2. The glutamic-alanine transaminase has an equilibrium constant of 1.4 at 25° and pH 7.5, and an optimum pH of 7.5.

3. The glutamic-aspartic enzyme has an equilibrium constant of 5.0 at 25° and pH 8 and an optimum pH between pH 8.0 and 8.5.

4. The effect of pyridoxal phosphate and some inhibitors on the transaminases suggests that the two systems may be catalysed by two different enzymes, as are the animal glutamic-alanine and glutamic-aspartic transaminase systems.

This work forms part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The authors wish to thank Professor C. S. Hanes, F.R.S., who initiated this study, and Professor G. E. Briggs, F.R.S., for his interest during the course of the work. We are grateful to the University of Adelaide, University of Cambridge, and Agricultural Research Council for financial assistance for one of us (D.H.C.).

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The Nature of the Link Between Protein and Carbohydrate of a Chondroitin Sulphate Complex from Hyaline Cartilage

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(Received 24 September 1957)

Chondroitin sulphate from hyaline cartilage has been prepared by several procedures which give products with physical properties differing according to the method used for extraction. Successively larger quantities can be extracted from fresh cartilage by water (Partridge, 1948*b*), neutral salts such as 30 % potassium chloride (Einbinder & Schubert, 1950) or 10 % calcium chloride (Meyer & Smyth, 1937; Blix & Snellman, 1945) and by alkali, which has been used extensively (Mörner, 1889; Levene & La Forge, 1913; Jorpes, 1928; Fürth & Bruno, 1937; Bray, Gregory & Stacey, 1944; Mathews, Roseman & Dorfman, 1951). Shrinkage of collagen in cartilage by heat-treatment increases the yield of water-soluble chondroitin sulphate (Partridge, 1948*b*), as does very

prolonged extraction with water (Shatton & Schubert, 1954). The yield from alkaline extractions is high, but extraction with neutral salt gives much smaller yields and the remaining polysaccharide can be extracted only with alkali (Blix & Snellman, 1945; Einbinder & Schubert, 1950). By making a solution of potassium chloride mildly alkaline with potassium carbonate Einbinder & Schubert (1950) increased the yield of chondroitin sulphate considerably. Alkali is therefore particularly effective in releasing soluble chondroitin sulphate from cartilage, but the polysaccharide is obtained in a comparatively degraded form as judged by the relatively low molecular weight of such preparations, although the values depend partly on the method of measurement. Values ranging from 975

(Fürth & Bruno, 1937) to 30 000 have been recorded (Meyer, Odier & Siegrist, 1948; Mathews & Dorfman 1953; Mathews, 1956). The material is polydisperse and can be separated by precipitation into fractions of larger and smaller molecular size (Mathews, 1956). Analyses of such preparations are generally in fairly good agreement with figures calculated for a simple polysaccharide and only traces of amino acids can be found (Mathews, 1956).

In contrast, extraction with 10% calcium chloride gives a viscous product, which has a molecular weight several times greater than that of the product of alkaline extraction, although only one-tenth of the total hexosamine is extracted (Blix & Snellman, 1945; Mathews & Dorfman, 1953; Mathews, 1956). Analyses of such preparations show them to contain more nitrogen and less hexosamine than would be calculated for the simple polysaccharide. This has generally been attributed to contamination by some protein other than collagen, as hydroxyproline is absent. In the present work, however, evidence is presented to show that the material is a chemically bound polysaccharide-protein complex containing approximately 10% of protein, the integrity of which is essential in maintaining its high viscosity. This material, which will be referred to as the chondroitin sulphate complex, may represent a small and more soluble component of cartilage, in which tissue the bulk of the polysaccharide is bound in some other complex which cannot be dissolved without disruption. Some of this work has already been presented in two preliminary communications (Muir, 1956, 1957*b*).

METHODS AND MATERIALS

Total nitrogen. This was determined by the micro-Kjeldahl method. Solutions containing 0.5–1 mg. of N were digested with 2 ml. of 10% (w/v) selenium in H_2SO_4 (M.A.R., British Drug Houses Ltd.) for 2 hr.

Hexosamine. Hexosamine was estimated by a modification of the Elson & Morgan (1933) method (Kraan & Muir, 1957). The polysaccharide was hydrolysed in sealed tubes at 100° for 8 hr. in 3.8*N*-HCl.

Sulphate. A modification of the method of Dodgson & Spencer (1953) was used. The precipitation of benzidine sulphate was carried out on sintered-glass funnels (7 cm. × 4 cm.; average pore diameter 2 μ) stuck into a large bung bored half-way through with holes fitting the tips of the funnels which were thus sealed at the bottom. After 16 hr. at 4° the precipitate was collected under slight suction and washed twice with 5 ml. of acetone saturated with benzidine sulphate and once with acetone, and then dissolved in 3 ml. of *N*-HCl and washed through with 3 ml. of water. The benzidine in the filtrate was estimated as described by Dodgson & Spencer (1953), except that 0.15% $NaNO_2$ was used, the coloured solution diluted 20 times and its intensity measured in 1 cm. cells at 490 $m\mu$. Formic acid (6*N*) was used for hydrolysis in sealed

tubes heated at 100° for 9 hr. The contents of the tubes were made up to volumes containing 50–150 μ g. of sulphate/ml. and the sulphate was estimated directly in 1 ml. samples. Formic acid does not interfere with the precipitation of benzidine sulphate. The values obtained were reproducible to within 1%.

