Studies on the Structure of Hyaluronic Acid

CHARACTERIZATION OF THE PRODUCT FORMED WHEN HYALURONIC ACID IS TREATED WITH ASCORBIC ACID

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Physical and chemical methods were used to characterize hyaluronic acid before (fraction HAIIBI) and after (fraction HA-AA) treatment with ascorbic acid. Fraction HA-AA was recovered with an almost quantitative yield and was shown to be chemically identical with fraction HAIIBI by all the methods used. These two materials, however, differed markedly in their molecular sizes and degree of polydispersity. By using sedimentation, diffusion and sedimentation-equilibrium analyses, weight-average molecular weights of about 1.2×10^6 and 6.5×10^4 respectively were obtained for fractions HAIIBI and HA-AA. It is concluded from these results that hyaluronic acid has a molecular weight of about 65 000 and that the polysaccharide chain of this molecule is not depolymerized by ascorbic acid. It is further proposed that hyaluronic acid molecules in the matrix of connective tissues are present either in an aggregated form or as subunits of heterogeneous macromolecules, and that it is the linkages responsible for the organization of these structures which are broken by ascorbic acid.

In an earlier paper (Swann, 1967) the results of studies concerned with the degradation of hyaluronic acid by ascorbic acid were reported. These studies indicated that hyaluronic acid was a heterogeneous structure and that the reaction involved certain reducible linkages, possibly in the protein moiety of the molecule. Subsequent work (Swann, 1968*b*), however, showed that approx. 90% of the amino acids initially present in the hyaluronic acid used in the degradation studies could be removed by procedures implying that they were not an intrinsic part of the molecule.

Although recent investigations have been carried out to determine the mechanism of this reaction (Niedermeier, Dobson & Laney, 1967*a*; Niedermeier, Laney & Dobson, 1967*b*), the structures of the hyaluronic acid used for degradation studies and the products formed during the reaction have never been satisfactorily characterized. Thus at the present time it is not known whether polysaccharide chains of the hyaluronic acid are randomly depolymerized or whether the molecule is degraded at certain specific locations.

It is the purpose of this paper to describe the physical and chemical properties of the product formed when hyaluronic acid is treated with ascorbic acid.

MATERIALS AND METHODS

Hyaluronic acid. Hyaluronic acid was prepared from rooster comb as described by Swann (1968*a*). Briefly the extracted hyaluronic acid was purified by repeated treatments with chloroform and by saturation with NaCl followed by centrifugation and precipitation with ethanol. This material was then further purified by a cetylpyridinium chloride precipitation procedure, ultracentrifugation and repeated precipitation with ethanol. The final product corresponds to a preparation described earlier (fraction HAIIB; Swann, 1968*b*) and is referred to below as fraction HAIIBI. The degree of polydispersity of this product was determined by the cetylpyridinium chloride fractionation procedure described by Laurent, Ryan & Pietruskiewicz (1960).

Ascorbic acid treatment. The reaction with L-ascorbic acid (Baker Chemical Co., Phillipsburg, N.J., U.S.A.) was carried out under two different conditions: (a) under acid conditions at about pH 3.0 in distilled water after the hyaluronic acid had been dialysed against Dowex 50 (H^+ form); (b) at pH 7.5 in solutions containing 0.1M-tris-HCl buffer and 0.2M-NaCl. Unless otherwise stated solutions containing equal quantities (by wt.) of ascorbic acid and fraction HAIIBI were used and the reaction was carried out at room temperature (22°) with gentle stirring. For (a) the ascorbic acid was dissolved in distilled water, whereas for (b) the ascorbic acid was dissolved in the 0.1M-tris-HCl buffer, pH 7.5, containing 0.2M-NaCl. These solutions were added directly to the hyaluronic acid solutions. The reaction was

allowed to proceed for 16 hr. and the product (fraction HA-AA) was then isolated by extensive dialysis against distilled water and freeze-drying or by precipitation with ethanol.

