The Chemistry of Connective Tissues

4. THE PRESENCE OF A NON-COLLAGENOUS PROTEIN IN CARTILAGE*

By S. M. PARTRIDGE and H. F. DAVIS
Low Temperature Station for Research in Biochemistry and Biophysics, University of Cambridge and Department of Scientific and Industrial Research

(*Received* 19 *June* 1957)

Work carried out some years ago (Partridge, 1948*b*) showed that the intercellular matter of hyaline cartilage is composed in very large part of two main components: chondroitin sulphate and collagen. Recently, however, the presence of a non-collagenous protein in cartilage has been reported by Shatton & Schubert (1954), who showed that aqueous extraction of fresh bovine nasal cartilage at 1° released a quantity of material which behaved as a rather stable compound of the new protein and chondroitin sulphate and was described by the authors as a mucoprotein. Soon afterwards Muir (1956) observed a marked reduction in the viscosity of an undegraded preparation of chondroitin sulphate from laryngeal cartilage on treatment with papain and suggested that such preparations may consist of polysaccharide units cemented by protein.

The extraction of chondroitin sulphate from cartilage in an undegraded form is a problem which has attracted many workers. The experience in the past has been that in order to effect the release of polysaccharide in good yield it is necessary to employ rather vigorous reagents and these give rise to extensive degradation. Good yields of chondroitin sulphate of high chemical purity may be obtained by extraction with caustic alkali, but the molecular weight of such preparations is considerably lower than that of extracts prepared under milder conditions. If undegraded preparations are required it is necessary to employ neutral reagents such as calcium chloride (Blix & Snellman, 1945). However, most of the effective reagents of this type reduce the temperature of thermal shrinkage of collagen and bring a great deal of the protein into solution (Partridge, 1948*b*). The molecular weight of preparations of chondroitin sulphate, prepared by neutral extraction, has been given as 200 000–300 000 by Blix & Snellman (from viscosity measurements) and 150 000 by Matthews (1955, light-scattering), while preparations prepared by the use of caustic alkali have molecular weights estimated to be in the range 10 000–50 000 by Meyer & Odier (1946) and 18 000–48 000 by Matthews.

* Part 3: Partridge & Davis (1955).

A considerable advance in the technique of extraction was made when Einbinder & Schubert (1950) introduced a new reagent consisting of a 30% (w/v) aqueous solution of potassium chloride containing 1% of potassium carbonate. Use of this solution extracts a product of high viscosity in good yield, but yet prevents extensive solubilization of collagen. Preliminary experiments carried out in this Laboratory showed that extraction of finely ground cartilage powder with this mildly alkaline reagent was effective in separating the bulk of the polysaccharide-containing material from the residual collagenous structure without causing observable degradation, and it was thought that an extract so prepared would offer a suitable starting-point for a further study of the composition of the ground substance of this tissue.

EXPERIMENTAL

Preparation of the cartilage powder. Fresh nasal cartilage from a group of young cattle was freed from connective tissue and chopped finely with a scalpel. The pieces were rapidly washed in 1% NaCl solution, followed by water, and were then introduced at once into acetone. After standing in two or three changes of solvent the pieces were dried in air at 70° and then ground to a fine powder in a hammer mill.

Extraction with potassium chloride–potassium carbonate solution. The procedure followed generally the description given by Einbinder & Schubert (1950). For the first extract (extract *A*, Table 1) the cartilage powder (30 g.) was suspended in 500 ml. of a solution containing 300 g. of KCl and 10 g. of K_2CO_3/l. and shaken for 48 hr. at room temperature (18–20°). A second extract (*B*, Table 1) was obtained by shaking the residue in a further quantity (500 ml.) of the same solution for 96 hr. Finally two further extracts, designed to exhaust the cartilage, were prepared by extracting the residue for 48 hr. with 30% KCl containing 1% of KOH (extract *C*), followed by 48 hr. with 30% KCl containing 2% of KOH (extract *D*). Each of the extracts was dialysed against repeated changes of distilled water until free from chloride; after samples for determination of dry weight and total hexosamine (after hydrolysis) had been taken, the solutions were frozen at −10° for storage. Analysis of a typical series of extracts is given in Table 1.

Zone electrophoresis. The apparatus was based on the design of Haglund & Tiselius (1950). A vertical jacketed

column packed with graded potato starch according to the procedure of Carlson (1954) was arranged over an automatic fraction changer so that at the end of the experiment the separated zones could be forced off the column by the slow passage of buffer. Two columns of different size (30 cm. × 1·1 cm. and 30 cm. × 2·9 cm.) were in use in these experiments; both columns were provided with water jackets. Silver–silver chloride electrodes were employed similar to those used in the standard Tiselius (1937) moving-boundary apparatus. Details of the electrode vessels and connecting bridges will be given in a later publication.