Sodium. Sodium was determined directly without combustion. A direct-reading flame photometer with gelatin filters was used.

Hydroxyproline. The method of Neuman & Logan (1950) was used to establish the absence of hydroxyproline.

Amino acids. Amino acids were identified by two-dimensional paper chromatography. The polysaccharide was hydrolysed with 6*N*-HCl in sealed tubes at 100° for 8 hr., and excess of acid removed by evaporating to dryness over solid NaOH. Samples calculated to contain approx. 140 μ g. of total amino acid in 10 μ l. of water were applied to Whatman no. 3 paper, which was irrigated with butanol-acetic acid-water (63:10:27) and then at right angles with phenol saturated with water (NH_3 atmosphere). Leucine and isoleucine were separated by substituting the phenol by butanol saturated with water (3% NH_3 atmosphere) (Consden, Gordon & Martin, 1944). Because of the presence of large amounts of amino sugar, basic amino acids were identified by chromatography in one direction with butanol-acetic acid-water followed by paper electrophoresis at right angles, in 0.04*M*-veronal buffer, pH 8.6, and with a potential gradient of 6 v/cm. for 5 hr. (Blass, Macheboeuf & Rebeyrotte, 1953). The chromatograms were stained with 0.1% (w/v) ninhydrin in butanol.

Quantitative estimation of amino acids was carried out on columns of Dowex 50 by the method of Moore & Stein (1951) as modified by Bowes, Elliott & Moss (1955). The chondroitin sulphate complex (200 mg.) was hydrolysed under reflux for 24 hr. in 750 ml. of constant-boiling HCl soln. The solution was evaporated to dryness and the residue taken up in 10 ml. of water; 1 ml. was applied to each column. Duplicate analyses were carried out. In one analysis of the chondroitin sulphate complex a known quantity of histidine was added to confirm the presence of histidine, which had not been definitely identified on paper. The small histidine peak was raised by the calculated amount. In one analysis of the papain-digested complex a known amount of aspartic acid was added as a marker.

Sugars. Amino sugars were identified by paper chromatography on Whatman no. 3 or 54 papers, irrigated with butanol-acetic acid-water (4:1:5) (Partridge, 1948*a*) for 44–48 hr. The polysaccharide was hydrolysed with 3.8*N*-HCl for 8 hr. at 100° and the excess of HCl removed at room temperature. Chromatograms run at the same time were stained with (a) 0.1% (w/v) ninhydrin in butanol; (b) benzidine (Harris & MacWilliam, 1954); (c) the Elson & Morgan reagent (Partridge, 1948*a*); (d) by dipping in a solution of 3 ml. of aniline in 75 ml. of acetone, to which had been added 25 ml. of 30% (v/v) phosphoric acid (A.R.) in water. Amino sugars were readily stained by (d) when the paper was heated at 95° for 5–7 min.

The presence of neutral sugars was sought by hydrolysing the chondroitin sulphate complex in sealed tubes at 100° in *N*-HCl for 10 hr. and in 0.5*N*-HCl for 16 hr. Excess of acid was removed *in vacuo* over solid NaOH at room temperature, and the residue examined for sugars by paper chromatography; irrigation was with butanol-acetic acid-water (Partridge, 1948*a*). Separate chromatograms were

stained with benzidine, naphtharesorcinol (Harris & MacWilliam, 1954) and aniline-phosphoric acid as described above. In some experiments 300 μ g. of hydrolysed material was used on one chromatogram, to enable less than 2% (by wt.) of neutral sugar to be detected by the stains employed.

Viscosity. Relative viscosities were measured in water or buffer solutions at $25 \pm 0.02^\circ$ in Ostwald viscometers (B.S. 188, no. 1), whose water-times were 196, 201 and 204 sec. In all enzyme experiments the flow-time of the appropriate buffer alone was measured and of the polysaccharide in the buffer.

Optical rotation. Optical rotations were measured in 2 dm. tubes at 25° . Aqueous solutions of the sodium salt of the polysaccharide were used.

Electrophoresis. Solutions (1%, w/v) of the preparations were examined in the Perkin-Elmer Tiselius electrophoresis apparatus at pH 7.4 in phosphate buffer (0.016M- $\text{Na}_2\text{HPO}_4 + 0.0335\text{M-NaH}_2\text{PO}_4$) and at pH 4 in acetate buffer (0.4M-sodium acetate + 1.6M-acetic acid). Ionic strengths were varied between 0.2 and 0.8 by adding sodium sulphate. Fig. 1 shows tracings of the boundaries at pH 7.4. In two experiments 1% (w/v) crystalline bovine-serum albumin was added to compare mobilities at pH 7.4 of the chondroitin sulphate complex and of that which had been digested with papain.

Animals. The tracheal and laryngeal cartilage of 6- to 8-month-old pigs was obtained within a few hours of death. Muscle and connective tissue were carefully removed and the cartilage was frozen with solid carbon dioxide and sliced. When it had thawed it was minced and extracted at once.

