Some Studies on the Occurrence of Sialic Acid in Human Cartilage

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Most studies on cartilage have involved investigations of the acid mucopolysaccharide components. There is evidence to suggest, however, that cartilage also contains significant amounts of high-molecular-weight neutral polysaccharide material that does not contain hexuronic acid or sulphate (Hisamura, 1938; Glegg, Eidinger & Leblond, 1954; Glegg & Eidinger, 1955; Herring & Kent, 1958).

In recent years there has been a growing interest in a family of compounds consisting of acylated neuraminic acids, the so-called 'sialic acids', first demonstrated by Blix (1936) in submaxillary gland. Compounds containing sialic acid, usually termed 'glycoproteins', have since been found in a wide variety of mammalian organs and secretions (for a review see Zilliken & Whitehouse, 1958) but few reports are available on the glycoproteins in connective tissue. Sialic acid has been detected in gelatin prepared from mammalian collagen (Courts, 1959, 1960), in the epiphyseal cartilage of young rabbits (Castellani, Ferri, Bolognani & Graziano, 1960) and in various other animal tissues by a histochemical technique (Diezel, 1955). No reports are available, however, on similar components in cartilage from human sources.

In view of the fact that sialic acid-containing glycoproteins possess a wide range of biological activities (for a review see Bettelheim-Jevons, 1958) and appear to play a part in many disease processes (Greenspan, 1955; Lockey, Anderson & MacLagan, 1956), the presence of these compounds in human cartilage has been investigated.

In the present work it soon became apparent that fractions prepared from human cartilage under mildly alkaline conditions gave strong positive reactions for sialic acid in the diphenylamine test (Dische, 1929). Since many methods of obtaining chondroitin sulphate involve a preliminary mild alkaline extraction of cartilage it would appear that, initially at least, such chondroitin sulphate preparations are contaminated with components containing sialic acid, in addition to peptide material.

It is now fairly well established that the difficulties in preparing protein-free chondroitin sulphate are due to the presence of relatively stable

protein-mucopolysaccharide complexes. Small amounts of these complexes can be extracted from bovine cartilage under neutral conditions (Shatton & Schubert, 1954; Muir, 1958) and larger amounts under mildly alkaline conditions (Partridge & Davis, 1958). Subsequent purification of mucopolysaccharides relies either on treatment with proteolytic enzymes (Webber & Bayley, 1956) or the use of relatively strong alkali or acid (Einbinder & Schubert, 1950) in order to split the protein-mucopolysaccharide bonds. The resulting protein-free mucopolysaccharide, however, is generally of a low molecular weight (Mathews & Dorfman, 1953; Mathews, 1956).

In view of the presence of sialic acid in human cartilage the question arose whether it was present in the form of sialoproteins similar to those in human urine (Anderson & MacLagan, 1955) and serum (Winzler, 1955), or whether it was connected in some way with the mucopolysaccharide-protein complexes of the type first reported by Shatton & Schubert (1954). It therefore appeared of interest to determine whether the difficulties attending the removal of peptide material from chondroitin sulphate preparations applied also to the removal of the sialic acid-containing components.

Three fractions have therefore been extracted from human cartilage: a major fraction at pH 10.6 and two smaller fractions at pH 11.6. The major fraction has been subjected to chemical and enzymic procedures designed to remove both protein and sialic acid-containing components. In the light of the results obtained possible relationships between sialic acid and the mucopolysaccharides present in human cartilage are discussed. A preliminary report of this part of the work has been published (Anderson, 1960).

METHODS AND MATERIALS

Chondroitin sulphate (sodium salt), prepared from bovine nasal septa (Analysis; N, 3.6; S, 2.4; ash, 7.2%), was bought from Mann Research Laboratories (New York); orosomucoid and *N*-acetylneuraminic acid were gifts from Dr R. J. Winzler.

The following three complexes were gifts from Dr H. Muir. Chondroitin sulphate A-peptide complex isolated

from pig tracheal cartilage (Muir, 1958). Chondroitin sulphate A from human aorta, isolated by Dr Muir as follows: the tissue was digested with pepsin (pH 2) and then with pancreatin (pH 7). The sulphated polysaccharides were purified by precipitating twice with 2-aminoacridine-HCl and then with ethanol. The chondroitin sulphate A was separated from the other polysaccharides by passage through a Celite-cellulose column, and elution with zinc acetate-propanol (Berenson, 1958). It was then converted into the calcium salt, precipitated with 40% (v/v) ethanol, reprecipitated with 2-aminoacridine-HCl and reisolated as the sodium salt. Chondroitin sulphate B, from human aorta, was isolated by Dr Muir in the same way as described for chondroitin sulphate A above as far as the Berenson procedure. After elution from the column it was separated from the other polysaccharides by glass-fibre electrophoresis at pH 7.3. Both chondroitin sulphates A and B from human aorta contained peptide fragments still intact after ultrafiltration, consisting chiefly of glycine and serine together with smaller amounts of glutamic acid and aspartic acid and traces of some other amino acids. The proportions of these amino acids differed in the two preparations.

Total nitrogen. Solutions containing 0.3-0.4 mg. of N were digested with 1.5 ml. of 1% (w/v) selenium in 50% (v/v) H_2SO_4 for 4 hr. N was determined by a modified nesslerization method (Koch & McMeekin, 1924).

Total hexose. The orcinol method, as described by Winzler (1955), was employed, equal amounts, by weight, of D-galactose and D-mannose being used as the standard.

Sulphate. A modification of the benzidine method of Dodgson & Spencer (1953) was used. Digestion of solutions containing 0.1-0.2 mg. of sulphate was carried out for 4 hr. in a mixture of conc. HNO_3 (1 ml.) and perchloric acid (sp.gr. 1.54; 1 ml.). After neutralization and adjustment of the digest volume to 10 ml. with water, 1 ml. was taken for assay. The intensity of an appropriate dilution of the final coloured product was measured in 1 cm. cells in a Unicam SP. 500 spectrophotometer at 500 $m\mu$.

