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The Chemistry of Connective Tissues

7. DISSOCIATION OF THE CHONDROITIN SULPHATE–PROTEIN COMPLEX OF CARTILAGE WITH ALKALI

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In the preceding paper a model was proposed for the constitution of the chondroitin sulphate–protein complex of cartilage in which at least 23 chondroitin sulphate chains are connected to a protein core by single linkages thought to be of covalent character. In the work now described, the dissociation of the complex in alkaline solution was studied more closely. Electrophoresis experiments in columns stabilized by a density gradient suggested that alkaline dissociation is irreversible and follows a time course related to the hydroxyl ion concentration. The protein core split off was isolated and analysed and was found to carry a second amino polysaccharide containing glucosamine and galactose.

METHODS

Chondroitin sulphate–protein complex. The complex was prepared from dried cartilage powder as described in the preceding paper. As it was important to decrease contamination with collagen to the lowest possible extent, treatment with ion-exchange resin (Amberlite CG 50) was repeated three or four times until the hydroxyproline content was less than 0·2% as judged by ion-exchange chromatography of the hydrolysed product.

Zone electrophoresis. The experiments were carried out with an electrophoresis column stabilized by a sucrose density gradient. The method used was generally similar to that described by Svensson, Hagdahl & Lerner (1957), but since the design of the apparatus was different, and has proved to be advantageous in some respects, a drawing of it (Fig. 1) is given here. The apparatus was symmetrical and consisted of two similar glass columns, A and A′, provided with ball-joints and arranged over a U tube B. Duplicate sets of columns with different diameters were provided so that the apparatus could be adjusted to suit the scale of the separation. The electrical circuit was completed by the provision of two vessels, C and C′ (diameter 7·5 cm.), containing large silver–silver chloride electrodes and connected through large-bore taps, D and D′. A bridge E was provided to secure hydrostatic equilibrium when the taps D and D′ were closed.

In the experiment described the columns were 2·7 cm. in diameter and the height between H and J′ was 34 cm. The columns were not provided with water jackets and the experiment was carried out at room temperature.

The apparatus was first filled with buffer (0·005N-NaOH containing 0·015N-NaCl) to a point above the level of the side arms D and D′. A reservoir filled with dense buffer (600 ml., 0·01N-NaOH containing 0·025N-NaCl and 50%, w/v, sucrose) was connected in series with a mixing chamber (capacity 550 ml.) and a metering pump. The mixing chamber was provided with a magnetic stirrer. The buffer stream of increasing density was pumped from the mixing chamber into the U tube through the tap I. The

flow rate was 10 ml./min. and pumping was continued until sufficient buffer had passed to displace the volume in the U tube to about 4 cm. above the level of the inlet tubes J and J′.

Tap D′ was then opened and D closed. Tap G′ was opened slowly to allow the collection of a few millilitres of buffer from the jet. This caused a sharp step in the density gradient at the point J′. The operation was then repeated on the opposite side with D′ closed and D open, collecting from the jet at G. Before introduction of the sample, D′ and D were both closed and F′ and F both opened to allow hydrostatic equilibration via the bridge.

The sample (52 mg.) was prepared as a solution (0·5%, w/v) in the buffer used to fill the apparatus, and was adjusted by the addition of a few drops of the sucrose buffer until its refractive index was the same as that of the mixed sample from the gradient which had been drawn out of the jet. This ensured that the sample was of the correct density to remain suspended in the interface at point J′. The sample was then drawn in through the inlet tube G and injected into the column at J′, by closing taps D and D′ and slowly opening tap I. To balance the column the same volume of buffer was drawn off from tap I with tap D opened and tap D′ closed. After closing tap D, saturated NaCl solution was introduced into the electrode vessels through funnels K and K′ to cover the exposed metal of the silver electrodes (L and L′) and the apparatus was again equilibrated by opening the bridge. This was closed before applying the current.