Sodium chondroitin sulphate complex

Minced cartilage (1 kg.) was homogenized in 200 g. portions in a Waring Blendor while suspended in 10% CaCl_2 adjusted to pH 6.8. The temperature was not allowed to rise above 25° but air was not excluded. The total volume of CaCl_2 was 3 l. The portions were combined, nitrogen was passed in for 45 min. and the suspension left to stand at 4° in an atmosphere of nitrogen for 2 days and shaken occasionally. The product was squeezed through several layers of cheese cloth and the semi-gelatinous residue again suspended in 1 l. of 10% CaCl_2 , homogenized in a Waring Blendor and left to stand under nitrogen at 4° for 24 hr. This material was also squeezed through cheese cloth and the filtrates were combined, stirred and carefully saturated with ammonium sulphate. After standing overnight at 4° the very thick precipitate was removed by filtration through Celite and washed twice with 500 ml. of saturated ammonium sulphate. Filtrate and washings were combined and dialysed against several changes of 20 l. of distilled water until no sulphate could be detected with barium chloride in the diffusate. The dialysis sacs were prevented from bursting with sheaths of silk net tied at both ends. A small amount of precipitate formed, which was removed by filtration through Celite and the Celite washed with a small volume of water. Filtrate and washings were combined (6 l.) and the chondroitin sulphate complex was precipitated with $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$ as described by Mathews & Dorfman (1953). It was converted into the sodium salt by their procedure and again precipitated with $[\text{Co}(\text{NH}_3)_6]\text{Cl}_3$, converted into the sodium salt and dialysed against four changes of 10 l. of distilled water at 4° . The resulting solution (1300 ml.) was treated with 4 ml. of

acetic acid and then 8 g. of sodium acetate. It was then slowly run into 4 l. of cold ethanol stirred by a stream of nitrogen. After standing for 2 days at 4° the precipitate was collected by centrifuging and was washed successively with 95% ethanol, ethanol and peroxide-free ether, and was finally dried *in vacuo* over P_2O_5 at 45° for 3 days. The yield was 7.1 g. The analysis of the product is given under (A) in Table 1.

Further purification of the chondroitin sulphate complex

A portion (3 g.) from the above preparation was dissolved in 1 l. of water and made acid to methyl orange with acetic acid. A saturated solution of 5-aminoacridine hydrochloride (British numbering) in water was slowly added with stirring until the precipitate flocculated, after which no more precipitate formed. The solution was left to stand overnight and the precipitate collected by centrifuging. It was washed three times by suspending in water and centrifuging. It was finally suspended in 300 ml. of 2.5% (w/v) sodium formate, and shaken with Zeo-Karb 225 (Na^+) (The Permutit Co. Ltd.) until the supernatant, which gradually became more viscous, showed no blue fluorescence. The resin was removed and washed four times with 100 ml. of water and the solution and washings were combined and dialysed against three changes of 5 l. of distilled water. The solution was again made acid with acetic acid and the polysaccharide reprecipitated with 5-aminoacridine hydrochloride. After conversion into the sodium salt as described above, the volume of the solution was reduced by dialysing under pressure, nitrogen at 12 lb./in.² being used, against three changes of 5 l. of distilled water. The dialysis sacs were supported by sheaths of silk net. The last traces of acridine were removed by placing some resin in the dialysis water. The polysaccharide was precipitated with ethanol and dried as described above. The yield was 2.7 g. The analysis of the sodium salt is given in Table 1 under (B) and the amino acid composition in Table 2. Aqueous solutions (1%, w/v) did not flocculate or show any opalescence when heated at 100° for 40 min., nor when treated with sulphosalicylic acid, uranyl acetate, tungstic acid, cadmium chloride or trichloroacetic acid.

Papain-treated chondroitin sulphate complex

Crude papain (100 mg.) was activated by incubating at 45° for 30 min. in 10 ml. of 0.02M-acetate buffer, pH 5.4, which contained cysteine and disodium ethylene diamine-tetra-acetate (Kimmel & Smith, 1954). The insoluble matter was removed by filtering and the filtrate added to a solution of 1 g. of purified chondroitin sulphate complex in 50 ml. of 0.02M-acetate buffer, pH 5.4. Some crystals of benzoic acid were added and the whole was incubated overnight at 45° . Some proteolytic activity was still present at the end of this time when tested against gelatin film. The solution was dialysed against six changes of 4 l. of distilled water for 48 hr. It was diluted to 500 ml. and the polysaccharide purified by precipitating twice with 5-aminoacridine hydrochloride as described above. This procedure did not precipitate papain from its solution in acetate buffer. After conversion into the sodium salt by shaking with Zeo-Karb 225 (Na^+) as described above, the polysaccharide was precipitated with ethanol and dried as previously described. The yield was 840 mg., of which

500 mg. was treated a second time with papain, twice precipitated with 5-aminoacridine and finally obtained as the sodium salt. The analysis of the final sample is given in Table 1 and amino acid composition in Table 2.

Alkali-treated chondroitin sulphate complex

The crude chondroitin sulphate (500 mg.) was dissolved in 50 ml. of 0.15N-NaOH. After 4 days at room temperature the solution was neutralized with HCl soln. and diluted to 250 ml. The polysaccharide was twice precipitated with 5-aminoacridine and finally obtained as the sodium salt, the analysis of which is given in Table 1.