Chemical methods. The hexuronic acid content was determined as described by Balazs, Berntsen, Karossa & Swann (1965) and glucosamine as described by Swann & Balazs (1966) and by ion-exchange chromatography (Gardell, 1953). Amino acid analyses by ion-exchange chromatography were performed on hydrolysates obtained by refluxing samples (about 30 mg.) of hyaluronic acid with 150 ml. of 6 M-HCl for 24 hr. under a continuous stream of N₂ as described by Swann (1968b). Dry weights were determined on freeze-dried samples that had been left to stand over P₂O₅ under vacuum at 40° until they reached a constant weight. All hyaluronic acid concentrations were obtained by multiplying the hexuronic acid value by the factor 1.95.

Physical methods. Intrinsic-viscosity measurements were performed on samples containing fractions HAIIBI and HA-AA at initial concentrations of 0.3 mg./ml. and 2.0 mg./ml. respectively in a Cannon-Ubbelohde semi-micro dilution viscometer (maximum shear rate 240 sec.⁻¹) and the relative viscosities in a Cannon-Manning semi-micro viscometer (solvent flow time 118.4 sec.), both at 25°. The sedimentation velocity, diffusion and sedimentation-equilibrium analyses were performed in a Beckman-Spinco model E analytical ultracentrifuge at 20° with 12 mm. cells. A single-sector aluminium centre piece and a rotor speed of 52640 rev./min. were used for the velocity measurements, and a synthetic-boundary valve-type aluminium centre piece and a rotor speed of 4609 rev./min. for the diffusion measurements. The area-maximum-height method was used to calculate the diffusion coefficients. The sedimentation-equilibrium analyses were carried out by the method of Yphantis (1964) by use of 2.5 mm. solution column in a Kel-F centre piece. These measurements in the ultracentrifuge were performed at several different initial concentrations of fractions HAIIBI and HA-AA. Mobility measurements were performed in a Beckman model H free-electrophoresis apparatus at 4° with the 11 cm. cell and solutions containing 3.5 mg. of fraction HA-AA/ml. and 1.5 mg. of fraction HAIIBI/ml. These physical measurements were carried out on samples dialysed extensively against 0.1 M-tris-HCl buffer, pH 8.0, containing 0.2 M-NaCl.

The i.r. spectra were measured with thin films of hyaluronic acid in a Perkin-Elmer model 237 infrared spectrophotometer. The u.v.-absorption measurements, with a Beckman model DK2 ratio recording spectrophotometer, and optical-rotation measurements, with a Perkin-Elmer model 141 polarimeter with the 589 nm. filter at 20°, were performed on sodium hyaluronate solutions in distilled water.

RESULTS

When ascorbic acid is added to hyaluronic acid there is a rapid fall in the viscosity of the hyaluronic acid. This is illustrated in Fig. 1, where the reaction was carried out at pH 3.0 in the absence of sodium chloride. Also shown in Fig. 1 are the extinctions at 242.5 nm. of the fraction HAIIBI and ascorbic acid solutions and the reaction mixture as a function of time. It is apparent that the rapid decrease in

viscosity is related to the decrease in extinction at the above wavelength. At neutral pH in the presence of 0.2 M-sodium chloride essentially similar observations were made, except that the relative viscosity changes were smaller owing to the presence of sodium chloride and the absorption maximum of ascorbic acid was at 265 nm. Under the conditions of the experiment shown in Fig. 1 the reactions at pH 3.0 and at pH 7.5 were essentially complete after 200 min. To ensure that the reaction between hyaluronic acid and ascorbic acid had proceeded to

Fig. 1. Changes in viscosity (○) and $E_{242.5}$ (▲, ■ and ●) during the reaction of ascorbic acid with fraction HAIIBI at pH 3.0. ▲, Ascorbic acid (78 µg./ml.); ■, fraction HAIIBI (800 µg./ml.); ● and ○, fraction HAIIBI (800 µg./ml.) + ascorbic acid (78 µg./ml.).

Fig. 2. Infrared spectra of fraction HAIIBI at pH 3.0 (upper curve) and fraction HA-AA (lower curve).

