Some Studies on the Relationship between Sialic Acid and the Mucopolysaccharide–Protein Complexes in Human Cartilage

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An earlier study showed that extraction of human cartilage with mild alkali yielded preparations containing sialic acid, in addition to chondroitin sulphate–protein (chondromucoprotein) complexes (Anderson, 1961*a*). Treatment of these preparations by mild chemical and enzymic procedures designed to reduce the nitrogen content failed to produce mucopolysaccharides completely free of protein and sialic acid. The further observation that sialic acid was present in electrophoretically homogeneous chondroitin sulphate A–peptide complexes obtained by Muir (1958) from pig trachea and human aorta, by methods specifically designed to remove contaminating material, suggested that sialic acid may be an integral part of the chondromucoprotein molecule. The possibility, however, that sialic acid in chondromucoprotein preparations may be a constituent of contaminating glycoprotein material of the type known to occur in serum (Winzler, 1958) and urine (Anderson, Lepper & Winzler, 1960) must be considered. There is no reason to believe that human-chondromucoprotein preparations are homogeneous. Johnson & Schubert (1960), for instance, have found that human nasal-cartilage chondromucoprotein can be fractionated ultracentrifugally into two distinct fractions.

The glycoprotein constituents, mannose, galactose, fucose and glucosamine, have been detected in cartilage from non-human sources (Hisamura, 1938; Glegg, Eidinger & Leblond, 1954; Glegg & Eidinger, 1955; Herring & Kent, 1958), and Bertelsen (1960) has reported the presence of glycoproteins in human aorta. The probable presence of sialic acid in these preparations was not investigated. These preparations may be artifacts since they were obtained by extraction of cartilage with strong alkali. Such conditions lead to degradative changes in chondromucoprotein and, as this present paper shows, to partial cleavage of sialic acid and probably other constituents.

This paper deals with the extraction of viscous chondromucoprotein preparations from human cartilage under mildly alkaline conditions which do not lead to marked solubilization of collagen or nucleic acids. Some properties of this crude fraction are reported, together with evidence of marked heterogeneity obtained by chromatography on ion-exchange cellulose. Sialic acid was detected in the fractions obtained and cleavage of this substance was observed with acid, alkali and neuraminidase. Experiments are also described which indicate that the nature of the human chondromucoprotein preparations is markedly altered after treatment with proteolytic enzymes and hyaluronidase. Some aspects of this work have been reported (Anderson, 1961*b*).

METHODS AND MATERIALS

Analytical methods

Total nitrogen, total hexose, sulphate, hexuronic acid and protein were determined as described by Anderson (1961*a*). Hexosamine was determined by the method of Elson & Morgan (1933), as described by Winzler (1955), hydrolysis being carried out in 4N-HCl for 4 hr. at 100°. Hydroxyproline was determined by the method of Neuman & Logan (1950), hydrolysis being carried out in 6N-HCl for 3 hr. at 100°.

The presence of sialic acid in the cartilage fractions was investigated as follows. *N*-Acetylneuraminic acid was used as the standard.

(1) The acid diphenylamine reaction of Dische (1947) was employed directly on the fractions, as described by Anderson (1961*a*).

(2) The fraction (15 mg.) was heated with 0·01N-HCl (6 ml., 80°, 1 hr.). The hydrolysate was dialysed against water (10 ml.) for 24 hr., the dialysis being repeated twice. The diffusates (30 ml.) were combined, freeze-dried, the residue was taken up in water (5 ml.) and sialic acid determined on 1 ml. of the solution, the diphenylamine reaction being used.

(3) The fraction (20 mg.) was heated with 0·1N-H_2SO_4 (5 ml., 80°, 1 hr.), the hydrolysate centrifuged and the liberated sialic acid freed of interfering substances with an anion-exchange resin (Svennerholm, 1958). It was then estimated by the resorcinol reaction (Svennerholm, 1957) and the diphenylamine reaction.

(4) (*a*) Total sialic acid: the fraction (containing 20–100 μg. of sialic acid) was heated with 0·1N-H_2SO_4 (2 ml., 80°, 1 hr.). Sialic acid was determined in 0·2 ml. of the hydrolysate by the thiobarbituric acid method of Warren (1959). (*b*) Free sialic acid: the fraction was dissolved in water and sialic acid estimated directly in portions (0·2 ml.) without preliminary hydrolysis. (*c*) Bound sialic acid: the

bound sialic acid in the fraction was calculated by subtracting the free sialic acid from the total sialic acid.

Unless otherwise stated, all analytical values for sialic acid were obtained with the thiobarbituric acid reaction.

Neuraminidase. Three sources of neuraminidase were used. (1) Receptor-destroying enzyme (RDE) from *Vibrio cholerae*, bought in portions (25 ml.) from Wellcome Research Laboratories, Beckenham, Kent, England. This enzyme solution was used without dilution. (2) Freeze-dried filtrate containing RDE from *V. cholerae*, bought from Philips-Duphar, Amsterdam, Holland. One ampoule was dissolved in calcium acetate buffer (50 mM, pH 5·5, 5 ml.). (3) Crystalline neuraminidase. One ampoule contained 30 000 units of biological activity, as defined by French & Ada (1959) and Ada, French & Lind (1961).

Ultracentrifuging. Cartilage fractions, at a concentration of 0·5–1·0% (w/v) in 0·2M-NaCl, were examined in a Spinco model E analytical ultracentrifuge at 52 000 rev./min. Sedimentation coefficients were corrected to $S_{20,w}$ values.

Tissue preparations

Disintegration of cartilage. Human cartilage was disintegrated in a mill in which the grinding chamber was surrounded by a jacket containing liquid nitrogen (Anderson, 1961*a*). The disintegrated cartilage was stored at −20° until used.

