to 7M in a solution of UFR and the solution left for 7 days at about 20°C, a precipitate of protein appeared which remained insoluble after dialysis against sodium chloride buffer. Removal of the precipitate by centrifuging left a hyaluronic preparation containing 7% protein. The viscosity

of this solution, and of a solution of hyaluronic acid with lowered protein content recovered from a DEAE-Sephadex column, were compared with that of UFR, of UFR treated with 4M potassium chloride, and of UFR treated with sulphite.

TABLE 1

ANALYTICAL VALUES FOR AMINO ACIDS IN EACH FRACTION OF PROTEIN, ISOLATED BY GRADIENT SEDIMENTATION OR BY CHROMATOGRAPHY ON DEAE-SEPHADEX

Values are expressed as $\mu\text{moles}/100 \mu\text{moles}$ of total amino acid estimated

Amino Acid	Amino Acid Composition of Fraction:						
	1	2a	2b	Whole Protein*	3	4	5
Lysine	8.4	6.3	5.4	4.8	6.2	7.3	5.7
Histidine	0.9	1.8	1.5	1.6	1.1	1.1	1.4
Arginine	1.4	3.7	4.7	3.8	3.8	4.7	4.7
Aspartic acid	7.8	8.4	9.1	8.8	10.3	10.1	10.1
Threonine	5.3	8.3	8.6	8.6	5.6	6.5	6.7
Serine	16.4	13.8	10.4	10.7	5.7	8.2	7.8
Glutamic acid	15.4	11.5	11.4	11.7	12.1	15.3	13.0
Proline	6.7	4.9	7.4	6.9	1.8	—	6.8
Glycine	14.7	8.4	8.3	7.9	11.2	11.2	10.4
Alanine	7.6	6.6	6.1	5.8	13.5	12.9	9.9
$\frac{1}{2}$ Cystine	—	—	—	1.3	—	—	—
Valine	4.6	9.4	8.9	9.0	7.2	8.8	7.0
Methionine	—	0.6	0.9	1.1	0.6	—	0.2
Isoleucine	3.0	3.0	3.8	3.6	4.9	3.7	4.5
Leucine	5.5	7.7	7.5	7.6	10.6	8.2	8.4
Tyrosine	—	2.4	2.7	3.4	0.8	—	—
Phenylalanine	2.3	3.2	3.3	3.4	4.6	2.0	3.5

* Values for protein prepared with DEAE-Sephadex, calculated from data of Preston, Davies, and Ogston (1965).

TABLE 2

SEDIMENTATION COEFFICIENTS OF VARIOUS PROTEIN PREPARATIONS

Lesser components shown in parentheses

Preparation	$S_{20,w}$ (S)	Preparation	$S_{20,w}$ (S)
Soluble fraction 2a from gradient sedimentation	34, 20	Protein separated by chromatography on DEAE-Sephadex then treated with sulphite (10 days, 2°C)	(15), 1.5
Soluble fraction 2a treated with sulphite, 24 hr, room temperature	6	Protein separated by chromatography on DEAE-Sephadex, treated with sulphite (10 days, 2°C), then oxidized for 10 days at 2°C	(15), 3
Protein separated by chromatography on DEAE-Sephadex	(32), (18), 2	Protein prepared by Curtin (1955) (Ogston 1955)	6-7

Measurements were made at velocity gradients 0 and 44 sec^{-1} and the ratio η_0/η_{44} was used as a measure of non-Newtonian viscosity (Silpananta, Dunstone, and

Ogston 1968). The results are shown in Figure 3. Potassium chloride neither precipitated protein nor affected the viscosity. Both the solutions with lowered protein

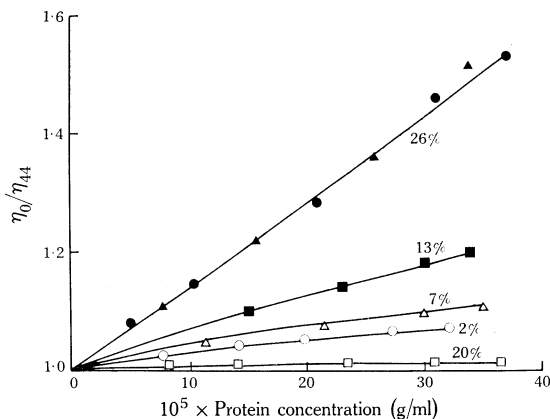


Fig. 3.—Plots of the ratio η_0/η_{44} (see text) against concentration for several preparations of hyaluronic acid. The numbers against the curves give the percentage of protein in each sample.

- Untreated UFR.
- ▲ UFR treated with 4M potassium chloride.
- Hyaluronic acid with reduced protein content prepared by chromatography on DEAE-Sephadex.
- △ Hyaluronic acid with protein content reduced by treatment with caesium chloride.
- Hyaluronic acid prepared by gradient sedimentation in caesium chloride.
- UFR treated with sulphite (see text).

content had reduced non-Newtonian viscosity corresponding to the degree of removal of the protein. Treatment with sulphite practically abolished the non-Newtonian viscosity.

IV. DISCUSSION

It has been shown that two mild procedures (treatment with caesium chloride and chromatography on DEAE-Sephadex) remove most of the residual protein from UFR; this makes it likely that most of the protein is not chemically bound. It remains possible, as with the 2% of protein that Sandson and Hamerman (1962) showed to be firmly bound to the hyaluronic acid of human synovial fluid, that some polypeptide or protein is chemically bound to the hyaluronic acid in ox synovial fluid. However, in view of the finding of Silpananta, Dunstone, and Ogston (1968) that undegraded hyaluronic acid can be separated with as little as 0.5% of protein, this amount must be very small.

Our viscosity experiments confirm those of Ogston and Stanier (1952) and of Silpananta, Dunstone, and Ogston (1968) in showing that the removal of protein progressively reduces the non-Newtonian viscosity of solutions of UFR (Fig. 3), without depolymerization of the hyaluronic acid. This suggests that the protein may help to stabilize the hyaluronic acid network *in vivo* and may therefore affect its physiological properties.

The separation of two lipoprotein zones in gradient sedimentation, with different densities and amino acid compositions, is consistent with the claim of Curtin (1955) that two serum-like lipoproteins are present. However, while his mixed separated proteins had $S_{20,w}$ values of 6–7 S (Ogston 1955), our separated samples have shown a wide variety of sedimentation coefficients. It appears that the processes of separation must cause aggregation or disaggregation to occur, and this fact makes it impossible, on the evidence available, to decide on the original molecular states of the proteins in synovial fluid or in UFR. The depolymerizing effect of

sulphite suggests that disulphide bonds may be involved in the changes of aggregation. Our failure to detect cystine in these preparations is probably due to our use of small amounts of peptide material for analysis, the determination of this amino acid as cystine and not as cysteic acid, and the presence of only small amounts of cystine in these protein fractions.

V. ACKNOWLEDGMENTS

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