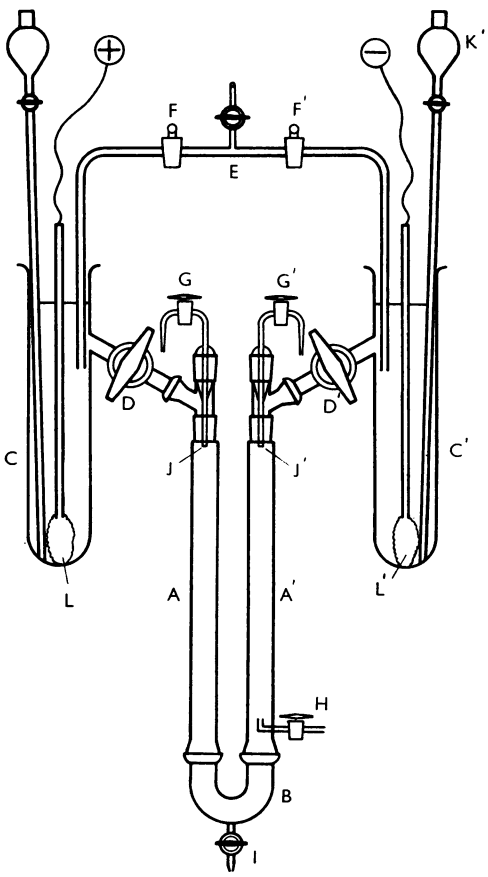

Fig. 1. Apparatus for zone electrophoresis stabilized by a sucrose density gradient.

The apparatus was allowed to stand for about 30 min. with taps D and D′ open to bring it into temperature equilibrium and to allow some diffusion of the lower edge of the zone. A current of 17 mA (370 v) was then applied and migration was allowed to proceed for 17 hr. Fractions (4 ml.) were then run off by hand into graduated tubes, buffer from vessel C being allowed to replace the solution in the column. The fractions were analysed for protein by the method of Lowry, Rosebrough, Farr & Randall (1951), with a solution of gelatin as standard, and for hexosamine by the method of Rondle & Morgan (1955), after hydrolysis with 4N-HCl for 16 hr. in sealed tubes at 100°.

Chromatographic fractionation of the core protein separated by treatment with alkali. The column (2·5 cm. × 15 cm.) was packed with the carboxylic acid resin Amberlite CG 50. The resin was passed through a sieve (200 mesh/in.) and prepared for use as described by Boardman & Partridge (1955). A freeze-dried preparation of the chondroitin sulphate–protein complex (2 g.) was dissolved in 200 ml. of 0·5N-KOH and allowed to stand at 25° for 20 hr. After neutralizing with acetic acid and dialysing against distilled water the solution was made up to 340 ml., potassium acetate being added to bring the concentration to 0·1M and acetic acid to pH 4·9.

The column was equilibrated to pH 4·9 by washing for 24 hr. with the acetate buffer, and the solution of the alkali-treated complex was then applied at 20 ml./hr., the effluent being collected as a single fraction. The column was provided with a buffer reservoir connected in series with a magnetically-stirred mixing chamber (500 ml. capacity). The reservoir and mixing chamber were filled with 0·25M-potassium acetate buffer, pH 4·9, and 140 ml. of this solution was passed through the column at 20 ml./hr., the effluent being collected in 20 ml. fractions. The solution in the reservoir was replaced with 1M-potassium acetate buffer of the same pH for 14 fractions and was then replaced with 0·4M-Na_2HPO_4. The fractions were estimated for protein by the method of Lowry *et al.* (1951), with a solution of gelatin as standard (Fig. 3).

Qualitative analysis of the neutral sugars in the core material. Fractions from the core protein (15 mg.) were dissolved in 1·2 ml. of 0·5N-H_2SO_4, sealed in a glass tube and hydrolysed at 100° for 4 hr. The solution was then neutralized to pH 5 with $Ba(OH)_2$ solution and the precipitated barium sulphate removed by centrifuging. The clear solution and washings were passed through a small column (100 mg.) of sulphonated polystyrene resin (Dowex-50) in its H^+ form, to remove amino acids and peptides. The column effluent and washings were concentrated by evaporation under reduced pressure and freeze-dried.

The product was then dissolved in 0·05 ml. of water. Single-dimensional paper chromatograms were prepared with butanol–acetic acid–water (Partridge, 1948) and ethyl acetate–pyridine–water (Jermyn & Isherwood, 1949). Aniline phthalate (Partridge, 1949) and naphtharesorcinol (Partridge, 1948) were used as spraying reagents for revealing the spots.

Estimation of hexosamines, hydroxyproline and reducing power. The methods used were those reported by Partridge *et al.* (1961).

Amino acid analysis. The chromatographic procedure was that of Moore & Stein (1951). For colour development the ninhydrin reagent of Yemm & Cocking (1955) was used.