Table 1. *Chemical properties of hyaluronic acid (fraction HAIIBI) and the product obtained after treatment with ascorbic acid (fraction HA-AA)*

Fraction	Dry wt. (g.)	Hexuronic acid		Glucosamine		$[\alpha]_D^{20}$
		(g.)	(% of dry wt.)	(g.)	(% of dry wt.)	
HAIIBI	2.0726	0.9755	47.1	0.8826	42.6	-76°
HA-AA	2.0200	0.9710	48.1	0.8700	43.1	-74
Recovery (%)	97.4	99.5	—	98.6	—	—

Table 2. *Physical properties of hyaluronic acid (fraction HAIIBI) and the product obtained after treatment with ascorbic acid (fraction HA-AA)*

Fraction	$10^5 \times$ Electrophoretic mobility at pH 8.0 (cm. ² v ⁻¹ sec. ⁻¹)		Intrinsic viscosity (ml./g.)	Sedimentation coefficient S^0 (s)	$10^7 \times$ Diffusion coefficient D^0 (cm. ² sec. ⁻¹)	$10^{-5} \times$ Molecular weight	
	Ascending	Descending				Sedimentation-diffusion	Sedimentation-equilibrium
HAIIBI	8.9	8.1	2000	6.6	0.41	11.7	12.0
HA-AA	7.9	7.0	100	1.94	2.05	0.686	0.61

Fig. 3. Dependence of the reciprocal of the sedimentation coefficient on concentration for fraction HAIIBI (●) and for fraction HAIIBI degraded by ascorbic acid at pH 7.5 (▲).

completion and that the fraction HA-AA to be used for physical measurements represented the final reaction product, the reaction was performed with equal quantities of fraction HAIIBI and ascorbic acid and was allowed to proceed for 16 hr. Under these conditions the viscosity of the reaction mixture decreased and reached a constant value in the presence of excess of ascorbic acid, and the viscosity of the isolated fraction HA-AA was not decreased by further treatment with ascorbic acid.

The results of chemical and physical measurements performed on samples of fraction HAIIBI and HA-AA are shown in Tables 1 and 2. There was a quantitative recovery of hexuronic acid and

glucosamine in fraction HA-AA as shown by colorimetric techniques, and 97.4% of the dry weight of the original fraction HAIIBI was recovered in the degraded product. It is not known to what extent this apparent loss of dry weight was caused by the initial and final products having different abilities to retain moisture in the drying procedure used. The optical rotations were essentially the same for the two samples, whereas the mobility of fraction HA-AA was slightly lower than that of the original fraction HAIIBI. These mobility measurements were not corrected for the different viscosities of the samples. There were also no detectable differences in the i.r. spectra of the initial fraction HAIIBI and fraction HA-AA (Fig. 2). The spectra shown in Fig. 2 were obtained from films made from fraction HAIIBI at pH 3.0 and from fraction HA-AA at pH 3.0 after the ascorbic acid had been removed by dialysis against distilled water.

The results of sedimentation and diffusion measurements on fraction HAIIBI and the product obtained after reaction with ascorbic acid are shown in Figs. 3 and 4. As was expected for fraction HAIIBI (Varga, 1955) there was a marked dependence of the experimental values on the concentration of sample, and therefore the sedimentation coefficient at zero concentration was obtained by plotting the reciprocals of the experimental values (Fig. 3). The values obtained for the diffusion coefficient were again dependent on concentration for fraction HAIIBI (Fig. 4), but for fraction HA-AA there were no significant differences in the determined values in the concentration range (1–6 mg./ml.) over which this material was examined. By using the results shown in Figs. 3 and 4 a

value of 6.6s is obtained for the sedimentation coefficient and $0.41 \times 10^{-7} \text{cm.}^2 \text{sec.}^{-1}$ for the diffusion coefficient of fraction HAIIBI. The corresponding values for fraction HA-AA are 1.94s and $2.05 \times 10^{-7} \text{cm.}^2 \text{sec.}^{-1}$. By substituting these values in the Svedberg formula and using a value of 0.66 ml./g. for the partial specific volume of both materials (Varga, 1955), molecular weights of 1.17×10^6 and 6.86×10^4 are obtained for fractions HAIIBI and HA-AA respectively. These two materials also gave very different intrinsic-viscosity values, as shown in Table 2. The range of values obtained for different preparations of fractions HAIIBI and HA-AA were 1800–2500 ml./g. and 75–150 ml./g. respectively.