Fractionation of disintegrated cartilage. Two methods of fractionation were employed. (*a*) The method described previously for the isolation of fraction A was followed (Anderson, 1961*a*), with the same mixture of human cartilage (articular 28%, semilunar 12%, costal 28% and tracheal 32%). Extraction was carried out in a glycine buffer (0·1M, pH 11·5), the final extracting pH being 10·6. The yield was 2·92 g./100 g. of wet cartilage. Analytical data and some properties of this fraction have been reported (Anderson, 1961*a*).

(*b*) Human cartilage, from a mixture obtained from articular (29%), semilunar (18%), costal (2%) and tracheal (51%) sources was disintegrated at the temperature of liquid nitrogen as described above. Portions (50 g.) of disintegrated cartilage were suspended in a glycine buffer (0·1M, pH 11·0, 250 ml.) and stirred mechanically at 4° for 24 hr. The final extracting pH was 10·1. After centrifuging, the supernatant fluid was adjusted to pH 7 and retained and the extraction repeated twice on the residue with the same volume of glycine buffer. The resulting residue was retained. The three neutral supernatant fluids were combined, filtered, dialysed against water for 5 days with frequent changes and freeze-dried. This product was termed fraction 1A. The yield was 1·40 g./100 g. of wet cartilage. The residue mentioned above was then suspended in 0·5N-NaOH (1 g. of residue to 3·6 ml. of alkali). After mechanical stirring overnight the final extracting pH was 12·8. The residue was centrifuged down and the supernatant fluid adjusted to pH 7 with acetic acid and retained. The extraction was repeated twice more on the residue with the same amount of alkali. The three neutral supernatant fluids were combined, filtered, dialysed against water for 5 days with frequent changes and freeze-dried. This produce was termed fraction 1B. The yield was 1·53 g./100 g. of wet cartilage.

All the cartilage fractions were stored over $CaCl_2$ *in vacuo* at 4°.

Ion-exchange chromatography of cartilage fractions

Chromatography was carried out with the procedure developed by Sober, Gutter, Wyckoff & Peterson (1956), diethylaminoethylcellulose (DEAE-cellulose) being used.

Preparation of diethylaminoethylcellulose. DEAE-cellulose (0·94 m-equiv. of ionizing groups/g., obtained from Brown Co., Berlin, New Hampshire, U.S.A.) was washed successively with N-NaOH, N-HCl and N-NaOH, filtration under suction being used each time. After washing to neutrality with water it was washed with buffer 1 (Table 1), the buffer in which the cartilage fraction was applied to the column (see below). The DEAE-cellulose was then poured as a slurry into a glass tube, the lower end of which contained a layer of glass wool. The column of cellulose, after it had settled by gravity, was further compacted to two-thirds of the original length by application of suction, from a water pump, to the lower outlet of the tube. The choice of buffers was determined by the following observations. When fraction A was applied to the column at pH 8·6 in a tris–citrate buffer (5 mM-citric acid adjusted to pH 8·6 with tris) about 40%, by wt., did not adsorb. This unadsorbed fraction was highly viscous and contained 8·7% of hexuronic acid. Adsorbed components of fraction A could be eluted step-wise with tris–citrate buffers of decreasing pH, and this elution was found to be sensitive to changes in the ionic strength of the eluting buffers. The following procedure was therefore adopted.

The fraction was suspended in buffer 1 (Table 1) at a concentration of about 2% (w/v). After incubation at 37° for 2 hr. the mixture was centrifuged and the residue resuspended in the same volume of buffer. This process was repeated once more. The highly viscous supernatant fluids were combined and enough buffer 1 was added to give a final concentration of about 0·5%. This solution was then applied to the column, when the flow-rate under gravity

Table 1. *Buffers used for the elution of subfractions after the adsorption of human-cartilage fractions on diethylaminoethylcellulose*

The buffers were prepared by adjustment to the appropriate pH with 2M-tris.

Buffer no.	Buffer composition	Buffer pH		Subfraction
		Fraction A	Fraction 1A	
1	5 mM-Citrate	8·6	8·6	1
2	20 mM-Citrate	7·4	7·4	2
3	50 mM-Citrate, 0·3M-NaCl	6·1	6·5	3
4	50 mM-Citrate, 0·7M-NaCl	6·1	6·5	4
5	50 mM-Citrate, 1·0M-NaCl	6·1	6·5	5
	Cellulose extracted with N-NaOH	11·2	11·7	6

decreased from 1·6 to 0·08 ml./min. owing to the high viscosity of the fraction. Buffer 1 was then run through the column and the effluent collected (in 5 or 10 ml. fractions) with a fraction collector at 4°. When the extinction values of the fractions measured in a 1 cm. cell at 280 mμ had dropped to less than 0·02 (when the flow-rate had increased to 1·3 ml./min.), buffer 2 was applied to the column, followed by buffers 3–5 (Table 1). The fractions eluting with buffers 1–5 (termed subfractions 1–5) were pooled, dialysed against water and freeze-dried. After elution with buffer 5 the cellulose was removed as a slurry and N-NaOH added to pH 11·2 (for fraction A) and pH 11·7 (for fraction 1A). The cellulose was filtered off and subfraction 6 obtained from the filtrate after dialysis and freeze-drying.

Viscosity studies

Flow-times of solutions (0·5 %) of the cartilage fractions, in appropriate buffers, were measured at 25° in Ostwald viscometers of 5 ml. capacities and water flow-times between 11 and 12 sec. The percentage decrease in flow-time after addition of the enzyme solution was calculated as follows.

If f_B = flow-time of buffer alone, f_S = flow-time of substrate in buffer, f_E = flow-time of substrate–enzyme mixture, then decrease in flow time (%) = $[100(f_S - f_E)]/(f_S - f_B)$. The effects of the following enzymes on the viscosities of solutions of the cartilage fractions were investigated.

Trypsin. Crystalline trypsin (50 mg., E. R. Squibb and Sons, N.Y.) was dissolved in barbiturate buffer (20 mM, pH 8·2, 10 ml.). A portion (0·2 ml.) of the solution was used for enzyme experiments.