RESULTS

Dissociation of the chondroitin sulphate–protein complex

It has already been reported (Partridge & Davis, 1958) that on zone electrophoresis (starch column) at pH 8·54 the chondroitin sulphate–protein complex migrates substantially as a single zone. Brief treatment with NaOH solution did not affect the stability of the complex in the electric field, but after treatment with 0·5N-NaOH for a fairly prolonged period the complex dissociated and on zone electrophoresis under the same conditions the material migrated as two zones, one consisting of protein and the other of protein-free chondroitin sulphate. The results of this and other similar experiments (cf. Muir, 1958) have been taken to indicate that the polysaccharide and protein in the complex are combined by primary valency. This, however, is not necessarily true since the complex may dissociate instantaneously in alkaline solution and this dissociation may be reversed when the solution is dialysed or neutralized in preparation for electrophoresis or ion-exchange treatment. This situation occurs not infrequently in investigations of protein–polysaccharide complexes and could be resolved if a separation technique were available which could be applied to highly alkaline solutions.

It was thought that zone electrophoresis, with a density gradient to stabilize against convection (Svensson *et al.* 1957), might provide such a technique. Accordingly this method was attempted with sucrose to provide the density gradient and 0·01–0·02M-NaOH as the electrolyte. Electrophoresis columns of this sort proved to be unsatisfactory owing to marked polarization which developed a few minutes after application of the current. In sucrose–NaOH systems the OH^- ion concentration is markedly decreased owing to the formation of sucrose anions, and a situation results in which the transport number of the cations (Na^+) is very much higher than that of the anions (sucrose$^-$ and OH^-). This results in the rapid depletion of Na^+ ions at the sucrose interface in the anode limb. (For theoretical discussion of concentration polarization of this type see Partridge & Peers, 1958.) It was found that polarization could be much decreased by the addition of a neutral salt to the solution, and accordingly the experiments described here have been performed with a mixture of NaOH (0·005–0·01M) and NaCl (0·015–0·025M).

Fig. 2 (*a*) shows the pattern obtained by electrophoresis of the chondroitin sulphate–protein complex at 370v for 17 hr. This should be compared with Fig. 2 (*b*), which shows the pattern obtained under as nearly as possible identical conditions by electrophoresis of the complex after treating it with 0·5N-NaOH for 20 hr. at 25°. In both cases 25 fractions each of 4 ml. were taken after completion of the run and samples from each were estimated for protein by the method of Lowry *et al.* (1951), and for chondroitin sulphate by measuring galactosamine after hydrolysis with 4N-HCl for 20 hr.

Fig. 2 (*a*) shows that the native complex migrated substantially as a single zone in dilute NaOH solution. However, the position of the protein and chondroitin sulphate peaks were skewed with respect to each other, indicating some degree of heterogeneity. This may have been due to partial hydrolysis during the electrophoresis experiment, or it may indicate the presence of a small amount of protein impurity in the original preparation. As will be shown later, contamination with a large amount of a second protein is unlikely, since the isolated protein component appears to be substantially homogeneous in amino acid composition.

The electrophoretic pattern obtained after a preliminary hydrolytic treatment with NaOH (Fig. 2*b*) shows the almost complete separation of

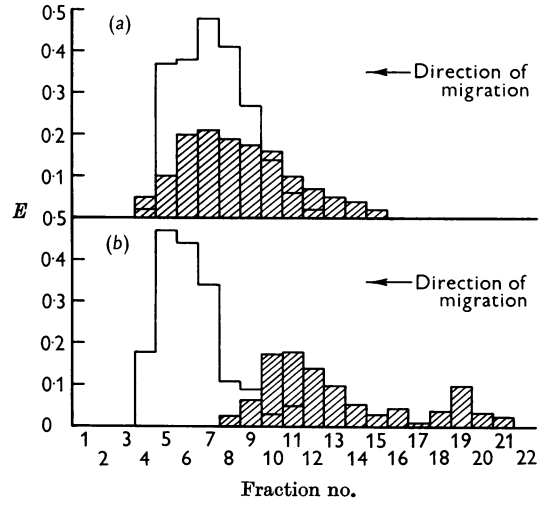

Fig. 2. Zone-electrophoresis pattern given by the chondroitin sulphate–protein complex at 370v for 17 hr. in NaOH–sucrose–NaCl buffer. (*a*) Pattern given by the native complex. (*b*) Pattern given by the complex after treatment with 0·5N-NaOH for 20 hr. at 25°. Hatched blocks, protein (Folin reagent). Open blocks, chondroitin sulphate (Elson & Morgan reagent).

protein from chondroitin sulphate and demonstrates the irreversible nature of the dissociation of the two components of the complex by the prolonged action of alkali (20 hr.). Pretreatment with 0·5 N-NaOH solution for shorter periods (8 or 16 hr.) provided only partial separation of the protein and carbohydrate components. In Fig. 2 (*b*) the zone due to protein shows considerable spread and the substance is clearly not homogeneous in charge density. This suggests that the protein is degraded to some extent; however, such degradation would be expected since it has been shown that similar treatment results in the hydrolysis of labile peptide bonds in proteins such as collagen (Bowes & Kenten, 1948) or gelatin (Saunders & Ward, 1955).