Trypsin-treated chondroitin sulphate complex

The purified complex (100 mg.) in 20 ml. of 0.02M-veronal buffer at pH 7.7 was treated with 400 μ g. of crystalline trypsin dissolved in 0.4 ml. of buffer. After 4 hr. at 25° a further 400 μ g. of trypsin was added. Proteolytic activity was still present 4 hr. later when the solution was tested with gelatin film. After 24 hr. the solution was dialysed against three changes of 150 ml. of distilled water for 24 hr., diluted to 50 ml., and the polysaccharide isolated with 5-aminoacridine and converted into the sodium salt as described above.

Ultrafiltration experiments

In duplicate experiments, 300 mg. samples of the complex and of the papain-digested complex were dissolved in 50 ml. and subjected to ultrafiltration through supported dialysis tubing by applying a pressure of 12 lb./in.². Volumes equal to about 80% of the original volume were collected and measured exactly. These ultrafiltrates were evaporated under reduced pressure and made up to 10 ml. Sulphate and hexosamine were determined in 2 ml. samples after hydrolysis, and the remainder was examined for amino acids after hydrolysis in 6N-HCl followed by evaporation to dryness *in vacuo*. The amino acids in this residue were extracted with 75% (w/v) aq. ethanol from salt which was also present in the ultrafiltrates. Control experiments were first carried out on each sample of untreated material. The volume in the sac was then restored to 50 ml. and the procedure repeated after treatment of the sample. The hexosamine content of the solutions was determined before the second ultrafiltration, to account for any losses during the control experiments. Ultrafiltrates were obtained from two types of experiment: (a) those in which the purified chondroitin sulphate complex and the papain-digested complex had been separately treated with 0.15N-NaOH for 4 days at room temperature and the solutions neutralized; (b) those in which the purified complex in 0.02M-acetate buffer at pH 5.4 had been treated with 50 mg. of activated papain and incubated at 45° overnight. Ultrafiltrates of solutions of polysaccharide with activators, but without papain, and of activated papain alone, were also examined. Those from papain were not analysed for amino acids. The results are given in Table 3.

Paper chromatography of chondroitin sulphate

The chondroitin sulphate complex after various treatments was examined on Whatman no. 2 paper which was irrigated with a mixture of 45% (v/v) propan-1-ol and 55% 0.2M-boric acid. A portion (10–30 μ g.) of the sodium

salt of the complex was applied to the paper. The polysaccharide was stained by dipping the dried paper in aq. 0.1% toluidine blue for 1 min. The paper was then hung in a bath of lukewarm water for 10 min., which reduced the background colour to a pale blue and left the polysaccharide stained a deep blue; 3 μ g./cm.² could be detected.

Precipitation of proteins and polyanions from aqueous solutions by 5-aminoacridine hydrochloride

Undiluted serum proteins formed a slight precipitate when treated with a saturated aqueous solution of 5-aminoacridine hydrochloride but redissolved immediately at pH 5.5. No precipitate was formed with neutral or acid solutions of 5% (w/v) gelatin, nor with acid-soluble collagen dissolved in dilute acetic acid. A precipitate was formed from 2% (w/v) solutions of hyaluronic acid at pH 4 but redissolved at pH 2. A 1% solution of yeast nucleic acid formed a precipitate which would not redissolve in 0.5N-HCl. Chondroitin sulphate was precipitated from very dilute solution containing 0.1 m-mole of sulphate groups.

RESULTS

Chemical composition

Table 1 shows the chemical analyses of the chondroitin sulphate complex as extracted with 10% CaCl₂ after various treatments, compared with the values calculated for the sodium salt of a simple polysaccharide made up of units of *N*-acetyl chondrosine sulphate and four molecules of water. All samples were dried under the same conditions, and it was assumed that each contained the same amount of water, as it is difficult to dehydrate chondroitin sulphate completely (Meyer *et al.* 1948). After purifying by precipitation with [Co(NH₃)₆]Cl₃ (Mathews & Dorfman, 1953), there remained an appreciable amount of non-hexosamine nitrogen, corresponding to approx. 15% of protein. This was reduced to about 10% when the complex was further purified by precipitating with 5-aminoacridine, which forms insoluble salts with organic sulphates (Dodgson, Rose & Spencer, 1954). This reagent did not precipitate undiluted serum proteins, gelatin or acid-soluble collagen.

Digestion with papain greatly reduced the protein nitrogen and raised the other analytical values to the calculated figures. Only the sodium value remained 10% below the calculated figure. Treatment of the crude complex (Table 1, A) with dilute alkali at room temperature, before the purification with 5-aminoacridine, gave a product entirely free of protein nitrogen (Table 1), and no amino acids were detected by paper chromatography, even when a large sample of hydrolysate was applied to the paper.

The protein associated with the polysaccharide in the complex was shown by paper chromatography and electrophoresis, and by quantitative chromatographic analysis (Moore & Stein, 1951), to

contain a considerable amount of serine and a large proportion of dicarboxylic and a much smaller proportion of basic amino acids. Aromatic amino acids were scarce and only traces of those containing sulphur could be detected. No hydroxyproline was found by the Neuman & Logan (1950) procedure. The results are given in Table 2. The whole complex contained only 1.77% of non-hexosamine nitrogen, of which 72% was accounted for. This result is relatively satisfactory as amide nitrogen was not determined and approximately two-thirds of the material consisted of carbo-

hydrate which may cause destruction of amino acids during hydrolysis (Block & Bolling, 1951; Martin & Synge, 1945).