To serve as a marker and as a control on the correct functioning of the column during elution, a small amount of bovine carbon monoxy haemoglobin was added to every sample; an experiment was accepted as satisfactory only if the red band due to carbon monoxy haemoglobin remained narrow and sharp after passage of the current, and if it were not distorted during the progress of elution. At pH 8·6 the migration of haemoglobin is very slow (about 0·05 cm./hr. at 8·5 v/cm.); it had the lowest mobility of any of the zones, and remained near the top of the column after passage of the current. During elution the arrival of the red band at the foot of the column was taken as an indication that the process was complete. For the separation of cartilage extracts the apparatus was used with water at room temperature, instead of the usual 2°, because the high viscosity of the extract at low temperature causes irregularity of electrophoretic migration and of elution.

A typical experiment with the 2·9 cm. diameter column was conducted as follows. A sample (2 ml.) of the freeze-dried extract (30 mg. in 1·5 ml. of buffer, to which was added 1·5 ml. of a 5% solution of carbon monoxy haemoglobin in the same buffer) was introduced on to the top of the column. The buffer used was sodium barbiturate (0·05 M) of pH 8·38 (glass electrode). The red haemoglobin band was first forced down the column to a position about 6 cm. from the top by application of buffer. The bridges connecting the electrode vessels were then put in place and the assembly filled with buffer. A potential of 400 v (12·4–12·8 mA) was then applied for 21 hr.; the bath temperature rose slowly from 21·8° to 23·8° during the course of the run. Through the combined effects of electrical transport and endosmosis the haemoglobin band migrated slowly towards the negative pole, finishing just below the top surface of the column at the end of the run. The bridge at the bottom of the column was then disconnected and buffer was applied from a reservoir arranged at a height sufficient to give a flow through the column of 5–10 ml./hr. Some forty fractions were taken at 10 min. intervals, up to the time of breakthrough of the red haemoglobin solution.

Estimation of hexosamine in the fractions. Since the analysis of some forty fractions was required for each experiment, it was necessary to adopt a simple and rapid procedure for hydrolysis. Complete hydrolysis or optimum colour yields was not required, the main object of the determination being to establish the position and shape of any zones due to hexosamine-containing polysaccharides. After preliminary trials the following procedure was adopted. Samples of solution (0·2 ml.) were taken from each fraction and introduced into a set of soda-glass test tubes, matched for photometric readings. 5 N-Hydrochloric acid (0·2 ml.) was then added to each. The tubes, supported in a rack and closed by means of glass bulbs, were then immersed in a boiling-water bath for 4 hr., at least two-thirds of each tube projecting from the water to provide condenser action. When it was cool, the solution was brought to near neutrality by the addition of 0·2 ml. of 4·8 N-NaOH, care being taken not to render the solution alkaline. Estimation of hexosamine was then continued with the same tubes by following the method of Elson & Morgan (1933) as modified by Blix (1948).

Estimation of protein in the fractions. Protein was estimated colorimetrically by the modified biuret method of Lowry, Rosebrough, Farr & Randall (1951), a commercial gelatin being used as the standard.

Other analyses carried out on the fractions. For the purpose of following the separation of the protein and polysaccharide components the estimation of hexosamine and 'total protein as gelatin' by the methods given above gave enough information. However, in a few of the experiments neutral polysaccharides were looked for by examining each fraction for 'sugars other than hexosamine' by the anthrone method of Dreywood (1946) as modified by Yemm & Willis (1954). In some experiments the fractions were also examined for uronic acid by the method of Anderson & Maclagan (1955), with galacturonic acid as a standard. The results obtained agreed with the view that the only major components of the system were proteins of collagenous and non-collagenous origin, together with chondroitin sulphate.

Removal of soluble collagen by treatment with an ion-exchange resin. The resin used was the fine-grade form (XE-64) of the carboxylic acid cation exchanger, Amberlite IRC-50 (obtainable from Charles Lenning and Co., London). Particles in the 150–200 mesh range were obtained by removing the fines. This was done by suspending the resin in water, allowing it to settle for 30 min. and removing the supernatant solution. The resin was passed twice through the sodium form by alternate treatments with 2 N-NaOH and 2 N-HCl, the resin being washed with distilled water between each treatment. The resin was finally air-dried in the H^+ ion form for storage.

The purified resin (1·2 g.) was suspended in 0·2 M-potassium acetate buffer of pH 5·0, allowed to settle and the supernatant removed. The process was then repeated until the pH of the supernatant was the same as that of the buffer. The dialysed cartilage extract (40 ml., containing about 1% of total solids) was mixed with 10 ml. of M-acetate buffer of pH 5. The equilibrated resin was then added and the mixture shaken gently for 30 min., after which the resin was collected in the centrifuge and washed with diluted buffer and the washings were added to the supernatant.

The resin was extracted three times with 1% (w/v) K_2CO_3 by stirring and centrifuging. The first extract required the addition of a few drops of 2 N-KOH to bring the mixture to about pH 10 before centrifuging. The alkaline extract from the resin contained protein, which from its hydroxyproline content was of collagenous origin. The protein was recovered by neutralizing the solution, and evaporating to small bulk after thorough dialyses against distilled water. The protein was then freeze-dried.