Estimation of the polysaccharide chain weight (*Cn*) by titration of the reducing end groups (Partridge, Davis & Adair, 1961) showed that *Cn* for the untreated complex was 46 800 and for the product of alkaline hydrolysis 24 100. After dialysis in Visking tubing, *Cn* for the alkali-treated product rose to 30 600. This indicated that the chondroitin sulphate moiety also suffered some degradation by chain rupture during alkaline treatment.

Homogeneity of the protein component

Thus far no means have been found whereby the protein component may be isolated under conditions that would be unlikely to cause secondary changes in the protein itself. The use of alkali, under as mild conditions as possible, is effective in separating chondroitin sulphate from the protein; but these same conditions can be expected to damage the protein by the rupture of easily hydrolysable peptide bonds or by liberation of ammonia from asparaginyl or glutamyl residues. The homogeneity of the protein prepared by alkaline hydrolysis was examined, and since only small amounts of the material were available a chromatographic procedure, with an ion-exchange-resin column, was adopted. Details of the procedure are given in the Methods section.

Fig. 3 shows the chromatogram obtained from the product of degrading the chondroitin sulphate–protein complex with 0·5 N-KOH for 20 hr. at 25°. The fractions from peak A were collected together and the product was recovered by dialysis followed by drying from the frozen state. The composition of the product [N, 2·8 %; galactosamine, 27·5 %; ash (as K_2SO_4), 30·1 %] showed it to be potassium chondroitin sulphate substantially free from protein. The protein moiety was not eluted at pH 4·9 at any concentration of salt up to 1 M, but when a pH gradient was provided by supplying Na_2HPO_4 to the mixing chamber it appeared as a series of partly resolved peaks (Fig. 3), which fell into two broad areas (labelled P 1 and P 2 in the Figure). The fractions grouped under P 1 and P 2 were isolated separately for analysis and Table 1 shows comparative analytical data for the two preparations.

The absence of hydroxyproline in both substances showed that neither was derived from collagen. Both materials showed similar u.v.-absorption curves (Fig. 4), and when the ratio of tyrosine to tryptophan was calculated from the equations of Goodwin & Morton (1946) the results for the two fractions were closely similar. Both materials contained substantial amounts of neutral sugar and the ratio of glucosamine to galactos-

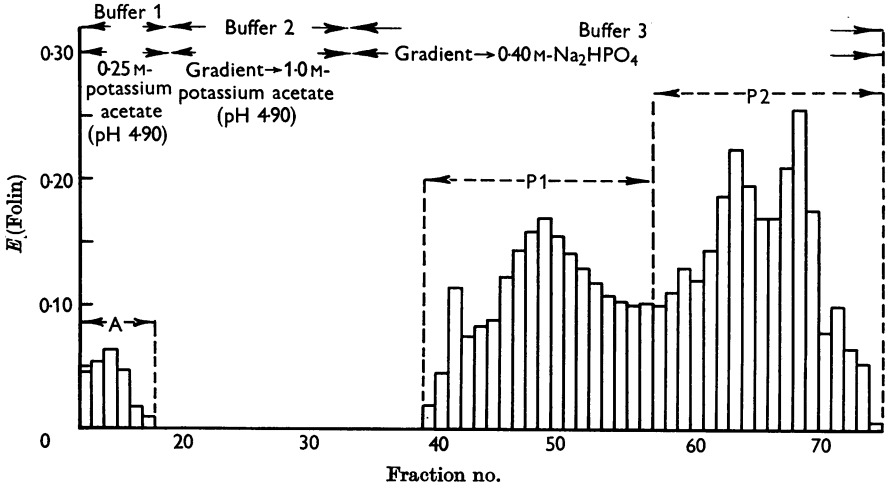

Fig. 3. Chromatographic fractionation of the core protein separated by treatment with alkali. Column 2·5 cm. × 15 cm.; resin Amberlite CG 50. The fractions were estimated for protein with the Folin reagent (Lowry, Rosebrough, Farr & Randall, 1951).