Serine was the only amino acid which was not greatly reduced by papain digestion, but remained in almost half the original amount (Table 2). Its proportion relative to other amino acids was thus greatly increased, so that it was prominent on paper chromatograms. The determination of the very small residue of protein nitrogen of the papain-digested material was unavoidably inaccurate and a complete chromatographic analysis

Table 1. *Analytical values of sodium chondroitin sulphate from pig tracheal cartilage*

Values were determined before and after purification with 5-aminoacridine, and after treatment with papain, and with dilute alkali followed by purification with 5-aminoacridine.

	Crude material (A)	After purifying with 5-aminoacridine (B)	(B) After treatment with papain	(A) After treatment with alkali	Calc. for $C_{14}H_{18}O_{14}NSNa_2 \cdot 4H_2O$
Nitrogen (%)	4.47	3.92	2.84	2.46	2.44
Hexosamine (%)	26.3	27.5	31.0	—	31.1
Non-hexosamine nitrogen by difference (%)	2.4	1.77	0.412	†	—
Sulphate (%)	14.1	14.5	16.6	15.7	16.68
Sodium (%)	6.0	6.62	7.2	7.2	8.01
Relative viscosity in water of 0.5% solutions					
(η/η_0) at 25°	4.63	4.5	1.39	1.37	—
$[\alpha]_D^{25}$	—	-33°	-27.6°	-26.2°	-28-32°*

* Meyer, Davidson, Linker & Hoffman, 1956.

† Paper chromatography showed no amino acids present.

Table 2. *Amino acid content of the chondroitin sulphate complex*

Amino acids remaining associated with the polysaccharide after digestion of the complex with papain are also listed.

	Chondroitin sulphate complex (1.77% of protein N)			Papain-digested complex (0.412% of protein N)		
	N (% protein N)	Amino acid (g./100 g. of complex)	Moles/ 50 000 mol.wt. of complex	N (% protein N)	Amino acid (g./100 g. of complex)	Moles/ 50 000 mol.wt. of complex
Glycine	7.07	0.67	4.47	7.55	0.181	—
Alanine	5.64	0.634	3.56			
Valine	4.46	0.66	2.82	2.9	0.10	0.43
Serine	6.38	0.848	4.04	11.9	0.367	1.75
Threonine	3.35	0.504	2.11	—	—	—
Leucine	6.26	1.04	3.96	—	—	—
Isoleucine	2.99	0.496	1.89	—	—	—
Phenylalanine	3.31	0.69	3.09	—	—	—
Tyrosine	1.87	0.424	1.17	—	—	—
Cystine*	Trace	—	—	—	—	—
Proline†	1.07	0.154	0.67	—	—	—
Hydroxyproline	0.0	0.0	0.0	—	—	—
Arginine	9.73	0.536	1.54	—	—	—
Histidine	2.87	0.187	0.6	—	—	—
Lysine	3.27	0.302	1.03	—	—	—
Aspartic acid	5.58	0.938	3.53	1.09	0.043	0.16
Glutamic acid	7.57	1.407	4.79	2.61	0.113	0.38
Unknown	0.82	—	—	—	—	—
	72.24					

* As cysteic acid.

† Value not accurate owing to proximity of large glutamic acid peak.

was not attempted, but only serine and those amino acids which were eluted in its vicinity were estimated. The amino acids present in lesser quantities in the original complex, namely phenylalanine, tyrosine, proline, threonine and the basic amino acids, could not be detected on paper chromatograms of the papain-digested complex. In contrast, digestion with trypsin did not change the amino acid pattern of the complex.

The purified complex did not contain any neutral sugars, as none could be detected by paper chromatography after hydrolysing with *N*- and 0.5*N*-acid. The amino sugar of the polysaccharide was identified as galactosamine on paper chromatograms after hydrolysis in hydrochloric acid, by comparison with an authentic specimen of the hydrochloride. A second spot was also obtained which had similar reactions to galactosamine but its *R_f* was very much smaller than that of either galactosamine or glucosamine in butanol-acetic acid-water (Partridge, 1948*a*). It was identified as due to the salt of galactosamine with sulphuric acid, which is more firmly bound than hydrochloric acid. It had been suggested that this substance might be the epimer of galactosamine (Muir, 1957*a*). However, there is sufficient sulphuric acid in hydrolysates of chondroitin sulphate to form considerable quantities of this salt, even in the presence of a large excess of hydrochloric acid. The free amino sugar was not liberated from its sulphuric acid salt by ammonia solution (sp.gr. 0.880) applied to the spot, and the hydrochloride could be obtained only by addition of barium chloride. Ammonium sulphate, when added to galactosamine hydrochloride, produced a similar spot. Such behaviour is in agreement with recent observations (Leaback & Walker, 1957). Both spots gave lyxose on oxidation with ninhydrin (Stoffyn & Jeanloz, 1954).

Electrophoresis

The purified chondroitin sulphate complex (Table 1, *B*), which contained 10% of protein, was electrophoretically homogeneous at ionic strengths from 0.2 to 0.8 in phosphate buffer, pH 7.4, and acetate buffer, pH 4. Fig. 1 shows tracings of patterns obtained at pH 7.4. The mobilities of the original material and of the papain-digested complex were identical when compared with an internal standard of bovine-serum albumin (Kunkel & Tiselius, 1951), and were 16.7×10^{-5} cm.²/sec./v at pH 7.4.