Hexosamine. The method of Elson & Morgan (1933) was used, as described by Winzler (1955), employing a standard of α -D-glucosamine-HCl, assay figures being expressed in terms of the free base. Hydrolysis was carried out in 3N-HCl for 6 hr. at 100°.

Hexuronic acid. The carbazole method of Dische (1955) was used, with glucuronic acid as the standard.

Protein. The method of Lowry, Rosebrough, Farr & Randall (1951) was employed, with reconstituted freeze-dried serum (Glaxo Laboratories Ltd., Greenford) of known protein content as the standard.

Sialic acid. The acid diphenylamine reaction of Dische (1929) was employed, as used by Anderson & MacLagan (1955). The test solution (1 ml.) was heated at 100° for 30 min. with a diphenylamine solution (2 ml.) consisting of 1 g. of diphenylamine (recrystallized twice from ethanol-water) dissolved in a mixture of conc. H_2SO_4 (A.R.; 10 ml.) and acetic acid (A.R.; 90 ml.). Absorption curves over the range 450-850 $m\mu$ were determined with 1 cm. cells in the spectrophotometer. N-Acetylneuraminic acid and human serum orosomucoid (containing 15% of N-acetylneuraminic acid) were used as standards. Sialic acid values (in terms of N-acetylneuraminic acid) were calculated from the differences in extinctions at 525 $m\mu$ in the

presence or absence of diphenylamine in order to correct for non-specific colour development.

Confirmatory evidence for the presence of sialic acid was obtained by the method developed by Svennerholm (1958). The test sample (10-30 mg.) was heated with 0.1N- H_2SO_4 (80°, 1 hr.), the hydrolysate was centrifuged and the supernatant fluid passed first through Dowex-50 $\times$ 8 (200-400 mesh, H^+ form) and then through Dowex-1 $\times$ 8 (100-200 mesh, acetate form). Sialic acid was eluted from Dowex-1 with an acetate buffer (1M, pH 4.6), the first 15 ml. of effluent being collected. Sialic acid was determined as follows. A portion (1 ml.) of effluent was heated at 100° for 15 min. with 2 ml. of resorcinol reagent (0.2 g. of resorcinol, A.R.) dissolved in a mixture of conc. HCl (A.R.; 80 ml.), water (20 ml.) and 0.1M- Cu_2SO_4 (0.25 ml.). The cooled digestion mixture was then shaken with isoamyl alcohol (5 ml.). Absorption curves of the chromogens extracted into the alcohol phase were determined over the range 450-750 $m\mu$ in the spectrophotometer. N-Acetylneuraminic acid, carried through the same procedure, was used as a standard.

Amino acids. The two-dimensional paper-chromatographic technique as modified by Bowden (1959) was used, employing the phenol solution of Datta, Dent & Harris (1950) as the first solvent and the ethyl methyl ketone-butanol-dicyclohexylamine-water mixture of Hardy, Holland & Naylor (1955) as the second solvent. The second solvent was found to give a cloudy solution and was therefore modified to a composition of ethyl methyl ketone (110 ml.), butanol (110 ml.), dicyclohexylamine (20 ml.) and water (50 ml.). This solvent system enabled amino acids to be identified not only by position but also by colour, after development of the chromatogram with ninhydrin. Hydrolysis of the fractions obtained from cartilage (see below) was carried out in stoppered tubes with 6N-HCl for 8 hr. at 100° in an oven. Excess of acid was removed *in vacuo* over solid NaOH.

Tissue preparations

Disintegration of cartilage. Human cartilage, from a mixture obtained from articular (28%), semilunar (12%), costal (28%) and tracheal (32%) sources, was collected from post-mortem cases restricted to subjects under 65 years, where death had resulted either from accident or pathological conditions unrelated to joint or cartilage diseases. After removal of adhering tissue and bone, the cartilage was washed briefly several times in 0.9% NaCl solution until white and stored frozen until used. To avoid the difficulties of homogenizing for long periods of time in large volumes of extracting solutions, the cartilage was first disintegrated in a mill (developed for crushing whole bone by Dr J. T. Scales, Department of Biomechanics and Surgical Materials, Institute of Orthopaedics, in association with Christy and Norris Ltd., Chelmsford, and British Oxygen Research and Development Ltd., London), in which the grinding chamber was surrounded by a jacket containing liquid nitrogen. By this means the cartilage was disintegrated into particles, less than 1.6 mm. in diameter, which readily dispersed in the aqueous solvents used for the extraction.

Fractionation of cartilage. Preliminary experiments were carried out to determine the effect of pH on the amount of material that could be extracted from the disintegrated

cartilage. All operations were performed at 4°. A known amount of cartilage was extracted successively with three equal volumes of a solution of known pH. Each extraction was performed by mechanical stirring overnight, followed by centrifuging. The three supernatant fluids obtained were combined, filtered through Whatman no. 1 filter paper and the pH was determined. Fractionation of the supernatant fluid was then carried out by the addition of ethanol solution [95% ethanol containing 1% (w/v) of potassium acetate and 1% (v/v) of acetic acid] in the following manner. After adjustment of the supernatant fluid to pH 7 with acetic acid, ethanol solution (2 vol.) was slowly added with constant stirring (final ethanol concentration 63%, v/v). After standing overnight the flocculent precipitate was centrifuged down and the supernatant fluid (S) retained. The precipitate was suspended in water and brought into solution by addition of alkali to pH 7. Reprecipitation was carried out by the addition of ethanol solution (2 vol.) and the product (fraction 1) washed with ethanol and ether and dried *in vacuo*. To the supernatant fluid (S) a further 2 vol. of ethanol solution was added (final ethanol concentration 84%). The flocculent precipitate, after standing overnight, was treated in the same manner as for fraction 1. The resulting product was termed fraction 2. Details of the yields of fractions 1 and 2, obtained at three different pH values, are shown in Table 1.