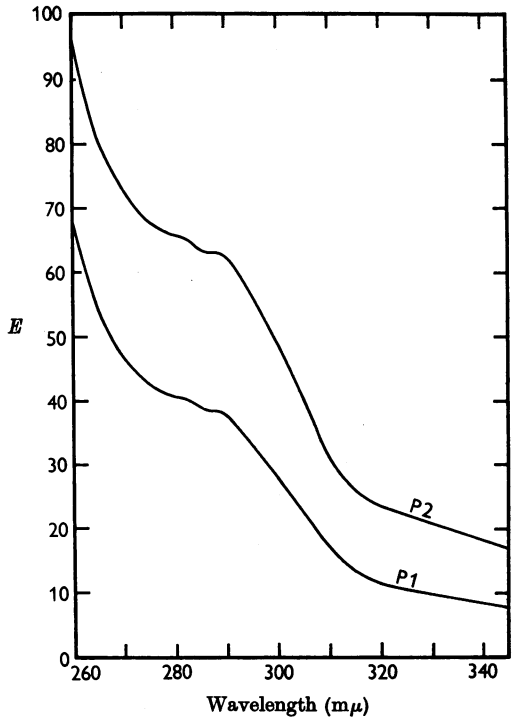

Fig. 4. Extinction (u.v.) of the head and tail fractions from the core protein separated by ion-exchange chromatography. Solvent, 0·1 N-NaOH. P1, 0·39 mg./ml. (N, 10·7%); P2, 0·41 mg./ml. (N, 11·81%).

amine, as measured by ion-exchange chromatography after acid hydrolysis, was similar in the two fractions. Table 2 shows the amino acid composition of fractions P1 and P2, estimated by ion-exchange chromatography of the products of acid hydrolysis. Whereas the results for a number of the amino acids show differences outside the normal range of error for the method, the pattern of amino acid distribution is similar for the two fractions. Comparison of the analytical results for the two fractions given in Tables 1 and 2 suggests that the material isolated by alkaline degradation has been derived from a single molecular species containing both protein and polysaccharide and that degradation has resulted in partial removal of

Table 1. *Analytical data for the head and tail fractions from the protein core after separation by ion-exchange chromatography*

	Fraction P1	Fraction P2
Weight of fraction (mg.)	62·6	40·0
Nitrogen (%)	10·66	11·81
Hydroxyproline (%)	Nil	<0·2
Tyrosine/tryptophan*	1·7	1·9
Neutral sugars (%) as glucose (anthrone)	6·1	3·5
Glucosamine (%)	2·5	1·24
Galactosamine (%)	0·54	0·44
Uronic acid (%) as glucuronic acid (carbazole)	0·48	0·24

* Moles of tyrosine and tryptophan in 1 g. of protein, determined from E at 280 and 294 mμ and the equations of Goodwin & Morton (1946).

Table 2. *Amino acid composition of the head and tail fractions resulting from chromatographic fractionation of the core protein*

	Head fraction P1		Tail fraction P2	
	Amino acid N (% of total N)	g. of amino acid/ 100 g. of dry ash-free sample	Amino acid N (% of total N)	g. of amino acid/ 100 g. of dry ash-free sample
Nitrogen	—	10·66	—	11·81
Aspartic acid	4·59	4·55	5·83	6·54
Threonine	2·10	1·90	2·51	2·52
Serine	2·51	2·00	3·34	3·00
Glutamic acid	9·26	10·4	7·30	9·08
Proline	8·87	7·78	5·20	5·10
Glycine	10·60	6·04	7·92	5·00
Alanine	4·88	3·31	4·60	3·40
Valine	3·18	2·83	4·30	4·20
Methionine	0·65	0·74	0·94	1·20
Isoleucine	2·32	2·31	4·50	3·10
Leucine	4·65	4·64	6·50	7·20
Tyrosine	2·83	3·90	2·30	3·50
Phenylalanine	1·41	1·77	2·80	3·95
Lysine	3·24	1·81	5·90	3·63
Histidine	2·57	1·01	4·00	1·73
Arginine	10·85	3·60	14·5	5·30
Hydroxyproline	Nil	Nil	<0·2	<0·2

polysaccharide and some loss of small peptides. Loss of carbohydrate was greater for the slower-running fractions grouped under P 2.