After extraction of the protein the resin was brought to the H^+ ion form and again equilibrated with buffer of pH 5. The cartilage extract was then treated again with the same batch of resin. The first treatment with resin removed 90–95% of the hydroxyproline-containing protein from the cartilage extract and the second treatment removed the remainder. Since it was found that components in the

cartilage extract other than hydroxyproline gave rise to a colour with the dimethylaminobenzaldehyde reagent of Newman & Logan (1950) this method was unsuitable for the estimation of the small amounts of collagenous protein which remain after resin treatment (cf. Miyada & Tappel, 1956). Removal of collagen after the treatments with the resin was therefore finally checked by showing the absence of a peak due to hydroxyproline in an elution chromatogram of the hydrolyses products carried out by the method of Moore & Stein (1951). As recommended by these authors, the pH of the sodium citrate buffer used for elution was lowered slightly to pH 3·04 in order to secure sharp separation of hydroxyproline from aspartic acid.

Isolation of the non-collagenous protein prepared by acid hydrolysis. A cartilage extract that had been treated with resin to remove collagenous protein as above was dialysed against several changes of distilled water to remove buffer salts. The solution was then concentrated to approximately 2% of solids by use of rotary evaporator (Partridge, 1951). To the concentrated solution (273 ml. containing 5 g. of solids) 4 ml. of acetic acid and 40 ml. of 1% (w/v) picric acid solution were added. The solution was then heated on a water bath, with an air condenser, for 6 hr. On cooling, the yellow flocculent precipitate of protein was separated by centrifuging, leaving a clear yellow supernatant. The supernatant solution was again heated for 3 hr. at 90°, when it developed a further light precipitate. This was removed by filtration and discarded. The precipitated protein was washed with dilute acetic acid–picric acid mixture, and dissolved in water with the addition of a few drops of NaOH solution to pH 9. It was then precipitated by adding 5 vol. of ethanol and adjusting the pH to 5 by the addition of acetic acid. This operation was repeated 2–3 times until the precipitate was colourless. In this condition the protein could be recovered quantitatively from alkaline solution in water by the addition of acetic acid to pH 4. The protein was finally washed with ethanol and 50% (w/v) ethanol–ether mixture and dried to a fine white powder (0·27 g.) under vacuum at 40°. Analyses carried out with this preparation are recorded in Tables 2 and 3.

Isolation of chondroitin sulphate prepared by acid hydrolysis. The supernatant from picric acid treatment was brought to pH 5 and 6 vol. of ethanol added. After standing overnight the precipitate was collected by centrifuging. The precipitate was then redissolved in 100 ml. of water and again precipitated with ethanol; a few crystals of potassium acetate were added to cause the precipitate to coagulate. The process was repeated twice more until the precipitate was colourless. The yield of crude polysaccharide was 3·4 g. Hydrolysis and two-dimensional paper chromatography [solvents: phenol–aq. NH_3 and butanol–acetic acid (Partridge, 1948*a*)] of a sample of the crude polysaccharide showed that amino acids were still present. Since this may have been due to uncombined protein or peptides which had escaped precipitation with picric acid, the solution was treated with the carboxylic acid resin at pH 5 according to the procedure described above. The supernatant solution was dialysed against four to five changes of distilled water and the polysaccharide recovered by ethanol precipitation with the addition of a little potassium acetate. The precipitate, which was then free from amino acids after hydrolysis, was redissolved in about 50 ml. of water, dialysed against distilled water to remove potassium acetate and freeze-dried.

Isolation of the non-collagenous protein prepared by alkaline hydrolysis. A preparation (4·5 g.) of the collagen-free protein–polysaccharide complex (preparation C, Table 2) which had been freeze-dried was dissolved in 0·5 N-KOH (200 ml.). The solution was allowed to stand in a constant-temperature room at 25° with occasional gentle shaking, and after 20 hr. was neutralized to pH 5·0 by the addition of acetic acid. The solution was then diluted to 1 l. and shaken for 2 hr. with 10 g. of IRC-50 resin, purified and equilibrated with buffer of pH 5 as described above for the removal of collagen. The resin was separated by centrifuging and the solution again shaken with a second 10 g. quantity of resin.

The two batches of resin were mixed, and washed on the centrifuge four times with 0·1 M-potassium acetate buffer of pH 5·0 (300 ml.), the washings being discarded. The resin was then suspended in water, and dilute KOH added dropwise to the stirred slurry to bring it to pH 8–9. A few millilitres of 1% (w/v) K_2CO_3 solution were then added and the mixture was centrifuged. The resin was extracted twice more with 1% (w/v) K_2CO_3 solution and the mixed supernatant collected for recovery of the protein. The supernatant solution was neutralized to pH 5–6 with acetic acid and dialysed against distilled water until the diffusate was free from potassium. The solution was then concentrated to small bulk at 40° and freeze-dried. The yield was 0·12 g.