The solute distributions obtained when sedimentation-equilibrium analyses were performed on fractions HAIIBI and HA-AA are shown in Fig. 5. With samples of fraction HA-AA a linear fringe-displacement curve was obtained, whereas with samples of fraction HAIIBI a sigmoidal curve indicating polydispersity and non-ideal behaviour was obtained. In this latter case the central linear portion of the curve was used for the molecular-weight calculations. When these measurements were repeated with different initial concentrations of fractions HAIIBI and HA-AA (Fig. 6), the molecular weight was dependent on the initial sample concentration for both samples. Although this effect was small with fraction HA-AA, it does indicate that polydispersity and non-ideal behaviour

also influenced the solute distribution shown in Fig. 5(a). It was difficult to extrapolate the results obtained with fraction HAIIBI to obtain a zero-concentration value because of the concentration-dependence and lack of results at very low concentrations, but the estimated value of 1.2×10^6 agrees well with the molecular weight obtained from the sedimentation and diffusion results. With fraction HA-AA, however, the values obtained were less dependent on and appeared to be linearly related to the concentration. A value of 6.1×10^4 is obtained by extrapolation of the results to zero concentration. The molecular size of fraction HA-AA was

Fig. 4. Dependence of the diffusion coefficient on concentration for fraction HAIIBI (●) and for fraction HAIIBI after degradation with ascorbic acid at pH 7.5 (▲).

Fig. 5. Net fringe displacement plotted against the square of the radius for: (a) an 0.0119% solution of fraction HA-AA after centrifugation at 27690 rev./min. in the Spinco model E ultracentrifuge; (b) an 0.0094% solution of fraction HAIIBI after centrifugation at 11272 rev./min. in the Spinco model E ultracentrifuge. Both measurements were performed at 20° after 16 hr. of centrifugation. In each case r_b^2 represents the base of the solution column.

Fig. 6. Dependence of molecular weight determined by sedimentation-equilibrium analysis on concentration for fraction HAIIBI (▲) and for fraction HAIIBI after degradation with ascorbic acid at pH 3.0 (●) and at pH 7.5 (○).

Table 3. Content of certain amino acids in hyaluronic acid (fraction HAIIBI) and the product obtained after treatment with ascorbic acid (fraction HA-AA)

Fraction ...	Content (μ moles/m-mole of hexuronic acid)		Content (residues/mol.)	
	HAIIBI	HA-AA	HAIIBI*	HA-AA†
Asp	0.93	1.00	2.71	0.15
Thr	0.41	0.44	1.20	0.07
Ser	0.82	0.90	2.39	0.14
Glu	0.96	1.30	2.80	0.20
Gly	0.96	1.09	2.80	0.16
Ala	0.62	0.63	1.81	0.10
Val	0.61	0.72	1.78	0.11
Ile	0.29	0.33	0.84	0.05
Leu	0.58	0.58	1.69	0.09

* Assuming a molecular weight of 1.2×10^6 and a hexuronic acid content of 47.1%.

† Assuming a molecular weight of 6.1×10^4 and a hexuronic acid content of 48.1%.

the same when the reaction was performed at pH 3.0 and pH 7.5.

The amino acid contents of fractions HAIIBI and HA-AA expressed as μ moles of amino acid/m-mole of hexuronic acid are shown in Table 3. Only certain of the acid and neutral amino acids were determined, because it was shown previously (Swann, 1968b) that these represented more than 80% of the total amino acids present in purified hyaluronic acid preparations.

The results of the cetylpyridinium chloride frac-

tation method (Laurent *et al.* 1960) carried out on one sample of fraction HAIIBI are shown in Table 4. When this procedure was performed on several occasions, some variations were found in the quantity and viscosity of the hyaluronic acid recovered in each fraction, but a distribution similar to the one shown was always observed. Several differences are apparent between the rooster-comb hyaluronic acid examined in this study and the bovine vitreous-body material studied by Laurent *et al.* (1960). The rooster-comb sample had a higher percentage content of the more viscous components; it contained a small amount (6.8%) of material with an intermediate viscosity (1540–1920 ml./g.) and the material from the bovine vitreous body with a viscosity of 250–295 ml./g. was apparently absent from the rooster-comb sample. There were also differences in the sodium sulphate concentration at which hyaluronic acid of a certain viscosity was precipitated. It is not known whether these were caused by sample differences or by the variability of the technique.

