Measurements of reducing end groups on bovine vitreous-humour hyaluronic acid by reaction with [^{14}C]cyanide

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Bovine vitreous-humour sodium hyaluronate was purified by precipitation with cetylpyridinium chloride, CsCl-density-gradient sedimentation and gel-permeation chromatography. The number of reducing end groups in two similarly prepared hyaluronate samples was determined by reaction with K^{14}CN , and measurements of intrinsic viscosity were performed to determine whether this reaction caused degradation of the hyaluronate. The intrinsic viscosity of one hyaluronate sample was 192 ml/g, compared with a value of 187 ml/g after reaction with [^{14}C]cyanide, which indicates that the labelling reaction did not cause depolymerization of the hyaluronate. The M_r calculated from these viscosity values is approx. 60 000. Fractionation of the [^{14}C]cyanide-labelled hyaluronate by gel chromatography showed that it was composed of a polydisperse population of molecules with calculated chain lengths, based on the ratio of [^{14}C]cyanide to uronic acid, ranging in molecular weight from 9000 to 264 000, with an average M_r of 63 200. On the basis of these measurements it is concluded that reaction with [^{14}C]cyanide does not cause degradation of bovine vitreous-humour hyaluronate polysaccharide chains and that reaction with [^{14}C]cyanide can be used to determine the molecular weight of this hyaluronate.

Hyaluronic acid, presumably mainly in the form of sodium hyaluronate, is a ubiquitous component of connective tissues, and, although differences have been reported in the molecular weight of hyaluronates isolated from different tissues, all hyaluronates appear to have the same primary chemical structure. The structure of hyaluronate has been discussed in detail elsewhere (Laurent, 1970; Swann, 1980). One of the uncertainties about the structure of hyaluronate is the length of the polysaccharide chains and whether the difference between low-molecular-weight hyaluronate [e.g. that from bovine vitreous humour, where M_r values less than 100 000 have been reported (Varga, 1955)] and high-molecular-weight hyaluronate [e.g. that from rooster comb, umbilical cord and synovial fluid, where M_r values as high as 12×10^6 have been obtained (Silpananta *et al.*, 1968)] is due solely to differences in the degree of polymerization. To resolve this question it is necessary to calculate molecular

weights by physical methods and chemical end-group measurements to determine the number and length of the polysaccharide chains.

In a previous study, Robert *et al.* (1962) showed that monosaccharides and hyaluronate hexasaccharide reacted with K^{14}CN and yielded products that contained the expected amount of radioactivity. This method should therefore be suitable to measure the number of end groups in hyaluronate preparations. When a commercial sample of hyaluronate was examined (Robert *et al.*, 1962), the calculated M_r value (340 000) was lower than values reported in the literature (Laurent & Gergely, 1955), and they concluded that the conditions of the cyanide addition reaction had caused degradation of the hyaluronate polysaccharide chains. Other studies (Swann, 1968b), however, showed that treatment with alkali caused little or no decrease in the viscosity of rooster-comb hyaluronate. We therefore decided to perform viscosity measurements on bovine vitreous-humour hyaluronate before and after reaction with [^{14}C]cyanide to determine if indeed this reaction causes degradation of hyaluronate.

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Materials and methods

Chemical methods

The uronic acid content of samples and column fractions was determined by using a carbazole reaction (Bitter & Muir, 1962), and hyaluronate concentrations were calculated from the uronic acid values by multiplying by 2.06. Glucosamine and amino acids were determined with an amino acid analyser as previously described (Swann & Mintz, 1979). Radioactivity measurements were performed on 100 μ l portions of samples, mixed with 3.5 ml of Bio-Fluor™ (New England Nuclear, Boston, MA, U.S.A.), with a liquid-scintillation counter.