In view of these preliminary experiments the following method of bulk extraction of the cartilage was employed. Portions (50 g.) of the disintegrated cartilage were suspended in a glycine buffer (0.1M, pH 11.5, 250 ml.) and stirred mechanically for 24 hr. The residue of cartilage remaining was centrifuged down, the supernatant fluid adjusted to pH 7 and retained, and the extraction was repeated twice more on the residue with the same volume of glycine buffer each time. The three neutral supernatant fluids were combined, filtered and ethanol solution (2 vol.) was added (final ethanol concentration 63%). After standing overnight the precipitate was centrifuged down, suspended in water (150 ml.) and brought into solution by adjustment to pH 7 with alkali. After dialysis of the solution against distilled water for 5 days with frequent changes, ethanol solution (2 vol.) was added. After standing overnight the precipitate was collected by centrifuging, washed with ethanol and ether and dried *in vacuo*. This product was termed fraction A.

The cartilage residue obtained after the three extractions with glycine buffer was then suspended in 0.5N-NaOH (250 ml.) and stirred mechanically overnight. After centrifuging, the supernatant fluid was adjusted to pH 7 and retained. The residue was extracted once more with the same volume of 0.5N-NaOH. The two neutral supernatant fluids were combined and ethanol solution (2 vol.) was added (final ethanol concentration 63%, v/v). After standing overnight the precipitate was centrifuged down and the supernatant fluid (S) retained. The precipitate was suspended in water (150 ml.), the mixture was adjusted to pH 7 and the resulting solution dialysed against water for 5 days with frequent changes. Reprecipitation was then carried out by the addition of ethanol solution (2 vol.) and the precipitate collected by centrifuging after standing overnight. After washing with ethanol and ether it was dried *in vacuo*. This product was termed fraction B₁. To the supernatant fluid (S) was added 2 vol. of ethanol solution (final ethanol concentration 84%, v/v). After standing

overnight the precipitate was centrifuged down and the residue suspended in water (60 ml.). After adjustment to pH 7 the solution was dialysed and reprecipitation carried out as for fraction B₁, except that ethanol solution was added to a final concentration of 84% (v/v). This product was termed fraction B₂. The yields of these three fractions are shown in Table 2.

EXPERIMENTAL AND RESULTS

Specificities of the diphenylamine and resorcinol reactions for sialic acid in the presence of protein and acid mucopolysaccharide components

Diphenylamine reaction. Fraction A gave a violet colour when heated with the diphenylamine reagent. Fractions B₁ and B₂ gave violet-blue colours. The absorption curves of the chromogens formed by these three fractions are shown in Fig. 1, after subtraction of the extinctions produced by a reagent blank consisting of water (1 ml.) in place of the test solution (1 ml.). The absorption curves of the light-blue chromogens formed with a sample of chondroitin sulphate, a chondroitin sulphate A-protein complex isolated from pig tracheal cartilage (Muir, 1958) and glucuronic acid are shown in Fig. 2. The absorption curves of the violet chromogens formed with *N*-acetylneuraminic acid and human-serum orosomucoid are shown for comparison in Fig. 2. L-Fucose (0.2 mg.), α -D-glucosamine-HCl (0.3 mg.), bovine-serum albumin (2 mg.), serine (0.2 mg.), glycine (0.2 mg.), aspartic acid (0.2 mg.), glutamic acid (0.2 mg.) and an equal mixture, by weight, of D-galactose and D-mannose (0.2 mg.), gave no visible colours with the diphenylamine reagent.

The percentages of sialic acid in cartilage fractions A, B₁ and B₂ are shown in Table 2, together with the results of analysis for other constituents. The sialic acid contents of the fractions extracted at three different pH values are shown in Table 1.

Mild acid hydrolysis of fraction A. Fraction A (20 mg.) was dissolved in 0.01N-HCl (6 ml.) and heated at 80° for 1 hr. A control, consisting of fraction A (20 mg.) in water (6 ml.), was treated similarly. Each solution was then dialysed against distilled water (10 ml.) for 24 hr., followed by a second dialysis against a further 10 ml. of water for the same period. The two diffusates obtained from the control gave no reaction when heated with the diphenylamine reagent. The two diffusates from the acid-hydrolysed fraction A, when caused to react quantitatively with the diphenylamine reagent, showed that 93% of the total sialic acid present initially in fraction A had diffused out in 48 hr. The absorption curves of the chromogens formed by these two diffusates are shown in Fig. 3.

The difference in the relative heights of the absorption maxima at 525 and 650 $m\mu$ shown by the first diffusate (Fig. 3) and that shown by authentic *N*-acetylneuraminic acid (Fig. 2) was probably due to the presence of the small amount of monosaccharides liberated from fraction A by mild acid hydrolysis. Courts (1959) has shown that some monosaccharides form chromogens with diphenylamine which absorb strongly at 650 $m\mu$. Fructose and deoxyribose, which both form chro-

mogens with diphenylamine absorbing at 650 $m\mu$, were absent from the diffusate, however, since heating the diffusate with the resorcinol reagent (see below) formed a chromogen identical with that produced by authentic *N*-acetylneuraminic acid. Both fructose and deoxyribose form chromogens

Fig. 1. Absorption curves of the chromogens obtained on heating three fractions (2 mg.) from human cartilage with the diphenylamine reagent: —, fraction A; ---, fraction B₁; — · —, fraction B₂.

Fig. 2. Absorption curves of the chromogens obtained on heating the diphenylamine reagent with: —, chondroitin sulphate (1 mg.); ---, glucuronic acid (0.3 mg.); — · —, chondroitin sulphate A-peptide complex (2 mg.) (isolated from pig tracheal cartilage; Muir, 1958); - - - -, *N*-acetylneuraminic acid (0.1 mg.); —○—, human-serum orosomucoid (1 mg.).