Hyaluronidase. Hyaluronidase (1500 i.u., Bengers Hyalase) was dissolved in phosphate–citrate buffer (150 mM, pH 5·0, 1·0 ml.). A portion (0·4 ml.) of the solution was used for enzyme experiments.

Papain. Crude papain (100 mg., Zimmermann and Co.) was activated by incubating at 45° for 30 min. in acetate buffer (20 mM, pH 5·5, 10 ml.), containing 10 mM-cysteine. Insoluble matter was removed by filtration and 0·2 ml. of the filtrate used for enzyme experiments.

Plasminogen. Plasminogen, prepared from human serum by the method of Kline (1953), was used as a solution in 2·5 mN-HCl. The activity, determined by the caseinolytic method of Norman (1957), was 0·205 *E* unit/ml., the unit of activity defined by Davidson (1960) being used.

Streptokinase. Dornokinase (Burroughs Wellcome and Co.) in Palitzsch borate buffer (pH 7·4, prepared as described by Norman, 1957) was used, at a concentration of 2000 units/ml.

Streptokinase-activated plasminogen. In a subsidiary experiment, by the casein-digestion method of Norman (1957), 0·05 ml. of the streptokinase solution was found to activate fully 0·4 ml. of the plasminogen solution. These optimum amounts were therefore used in enzyme experiments involving activation of plasminogen by streptokinase, streptokinase being added 15 min. after the addition of plasminogen.

EXPERIMENTAL AND RESULTS

Chromatography of cartilage fractions

Fig. 1 shows a typical chromatogram obtained from fraction A with the buffer system shown in Table 1. A similar chromatogram was obtained from fraction 1A. Table 2 shows the composition of fractions A and 1A and of the subfractions (1–6) obtained from fractions A and 1A after chromatography.

The recovery of fractions A and 1A, by weight, was between 70 and 75 %. Subfractions 1–6 comprized, by weight, 48, 3, 3, 12, 2 and 5 % (respectively) of the total amount of fraction A chromatographed. Subfractions 1 and 4 were completely water-soluble, forming strongly opalescent solutions. The other subfractions were almost completely water-soluble and formed clear solutions. A solution of subfraction 1 in water (0·5 %) formed the most highly viscous solution, the flow-time in an Ostwald viscometer (of water flow-time 11·1 sec.) being 109·0 sec. The flow-times of fractions A and 1A, under the same conditions, were 30·2 and 33·4 sec. respectively.

All the subfractions gave positive reactions for sialic acid. Table 3 shows the percentages of sialic acid present in fraction A and subfractions 1 and 4, as determined by the methods described in the Methods and Materials section. Mild acid hydrolysis (0·01 N-HCl, 80°, 1 hr.) of all the subfractions liberated sialic acid quantitatively.

The ultracentrifugal patterns for subfractions 1 and 4, obtained from fraction 1A, are shown in Fig. 2. The two fractions had sedimentation coefficients of 5·07 and 1·57 respectively. Neither peak was symmetrical and both subfractions must be to some extent heterogeneous.

Fraction 1B, fractionated on DEAE-cellulose under the same conditions, gave a similar chro-

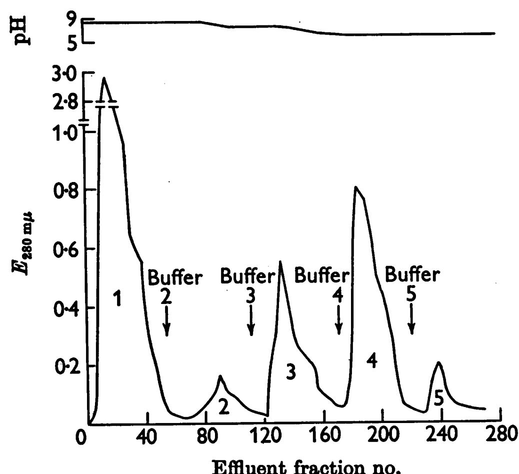

Fig. 1. Chromatogram obtained from the fractionation on DEAE-cellulose of fraction A (1200 mg.) isolated from human cartilage at pH 10·6. The effluent was collected in 5 ml. fractions. The compositions of the eluting buffers are described in Table 1.

Table 2. *Percentage composition of subfractions from fractions A and 1 A obtained by chromatography on diethylaminoethylcellulose*

Percentage concentration of proteins (column 2) was obtained in two ways: (*a*) by the method of Lowry, Rosebrough, Farr & Randall (1951); (*b*) calculated by multiplying the total nitrogen, after subtracting hexosamine N (7·8%) and sialic acid N (4·54%), by 6·25. Percentage concentration of collagen (column 9) was obtained by multiplying percentage concentration of hydroxyproline by 7·15 (assuming collagen contains 14% of hydroxyproline). Sialic acid (column 10) was estimated by the thiobarbituric acid reaction (Warren, 1959).

Fraction	Protein (%) (*a*) A	Protein (%) (*a*) 1A	Protein (%) (*b*) A	Protein (%) (*b*) 1A	Total nitrogen (%) A	Total nitrogen (%) 1A	Hydroxyproline (%) 1A	Collagen (%) 1A	Sialic acid (%) A	Sialic acid (%) 1A	Hexuronide (%) A	Hexuronide (%) 1A	Hexosamine (%) A	Hexosamine (%) 1A	Hexose (%) A	Hexose (%) 1A	Sulphate (%) A	Sulphate (%) 1A
Fraction before chromatography	34·0	33·9	46·0	48·3	8·3	8·9	0·5	3·6	2·1	1·6	8·6	8·8	13·9	14·5	14·6	8·5	10·7	7·6
Subfraction																		
1	25·5	28·6	45·0	44·4	8·4	8·4	0·3	2·1	2·1	0·6	8·7	11·0	14·0	16·3	10·8	8·8	7·0	9·5
2	—	39·6	—	58·8	—	9·7	0·1	0·7	—	0·8	—	0	—	2·6	—	4·7	—	3·2
3	—	50·2	—	77·4	—	12·8	0·05	0·4	0·5	1·4	1·2	0·5	1·2	3·8	2·1	6·4	—	5·0
4	18·5	18·8	25·2	35·3	5·6	7·1	0·05	0·4	1·4	1·2	18·1	12·6	18·8	17·6	11·4	8·9	10·5	10·8
5	—	14·0	—	23·3	—	4·3	0·01	0·1	1·0	1·3	0·8	3·2	4·0	6·6	5·3	8·9	—	4·7
6	—	28·4	—	37·2	—	6·3	0·1	0·7	1·0	0·8	2·0	2·4	3·8	4·0	5·1	5·9	—	4·5