Preparation of bovine vitreous-humour hyaluronate

Hyaluronate was prepared from pooled adult bovine vitreous humour. Eyes were collected from freshly killed adult (5–12-year-old) cattle and transported to the laboratory on ice. After equatorial section of the globe the posterior-peripheral vitreous humour was removed with forceps. The 'residual protein' fraction (Pirie *et al.*, 1948) was removed by centrifugation [at 125 000 g (r_{av} , 6.3 cm) for 30 min at 4°C], and hyaluronate was isolated from the supernatant fraction by precipitation with cetylpyridinium chloride (Scott, 1960), solubilization of the cetylpyridinium chloride-hyaluronate precipitate with 0.3 M-NaCl followed by centrifugation [at 125 000 g (r_{av} , 6.3 cm) for 60 min at 4°C] and precipitation with ethanol (Swann, 1968a). The hyaluronate was then dissolved in distilled water and fractionated by CsCl-density-gradient sedimentation (Silpananta *et al.*, 1967). The hyaluronate fraction obtained by density-gradient sedimentation was re-fractionated by gel-permeation chromatography on a Bio-Gel A-50m (Bio-Rad Laboratories, Richmond, CA, U.S.A.) column (100 cm $\times$ 4.5 cm) eluted with 1 M-NaCl/50 mM-sodium phosphate buffer, pH 7.2 (Fig. 1a). The column fractions indicated in Fig. 1(a) were pooled, dialysed extensively against distilled water and freeze-dried to yield hyaluronate fraction HA-I. Two samples of fraction HA-I prepared by the above sequence of fractionation steps were used. These are labelled preparations HA-IA and HA-IB for convenience.

Preparation and fractionation of oligosaccharides

Oligosaccharides were prepared from hyaluronate preparation HA-IA by partial digestion with an excess amount of hyaluronidase (HSEP; Worthington Biochemicals, Freehold, NJ, U.S.A.). Preparation HA-IA (200 mg) was dissolved in 5 ml of 0.1 M-sodium acetate buffer, pH 5.0, containing 0.1 M-NaCl and incubated with 3745 units of enzyme (activity as defined in the Worthington Manual) for 5 h at 37°C. The digestion mixture was fractionated by gel-permeation chromatography on

Sephadex G-50 (superfine grade) (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) column (200 cm $\times$ 2.5 cm) equilibrated and eluted with 0.1 M-acetic acid (Fig. 2). The oligosaccharide fractions A and B indicated in Fig. 2 were pooled, freeze-dried and rechromatographed on the same Sephadex G-50 column. The A and B fractions were again pooled and freeze-dried. Fraction A was characterized as a hexasaccharide and fraction B as a tetrasaccharide by paper chromatography (Weissman *et al.*, 1954) and by gel chromatography on an analytical Bio-Gel P-4 column (112 cm $\times$ 1.0 cm) eluted with 0.1 M-ammonium formate buffer, pH 6.8.

Reaction with $K^{14}CN$

The samples of preparations HA-IA and HA-IB and oligosaccharides were dissolved in 0.2 M- $Na_2CO_3/NaHCO_3$ buffer, pH 9.1 (buffer A), to give a uronic acid concentration of about 2.5 μ mol/ml of

Fig. 1. Gel-permeation chromatography on a Bio-Gel A-50m column (100 cm $\times$ 4.5 cm) eluted with 1 M-NaCl/50 mM-phosphate buffer, pH 7.2, of the hyaluronate fraction (250 mg) obtained by CsCl-density-gradient sedimentation (a) and 25 mg of [^{14}C]cyanide-labelled preparation HA-IA (b)

For full experimental details see the text. The HA-I fraction indicated in (a) was used in the reaction with $K^{14}CN$. The column fractions indicated in (b) were pooled to yield [^{14}C]cyanide-labelled HA-IA subfractions a-e: ●, A_{530} ; ▲, radioactivity. The arrows marked 1 and 2 indicate the first elution position of Blue Dextran 2000 (Pharmacia Fine Chemicals) and the peak elution position of bovine serum albumin respectively.

Fig. 2. Gel-permeation chromatography of oligosaccharides produced by partial digestion of preparation HA-IA (200mg) with hyaluronidase on a column (200 cm $\times$ 2.5 cm) of Sephadex G-50 (superfine grade) eluted with 0.1 M-acetic acid

For full experimental details see the text. The column fractions indicated were pooled and freeze-dried to yield oligosaccharides A and B. V_0 indicates the elution positions of Blue Dextran 2000 and V_i the elution position of glucuronolactone.

solution. The uronic acid content of these solutions was determined, and an equimolar concentration of $K^{14}CN$ (60 $\mu Ci/ml$ in buffer A) was then added. The mixture was incubated at 22°C for 36 h, after which time unlabelled KCN (equivalent to 4 times the uronic acid concentration) in buffer A was added. After an additional 12 h the reaction was terminated by addition of acetic acid to give a pH of 4.0.