Table 1. *Extraction of human cartilage at three different pH values*

Amount of wet disintegrated cartilage extracted (g.)	Extraction medium*	pH of combined extracts	Yield (mg./100 g. of wet cartilage)		Sialic acid content of fraction 1 (%)
			Fraction 1†	Fraction 2‡	
30	0.5N-NaOH (100 ml.)	11.6	4100	320	3.6
10	Glycine buffer (0.1M, pH 11.5, 50 ml.)	10.6	2230	70	3.7
20	Glycine buffer (0.1M, pH 9.5, 50 ml.)	9.0	563	Trace	3.7

* The cartilage was extracted three times with the extraction medium and the extracts were combined.

† Obtained by precipitation of the combined extracts with ethanol solution (final ethanol concentration 63%, v/v).

‡ Obtained by ethanol precipitation of the supernatant fluid remaining after removal of fraction 1 (final ethanol concentration 84%, v/v).

Table 2. *Yields and analysis of fractions A, B₁ and B₂ from human cartilage*

Fraction A was obtained from cartilage by extraction at pH 10.6 followed by ethanol precipitation (63%, v/v). Fractions B₁ and B₂ were obtained by extraction of the remaining cartilage at pH 11.6, followed by ethanol precipitation to 63% (v/v) and 84% (v/v) respectively. Protein was (1) determined by the method of Lowry, Rosebrough, Farr & Randall (1951), or was (2) calculated by multiplying total N by 6.25 after subtracting hexosamine N (7.8%) and sialic acid N (4.5%).

Fraction	Yield (mg./100 g. of wet cartilage)	N (%)	Protein (%)		Sialic acid (%)	Total hexose (%)	Hexosamine (%)	Hexuronic acid (%)	Sulphate (%)
			(1)	(2)					
A	2915	8.3	34.0	46.0	3.7	14.6	10.9	8.6	10.7
B ₁	608	9.2	42.3	53.0	4.1	14.0	6.3	7.3	7.7
B ₂	151	9.5	43.3	55.0	6.4	11.6	6.5	0.3	5.6

with the resorcinol reagent which absorb strongly in the region 450–500 $m\mu$.

The acid-hydrolysed fraction A, after prolonged dialysis against water, gave a colourless product when heated with the diphenylamine reagent (Fig. 3). The analysis for native fraction A, and for fraction A after acid hydrolysis followed by prolonged dialysis against water, is shown in Table 3.

Resorcinol reaction. Fraction A formed a brown chromogen, the absorption curve of which is shown in Fig. 4. Fractions B₁ and B₂ behaved similarly. The high absorption in the region 450–500 $m\mu$ was found to be due to the hexose and hexuronic acid constituents in the fractions, both of which form orange-yellow chromogens with resorcinol. The following substances were found to give negative reactions: α -D-glucosamine-HCl (0.1 mg.), serine (0.2 mg.), glycine (0.2 mg.), glutamic acid (0.2 mg.),

aspartic acid (0.2 mg.). The chromogen produced with *N*-acetylneuraminic acid gave a strong absorption at 575 $m\mu$, with an inflexion in the curve at 612 $m\mu$ (Fig. 4).

After the following pretreatments fractions A, B₁ and B₂ formed blue chromogens with the resorcinol reagent with absorption spectra identical with that produced by *N*-acetylneuraminic acid: (a) mild acid hydrolysis followed by dialysis (for experimental details see above); (b) treatment by the procedure described by Svennerholm (1958) (see under Methods and Materials; the absorption curve of the chromogen produced with fraction A after this treatment is shown in Fig. 4).

These results indicate that the resorcinol reaction, unlike the diphenylamine reaction, can be used for estimating sialic acid only after removal of interfering hexoses and hexuronic acid.

Fig. 3. Absorption curves of the chromogens obtained on heating the diphenylamine reagent with: —, fraction A (2 mg.) after acid hydrolysis (0.01 *N*-HCl, 80°, 1 hr.) and prolonged dialysis against water; ---, the first diffusate (1 ml.) obtained from acid-hydrolysed fraction A after dialysis against 10 ml. of water during the period 0–24 hr.; — · —, the second diffusate (1 ml.), obtained from acid-hydrolysed fraction A after dialysis against a further 10 ml. of water during the period 24–48 hr.

Fig. 4. Absorption curves of the chromogens obtained on heating the resorcinol reagent with: —, *N*-acetylneuraminic acid (0.1 mg.); ---, fraction A (2 mg.); — · —, fraction A after acid hydrolysis (0.1 *N*-H₂SO₄, 80°, 1 hr.) followed by purification of sialic acid by the ion-exchange method of Svennerholm (1958).

Table 3. *Estimation of constituents in fraction A (20 mg.) before mild acid hydrolysis and after mild acid hydrolysis followed by prolonged dialysis against water*

Hydrolysis of fraction A was carried out with HCl (0.01 *N*, 80°, 1 hr.).

Constituent	Before hydrolysis (mg.)	After hydrolysis and prolonged dialysis (mg.)	Decrease after hydrolysis and dialysis (%)
Hexuronic acid	1.7	1.7	0
Hexosamine	2.1	2.1	0
Protein*	6.8	6.0	12
Total hexose	2.9	2.6	10
Sialic acid	0.7	0	100
Complete hydrolysis (5 <i>N</i> -HCl, 100°, 6 hr.)	Large amount of humin	Small amount of humin	—
Solubility in water	Complete	Partial	—

* Method of Lowry *et al.* (1951).

Amino acid composition of fractions A, B₁ and B₂

Chromatography of 1 mg. of the hydrolysed fractions revealed the presence of aspartic acid, glutamic acid, glycine, alanine, serine, threonine, valine, leucine (isoleucine), arginine, lysine, phenylalanine, tyrosine, proline, hydroxyproline and phosphoserine. In addition, a strong spot was detected in the position corresponding to hexosamine. Glucosamine and galactosamine were not separated with the solvent system employed. Chromatography of 0.5 mg. of the hydrolysed fractions revealed the presence of the same amino acids in all three fractions, except that in fractions A and B₂ no hydroxyproline could be detected. One unidentified component reacting with ninhydrin was present in fraction A and two unknown components were in fractions B₁ and B₂.