Effect of proteolytic enzymes, alkali and heat on the viscosity of the chondroitin sulphate complex

The effects of different proteolytic enzymes on the viscosity of the complex were approximately compared by measuring the flow-time of solutions

of chondroitin sulphate in the appropriate buffer before and after addition of enzyme. The large variations in the viscosity of chondroitin sulphate in the presence of salts (Mathews, 1953) were thus approximately allowed for.

Trypsin and chymotrypsin reduced the flow-time of solutions of the crude complex by 20–25%. No further effect of trypsin was observed when fresh enzyme was added after 4½ hr., although proteolytic activity could still be detected in the solution 2 hr. later, when tested against gelatin film. Pepsin at pH 1.9 almost halved the flow-time, but was without effect at pH 6.8, and carboxypeptidase had almost no effect. These results are illustrated in Fig. 2. In contrast with these enzymes, papain caused a rapid and large fall in the flow-time of solutions of the complex, as shown in Fig. 3, even though the relative substrate concentration was greater than that used in the experiments with trypsin, chymotrypsin and pepsin. Crude papain contains at least two proteolytic enzymes (Kimmel & Smith, 1954), but the

Fig. 1. Tracings of electrophoresis scanning photographs of ascending and descending boundaries; Perkin-Elmer Tiselius apparatus model 38. Chondroitin sulphate complex 1% (w/v) in 0.02*M*-phosphate buffer, pH 7.4, plus 0.21*M*-sodium sulphate, *I* 0.7; potential gradient 4v/cm. (1) Ascending boundary, after 60 min.; (2) descending boundary, after 60 min.; (3) ascending boundary, after 120 min.; (4) descending boundary, after 120 min. Direction of migration is shown by arrows.

purified crystalline enzyme was more active than the crude enzyme and reduced the flow-time by 80% within a few minutes, as shown in Fig. 3. *p*-Chloromercuribenzoate completely inhibits papain at a concentration of 0.1 mM, and this concentration also inhibited its effect on the viscosity of solutions of the complex. In this experiment the activated enzyme was dialysed for 16 hr. to remove excess of cysteine and ethylenediaminetetraacetate which had been used to activate it, so that it had lost some of its activity, as can be seen from Fig. 3.

Very dilute alkali at 25° also caused a decrease in the viscosity of solutions of the complex. The effect of 0.015*N*-NaOH was to reduce the relative viscosity from 4.37 to 3.8 in 1 hr.; this continued to fall gradually to 1.48 in 36 hr. Although 0.01*N*-alkali also reduced the viscosity, 0.0075*N*-NaOH was without effect after 5 hr.

Digestion of the complex with papain or treatment with 0.15*N*-NaOH resulted in products which had similar relative viscosities; both were low compared with that of the untreated complex, as shown in Table 1.

Heat-treatment of the chondroitin sulphate complex did not significantly effect its viscosity. The relative viscosity of 0.5% solutions in water

was reduced by only 1.5% after heating for 40 min. at 100°. The solution did not flocculate or become opalescent.

Effect of proteolytic enzymes, alkali and heat on the chromatographic behaviour of the chondroitin sulphate complex

Changes in the behaviour of the chondroitin sulphate complex, on paper chromatograms irrigated with propan-1-ol-boric acid paralleled changes in its viscosity after various treatments. The untreated complex remained at the origin, as did that which had been heated or treated with those enzymes which did not greatly affect its viscosity, namely trypsin, chymotrypsin and carboxypeptidase. On the other hand, papain and alkali, which caused a large decrease in viscosity, also changed the chromatographic behaviour of the polysaccharide, which now travelled with R_f 0.6. Neither papain inhibited by *p*-chloromercuribenzoate nor 0.1*N*-NaOH acting for less than 5 min. had this effect. After longer exposures to alkali, the proportion of material of R_f 0.6 increased with time until no material remained at the starting line, but there was no change of optical rotation during this process. The effect of treatment with pepsin, which approximately halved the flow-time of solutions of the complex, was similar to

Fig. 2. Effect of proteolytic enzymes at 25° on flow-times measured in Ostwald viscometers of solutions of the crude chondroitin sulphate complex. The percentage decrease in flow-time is calculated from the formula $100(x-y)/x$, where x is the flow-time for untreated complex and y the flow-time for substrate after addition of enzyme. ●, Solution (1%) in 0.02*M*-citrate buffer, pH 1.9, plus pepsin (10 μ g./ml.); ○, 0.5% solution in 0.02*M*-veronal buffer, pH 7.7, plus trypsin (10 μ g./ml.); ▲, 0.5% solution in 0.02*M*-veronal buffer, pH 7.7, plus chymotrypsin (10 μ g./ml.); ■, 0.5% solution in 0.02*M*-veronal buffer, pH 7.7, plus carboxypeptidase (10 μ g./ml.).