matogram to fractions A and 1A, except that the highly viscous subfraction 1 was absent, indicating that all of fraction 1B was adsorbed. A solution of fraction 1B in water (0·5%) had a flow-time of only 13·7 sec., indicating the presence of only low-viscosity components. Fraction 1B contained 0·17% of hydroxyproline and 1·9% of sialic acid. There was no evidence, from the thiobarbituric acid reaction, for the presence of 2-deoxyribose. The presence of a component in fraction 1B rich in sialic acid and low in hexuronic acid was shown as follows. Fraction 1B (900 mg.) was dissolved in water (90 ml.) and ethanol solution added to 63% (v/v). The precipitate was centrifuged down, dissolved in water and after dialysis was reprecipitated by the addition of ethanol solution (63%, v/v). The precipitate was centrifuged down, washed with ethanol and ether and dried *in vacuo*. The yield was 648 mg. Analysis: sialic acid, 1·4; hexuronic acid, 9·6%. Ethanol solution was added to the supernatant fluid (to 83%, v/v) and the precipitate centrifuged down, dissolved in water, and

Table 3. *Estimation of sialic acid in fraction A and in subfractions 1 and 4, obtained by chromatography of fraction A on diethylaminoethylcellulose*

The following methods were used: (*a*) the diphenylamine reaction of Dische (1947), as described by Anderson (1961*a*); (*b*) and (*c*) the diphenylamine and resorcinol (Svennerholm, 1957) reactions respectively after hydrolysis and purification of the freed sialic acid by the method of Svennerholm (1958); (*d*) the thiobarbituric acid method of Warren (1959).

Fraction	Sialic acid (%) (*a*)	(*b*)	(*c*)	(*d*)
Fraction A	3·7	2·7	2·2	2·1
Subfraction 1	3·3	4·0	1·2	2·1
Subfraction 4	3·1	2·1	0·8	1·4

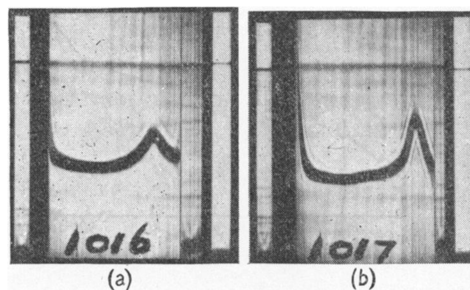

Fig. 2. Sedimentation patterns of cartilage fractions isolated from fraction 1A on DEAE-cellulose. Speed 52 000 rev./min. The fractions were suspended in 0·2M-NaCl, incubated at 37° for 3 hr. and the small amount of insoluble material was removed by centrifuging. Sedimentation (right to left) for: (*a*) subfraction 1 (0·5%) after 40 min.; (*b*) subfraction 4 (1·0%) after 87 min.

the solution was dialysed and precipitation repeated at the same ethanol concentration. The yield was 14 mg. Analysis: sialic acid, 4·0; hexuronic acid, 2·5 %.

Effect of enzymic treatment on the viscosity of solutions of cartilage fractions

The effects of papain, trypsin and hyaluronidase on the flow-times of solutions of fraction A and subfraction 1 are shown in Fig. 3. The effects of plasminogen and streptokinase-activated plasminogen on fraction A and subfraction 1, together with the slight inhibitory effect of ϵ-aminocaproic acid on the activation of plasminogen by streptokinase, are shown in Fig. 4.

Removal of sialic acid from cartilage fractions by neuraminidase

Fraction 1*A*. Method (*a*). Fraction 1 A (12·5 mg.) was dissolved in calcium acetate buffer (50 mM, pH 5·5, 2·5 ml.) and 0·2 ml. of *V. cholerae* filtrate (Philips-Duphar) was added. The mixture was incubated at 37°. Portions (0·2 ml.) were removed at intervals and added immediately to the periodate–phosphoric acid mixture (0·1 ml.) used in the thiobarbituric acid reaction, to inactivate the enzyme. Free sialic acid was then determined. The rate of release of sialic acid is shown in Fig. 5.

A similar experiment showed that crystalline neuraminidase (800 units) released 92 % of the sialic acid from 1·5 mg. of subfraction 1, obtained from fraction 1 A by chromatography on DEAE-cellulose, after incubation for 18 hr. at 37°.

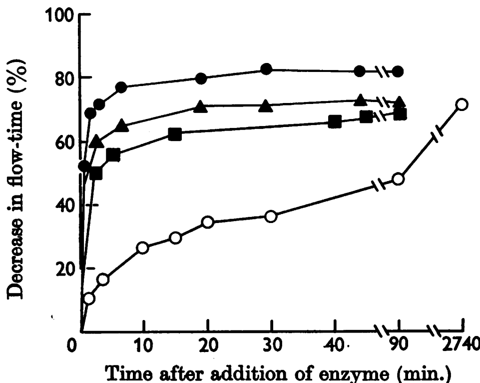

Fig. 3. Effect of enzymes at 25° on flow-times measured in an Ostwald viscometer of solutions (0·5 %) of fraction A and subfraction 1, isolated from fraction A by chromatography on DEAE-cellulose: ●, subfraction 1 plus papain; ▲, fraction A plus papain; ■, subfraction 1 plus trypsin; ○, fraction A plus hyaluronidase. The buffers and quantities of enzymes used are described in the Methods and Materials section.