Non-enzymic fractionation procedures on fraction A

Deproteinizing procedures. The procedure used by Webber & Bayley (1956) to decrease the N content of chondroitin sulphate preparations was followed. Fraction A (100 mg.) was dissolved in water (20 ml.) and 5 ml. of a solution of 5% (w/v) phosphotungstic acid in 2N-HCl added. The precipitate that formed was centrifuged down and the supernatant fluid (S) retained. The precipitate was suspended in water (20 ml.) and brought into solution by adjustment to pH 7 with alkali. After prolonged dialysis against water the precipitate was recovered by addition of ethanol solution (2 vol.), followed by washing with ethanol and ether. The product (R₁) was dried *in vacuo*. The supernatant fluid (S) was neutralized with alkali and freed of phosphotungstic acid by dialysis. Ethanol solution (2 vol.) was added to the dialysed solution and the precipitate (R₂) was washed with ethanol and ether and dried *in vacuo*. The yields and analysis of R₁ and R₂ are given in Table 4. A control experiment showed that chondroitin sulphate was completely soluble in the phosphotungstic acid reagent.

Although a large proportion of the mucopolysaccharides in fraction A was concentrated in the R₂ fraction, a considerable amount of material which contained sialic acid was also present. Treatment of R₂ with trypsin, by the method of Shatton & Schubert (1954), degraded 34% of the peptide material to dialysable fragments but did not result in the liberation of dialysable sialic acid. Attempts to adsorb the sialic acid-containing components of R₂ with Lloyd's reagent (amorphous aluminium silicate) by the method of Pearce & Watson (1949) were unsuccessful.

Similarly, although shaking an aqueous solution of fraction A with chloroform-amyl alcohol (Sevag, 1934) removed 22% of the fraction in the

form of insoluble material, the remainder still contained both hexuronic acid and sialic acid.

Ammonium sulphate fractionation. Fraction A (100 mg.) was dissolved in water (20 ml.) and 20 ml. of a sat. aq. solution of (NH₄)₂SO₄ was added. After standing 6 hr. at 4° the mixture was centrifuged and the precipitate (R₁) treated as described below. Solid (NH₄)₂SO₄ was added to the supernatant fluid until fully saturated and the mixture centrifuged after standing overnight. The residue (R₂) was treated as described below. The supernatant fluid was dialysed against distilled water, solid NaCl was added to 0.2M and the solution poured into acetone (5 vol.). After standing overnight the precipitate (R₃) was centrifuged down. R₁, R₂ and R₃ were each dissolved in water (20 ml.) at pH 9 and the solutions were dialysed against distilled water. Reprecipitation was carried out with NaCl and acetone as described above, the products being washed with ethanol and ether and dried *in vacuo*. Yields and analysis of the three fractions are shown in Table 5. A control experiment showed that chondroitin sulphate was completely soluble in sat. aq. (NH₄)₂SO₄.

Adsorption on to benzoic acid. The method of Anderson & MacLagan (1955) for the isolation of the sialic acid-containing glycoproteins in urine was used. All operations were performed at 4°. Fraction A (50 mg.) was dissolved in water (500 ml.). 2M-Sodium benzoate (62.5 ml.) was added, followed by the slow addition with constant stirring of

Table 4. Phosphotungstic acid precipitation of fraction A

Fraction A (100 mg.) was treated with a solution of phosphotungstic acid in 2N-HCl (5%, w/v). R₁ and R₂ represent the insoluble and soluble fractions respectively.

Component	Yield (mg.)	Sialic acid (%)	Hexuronic acid (%)	Protein*
R ₁	38	1.9	2.5	50.0
R ₂	64	4.3	11.9	24.0

* By the method of Lowry *et al.* (1951).

Table 5. Yields and analysis of the residues obtained from fraction A (100 mg.) after ammonium sulphate fractionation

R₁ and R₂ were the fractions insoluble at half and full saturation with (NH₄)₂SO₄ respectively. R₃ was the residue soluble in fully saturated (NH₄)₂SO₄ solution.

Fraction	Yield (mg.)	Sialic acid (%)	Hexuronic acid (%)	Protein*
R ₁	21	3.8	2.8	37.6
R ₂	65	4.1	9.0	31.8
R ₃	16	0.3	11.7	3.4

* By the method of Lowry *et al.* (1951).

2N-HCl (50 ml.). After standing for 1 hr. the benzoic acid precipitate was filtered off under suction, drained thoroughly and treated with acetone (100 ml.). The residue remaining was centrifuged down, washed once with acetone and suspended in water (25 ml.). The residue dissolved completely after adjustment to pH 9 with alkali. Reprecipitation was carried out by addition of solid NaCl, to 0.2M, and acetone (5 vol.). The precipitate was dialysed against distilled water and reprecipitated in the same manner. The yield, after washing with ethanol and ether and drying *in vacuo*, was 34 mg. (Analysis: sialic acid, 3.7; hexuronic acid, 6.0; protein, 34%). The characteristics of the absorption curve of the chromogen formed by this product with the diphenylamine reagent were identical with the curve shown in Fig. 1 for fraction A.

A control experiment with a sample of chondroitin sulphate (50 mg.) showed that less than 2% was adsorbed on to benzoic acid. The remainder could be recovered from the benzoic acid filtrate on addition of ethanol solution (2 vol.).

Enzymic fractionation procedures on fraction A

Papain digestion. Crude papain (100 mg.) was activated by incubating at 45° for 30 min. in an acetate buffer (0.02M; pH 5.4; 10 ml.) containing 0.01M-cysteine. After filtering off the insoluble material, 1 ml. of filtrate was added to fraction A (100 mg.) contained in the acetate buffer (5 ml.). The mixture was incubated overnight at 45°. A control, consisting of a mixture of 1 ml. of papain filtrate in 5 ml. of acetate buffer, was treated similarly. Each mixture was then dialysed against water and the non-dialysable components were precipitated by the addition of solid NaCl (to 0.2M) and ethanol solution (3 vol.). The precipitates were washed with ethanol and ether and dried *in vacuo*. Fraction A, after digestion, yielded 61 mg. The residue from the control experiment was too slight to collect and estimations on this residue were performed on portions after dissolving the residue in water (10 ml.) [Analysis for fraction A after digestion (corrected for the papain control): sialic acid, 4.7; hexuronic acid, 15.2; protein, 14.0%]. The amino acid composition was similar to that of native fraction A, except that no phosphoserine could be detected.