Isolation of chondroitin sulphate prepared by alkaline hydrolysis. The hydrolysis product, after treatment with resin, contained chondroitin sulphate substantially free from protein. A further treatment with resin was given to ensure complete removal of protein, and the supernatant, which was a pale-amber colour, was concentrated to 500 ml. and dialysed against distilled water. It was then further concentrated to 125 ml. and adjusted to pH 7 with a few drops of KOH solution. The solution was again dialysed against several changes of distilled water until the diffusate contained less than 1·5 p.p.m. of K^+ ions. The product (3·2 g.) was freeze-dried.

Determination of hexosamine in purified preparations of chondroitin sulphate. Optimum conditions for the hydrolysis of chondroitin sulphate were investigated by hydrolysing samples in HCl of various concentrations and for varying lengths of time. The polysaccharide proved to be more resistant to hydrolysis than many mucoids, and in order to complete the reaction in a reasonable time it was necessary to use the acid at a rather high concentration. Employing 4 N-HCl at 100°, maximum colour yield with the Elson & Morgan (1933) reagents was obtained after 16 hr. hydrolysis, and thereafter the colour yield fell slowly (cf. Smith & Zwartouw, 1956). Both glucosamine and galactosamine are slowly destroyed when they are maintained at 100° in the presence of 4 N-HCl, but whereas with glucosamine only about 5% was lost after 16 hr. treatment (cf. Boas, 1953) the loss with a pure sample of galactosamine proved to be 10–14% in different experiments. When equimolecular solutions of galactosamine and galacturonic acid were heated with HCl under the same conditions the loss of galactosamine was about the same.

RESULTS

Extraction of the cartilage powder. A series of preliminary experiments were carried out on the course of extraction of material containing hexos-

amine by 30% (w/v) potassium chloride solution containing alkali. The results of extraction at 18–20° are summarized in Table 1. Of the hexosamine available in the cartilage 56% was extracted into KCl–K_2CO_3 solution over a period of 6 days. Towards the end of this period extraction became very slow, and more alkaline solutions were needed to release the remainder of the hexosamine-containing material. Final exhaustion of the cartilage powder was obtained by employing 30% potassium chloride solution containing 2% (w/v) of potassium hydroxide; but the last extract contained only 1·9 g. of hexosamine/100 g. of dry solids, showing that these severely alkaline conditions resulted in extensive dissolution of collagen.

Column 7 of Table 1 shows that the successive extracts gave material which was progressively poorer in hexosamine, and since the aim was to isolate the components of the tissue which contain a high proportion of polysaccharide a single 48 hr. extract in potassium chloride–potassium carbonate solution was employed for further study. Table 2 gives analyses of material extracted into potassium chloride–potassium carbonate solution at room temperature (extract A) and at 0° (extract B). Judged by the hydroxyproline contents, extract A contained about 39% of protein derived from collagen, whereas B contained only 10%. All subsequent experiments were therefore carried out with material extracted at 0°.

Zone electrophoresis. Fig. 1 (*a*) shows the zone-electrophoresis pattern given by extract B (Table 2). The points representing hexosamine content (solid circles) were determined after partial hydrolysis (see experimental section) and the values, while showing the position of the zones due to chondroitin substrate, do not have quantitative significance. It will be seen that while part of the protein (peak *Z*) migrated only slowly in the electric field fractions 1–15 (peaks *X* and *Y*), which contained hexosamine, also contained protein. The pooled fractions containing hexosamine were found to be free from hydroxyproline after hydrolysis, showing that the protein which migrated with chondroitin sulphate was not of collagenous origin. Fig. 1 (*b*) shows the zone-electrophoresis pattern obtained under the same experimental conditions with the same batch of extract after treatment with 0·5N-sodium hydroxide for 4 hr. at 25°. The alkali-degraded material separated sharply into two zones, one of which (peak *X*) contained polysaccharide uncontaminated with

Table 1. *Yields and analytical data of products from successive extracts of* 30 *g. of dry cartilage powder*

Method of extraction	Extract	Vol. of extract (ml.)	Total solid (by dry wt., g.)	Hexosamine (mg./ml. of extract)	Total hexosamine in extract (g.)	Hexosamine in extracted solid (%)	Proportion of original hexosamine extracted (%)
48 hr. 30% KCl–1% K_2CO_3	*A*	500	3·45	0·875	0·428	12·7	37·7
96 hr. 30% KCl–1% K_2CO_3	*B*	550	1·97	0·387	0·212	10·8	18·3
48 hr. 30% KCl–1% KOH	*C*	490	3·76	0·66	0·324	8·6	28·0
48 hr. 30% KCl–2% KOH	*D*	610	4·78	0·149	0·091	1·9	7·8
Total	—	—	13·96	—	1·055	—	91·8
Residue	—	—	—	—	0·051	—	4·4

Table 2. *Composition of preparations from ox cartilage* (septum nasi)