Method (*b*). Three tubes were set up, each containing fraction 1 A (15 mg., 240 μg. of sialic acid) in calcium acetate buffer (5 ml.). To tube 1 was added 0·6 ml. of RDE (Wellcome Research Laboratories); to tube 2, 0·6 ml. of RDE after heat-treatment (100°, 10 min.); to tube 3, 0·6 ml. of water. The contents of the tubes were incubated at 37° for 5 hr., dialysed exhaustively against water and the resulting volumes were made up to 9 ml. with water. Portions (3 ml.) from each tube were

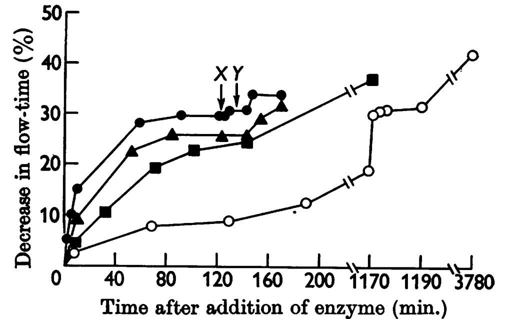

Fig. 4. Effect of human plasmin and plasminogen at 25° on flow-times, measured in an Ostwald viscometer, of solutions (0·5 %) of fraction A and subfraction 1, isolated from fraction A by chromatography on DEAE-cellulose: ●, subfraction 1 plus plasminogen, activated at 0 min. with streptokinase; ▲, fraction A plus plasminogen, activated at 0 min. with streptokinase; ■, subfraction 1 plus plasminogen in the presence of 0·05 M-ϵ-aminocaproic acid, activated at 0 min. with streptokinase; ○, subfraction 1 plus plasminogen, activated at 1170 min. with streptokinase. To the incubation mixtures, represented by ● and ▲, a second equal addition of plasminogen was made after 122 min. (at *X*), followed by a second equal addition of streptokinase after a further 12 min. (at *Y*). The buffers and quantities of enzymes used are described in the Methods and Materials section.

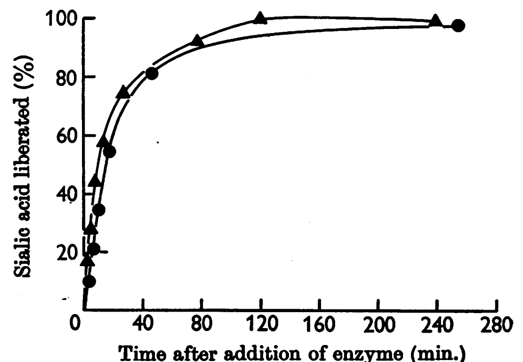

Fig. 5. Cleavage of sialic acid from chondromucoprotein by RDE at 37°: ●, fraction 1 A from human cartilage; ▲, chondroitin sulphate A–peptide complex from pig tracheal cartilage (Muir, 1958).

taken, N-H_2SO_4 (0·33 ml.) was added and the mixtures were hydrolysed (80°, 1 hr.). The free sialic acid in portions (0·2 ml.) of the hydrolysates (equivalent to 0·33 mg. of fraction 1A) was then estimated. Absorption curves of the chromogens obtained are shown in Fig. 6. The absorption curves given by fraction 1A without enzyme addition (tube 3) and with addition of heated enzyme (tube 2) were identical. Incubation of fraction 1A with unheated enzyme followed by dialysis (tube 1) gave an absorption curve very similar to 2-deoxyribose, indicating that if fraction 1A contains 2-deoxyribose the amount present (based on readings of E at 520 mμ) must be less than 0·04%. Calculation for sialic acid in 15 mg. of fraction 1A after treatment with unheated and heated enzyme followed by dialysis (based on readings of E at 535 mμ) gave 23 and 195 μg. respectively.

Effect on viscosity of subfraction 1. Subfraction 1 (25 mg., 150 μg. of sialic acid), obtained from chromatography of fraction 1A, was dissolved in calcium acetate buffer (5 ml.). The flow-time at 25° in an Ostwald viscometer (of buffer flow-time 11·3 sec.) was 16·9 sec. A portion (0·4 ml.) of RDE (Wellcome Research Laboratories) was then added and the contents were mixed. After incubation at 25° for 21 hr. the flow-time had decreased to 15·5 sec. A subsidiary experiment indicated that this decrease in flow-time was no greater than the

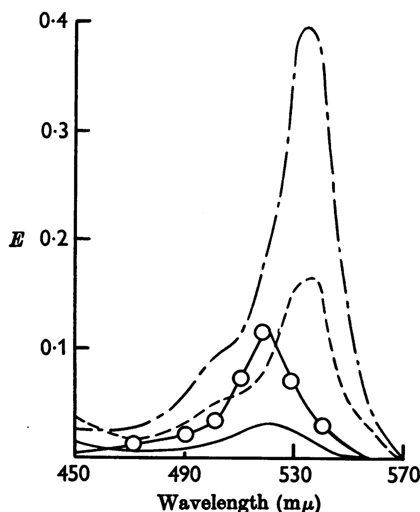

Fig. 6. Absorption curves of the chromogens formed by fraction 1A in the thiobarbituric acid reaction after: —, treatment with unheated RDE; – – –, treatment with heated RDE (100°, 10 min.). Before estimation, both incubation mixtures were dialysed exhaustively against water. -·-·-, N-acetylneuraminic acid (10 μg.); o–o–o, 2-deoxyribose 0·5 μg.).

spontaneous decrease observed after incubation of the untreated fraction in buffer alone. The amount of free sialic acid present after this time-interval in the presence of RDE was estimated at 160 μg.

Chondroitin sulphate A–peptide complex. The chondroitin sulphate A–peptide complex isolated from pig tracheal cartilage (Muir, 1958) was used. The complex contained 0·55, 0·44 and 0·48% of sialic acid (as N-acetylneuraminic acid) as estimated in the diphenylamine, resorcinol and thiobarbituric acid reactions respectively. The complex (30 mg.) was dissolved in calcium acetate buffer (4 ml.). A portion (1 ml.) of RDE (Wellcome Research Laboratories) was added and the mixture incubated at 37°. Portions (0·2 ml.) were removed at intervals and the free sialic acid was estimated. The rate of release of sialic acid is shown in Fig. 5. The absorption curve of the released chromogenic material over the range 450–570 mμ was identical with the chromogen produced by N-acetylneuraminic acid.