Hyaluronidase digestion. Fraction A (100 mg.) was incubated with hyaluronidase (Benger's Hyalase, 1500 i.u.) in a phosphate-citrate buffer (0.15M-phosphate, pH 5.0, 25 ml.) for 90 hr. at 25°. After dialysis of the incubation mixture, 79 mg. of non-dialysable material was precipitated after addition of acetone (5 vol.) and solid NaCl (to 0.2M.) (Analysis: sialic acid, 4.6; hexuronic acid, 8.8%). Further incubation of this product with

hyaluronidase (4500 i.u., 72 hr., 25°), followed by dialysis and precipitation of the incubation mixture as described above, yielded 53 mg. (Analysis: sialic acid, 4.9; hexuronic acid, 7.4%). Both the yields and analysis were obtained after correction for the hyaluronidase present. The activity of the hyaluronidase was checked as follows. Incubation of chondroitin sulphate (20 mg.) with hyaluronidase (1800 i.u., added at 2-day intervals in three equal amounts over a 7-day period) resulted in the diffusion of 0.13 mg. of hexuronic acid after dialysis of the incubation mixture against water (10 ml., 24 hr.).

Treatment with 5-aminoacridine hydrochloride

5-Aminoacridine-HCl, which forms insoluble salts with organic sulphates (Dodgson, Rose & Spencer, 1954), has been used by Muir (1958) to decrease the protein content of a chondroitin sulphate-protein complex obtained under neutral conditions from cartilage. This procedure was used to determine if a chondroitin sulphate preparation free of sialic acid could be precipitated from fraction A.

Fraction A (50 mg.) was dissolved in water (17 ml.) and the solution made acid to methyl orange with acetic acid. After centrifuging off and discarding the small amount of insoluble material, a sat. aq. solution of 5-aminoacridine-HCl was added slowly until no more precipitate had formed. After standing overnight at 4° the precipitate was centrifuged down, washed three times with water and suspended in 2.5% (w/v) sodium formate (8 ml.). The suspension was shaken with Zeo-Karb 225 (Na⁺) (The Permutit Co. Ltd.) until the supernatant fluid showed no blue fluorescence. The resin was removed and washed four times with 3 ml. portions of water. The supernatant fluid and washings were combined, dialysed against distilled water and precipitation with the hydrochloride was repeated. The final product was precipitated from the dialysed solution by the addition of ethanol solution (4 vol.), followed by washing with ethanol and ether. The yield, after drying *in vacuo*, was 27 mg. (Analysis: sialic acid, 4.2; hexuronic acid, 4.2; protein, 27.0%). The amino acid composition was similar to that of fraction A, except that no phosphoserine could be detected. A control experiment showed that orosomucoid (2 mg./ml.) and N-acetylneuraminic acid (1 mg./ml.) formed no precipitate under these conditions.

Treatment with alkali

Fraction A (40 mg.) was dissolved in NaOH (0.2N, 5 ml.) and incubated at 37° for 33 hr. To the resulting non-viscous, slightly yellow solution, acetic acid was added, to pH 5.4, and the solution was dialysed against water (10 ml.) for 24 hr.

Analysis of the diffusate showed that 44, 12 and 2% of the sialic acid, peptide material and hexuronic acid respectively had diffused out. The dialysate was then exhaustively dialysed against water and the barium salt of chondroitin sulphate was isolated from the dialysed solution by the method of Malawista & Schubert (1958) as follows. Barium chloride (50 mg.) was added to the dialysate, followed by ethanol to 20% (v/v). The precipitate (R_1) was collected, washed with ethanol and ether and dried *in vacuo*. This yielded 7.6 mg. of a light-brown material (Analysis: protein, 41; hexuronic acid, 5.7; sialic acid, 0%). Ethanol was added to the supernatant fluid to 50% (v/v) and the precipitate (R_2) centrifuged down. The supernatant fluid (S) was retained. R_2 was redissolved in water (5 ml.), the reprecipitation with ethanol to 50% (v/v) in the presence of barium ions was repeated and the product washed with ethanol and ether. Ethanol (3 vol.) was added to the supernatant fluid (S) and the precipitate (R_3) washed with ethanol and ether. The amounts of R_2 and R_3 were too small to collect, and they were therefore dissolved in water (3 ml.) for purposes of analysis. The amounts of protein, sialic acid and hexuronic acid present in R_1 , R_2 and R_3 are shown in Table 6.

Examination of chondroitin sulphate-protein complexes for the presence of sialic acid

The three chondroitin sulphate-protein complexes described under Methods and Materials were investigated. Portions (70 mg.) of each fraction

Table 6. *Composition of fractions prepared from alkali-digested fraction A by fractional precipitation with ethanol*

Fraction A (40 mg.) was treated with NaOH (0.2N, 37°, 33 hr.). Fractional ethanol precipitation of the dialysed mixture was then carried out in the presence of Ba^{2+} ions. R_1 , R_2 and R_3 were precipitated at ethanol concentrations of 20, 20-50 and 50-80% (v/v) respectively.

Fraction	Protein* (mg.)	Sialic acid (mg.)	Hexuronic acid (mg.)
R_1	3.1	0	0.43
R_2	0.3	0	1.13
R_3	0.6	0.5	0.11

* Method of Lowry *et al.* (1951)

were suspended in 0.01N-HCl (5 ml.) and the mixtures adjusted to pH 3 with 0.1N-HCl. The mixtures were then heated at 80° for 1 hr. Each hydrolysate was then dialysed against water (10 ml.) for 24 hr., followed by a second dialysis against a fresh 10 ml. portion of water for a further 24 hr. The diffusates (20 ml.) were combined, adjusted to pH 7 with 0.1N-NaOH and freeze-dried. The residues were dissolved in water (4 ml.) and 1 ml. was used for colorimetric estimation of sialic acid with both the diphenylamine and resorcinol reactions. The results are shown in Table 7. The absorption curves over the range 450-850 m μ of the chromogens formed after heating chondroitin sulphate A (pig trachea) and chondroitin sulphate A (human aorta) complexes, after the above-described treatment, with both the diphenylamine and resorcinol reagents, were identical with the chromogens produced with authentic *N*-acetylneuraminic acid.