DISCUSSION

The various agents that decrease the molecular size of hyaluronic acid in the demonstrable absence of known enzymes are usually assumed to operate via similar, if not identical, mechanisms, and as such they are discussed under the title of non-enzymic oxidation-reduction reactions (Sundblad & Balazs, 1966; Matsumura, Herp & Pigman, 1966). It is apparent, however, that, although all of these reactions may have certain features in common, there must be a clear distinction between those reactions that are induced by radiation from a high-energy source, when the random cleavage of glycosidic bonds and/or the random destruction of the constituents is to be expected (Balazs, Davies, Phillips & Young, 1967), and the reaction carried out only in the presence of ascorbic acid and oxygen, where no diffusible products are observed and there is no demonstrable destruction of the constituent sugars (Pigman & Rizvi, 1959; Swann, 1967). On this basis alone it is essential that the degradation products be adequately characterized before any one mechanism is assigned to these reactions. The present analyses were performed on the product obtained after degradation of hyaluronic acid with ascorbic acid, because of the possible physiological importance of this reaction.

The present understanding of the structure of hyaluronic acid is based on the chemical investigations, which showed the polysaccharide chain to be composed of repeating units of 2-acetamido-2-deoxy-3-*O*- β -D-glucopyranosyluronic acid-D-glucose linked by β -(1 $\rightarrow$ 4)-glycosidic linkages (see Jeanloz, 1955; Meyer, 1958), and the physical

Table 4. *Fractionation of hyaluronic acid by precipitation with cetylpyridinium chloride at known concentrations of sodium sulphate*

Results obtained in the present work for rooster-comb hyaluronic acid (fraction HAIIBI) are compared with those obtained by Laurent *et al.* (1960) for bovine vitreous-body hyaluronic acid.

Fraction	Concn. of Na ₂ SO ₄ at precipitation (M)	Fraction HAIIBI		Bovine vitreous-body hyaluronic acid	
		Percentage of starting material	Limiting viscosity number (ml./g.)	Percentage of starting material	Limiting viscosity number (ml./g.)
1	0.250	—	—	—	—
2	0.190	—	—	0.9	—
3	0.174	—	—	4.7	2450
4	0.165	31.9	2500	10.9	2340
5	0.155	8.2	2400	20.9	875
6	0.130	6.3	1920	12.3	700
7	0.060	0.5	1540	2.2	—
	0.030	—	—	—	—
8A	—	42.8	880	11.6	295
8B	—			15.5	250

studies, which have characterized the molecule as a polymer with a random-coil configuration which occupies a somewhat spherical domain in solution (see Laurent, 1957; Balazs, 1958; Blumberg & Ogston, 1958). There are, however, no results available at the present time to indicate that the macromolecule examined in the physical studies is composed entirely of the constituents and linkages characterized in the chemical investigations. Therefore the description of the reaction between hyaluronic acid and ascorbic acid as a depolymerization process (Niedermeier *et al.* 1967a; Matsumura & Pigman, 1965; Matsumura *et al.* 1966) is an oversimplification and one that, judged from the results presented here, is inappropriate.

All of the results described indicate that the reaction is a specific one in that the product, which was obtained in near quantitative yield, had a molecular size of 60000–70000 and a chemical composition identical, within experimental error, with that of the original hyaluronic acid (Tables 1 and 2). In addition, repeated treatment with ascorbic acid failed to decrease the molecular size of fraction HA-AA. It is therefore apparent that, to the extent that hyaluronic acid has been shown to be a polymer, fraction HA-AA must also be so defined, and this being the case the reaction with ascorbic acid cannot be described as a depolymerization process.