Fractionation of $[^{14}C]$ cyanide-labelled hyaluronates and oligosaccharides

In Expt. I hyaluronate preparation HA-IA (25 mg) and tetrasaccharide (6 mg) were used. The $[^{14}C]$ cyanide-labelled preparation HA-IA was separated into subfractions by chromatography of the acidified reaction mixture on the Bio-Gel A-50m column (100 cm $\times$ 4.5 cm) eluted with 1 M-NaCl/50 mM-sodium phosphate buffer, pH 7.2 (Fig. 1b). After determination of the distribution of uronic acid and radioactivity in the column effluent, the column samples were pooled to obtain five subfractions a-e (Fig. 1b). The subfractions a-e were dialysed extensively against distilled water, freeze-dried, and

Fig. 3. Gel-permeation chromatography of the $[^{14}C]$ -cyanide-labelled preparation HA-IB on a column (16 cm $\times$ 1.5 cm) of Sepharose CL-2B eluted with 3 M-guanidinium chloride

For full experimental details see the text. The arrows marked 1 and 2 indicate the first elution position of Blue Dextran 2000 and the peak elution position of bovine serum albumin respectively. ●, A_{530} ; ▲, radioactivity.

then portions were dissolved in water and the content of uronic acid and radioactivity determined. Viscosity measurements were also performed on the HA-IA subfractions a-e. The $[^{14}C]$ cyanide-labelled tetrasaccharide was rechromatographed on the Sephadex G-50 (superfine grade) column and subfraction B (Fig. 2) was freeze-dried. A portion of subfraction B was dissolved in water and the content of uronic acid and the radioactivity were determined.

In Expt. II hyaluronate preparation HA-IB (7 mg) and hexasaccharide (6 mg) were used. The sample of $[^{14}C]$ cyanide-labelled preparation HA-IB was dialysed extensively against distilled water and then fractionated on a column (16 cm $\times$ 1.5 cm) packed with Sephadex CL-2B (Pharmacia Fine Chemicals) and eluted with 3 M-guanidinium chloride. Uronic acid and radioactivity measurements were performed on the individual column fractions (Fig. 3). The $[^{14}C]$ cyanide-labelled hexasaccharide was re-fractionated by chromatography on a column (112 cm $\times$ 1.0 cm) packed with Bio-Gel P-4 and eluted with 0.1 M-ammonium formate buffer, pH 6.8 (Fig. 4).

Fig. 4. Gel-permeation chromatography of the [^{14}C]-cyanide-labelled oligosaccharide (A) on a column (112 cm $\times$ 1.0 cm) of Bio-Gel P-4 eluted with 0.1 M-ammonium formate buffer, pH 6.8

For full experimental details see the text. The arrows marked 1, 2, 3, 4 and 5 indicate the peak elution positions of Blue Dextran 2000, hyaluronate hexasaccharide, hyaluronate tetrasaccharide, hyaluronate disaccharide and glucuronolactone respectively.

Viscosity measurements

Viscosity measurements were performed on hyaluronate solutions that had been dialysed extensively against 0.2 M-NaCl/50 mM-Tris/HCl buffer, pH 7.2. A Cannon-Ubbelohde semi-micro dilution viscometer (solvent flow time 132.6 s) was used to determine the viscosities of serial dilutions of the hyaluronate solutions. The intrinsic viscosity was calculated from a plot of the reduced viscosity versus the hyaluronate concentration by extrapolation to zero concentration.

Results and discussion

When the hyaluronate fraction obtained by CsCl-density-gradient sedimentation was fraction-

ated by gel chromatography (Fig. 1a), uronic acid-containing constituents were distributed throughout the column volume. On rechromatography on the same column, the HA-I fraction was not eluted within the K_{av} limits shown in Fig. 1(a) but was again distributed throughout most of the column volume. The range of K_{av} values over which hyaluronate fractions were eluted depended on the amount of sample applied to the column. This indicates that there was considerable interaction between hyaluronate molecules and that chromatography on the Bio-Gel column, when a sample of 200 mg was applied, achieved only a partial molecular-weight fractionation. The HA-I fractions used in the labelling experiments therefore probably contained a similar distribution of molecules to those of the density-gradient hyaluronate fractions. Analysis of the freeze-dried HA-IA preparation showed that the uronic acid content was 47.2% (w/v), the glucosamine content was 44.6% (w/w) and total amino acid content was 1.8% (w/w). The intrinsic viscosities of the HA-IA and HA-IB preparations were 200 ml/g and 192 ml/g respectively.