DISCUSSION

Mechanical disintegration of cartilage at -196° produced a finely divided product, which, after warming to above 0°, dispersed readily in the solvents used for extraction. This procedure obviated the disadvantages attending high-speed homogenization of the tissue in large volumes of water. Extraction of the disintegrated cartilage at three different pH values (Table 1) showed, in agreement with results of other workers, that increasing amounts of material could be extracted as the pH was increased. Although alkali is particularly effective in releasing soluble cartilage constituents a certain amount of degradation undoubtedly occurs, as judged by the relatively low molecular weights of some chondroitin sulphate preparations (Mathews & Dorfman, 1953). On the other hand, less than 10% of the chondroitin sulphate in cartilage is extracted by neutral calcium chloride solutions (Mathews & Dorfman, 1953), and this material may not be entirely representative of the whole. In the bulk extraction of cartilage described in the present work a compromise was therefore reached whereby the pH was kept as low as possible, at the same time assuring a reasonably high yield of water-soluble constituents.

Table 7. *Sialic acid content of chondroitin sulphate-peptide complexes*

Methods used in isolating the complexes are given under Methods and Materials.

Complex	Source	Sialic acid (%)	
		Diphenylamine reaction	Resorcinol reaction
Chondroitin sulphate A	Pig tracheal cartilage	0.55	0.44
Chondroitin sulphate A	Human aorta	0.29	0.17
Chondroitin sulphate B	Human aorta	0	0

Under the mildly alkaline conditions used, the total yield from 100 g. of wet cartilage was 3.7 g. and analysis (Table 2) showed that 85% of the material could be accounted for on the basis of protein, sulphate, sialic acid, hexosamine and total non-nitrogenous hexose. The results for sulphur analysis were expressed in terms of sulphate since no sulphur-containing amino acids could be detected in any of the three fractions. The small amount of hydroxyproline in fraction A indicated that collagen was not a major protein constituent of this fraction. The most marked chemical difference between these fractions was the absence of appreciable amounts of hexuronic acid in fraction B₂, indicating the absence of chondroitin sulphates. Glegg *et al.* (1954) and Glegg & Eidinger (1955) have reported similar chondroitin sulphate-free fractions from connective tissue and demineralized bone from cattle.

The diphenylamine reaction for estimating sialic acid has been investigated by several workers (Anderson & MacLagan, 1955; Courts, 1959), and the violet chromogen produced, with a typical absorption maximum at 525 m μ , is thought to be specific for both the free and bound compound. The three fractions obtained from cartilage gave a similar chromogen with a strong absorption at 525 m μ (Fig. 1), which could not have been due to the presence in these fractions of non-nitrogenous hexoses or hexuronic acid, both of which gave small absorption maxima at 625 m μ . The absorption maxima (620–630 m μ) shown by a commercial sample of chondroitin sulphate and a chondroitin sulphate A–peptide complex isolated from pig tracheal cartilage (Muir, 1958) (Fig. 2) were no doubt due to the large amount of hexuronic acid present in these fractions. The second maxima (625–675 m μ) shown by the three cartilage fractions (Fig. 1) were probably due to their content of non-nitrogenous hexose and hexuronic acid.

Additional confirmation for the presence of sialic acid in fraction A was obtained after mild acid hydrolysis (Fig. 3). These results were similar to those obtained after mild acid hydrolysis of other sialic acid-containing fractions, particularly human-serum orosomucoid (Winzler, 1958), human-plasma α_1 -acid glycoprotein (Schmid, Bencze, Nussbaumer & Wehrmüller, 1959), erythropoietin (Rambach, Shaw, Cooper & Alt, 1958) and bovine salivary glycoprotein (Gottschalk & Graham, 1959), where complete cleavage of sialic acid has been found associated with only traces of hexoses, more drastic hydrolysis being needed for the complete cleavage of hexoses and for the liberation of hexosamines. The chromogen formed on heating the non-dialysable components of hydrolysed fraction A with the diphenylamine reagent showed no significant maximum at 525 m μ (Fig. 3), indicating

the complete removal of sialic acid. The modified non-dialysable product was partially denatured, as judged by solubility in water, and produced only a slight trace of humin material when heated with 5N-hydrochloric acid. Both free and bound sialic acid are known to produce considerable amounts of humin material when heated with strong mineral acid.

In the resorcinol reaction, although the chromogens produced with hexoses and hexuronic acid do not show absorption maxima at the wavelength (575 m μ) characteristic for sialic acid they absorb strongly enough at 450–500 m μ to interfere seriously with the colorimetric estimation of sialic acid (Fig. 4). However, mild acid hydrolysis of fraction A, which does not liberate dialysable hexuronic acid, and only small amounts of hexoses (Table 3), does liberate dialysable material which forms a chromogen identical to that formed by authentic *N*-acetylneuraminic acid. Hydrolysis of the cartilage fractions under more acid conditions, followed by removal of interfering substances by the method developed by Svennerholm (1958), also confirms the presence of sialic acid with the resorcinol (Fig. 4) and the diphenylamine reactions.

The amount of sialic acid thus found present in human cartilage would indicate that sialic acid-containing components form a substantial part of the polysaccharide fraction of the ground substance, increasing amounts of which could be extracted as the pH of the extracting medium was raised (Table 1).

Treatment of fraction A by mild chemical and enzymic procedures which have been used to decrease the nitrogen content of mucopolysaccharide preparations failed to separate completely the protein and sialic acid-containing components from the mucopolysaccharides. Moreover, the distribution of these components in the various fractions obtained by these methods was often at variance with the results obtained with purified chondroitin sulphate preparations from cartilage and glycoprotein preparations from human urine and serum.