Dissociation of human chondromucoprotein in cartilage fractions by mild acid and alkaline hydrolysis

Subfraction 1, obtained by treatment of fraction 1A with DEAE-cellulose, was used. The fate of sialic acid, various chemical methods being used to dissociate chondromucoprotein, was investigated.

Hot acid hydrolysis. The method used by Partridge & Davis (1958) was followed. Subfraction 1 (40 mg.) was dissolved in water (4 ml.). Acetic acid (0·06 ml.) and picric acid (1%, w/v; 0·6 ml.) were added and the clear solution was heated (90°, 6 hr.). On cooling, the yellow flocculent precipitate of protein–picrate was centrifuged down and the supernatant fluid retained. The precipitate was washed with acetic acid–picrate mixture, dissolved in water (2 ml.), the solution adjusted to pH 9 and ethanol (5 vol.) added. After adjustment to pH 5 with acetic acid the flocculent protein precipitate that formed was centrifuged down, dissolved in water and the reprecipitation repeated twice to free the protein from picric acid. Yield: 8·6 mg. Analysis: hexuronic acid 2·3; sialic acid, 0%. The original supernatant fluid was adjusted to pH 5, solid NaCl added to 0·2 M and ethanol (5 vol.) added. The precipitate of mucopolysaccharide was centrifuged down, dissolved in water and the precipitation repeated twice. Yield: 15·0 mg. Analysis: hexuronic acid, 21·9; sialic acid, 0%.

Cold acid hydrolysis. Subfraction 1 (30 mg.) was dissolved in 0·5 N-HCl (3 ml.) and the solution incubated at 25°. Portions (0·2 ml.) were removed at intervals (between 2 and 92 hr.) and caused to react, without preliminary hydrolysis, in the thiobarbituric acid reaction. The absorption curves of the chromogens produced were identical with the

absorption curve produced by *N*-acetylneuraminic acid, indicating release of free sialic acid to the extent of 14 and 49 % after 2 and 92 hr. respectively of the total bound amount originally present. A subsidiary experiment showed that the flow-time in an Ostwald viscometer at 25° (of water flow-time 12·0 sec.) of a solution of subfraction 1 in water (0·5 %) dropped from 102·3 to 16·2 sec. in 5 min. after addition of HCl to 0·5 N, indicating extensive dissociation of the chondromucoprotein.

Hydrolysis with potassium hydroxide. The method used by Partridge & Elsden (1961) was followed. Subfraction 1 (80 mg.) was dissolved in 0·5 N-KOH (8 ml.) and the solution incubated at 25°. The final pH was 12·4. In a subsidiary experiment the drop in viscosity of subfraction 1 under these alkaline conditions was similar to that observed with 0·5 N-HCl reported above. Portions (0·2 ml.) of the incubation mixture were removed at intervals (from 2 to 92 hr.) and caused to react directly, without preliminary hydrolysis, in the thiobarbituric acid reaction. The characteristics of the absorption curves ($E_{max.}$ at 520 mμ) were unlike that produced by *N*-acetylneuraminic acid, indicating that only traces of free sialic acid were split off after this alkaline treatment.

After incubation for 20 hr. at 25°, 5 ml. of the incubation mixture was removed (equivalent to 50 mg. of subfraction 1, containing 300 μg. of sialic acid), the pH adjusted to 7 with acetic acid and the neutral solution dialysed against three 10 ml. portions of water, each for 24 hr. The three diffusates were combined, freeze-dried and the resulting diffusible residue (D) was retained for further investigation. The dialysed solution was then exhaustively dialysed against water and the barium salt of chondroitin sulphate isolated by the method of Malawista & Schubert (1958) as follows. Solid $BaCl_2$ (60 mg.) was added to the dialysate, followed by ethanol to 50 % (v/v). The resulting precipitate of chondroitin sulphate was centrifuged down, washed with ethanol and ether and dried *in vacuo*. Yield: 32 mg. Analysis: sialic acid, 0·24 %. Ethanol (3 vol.) was added to the supernatant fluid, followed by solid NaCl to 0·2 M. The precipitate was centrifuged down, washed with ethanol and ether and dried *in vacuo*. Yield: 4·7 mg. Analysis: sialic acid, 1·1 %. Both these precipitates gave chromogens in the thiobarbituric acid reaction which were identical with that produced by *N*-acetylneuraminic acid.

The recovery of sialic acid in the non-diffusible fraction (129 μg.) was therefore only 43 % of the total amount originally present in subfraction 1. The possibility arose that, although significant amounts of free sialic acid were not cleaved from subfraction 1 by this mild alkaline treatment, bound sialic acid in a diffusible form, which would not react directly in the thiobarbituric acid reaction, might be present. This was investigated as follows. The freeze-dried diffusate (D) was dissolved in 3 ml. of calcium acetate buffer (50 mM, pH 5·5). Three tubes were set up in duplicate. Tube 1: 0·8 ml. of diffusate solution, 0·4 ml. of water. Tube 2: 0·8 ml. of diffusate solution, 0·4 ml. of RDE (Wellcome Research Laboratories). Tube 3: 0·8 ml. of calcium acetate buffer, 0·4 ml. of RDE. The contents of each tube were incubated at 37° for 5 hr. Portions (0·4 ml.) were then made to react, without preliminary hydrolysis, in the thiobarbituric acid reaction. The control tube 3 gave no chromogen in the reaction. Inspection of the absorption curve given by the diffusate in the absence of RDE (tube 1) showed negligible amounts of free sialic acid. After treatment of the diffusate with RDE (tube 2), however, the absorption curve was identical with that produced by *N*-acetylneuraminic acid. Subtraction of the extinctions of the chromogens formed in the reaction mixture in the absence of RDE (tube 1) from the extinctions formed after enzyme treatment (tube 2) is a measure of the bound diffusible sialic acid. From the readings of E at 535 mμ this calculation showed that 48 μg. of bound sialic acid was present in the total diffusate. The amount of bound diffusible sialic acid released from 50 mg. of subfraction 1 by mild alkaline treatment (0·5 N-KOH, 25°, 20 hr.) was therefore 16 % of the total.