The reaction with K^{14}CN was performed essentially as described by Robert *et al.* (1962) under conditions where the reaction with hyaluronate hexasaccharide was shown to proceed to completion. To minimize contamination of the labelled products with free [^{14}C]cyanide, additional unlabelled KCN was added to the reaction mixture, and after acidification the labelled products were re-isolated by column chromatography. The reaction was also performed with constant ratios of [^{14}C]cyanide to uronic acid for both the hyaluronate and reference oligosaccharides.

These reaction conditions were chosen because hyaluronate samples are polydisperse and contain different concentrations of individual molecules. The ratio of [^{14}C]cyanide to end groups and the rates of reaction therefore will vary for different molecules in the mixture. Experiments with mixtures of oligosaccharides (obtained by digestion with testicular hyaluronidase, labelled with an equimolar ratio of [^{14}C]cyanide to uronic acid and then isolated by gel chromatography), however, showed the expected ratios of incorporated radioactivity. This indicates that the relative uptake of [^{14}C]cyanide did not depend on the varied proportions of oligosaccharides and rates of reaction. It is therefore to be expected that polydisperse hyaluronate molecules will react in a similar manner.

After reaction with K^{14}CN , the HA-IA preparation was eluted over a large portion of the column volume (Fig. 1b). Radioactivity and uronic acid measurements showed that the ratio of radioactivity (c.p.m.) per μg of uronic acid increased with increasing K_{av} , and the column effluent was divided

Table 1. *Viscosity measurements and incorporation of [^{14}C]cyanide into hyaluronate and oligosaccharide fractions*
 For full experimental details see the text. For Expt. II the uronic acid values expressed as percentages of the total recovered in fractions 12–19 are given in parentheses in the column headed 'Radioactivity incorporated'. Molecular weights based on viscosity measurements were calculated by using the formula $[\eta] = 0.036 M^{0.78}$ (Laurent *et al.*, 1960); those based on [^{14}C]cyanide labelling were calculated by using values of 820 and 1221 for the molecular weight of the tetrasaccharide (preparation HA-IA subfractions) and hexasaccharide (preparation HA-IB chromatography fractions) respectively.

Expt. I Prepn. HA-IA subfractions (Fig. 1b)	Intrinsic viscosity (ml/g)	Radioactivity incorporated (ng of uronic acid/c.p.m.)	$10^{-3} \times \text{Calc. } M_r \text{ value}$	
			From $[\eta]$ measurement	From [^{14}C]cyanide labelling
a	406	188.5	158	155
b	273	89.1	94	76
c	182	83.1	56	68
d	161	58.3	48	48
e	113	43.4	30	36
Tetrasaccharide		1.0		0.82
Expt. II				
Prepn. HA-IB chromatography fractions (Fig. 3)				
12		216.5 (2.9)		264
13		107.0 (7.1)		131
14		75.0 (14.5)		92
15		53.0 (22.2)		65
16		38.8 (23.5)		47
17		25.3 (19.9)		31
18		11.6 (8.2)		14
19		7.3 (1.6)		9
Hexasaccharide		1.0		1.22

up to yield five subfractions (Fig. 1b). The plots of reduced viscosity versus concentration obtained with these subfractions were linear and gave intrinsic-viscosity values of 406 to 113 ml/g. By use of the formula derived by Laurent *et al.* (1960), the calculated molecular weights of the subfractions a–e ranged from 158 000 to 30 000 (Table 1). The molecular weights of these subfractions calculated from the ratio of radioactivity incorporated into the hyaluronate fractions to incorporation into the tetrasaccharide (M_r 820) ranged from 155 000 to 36 000.