Thus deproteinization attempts with phosphotungstic acid, followed by trypsin-treatment of the soluble fraction (R₂, Table 4), resulted in a product still containing protein and sialic acid-containing components. These results are similar to those of Webber & Bayley (1956), who found it impossible, with the same method, to obtain protein-free chondroitin sulphate from bovine nasal-septa extracts. Two other deproteinization treatments of fraction A, with chloroform–amyl alcohol and Lloyd's reagent, similarly failed to remove peptide material and sialic acid-containing components from the mucopolysaccharides.

Anomalous behaviour was also noticed when

fraction A was subjected to the benzoic acid-adsorption method, which has been used to obtain urinary glycoproteins virtually free of mucopolysaccharides (Anderson & MacLagan, 1955). It was found that adsorption on to benzoic acid of purified chondroitin sulphate was negligible.

Only when fraction A was treated with relatively strong alkali was it found possible to remove all the sialic acid and most of the peptide material from the mucopolysaccharide constituents. Such treatment did not result in the liberation of dialysable hexuronide. These results indicate, in agreement with other workers, that protein-free chondroitin sulphate preparations can only be obtained if the cartilage extract is first subjected to treatment with relatively strong alkali. It must also be concluded that similar difficulties exist in the removal of the sialic acid-containing components.

Three explanations are possible for this close relationship between sialic acid and hexuronic acid in cartilage extracts. Firstly, the protein moiety of the mucopolysaccharide-protein complex may be a sialoprotein of a type similar to those present in urine and serum. Partridge & Davis (1958) have detected glucosamine in the protein moiety of a mucopolysaccharide-protein complex extracted under mild alkaline conditions from bovine nasal septum. They concluded that the low nitrogen and amino acid content of this moiety indicated that only two-thirds was polypeptide material, and that the remainder must therefore contain components other than amino acids. Secondly, sialic acid may be involved in the linkage of the mucopolysaccharide molecule to the protein component, since theoretically sialic acid can enter into combination with sugars and amino acids through both glycosidic and peptide bonds. Lastly, sialic acid may be part of a glycoprotein, or mixture of glycoproteins, quite unrelated to any mucopolysaccharide-protein complex. In this case, failure to separate the glycoproteins from mucopolysaccharide material would merely be due to non-specific coprecipitation.

The presence of sialic acid in chondroitin sulphate A-peptide complexes isolated from pig trachea and human aorta by rigorous methods designed specifically to remove contaminating material would seem to indicate that sialic acid is, in fact, an integral part of these molecules. Further, the absence of sialic acid in the chondroitin sulphate B-peptide complex isolated from human aorta by similar methods indicates that contaminating sialic acid-containing components associated with this fraction have been effectively removed. The presence of sialic acid in the two preparations of chondroitin sulphate A and its absence in the chondroitin sulphate B preparation may reflect some fundamental differences in the

nature of either the mucopolysaccharide-peptide links or in the peptide fragments themselves.

Both these chondroitin sulphate A complexes were isolated below pH 7.3. When subjected to pH values above 8.5, followed by ultrafiltration, amino acid-free polysaccharides can be isolated, the amino acid-containing portion appearing in the ultrafiltrate (H. Muir, personal communication). It is unlikely therefore that fraction A, isolated from cartilage at pH 10.6, contains the same complexes. However, the presence of more alkali-stable sialic acid-containing chondroitin sulphate complexes, which can only be isolated under more alkaline conditions, cannot be excluded.

SUMMARY

1. The specificities of the diphenylamine and resorcinol reactions for sialic acid in the presence of protein and acid mucopolysaccharide components were investigated, and the violet and blue chromogens absorbing strongly at 525 m μ and 575 m μ respectively were found specific for this compound.
2. Disintegration of human cartilage was carried out mechanically at -196° and the amounts of water-soluble material extractable at three pH values were determined. Sialic acid-containing components were present in these fractions, increasing amounts of which could be extracted from cartilage as the pH was increased.
3. Three fractions were extracted from the disintegrated cartilage: a major fraction at pH 10.6 and two smaller fractions at pH 11.6. Analysis for protein, sulphate, sialic acid, hexosamine and total non-nitrogenous hexoses accounted for 85% by weight of the total solids. No sulphur-containing amino acids were detected.
4. Sialic acid was detected in certain mucopolysaccharide-peptide complexes.
5. The difficulties attending the separation of protein from acid mucopolysaccharide preparations were found to apply equally to the removal of the sialic acid-containing components. The significance of this is discussed.

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The Preparation and Properties of Cystine Esterase

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Hooper & Jessup (1959) showed that placenta contain separate enzyme systems destroying oxytocin and vasopressin. During the purification of the enzymes, it seemed desirable to replace the biological estimation of oxytocinase by a chemical method using synthetic substrates. Certain properties of oxytocinase were revealed by inhibitors (Hooper, 1959), and it was concluded that the enzyme is a peptidase with esterase activity. Attempts have been made to find a chemical method of assay based on the presumed esterase activity of oxytocinase. A variety of esters was incubated with crude preparations of the enzyme and an additional enzyme which hydrolysed esters

of cystine and of *S*-benzylcysteine, which has been called cystine esterase, was found. This report describes some of its specificities and its partial purification.

METHODS

Estimation of amino acids. Amino acid ester hydrochlorides were incubated with enzyme as described below. Samples equivalent to approx. 100 μ g. of original substrate were chromatographed on Whatman no. 1 paper with butan-1-ol-acetic acid-water (5:1:4, by vol.). After spraying with ninhydrin solution (0.1% in butan-1-ol-2% acetic acid) the colour was developed at room temperature for 1 hr., followed by heating at 80° for 5 min. Spots produced by amino acids were cut out, the colour was eluted with 3 ml. of propan-1-ol-water (1:1, v/v), and the extinction at 580 m μ measured. Determinations were done

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