The total recovery of sialic acid in the diffusible and non-diffusible components of subfraction 1 after this alkaline treatment was therefore 59 %. A subsidiary experiment showed that *N*-acetylneuraminic acid, subjected to these alkaline conditions, lost 18 % of its chromogenic power in the thiobarbituric acid reaction, which probably accounts for part of the low recovery of 59 %.

Hydrolysis with barium hydroxide. Subfraction 1 (40 mg., 240 μg. of sialic acid) was dissolved in barium hydroxide (10 %, w/v; 4 ml.) and the solution incubated for 20 hr. at 25°. The final pH was 12·4. The mixture was then dialysed against three 10 ml. portions of water, each for 24 hr. The diffusates were combined and freeze-dried (diffusate D). After prolonged dialysis against water the non-diffusible material was precipitated by the addition of ethanol (4 vol.). After centrifuging, the precipitate was washed with ethanol and ether and dried *in vacuo*. Yield: 31 mg. Analysis: sialic acid, 0·52 %. This represented 67 % recovery of the total bound sialic acid originally present in subfraction 1. The possibility that the freeze-dried diffusate (D) contained neuraminidase-susceptible bound sialic acid was investigated as described above ('Hydrolysis with potassium hydroxide'). Treatment of the freeze-dried diffusate with RDE showed that 15 % of the sialic acid in subfraction 1 was in fact

released in a neuraminidase-susceptible bound diffusible form after treatment with barium hydroxide.

DISCUSSION

The method used in this study for isolating human chondromucoprotein resulted in a product containing only traces of deoxyribonucleic acid and collagen, since only small amounts of deoxyribose (Fig. 6) and hydroxyproline (Table 2) were detected. Chromatography on DEAE-cellulose of the crude human chondromucoprotein preparations showed evidence of marked heterogeneity (Fig. 1). A highly viscous component (subfraction 1), amounting to about half the crude fraction, was not adsorbed, whereas elution of the adsorbed components resulted in subfractions (2–6) forming solutions of much lower viscosity. This chromatographic method is therefore suitable for removing those components that do not contribute to the high viscosity of chondromucoprotein preparations. Of the total chondroitin sulphate contained in the crude fractions, 97 % was recovered in subfractions 1 and 4, as judged by hexuronide content. The close association of chondromucoprotein with sialic acid (Anderson, 1961*a*) is also evident after chromatography since no subfraction was obtained which contained chondroitin sulphate completely free of sialic acid.

In subfractions 2, 3, 5 and 6, where only traces of chondroitin sulphate were present, the high ratios of sulphate to hexuronide indicated the presence of keratosulphate, which is known to occur in human cartilage (Meyer, Hoffman & Linker, 1958). Amino acids did not contribute to the sulphate assay since human chondromucoprotein fractions are deficient in sulphur-containing amino acids (Anderson, 1961*a*). The presence of glycoprotein in subfractions 2, 3, 5 and 6 was suggested by the high ratios of sialic acid and protein to hexuronide.

A further fraction (fraction 1B) was extracted from cartilage at a higher pH, and 2 % of this fraction consisted of material containing 4 % of sialic acid and only 2·5 % of hexuronic acid. Fractions prepared at a similar high pH from ox tracheal cartilage (Glegg *et al.* 1954; Herring & Kent, 1958) have been shown to contain typical glycoprotein constituents (mannose, galactose, fucose and hexosamine) although the probable presence of sialic acid in these fractions was not investigated.

An indication of the type of attachment of sialic acid to the parent molecule can be obtained from the following considerations. Since the removal of sialic acid by neuraminidase did not decrease the viscosity of subfraction 1, sialic acid cannot be involved directly in the link between chondroitin sulphate and protein. It is more probable that sialic acid is terminally attached to the parent molecule by a labile glycosidic bond since free sialic acid was readily liberated with mild acid. A glycosidic link is also indicated since free sialic acid was cleaved by neuraminidase (Fig. 5), the action of this enzyme having been defined by Gottschalk (1960) as the 'hydrolytic cleavage of the glycosidic bond joining the keto group of neuraminic acid to D-galactose or D-galactosamine and possibly to other sugars'. Also, hydrolysis with mild alkali, under conditions which would not be expected to disrupt a glycosidic bond, liberated sialic acid in a bound diffusible form. Only after treatment of this diffusible split product with neuraminidase could free sialic acid be detected.

If sialic acid is an integral part of chondroitin sulphate–protein complexes the present findings are not inconsistent with the model proposed by Partridge, Davis & Adair (1961), in which at least 23 chondroitin sulphate chains are each connected to a protein core by a single link. Since they found the protein core, obtained by mild alkaline hydrolysis, to contain galactose and glucosamine, with traces of glucose and mannose, they suggested the presence of keratosulphate still linked to core protein by bonds more resistant to alkali than those binding chondroitin sulphate. An alternative hypothesis to account for the presence of glucosamine and neutral sugars in preparations of chondroitin sulphate–protein complexes, and one which would also account for the presence of sialic acid, is that the protein core of the complex is a glycoprotein (or sialoprotein). Human urine and serum glycoproteins contain, in addition to protein and sialic acid, both glucosamine and neutral sugars (Winzler, 1958; Anderson *et al.* 1960) in which the sialic acid is believed to be terminal and linked by acid-labile, neuraminidase-susceptible bonds (Klenk, Faillard & Lempfrid, 1955).