Similar findings were obtained in Expt. II when the [^{14}C]cyanide-labelled HA-IB preparation was fractionated on a Sepharose CL-2B column (Fig. 3) and the molecular weights were calculated from the ratio of radioactivity incorporated into the hyaluronate fractions to incorporation into hexasaccharide (M_r 1221). In this case analyses were performed on the individual column fractions and a wider spread of molecular weights (264 000 to 9000) was obtained. The molecular weight calculated from the [^{14}C]cyanide uptake into the different column fractions and the percentage of total uronic acid in each fraction (Table 1) was 63 200.

The accuracy of the M_r values calculated from uptake of [^{14}C]cyanide depends on five main factors: (1) the errors involved in uronic acid and radio-

activity measurements, (2) the ratio of [^{14}C]cyanide specific radioactivity to uronic acid being identical for both the hyaluronate and reference oligosaccharide samples, (3) the identity and homogeneity of the oligosaccharides, (4) the reaction conditions used did not degrade the hyaluronate polysaccharide chains and (5) the assumption that the end groups of the hyaluronate and oligosaccharide samples react in a similar manner.

The errors in points (1) and (2) above were minimized by paying attention to the analytical procedures. The identities of the tetrasaccharide used in Expt. I and the hexasaccharide used in Expt. II were determined by their elution positions after gel chromatography and their mobilities when analysed by paper chromatography (Weissman *et al.*, 1954). The labelled oligosaccharides were re-fractionated by gel chromatography to ensure minimal contamination with adjacent lower and higher oligomers. The homogeneity of the hexasaccharide was also confirmed by the constant ratio of radioactivity to uronic acid when it was analysed by chromatography on the analytical Bio-Gel P-4 column (Fig. 4). The ratio of radioactivity (c.p.m.) per μg of uronic acid for four consecutive hexasaccharide-containing fractions eluted from the Bio-Gel P-4 column were 2895, 2851, 2903 and 2866.

The question of degradation was addressed by

performing viscosity measurements before and after reaction with $K^{14}CN$. Before reaction the intrinsic viscosity of preparation HA-IB was 192 ml/g, compared with a value of 187 ml/g for the $[^{14}C]$ -cyanide-labelled sample. These viscosity values are identical within experimental error and give a calculated M_r of approx. 59 000 (Laurent *et al.*, 1960). The molecular weight of labelled preparation HA-IB calculated from the cyanide-uptake data (Table 1) was 63 200. On the basis of these measurements the reaction with cyanide did not cause depolymerization of the hyaluronate polysaccharide chains. It is more difficult to test the assumption raised in point (5) above, but the fact that the molecular weights calculated from the $[^{14}C]$ -cyanide-uptake data with oligosaccharides as reference substances agree closely with the values calculated from viscosity measurements indicates that it is a valid one. It seems therefore reasonable to conclude that reaction with $[^{14}C]$ -cyanide does not cause degradation of hyaluronate polysaccharide chains and that this reaction can be used to determine the number of polysaccharide chain end groups in bovine vitreous-humour hyaluronate.

General discussion

A broad range of molecular-weight values has been reported for bovine vitreous-humour hyaluronate. Varga (1955) calculated a value of 86 600 based on sedimentation and diffusion measurements, whereas light-scattering studies (Laurent, 1955) gave molecular weights of 340 000 to 500 000. In later experiments values of 178 000 (Varga *et al.*, 1959a) and 289 000 (Varga *et al.*, 1959b) were obtained. Rowen *et al.* (1956) reported a molecular weight of 1.27×10^6 , and Laurent *et al.* (1960) obtained different hyaluronate fractions with molecular weights that ranged from 77 000 to 1.7×10^6 . Further evidence for the occurrence of discrete types of hyaluronate from both the central and cortical vitreous humour was reported by Berman (1963). It can be concluded from these earlier studies that bovine vitreous-humour hyaluronate has a lower molecular weight than has hyaluronate purified from other tissues (Laurent, 1970), but the reason for the variations reported is not clear. The calculated molecular weights obtained in the present study again showed a wide variation of chain lengths (Table 1), but we did not

detect the presence of discrete types (Berman, 1963) or high-molecular-weight components (Laurent *et al.*, 1960). The fractionation procedure used in the present work, however, may have eliminated the occurrence of high-molecular-weight molecules from the samples used in the labelling reaction. Our findings indicate that the samples of purified bovine vitreous-humour hyaluronate were composed of a polydisperse population of molecules with molecular weights in the range 9000–264 000.

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