Fig. 3. Effect of activated papain (Kimmel & Smith, 1954) at 25° on flow-times measured in Ostwald viscometers, of solutions of the chondroitin sulphate complex in 0.02*M*-acetate buffer at pH 5.5. The percentage decrease in flow-time is calculated as in Fig. 2. ●, Solution (0.4%) plus crystalline papain (1 μ g./ml.); ○, 1% solution plus crude papain (10 μ g./ml.); ▲, 0.4% solution plus crude papain (10 μ g./ml.), dialysed for 16 hr. to remove excess of activators; ■, 0.4% solution plus crude papain (10 μ g./ml.), dialysed for 16 hr. plus *p*-chloromercuribenzoate (0.1 mM).

that obtained by treatment for 15–30 min. with 0.1N-NaOH. Some of the material moved with R_f 0.6 and some remained at the origin, and there appeared intermediate streaks. Pepsin had no effect at pH 6.8.

Ultrafiltration

Experiments of type (a) (see Methods and Materials).

No amino acids and negligible amounts of hexosamine and sulphate were found in hydrolysed ultrafiltrates obtained either from solutions of the untreated purified complex or from solutions of the papain-digested complex which had been reisolated and purified. However, after treatment with dilute alkali, the hydrolysed ultrafiltrates from both these materials contained amino acids in the same relative proportions as were present in the starting materials. An appreciable amount of hexosamine, and roughly twice this amount of sulphate, also became diffusible, as shown in Table 3. Part of the diffusible sulphate was free inorganic sulphate and could be precipitated by barium chloride without previous hydrolysis. Barium chloride does not precipitate chondroitin sulphate from dilute solutions. Some inorganic sulphate was also slowly released from commercial chondroitin sulphate by 0.15N-alkali.

Experiments of type (b) (see Methods and Materials).

Treatment of the purified complex with papain gave rise to rather less diffusible hexosamine and sulphate (Table 3).

DISCUSSION

The chondroitin sulphate extracted from pig tracheal cartilage was not contaminated by other polysaccharides, since during purification the molar ratio of hexosamine to sulphate remained

unity, no neutral sugars were found and galactosamine was the sole amino sugar present. The sodium analyses were, however, low, even of preparations containing very little non-hexosamine nitrogen. This may be due to the presence of some lactone rings (Meyer & Rapport, 1951), which would not have been opened as alkaline conditions were carefully avoided.

The small protein residue which could not be removed by further purification is unlikely to have been held by electrostatic forces or hydrogen bonds to the chondroitin sulphate because the complex was extracted with neutral calcium chloride and subsequently saturated with ammonium sulphate. The purified chondroitin sulphate complex migrated as a single boundary on electrophoresis and did not dissociate even when the ionic strength was increased by addition of sodium sulphate, a salt which is particularly effective in dissolving insoluble salts of chondroitin sulphate with complex cations (Vouras & Schubert, 1957), and which might be expected to displace chondroitin sulphate from its salts with protein. Furthermore, the protein component of the complex contained a large proportion of dicarboxylic amino acids, but only a small proportion of basic amino acids. Nevertheless, the difficulty of removing traces of protein contaminating preparations of acidic polysaccharides is well known. The residual protein of the complex was not collagenous, since it contained no hydroxyproline and less glycine and arginine than collagenous proteins (Bowes *et al.* 1955), but serum proteins have also been found in connective tissue (Boas, 1955; Humphrey, Perkins & Neuberger, 1957) and some non-collagenous protein is present in cartilage (Shatton & Schubert, 1954). Although 5-aminoacridine did not itself precipitate

Table 3. *Analysis of ultrafiltrates*

Two types of experiment are reported: (a) after the effect of 0.15N-NaOH on the chondroitin sulphate complex, and on the papain-digested complex; (b) after the effect of papain on the complex. The diffusible hexosamine and sulphate, determined after hydrolysis, are calculated as % of the total in starting materials. Amino acids were identified after hydrolysis. —, Not estimated.

No. of experiment	Hexosamine (% of total)		Sulphate (% of total)		Amino acids		
	I	II	I	II	I	II	III
(a)							
Chondroitin sulphate complex treated with NaOH and neutralized	6.77	6.65	14.1†	14.2	Same relative proportions as in complex		
Complex alone	—	0.3	1.8	1.8	None	None	None
Papain-digested complex treated with NaOH and neutralized	14.32	9.52	21.0	14.82	Serine and other residual acids		
Papain-digested complex alone	2.9	0.8	0	0	None	None	None
(b)							
Complex + papain and activators*	6.09	4.23	—	5.6	—	—	—
Complex + activators	0.9	—	—	—	—	—	—
Activators + papain	0	—	—	—	—	—	—

* Ethylenediaminetetra-acetate + cysteine (Kimmel & Smith, 1954).

† Free inorganic sulphate determined without hydrolysis was 6.1%.

serum proteins, there remained the possibility that the protein residue had become adsorbed on to the precipitate. This was unlikely, however, because when the protein had been previously detached from the polysaccharide by treatment with alkali, but was still present in solution during the precipitation, a protein-free product was obtained by the same procedure that had been used to purify the complex. The low viscosity of this product appeared to be mainly due to removal of the protein, since a product of similar low viscosity resulted from papain treatment, which left only a small residue of protein nitrogen. The presence of contaminating papaya lysozyme cannot explain the effect of papain, which was inhibited by a sulphhydryl reagent that does not greatly affect lysozyme (Smith, Kimmel, Brown & Thompson, 1955).