	Nitrogen (%)	Hexosamine (%)	Ash as K_2SO_4 (%)	Hydroxyproline (%)
A. Material extracted into KCl–K_2CO_3 solution 48 hr. at 18–20°	10·04	11·6	13·1	5·5
B. Material extracted into KCl–K_2CO_3 solution 48 hr. at 0°	6·17	18·3	22·5	1·44
C. Extract B after treating with resin to remove collagen	4·13	24·0	18·6	Nil
D. Non-collagenous protein prepared by acid hydrolysis of C	13·7	6·9	5·5	Nil
E. Non-collagenous protein prepared by alkaline hydrolysis of C	13·3	2·03	0·5	Nil
F. K salt of chondroitin sulphate prepared by acid hydrolysis of C	2·8	27·4	29·9	—
G. K salt of chondroitin sulphate prepared by alkaline hydrolysis of C	2·8	27·2	30·2	—
H. Calc. for chondroitin sulphate, $C_{14}H_{19}O_{14}NSK_2$	2·62	33·5	32·5	—

protein. This is in accord with the usual experience that alkali-treated or alkali-extracted chondroitin sulphate can readily be separated from protein by purely physical methods.

It will be observed that in Fig. 1 (*a*) the composite peak $X + Y$ is not symmetrical and is not uniform in composition. The shape of the composite peak suggests that it has resulted from the summation of two components of high mobility, the slower-moving of the two migrating as a rather broad zone and containing most or all of the protein. The faster-moving component may be identified, as regards position, with peak X in Fig. 1 (*b*) and may be due to free chondroitin sulphate. The slower-moving component (Y) carries the non-collagenous protein and disappears after hydrolytic treatment with sodium hydroxide: it is therefore considered to be due to a protein–polysaccharide complex or mucoprotein. The small protein peak in Fig. 1 (*b*) covering fractions 10–15 may be a residue of the mucoprotein which has escaped hydrolysis. Peak Z in Fig. 1 (*a*) contained hydroxyproline in the proportion which would be expected if it were derived from collagen and the peak was therefore identified with this protein. Peak Z in Fig. 1 (*b*) was considerably increased in amount after the hydrolytic treatment and is regarded as consisting of a mixture of collagenous and non-collagenous protein.

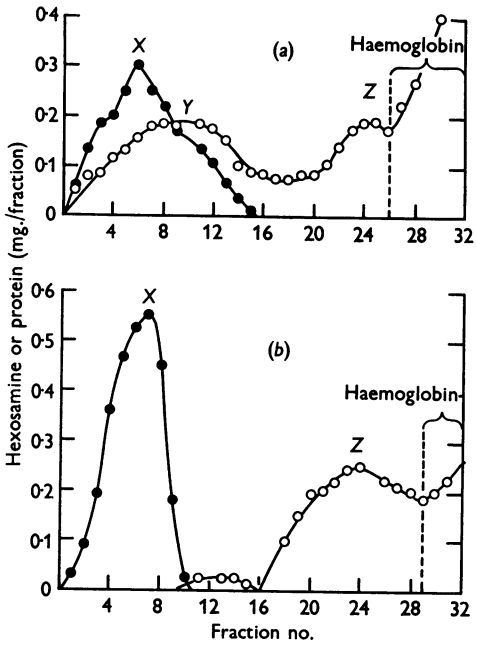

Fig. 1. (*a*) Zone-electrophoresis pattern of an extract of cartilage powder in KCl–K_2CO_3 solution (preparation B in Table 2). (*b*) Pattern given by the same extract after treatment with 0·5 N-NaOH for 4 hr. at 25°. A column 30 cm. × 1·1 cm. was packed with graded potato starch; the buffer was 0·5 M-sodium barbiturate, pH 8·54; haemoglobin was added to both samples as marker. ○, Protein estimated against a gelatin standard (Lowry *et al.* 1951); ●, hexosamine estimated after partial hydrolysis (Elson & Morgan, 1933).

Isolation of the mucoprotein. The interpretation of the zone-electrophoresis experiments given above was confirmed by the isolation of the polysaccharide–protein complex assumed to be in admixture or loosely bound with soluble collagen. Boardman & Partridge (1955) showed that below pH 6·5 many proteins were very strongly adsorbed on the fine-mesh form of the carboxylic acid resin IRC-50, and most could readily be desorbed again by treatment with alkaline buffers. It was found that a single treatment of the cartilage extract (B in Table 2) with the resin removed 90–95 % of the hydroxyproline-containing protein and that two treatments completed the removal. The mucoprotein complex which remained contained N, 4·13; hexosamine, 24·0 %; and its protein content was thus about 10 %. Since 3–4 further treatments with ion-exchange resin failed to reduce the nitrogen content of the complex it seemed clear that the non-collagenous protein was firmly bound and possibly in chemical combination with the polysaccharide. An attempt was made to dissociate the mucoprotein by mild hydrolysis, and a series of trials showed that the complex readily yielded to degradation with dilute organic acids at 95° or dilute aqueous sodium hydroxide at 25°.