An explanation for the present findings could be that when fraction HAIIBI is degraded constituents and/or covalent linkages (which have not yet been identified) are cleaved by the ascorbic acid, possibly in a manner similar to that described by Caygill (1968). Under these conditions hyaluronic acid could be envisaged as a heterogeneous structure containing linkages sensitive to oxidation. On the

basis of earlier findings a structure similar to this was proposed (Swann, 1967, 1968c). It was, however, thought at that time that hyaluronic acid contained a number of peptide moieties with reducible linkages that might be the site of the reaction. However, the results in Table 3 show that, although fractions HAIIBI and HA-AA contain approximately the same quantities of the determined amino acids/mole of hexuronic acid, no single amino acid was present in fraction HA-AA in sufficient amounts, on a residues/mole basis, for it to be considered as the site of the degradation reaction. Arabinose has been shown to be present in hyaluronic acid preparations (Wardi, Allen, Turner & Stary, 1966), but there is no direct evidence that it is a constituent in the hyaluronic acid molecule as such.

In contrast with the chemical similarity of fractions HA-AA and HAIIBI, it is clear from the ultracentrifuge results (Table 2) that the ascorbic acid treatment caused about a 20-fold decrease in the average particle size of the hyaluronic acid. Molecular weights of about 1.2×10^6 and 6.5×10^4 were obtained by equilibrium and sedimentation-diffusion measurements for fractions HAIIBI and HA-AA respectively. In view of the polydispersity of fraction HAIIBI (Table 4) these numbers are, however, difficult to interpret. Judged from the sedimentation-equilibrium experiments, fraction HA-AA always appeared to be much less heterogeneous. When the solute distributions obtained by equilibrium ultracentrifugation are examined (Fig. 5) fraction HAIIBI gave evidence of polydispersity, but to a much smaller extent than was indicated by the fractionation procedure (Table 4). This material also showed non-ideal behaviour even

at low initial concentrations. In contrast fraction HA-AA showed a linear distribution. It is thought, because of the much smaller molecular size of this material and the more limited influence of concentration on the determined molecular parameters (Figs. 3, 4 and 6), that the solute distribution shown in Fig. 5 will be more representative of the actual molecular-size distribution of fraction HA-AA than is the case for fraction HAIIBI.

In the light of these results it is suggested that, if hyaluronic acid is defined as a polysaccharide composed of 2-acetamido-2-deoxy-3-*O*- β -D-glucopyranosyluronic acid-D-glucose linked by β -(1 $\rightarrow$ 4)-glucosidic linkages, then this polysaccharide chain has a molecular weight of about 65000. With this definition for hyaluronic acid, material with a higher molecular weight should be considered to be either a heterogeneous macromolecule containing hyaluronic acid subunits, if the unidentified structures disrupted by ascorbic acid involve covalent linkages, or an aggregate, if the linkages involved in the reaction with ascorbic acid are non-covalent in nature. In the first instance the reaction with ascorbic acid would involve the cleavage of specific linkages in the heterogeneous macromolecule, in which case the hyaluronic acid subunits of this macromolecule would have a molecular weight of about 65000. If on the other hand the reaction involves an irreversible dissociation of aggregates into the component molecules, this molecular weight would refer to the hyaluronic acid molecule as such. Although there are no data to distinguish between these two possibilities at the present time, there is evidence that the conformation of the polysaccharide chains and the interaction between these moieties may play an important role in the structure of the macromolecule (Swann & Nagy, 1969). The demonstration that rooster-comb extracts contain weak bonds and/or interactions that are responsible for their extremely high viscosity (Swann, 1969) also supports the view that hyaluronic acid occurs in the connective-tissue matrix as an organized aggregate or macromolecular structure. On this basis the material isolated from bovine synovial fluid and shown to have a molecular weight of 10^7 by Silpananta, Dunstone & Ogston (1968) would refer to the organized structure in which the hyaluronic acid occurs in that tissue and would not be a measure of the size of hyaluronic acid as a molecular entity.

Although it is important to examine the structures that actually occur in the connective-tissue matrix, it is obviously of equal importance to try to determine the size of the molecular entities that the cell synthesizes and from which the extracellular structures are elaborated. It is noteworthy that hyaluronic acid prepared from the bovine vitreous body by Varga (1955) had a molecular weight of

86600, a value close to that found for fraction HA-AA in the present study. The results obtained *in vitro* reported here may therefore have relevance to the structure of the hyaluronic acid molecule *in vivo*.

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