The similar electrophoretic mobility of the complex and papain-digested product is to be expected, because the protein component amounted to only one-tenth of the total complex and contained few basic amino acids. Mathews & Dorfman (1953) found no difference between the mobilities of chondroitin sulphate extracted with alkali, which contained only traces of amino acids, and that extracted with calcium chloride.

The protein moiety of the complex appeared to be bound to the polysaccharide by a bond as sensitive to alkali as an ester, because very dilute alkali caused a loss of viscosity. The residue which remained after digestion of the complex with papain was also labile to alkali. Since the greater part of this residue was serine, it would seem that the protein moiety was linked to chondroitin sulphate through serine, which may be combined with an acidic group of chondroitin sulphate as phosphate is attached to the hydroxyl group of serine in phosphoproteins. These are labile to alkali but more stable to acid (Perlmann, 1955). Blix & Snellman (1945) found that the viscosity of a chondroitin sulphate preparation extracted with calcium chloride was not greatly affected by acid but was reduced by dilute alkali.

Chondroitin sulphate complexes appear to be linear (Webber & Bayley, 1956; Bernardi, 1957) and it has been suggested that the polysaccharide chains are cemented together by protein (Muir, 1956) in an end-to-end arrangement (Webber & Bayley, 1956). If the complex from pig cartilage has a molecular weight of 50 000, as does that from ox cartilage (Mathews, 1956), four molecules of serine would be present in each molecule, while after papain digestion almost two would remain, so that the protein moiety could be attached at both ends to chondroitin sulphate molecules, in the manner suggested. The protein moiety was small enough to diffuse through dialysis tubing, so that

its removal should not greatly affect the viscosity unless it joined together longer carbohydrate chains. Its small size also makes it unlikely that the effect of papain can be explained by the removal of an impurity of very high molecular weight, to which Mathews (1956) attributes the effect of proteolytic enzymes on the viscosity of chondroitin sulphate from ox cartilage.

Mild extraction procedures which cause no breakdown should select only smaller molecules, leaving most of the chondroitin sulphate in insoluble complexes of very large molecular weight. Larger yields are obtained after autolysis (Einbinder & Schubert, 1950), and digestion with papain is known to release most of the polysaccharide sulphate from various tissues (Slack, 1955). It was therefore to be expected that good yields of chondroitin sulphate of low viscosity would be obtained by the methods described above when the cartilage had first been treated with papain. Since good yields are also obtained by alkaline extraction of other tissues, chondroitin sulphate to protein bonds which are alkali sensitive should occur generally. Alkali, however, causes breakdown in addition to the detaching of protein, because more diffusible carbohydrate appears when the papain-digested complex is treated with alkali. Mathews (1956) similarly found that dilute alkali degrades chondroitin sulphate to smaller units. The slow liberation of inorganic sulphate by alkali might explain why some preparations of chondroitin sulphate extracted with alkali are low in sulphate (Bray *et al.* 1944; Einbinder & Schubert, 1950; Fürth & Bruno, 1937). On the other hand, Blix & Snellman (1945) observed that although new acidic groups appear when chondroitin sulphate is treated with alkali, only a fraction can be attributed to the hydrolysis of ester sulphate.

The chondroitin sulphate complex of pig tracheal cartilage differs from complexes obtained from ox cartilage by Shatton & Schubert (1954), Mathews (1956) and Webber & Bayley (1956) in containing rather less protein and in being resistant to trypsin and chymotrypsin. This resistance may be due to the low aromatic and basic amino acid content (Green & Neurath, 1954) and may depend upon a species difference, since glutamic acid, a prominent feature of the complex from pig cartilage, was absent from material from ox cartilage (Webber & Bayley, 1956).

SUMMARY

1. A chondroitin sulphate complex was extracted from pig tracheal cartilage with calcium chloride. After purifying there remained nearly 10% of protein. Contamination by other polysaccharides was excluded.

2. The complex was electrophoretically homogeneous and did not dissociate at high ionic strengths.

3. Its high viscosity depended upon the integrity of the protein component, which was destroyed by papain but which was resistant to trypsin and chymotrypsin, and partially resistant to pepsin.

4. Dilute alkali also brought about a large fall in viscosity, after which chondroitin sulphate entirely free of amino acids was obtained from the crude material by the same procedure as had been used to purify the complex.

5. Quantitative amino acid analysis showed that the complex contained considerably more dicarboxylic than basic amino acids and a large amount of serine.

6. After digestion with papain half the original amount of serine was left, whereas the quantities of the other amino acids which remained were very much less. A small proportion of the total hexosamine and inorganic sulphate appeared in ultrafiltrates.

7. Ultrafiltrates obtained from the purified complex and from that which had been isolated again after digestion with papain contained no hexosamine sulphate or amino acids, until treated with dilute alkali, when amino acids appeared in the same relative proportions as were present in the original materials, together with some sulphate and hexosamine.

8. It is concluded that the complex consists of polysaccharide chains of variable but moderate length, cemented together by polypeptide units attached to carbohydrate by a bond as sensitive to alkali as an ester or lactone.

I am extremely grateful to Dr J. H. Bowes and Mr A. J. Moss, of the British Leather Manufacturers' Research Association, who very kindly carried out the quantitative amino acid analyses which have contributed so much to this work, and to Mr W. Cook for assistance in modifying the method for estimating sulphate. I am also indebted to the Empire Rheumatism Council for a Fellowship.

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