Hydrolysis with acid. On heating solutions of the mucoprotein in 1 % acetic acid solution, the slightly opalescent solution became increasingly opaque and addition of picric acid resulted in the immediate separation of a flocculent yellow precipitate. It proved convenient to carry out the hydrolysis in a mixture of picric acid and acetic acid, when the bulk of the liberated protein could be removed as insoluble picrate after about 6 hr. at 95°, when 80–90 % of the reaction was complete. Longer periods of heating resulted in the gradual destruction and resolution of the protein picrate.

The crude protein, after removal of picric acid, had N, 13·7; ash, 5·5 %; P, absent. It dissolved to form a clear colourless solution in aqueous sodium hydroxide and was precipitated quantitatively by adjustment of the pH to 4·5. Determination of the hexosamine content (colorimetric) gave 6·9 % of hexosamine, showing that the liberation of the polysaccharide moiety was not complete. No conditions for the acid hydrolysis of the mucoprotein could be found which would result in the clear-cut separation of the protein in high yield, and all preparations contained some polysaccharide. In Table 3 a partial amino acid analysis of a preparation of the crude protein is given (method of Moore & Stein, 1951).

Table 3. *Amino acid composition of the protein moiety of the mucoprotein compared with ox-bone collagen*

	Prepared by acid hydrolysis of the mucoprotein		Prepared by alkaline hydrolysis of the mucoprotein		Ox-bone collagen analysis by Eastoe (1955)
	g. of N/100 g. of protein N	g. of amino acid/100 g. dry wt.	g. of N/100 g. of protein N	g. of amino acid/100 g. dry wt.	g. of amino acid/100 g. dry wt.
Hydroxyproline	0·03	0·04	Nil	Nil	14·1
Aspartic acid	6·12	7·96	5·71	7·3	7·1
Threonine	2·74	3·19	2·86	3·27	2·52
Serine	3·16	3·25	2·64	2·65	4·24
Glutamic acid	7·0	10·0	8·80	12·4	11·9
Proline	5·37	6·05	6·94	7·86	14·7
Glycine	7·55	5·54	4·79	3·46	25·3
Alanine	4·88	4·24	4·79	4·11	10·5
Valine	3·92	4·49	4·05	4·54	2·65
Methionine	0·84	1·23	0·87	1·22	0·80
Isoleucine	3·05	3·91	2·77	3·50	1·73
Leucine	6·60	7·69	6·15	7·73	3·93
Tyrosine	2·47	4·38	2·60	4·54	0·56
Phenylalanine	3·17	5·12	4·67	7·42	2·88
Histidine	—	—	3·67	1·83	0·96
Lysine	—	—	5·04	3·50	4·11
Arginine	—	—	5·57	2·3	9·2
Glucosamine	—	—	0·35	0·61	—
Galactosamine	—	—	0·33	0·56	—
Unknown amino sugar	—	—	—	0·33	—

Crude chondroitin sulphate was recovered from the supernatant solution by precipitation with ethanol and the product purified by further reprecipitation. The analysis of a typical sample is given in Table 2. The value for nitrogen (2·84 %) indicated that the protein content of the material was small and this was confirmed by paper chromatograms carried out on hydrolysed samples. These showed only traces of two or three amino acids.

Hydrolysis with alkali. The polysaccharide prepared by treatment for 4 hr. under the conditions given had N, 3 %; since paper chromatograms carried out after hydrolysis with 5·5N-hydrochloric acid showed the presence of spots due to seventeen amino acids, it was concluded that the preparation still contained mucoprotein and that alkaline hydrolysis was incomplete. Analysis of the 20 hr. and 40 hr. preparations showed them to be almost identical in composition, both having N, 2·8; hexosamine, 26·3 %. Samples of both preparations were hydrolysed and tested for the presence of amino acids by paper chromatography. Apart from the very large spots due to chondrosamine, only one or two traces of ninhydrin-reactive substances were observed, and the preparations were regarded as substantially free from protein.

Further purification of the polysaccharide from a 40 hr. alkaline hydrolysis was attempted by employing a solvent-fractionation procedure. A centre fraction from ethanol precipitation was refractionated by step-wise addition of 90 % (w/v) aqueous phenol. The analysis of the centre fraction from phenol precipitation is given in Table 2 (preparation G) and shows little change in composition from the crude material.

Purified samples of chondroitin sulphate prepared by acid and alkaline degradation had almost identical composition, but the analytical results show that the integral ratios (g.atom or g.mol./g.mol. of $C_{14}H_{19}O_{14}NSK_2$) for nitrogen, hexosamine and potassium sulphate deviated from unity; these ratios were 1·08, 0·82 and 0·92 respectively. The high value for nitrogen is understandable and may be due to contamination with traces of peptides, but the low hexosamine figure is outside the range of expectable error. Less than unit integral ratios for hexosamine have been reported by other workers. Thus Einbinder & Schubert (1950) gave hexosamine contents averaging at 23·6 %, whereas Blix & Snellman (1945) reported 24–25 % for highly purified preparations; on the other hand, Meyer & Smyth (1937), analysing the acid calcium salt, gave higher figures (28–30 %). All the galactosamine determinations reported here have been carried out with material hydrolysed for 16 hr. with 4N-hydrochloric acid at 100° and, as indicated in the Experimental section, the results may be assumed to be about 12 % low owing to loss during hydrolysis. Correction for this loss brings the integral ratio for galactosamine to 0·93, a figure which agrees well with the corresponding ratio (0·92) for potassium sulphate derived from estimation of the ash. Chromatographic analysis of the hydrolysis products of both preparations of chondroitin sulphate (F and G in Table 2) by the procedure of Gardell (1953) showed the absence of amino sugars

other than galactosamine, and the quantitative recoveries were identical with those obtained by direct colorimetric estimation. The analytical results confirm the accepted formulation for chondroitin sulphate and suggest that, apart from a small contamination with material of peptide character (and possibly with bound water), the preparations were substantially pure.