The present study shows that the total amount of bound sialic acid extractable from human cartilage at pH 12·8 is about 50 mg./100 g. of wet tissue, and the chromatographic results indicate that it is present in several distinct components, some of which are closely associated with mucopolysaccharide–protein complexes. The instability of bound sialic acid towards dilute acid and alkali establishes it as probably the most labile cartilage component and the one most readily lost when cartilage is extracted under those conditions required for the isolation of pure mucopolysaccharides.

Action of proteolytic enzymes

The high viscosity of the cartilage fractions isolated by mild alkali (fractions A and 1A) is due to the presence of chondroitin sulphate–protein complexes in which the link between chondroitin sulphate and protein is most probably covalent

(Muir, 1958; Partridge & Elsden, 1961). Extraction of cartilage with stronger alkali (at pH 12·8) resulted in a degraded product of low viscosity (fraction 1B). The viscosity of the human-cartilage fractions was also found to decrease after incubation with papain, trypsin, hyaluronidase and streptokinase-activated plasminogen (Figs. 3 and 4). Trypsin has similarly been found to decrease the viscosity of bovine-cartilage chondromucoprotein (Shatton & Schubert, 1954; Mathews, 1956; Webber & Bayley, 1956), although it has no effect on chondromucoprotein extracted from pig tracheal cartilage (Muir, 1958).

The slow viscosity decrease caused by unactivated plasminogen was due either to traces of plasmin in the plasminogen preparation or to slow autocatalytic activation (Alkjaersig, Fletcher & Sherry, 1958). The presence of ϵ-aminocaproic acid, reported to be an inhibitor of the streptokinase activation of plasminogen (Alkjaersig, Fletcher & Sherry, 1959), resulted in a slightly less rapid drop in viscosity.

Enzymic breakdown of cartilage chondromucoprotein can occur both *in vitro* and *in vivo*. Incubation of cartilage slices with papain, trypsin and plasmin leads to liberation of chondroitin sulphate (or low-molecular-weight chondroitin sulphate–peptide complexes) into the surrounding medium (Lack & Rogers, 1958). They conclude that, since certain bacterial kinases (from *Streptococcus* and *Staphylococcus*) activate plasminogen to plasmin, this mechanism may be relevant to the chondrolysis observed after joint infections by these organisms, assuming sufficient plasminogen is present in the vicinity of cartilage *in vivo*. Similarly, injection of unactivated papain into rabbits leads to loss of cartilage matrix, ear collapse and the rapid appearance of chondroitin sulphate in blood and urine (Thomas, 1956; Spicer & Bryant, 1958). The concentrations of both serum (Crosti, Pirani & Catchpole, 1960) and urine (A. J. Anderson, unpublished, estimated by the method of Anderson & Maclagan, 1955) sialic acid-containing glycoproteins, which do not contain significant amounts of hexuronic acid, are also raised. Some of these components may originate from cartilage matrix. Similar results follow injection of plasmin into rabbits, although appearance of increased urinary chondroitin sulphate is more delayed and ear collapse less marked compared with the effects after papain injection (Lack, Anderson & Ali, 1961). These later findings conform with the experiments described above, where proteolytic attack on chondromucoprotein by plasmin (Fig. 4) was slower and less extensive than the attack with papain (Fig. 3).

The significance of the sialic acid-containing components in human cartilage, their possible contribution to the mechanical and biological properties of this tissue and their role in enzymic and pathological processes are unknown.

SUMMARY

1. Crude chondromucoprotein preparations, extracted from human cartilage with mild alkali, were fractionated on diethylaminoethylcellulose, with tris–citrate buffers of decreasing pH and increasing ionic strength. Evidence for the presence of sialic acid in the subfractions obtained is reported. Analytical data indicated the presence in cartilage of protein and glycoprotein material unrelated to chondroitin sulphates.

2. About 50% of the crude preparation was not adsorbed on the ion-exchanger. This major subfraction was highly viscous and was probably the fraction responsible for the high viscosity of the chondromucoprotein preparations.

3. The effects of papain, trypsin, hyaluronidase and plasmin on the viscosities of the crude preparations and the major subfraction were investigated.

4. Cleavage of sialic acid in the major subfraction was detected after dissociation of the complex with acid and alkali. The mechanism of release by both reagents appeared different.

5. The cleavage of sialic acid from the various fractions by receptor-destroying-enzyme preparations and crystalline neuraminidase was investigated. The sialic acid in a chondroitin sulphate A–peptide complex was also found to be susceptible to neuraminidase action.

6. Possible relationships between sialic acid and chondromucoprotein are discussed and the hypothesis is advanced that the protein component of the chondromucoprotein molecule is a sialoprotein.

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The Metabolism of Exogenous Adenine and Purine Analogues by Fungi

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Cordycepin, a metabolic product of the fungus *Cordyceps militaris* (Linn.) Link, has been shown to be 9-cordyceposidoadenine, the sugar cordycepose being a 3-deoxy branch-chain pentose (Cunningham, Hutchinson, Manson & Spring, 1951; Bentley, Cunningham & Spring, 1951). *C. militaris* has been grown on media containing [8-^{14}C]adenine, and the cordycepin isolated found to be radioactive (Kredich & Guarino, 1961). We have done similar experiments with two concentrations of [8-^{14}C]adenine. Increasing the concentration of added adenine increased both the yield and the radioactivity/mole of the cordycepin produced. With 500 mg. of adenine in 1 l. of the medium, the counts/mole in the cordycepin isolated were 73 % of those in the adenine. It thus seems probable that the adenine supplied was incorporated as such into the cordycepin.

It was consequently thought that analogues of purines might be similarly incorporated, so *C. militaris* was grown on media containing such compounds and new derivatives were sought in the culture filtrates by paper chromatography. No new cordyceposides have been found, but from an isomer of adenine two ribosides have been obtained, and three aromatic amines have been acetylated.

The isomer of adenine, 4-amino-1*H*-pyrazolo-[3,4-*d*]pyrimidine [4-amino-(I)], has been converted into a riboside and into 4-hydroxypyrazolo-