The protein which was released during alkaline hydrolysis was recovered from the ion-exchange resin by elution with alkaline buffer after exhaustive washing. The analysis of a preparation from a 40 hr. hydrolysate is given in Table 2 (preparation E). The protein contained 2·03% of hexosamine (colorimetric) and 0·5% ash, showing that even after prolonged hydrolytic treatment some mucoprotein was still present. A complete amino acid analysis (obtained by the method of Moore & Stein, 1951) is given in Table 3 (main column 2). Hydroxyproline is absent and the proportions of the other amino acids are very different from those found in collagen (Table 3, main column 3). The high content of tyrosine (4·54%) in the new protein is of particular interest. Ion-exchange chromatography of the protein hydrolysate carried out according to the method of Gardell (1953) showed three peaks due to material reacting with the Elson & Morgan (1933) reagent. The first peak (fractions 74–78) was due to an unknown substance which, estimated as a hexosamine against a glucosamine standard, represented 0·33% of the protein dry weight. The second peak (fractions 81–86) was glucosamine (0·61%) and the third (fractions 98–102) was galactosamine (0·56%). Since pure chondroitin sulphate contains no glucosamine it is clear that a large part of the polysaccharide content of the protein must have another origin. Glucosamine was found in all preparations of the protein, whether from acid or from alkaline degradation or the original mucoprotein, but its origin remains unknown. The nitrogen content of the protein is very low (about 13·5%) and only 68% of the nitrogen was recovered as amino acids in the Moore & Stein chromatograms. This indicates that only about two-thirds of the substance is of polypeptide character and that the remainder of the material must contain nitrogenous and non-nitrogenous components other than amino acids. The amount of material obtainable thus far has been insufficient for further analysis, but work on the problem is continuing.

DISCUSSION

The discovery by Shatton & Schubert (1954) of a hitherto unrecognized protein component in combination with chondroitin sulphate in cartilage is clearly of importance in any attempt to understand the structure of the tissue, and the object of the work now described was to confirm and extend this observation by the isolation of the complex and the examination of its degradation products. From the outset it appeared unlikely that all the polysaccharide could exist in chemical combination with protein, since many workers have isolated chondroitin sulphate of quite high purity from cartilage by partial extraction under conditions which would not be expected to degrade a stable compound (see particularly Blix & Snellman, 1945). However, in these experiments the yields of pure polysaccharide have always been low. Much higher yields of pure material are obtainable by extraction with sodium hydroxide (cf. Meyer & Odier, 1946) but here the conditions employed are sufficiently hydrolytic to account for the dissociation of any pre-existing mucoprotein. High yields of free chondroitin sulphate may also be obtained if the fresh tissue is allowed to autolyse for some time before extraction (Einbinder & Schubert, 1950).

The evidence that part at least of the chondroitin sulphate in cartilage is in firm combination with a non-collagenous protein is derived from two independent types of experiment. These were the isolation of the complex by use of an ion-exchange resin and zone-electrophoresis experiments carried out on the degraded and undegraded extracts. When the initial extract was treated with an ion-exchange resin under conditions which were known to result in the adsorption of free protein, collagen was removed quantitatively but the remaining material still contained a protein which was free from hydroxyproline. Treatment of this residue with sodium hydroxide solution at room temperature effected the release of the non-collagenous protein, and when the solution was re-treated with the same resin the liberated protein was adsorbed, leaving chondroitin sulphate in a substantially pure form. The non-collagenous protein was then recovered by eluting it from the resin with alkaline buffer, and amino acid analysis confirmed that its composition was quite different from that of collagen. The zone-electrophoresis experiments carried out on the initial extract showed that the non-collagenous protein migrated with chondroitin sulphate as a zone of high mobility which was well separated from the zone due to collagen. A quite different pattern was obtained when extracts which had been treated with dilute aqueous sodium hydroxide were submitted to electrophoresis under the same experimental conditions. The chondroitin sulphate was then found to migrate as a sharp zone free from protein and the slower-moving material consisted of a mixture of collagen with the non-collagenous protein.

The results of electrophoresis in free solution (Partridge, 1948*b*) had previously indicated that in

undegraded extracts soluble collagen exists as an equilibrium mixture with chondroitin sulphate and a loose complex formed between the two. It was not then observed that chondroitin sulphate was itself combined with a second protein. Since it has been shown that the high viscosity associated with 'undegraded chondroitin sulphate' is rapidly reduced by the action of commercial trypsin (Shatton & Schubert, 1954) or papain (Muir, 1956), it appears that the protein content of these preparations is concerned in the formation of molecular aggregates. Preparations of chondroitin sulphate lose their capacity to combine reversibly with collagen when they are degraded by heating or by the action of dilute alkali (Partridge, 1948*b*). It therefore seems probable that the species responsible for the linkages with collagen that determine the integrity of the native tissue is the protein–polysaccharide macromolecule and not free chondroitin sulphate.

The possibility that the mucoprotein arises as an artifact during the process of extraction must be considered. Although cartilage from nasal septum contains only a small number of cells, these could themselves be a source of non-collagenous protein, and it is possible that such a protein could become associated with chondroitin sulphate as a result of the disorganization arising from extraction. However, this seems to be unlikely in view of the indications that the protein is chemically combined. Intervention of plasma proteins also seems to be unlikely in view of the small size of the free pore spaces in the cartilage gel. These have been given by Paulson, Sylvén, Hirsch & Snellman (1951) as of the order of 10–15 Å in bovine nucleus pulposus, suggesting that this system is permeable to simple sugars and amino acids but is impermeable to large molecules.

SUMMARY

1. The product of extracting cartilage powder prepared from bovine *septum nasi* with 30% potassium chloride–1% potassium carbonate solution has been examined.

2. Zone electrophoresis showed the extract to contain soluble collagen, a mucoprotein complex consisting of chondroitin sulphate combined with a non-collagenous protein and probably some free chondroitin sulphate.

3. The extract was freed from collagen by treatment with a carboxylic acid ion-exchange resin at pH 5.

4. Mild hydrolysis of the collagen-free material with dilute aqueous potassium hydroxide at 20° or acetic acid–picric acid mixture at 95° dissociated the complex and liberated the non-collagenous protein.

5. After release by alkaline hydrolysis, the new protein became available for adsorption on the same resin as was used for removal of collagen, and could be recovered from the resin by elution at pH 9.

6. The amino acid composition of the non-collagenous protein is given.

The work described in this paper was carried out as part of the programme of the Food Investigation Organization of the Department of Scientific and Industrial Research. The amino acid analyses were carried out by Mr D. F. Elsden.

REFERENCES

Anderson, A. J. & Maclagan, N. F. (1955). *J. biol. Chem.* **59**, 638.
Blix, G. (1948). *Acta chem. scand.* **2**, 467.
Blix, G. & Snellman, O. (1945). *Ark. Kemi Min. Geol.* **19A**, no. 32.
Boardman, N. K. & Partridge, S. M. (1955). *Biochem. J.* **59**, 543.
Boas, N. F. (1953). *J. biol. Chem.* **204**, 553.
Carlson, L. A. (1954). *Acta chem. scand.* **8**, 510.
Dreywood, R. (1946). *Industr. Engng Chem.* (*Anal.*), **18**, 499.
Eastoe, J. E. (1955). *Biochem. J.* **61**, 589.
Einbinder, J. & Schubert, M. (1950). *J. biol. Chem.* **185**, 725.
Elson, L. A. & Morgan, W. T. J. (1933). *Biochem. J.* **27**, 1824.
Gardell, S. (1953). *Acta chem. scand.* **7**, 207.
Haglund, H. & Tiselius, A. (1950). *Acta chem. scand.* **4**, 957.
Lowry, C. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Matthews, M. B. (1955). *Fed. Proc.* **14**, 252.
Meyer, K. & Smyth, E. M. (1937). *J. biol. Chem.* **119**, 507.
Meyer, K. H. & Odier, M. (1946). *Experientia*, **2**, 8.
Miyada, D. S. & Tappel, A. L. (1956). *Analyt. Chem.* **28**, 909.
Moore, S. & Stein, W. H. (1951). *J. biol. Chem.* **192**, 663.
Muir, H. (1956). *Biochem. J.* **62**, 26P.
Newman, R. E. & Logan, M. A. (1950). *J. biol. Chem.* **184**, 299.
Partridge, S. M. (1948*a*). *Biochem. J.* **42**, 238.
Partridge, S. M. (1948*b*). *Biochem. J.* **43**, 387.
Partridge, S. M. (1951). *J. sci. Instrum.* **28**, 28.
Partridge, S. M. & Davis, H. F. (1955). *Biochem. J.* **61**, 21.
Paulson, S., Sylvén, B., Hirsch, C. & Snellman, O. (1951). *Biochim. biophys. Acta*, **7**, 207.
Shatton, J. & Schubert, M. (1954). *J. biol. Chem.* **211**, 565.
Smith, H. & Zwartouw, H. T. (1956). *Biochem. J.* **63**, 447.
Tiselius, A. (1937). *Trans. Faraday Soc.* **33**, 524.
Yemm, E. W. & Willis, A. J. (1954). *Biochem. J.* **57**, 508.