Composition of the carbohydrate portion of the core material

Fractions P 1 and P 2 were hydrolysed with dilute H_2SO_4 for identification of the neutral sugars by paper chromatography. After removal of amino acids, peptides and amino sugars with sulphonated polystyrene resin, chromatograms were prepared with butanol–acetic acid–water and pyridine–ethyl acetate–water. Both P 1 and P 2 gave heavy spots for galactose but traces only of glucose and mannose. The polysaccharide moiety of the core protein thus contained glucosamine and galactose in relatively large amounts together with smaller quantities of galactosamine and uronic acid. This suggests the presence of keratosulphate (Meyer, Linker, Davidson & Weissmann, 1953) as the major alkali-stable component, together with a small amount of galactosamine and uronic acid as a residue of the chondroitin sulphate chains split off by the action of alkali.

DISCUSSION

Warner & Schubert (1958), using the boundary-electrophoresis method of Tiselius, showed that the chondroitin sulphate–protein complex of bovine nasal cartilage migrated as a single peak at all values over the range pH 2–10. At pH 11 a very small component with a lower mobility appeared but in sodium hydroxide solution of pH 12·5 the boundary split into two parts, the slower-moving part representing about 30 % of the pattern area. The dissociation was not reversible since when the solution exposed to pH 12·5 was subsequently examined at pH 3·3 the two components were still evident. Analysis results for the separated components were not available, but Warner & Schubert (1958) pointed out that the high negative mobility of the smaller component showed that it could not be purely protein in character and that exposure to alkali under these conditions did not result in a clean split between protein and polysaccharide. It appears rather that the slower-moving component contained the protein part of the complex with some sulphated polysaccharide still attached.

In the zone-electrophoresis experiments we report here the hydroxyl ion concentration of the buffer solution was less than that of a sodium hydroxide solution of the same concentration, owing to the presence of sucrose, and values obtained with the glass electrode were near pH 10·8. Substantial degradation by alkali during the course of the run, as reported by Warner & Schubert (1958), would therefore not be expected. It was found that, as minimum conditions, treatment with 0·5 N-sodium hydroxide for 20 hr. at 25° was necessary in order to produce complete hydrolysis. Under these conditions the dissociation was irreversible, and pure chondroitin sulphate and a protein fraction could be isolated readily from the product by zone electrophoresis. The protein fraction prepared in this way contained up to 0·5 % of galactosamine and 0·5 % of uronic acid, suggesting that even after the severe alkaline treatment it still contained chondroitin sulphate to the extent of 1–2 %. The results in the preceding paper show that in the native chondroitin sulphate–protein complex the chondroitin sulphate chains are linked to a protein core at a single point or small area, and taking this information together with the present demonstration of the irreversible nature of the alkaline dissociation, the evidence strongly suggests that the polysaccharide chains are combined to protein by covalent bonds which are fairly readily hydrolysed by alkali. Attempts are now being made to determine the nature of these bonds.

The high degree of spreading of the protein zone during electrophoresis (Fig. 2*b*) suggests that the protein liberated by alkaline treatment is heterogeneous at least as regards charge density, and this was confirmed by chromatographic fractionation with an ion-exchange column (Fig. 3). In the column experiment the protein was eluted as a group of poorly resolved peaks, suggesting a degraded product. Head and tail fractions were analysed separately and it was found that both fractions were free from hydroxyproline, showing the absence of collagen-degradation products: both fractions had similar ratios of tyrosine to tryptophan and similar amino acid analysis, showing that they were most probably derived from a single native protein. However, the content of carbohydrate in the two fractions, though of the same composition, varied considerably in amount.

The analytical results as a whole suggest that the protein fractions were derived from an original polysaccharide-bearing protein contained in the core of chondroitin sulphate–protein complex, and that the variations in polysaccharide content and net charge were due to varying degrees of degradation suffered during the treatment with alkali used to dissociate the complex. Both protein fractions, besides containing a small amount of galactosamine and uronic acid, which may represent a remaining trace of chondroitin sulphate, also contained considerable amounts of glucosamine and galactose. This result is strongly suggestive of the presence of keratosulphate and it is possible that the chondroitin sulphate–protein complex contains keratosulphate bound to the protein core by links that are more resistant to alkali than those binding chondroitin sulphate.

There remains the possibility that keratosulphate is present as an independent protein complex and that the initial material is a mixture. From the results given this seems unlikely. The ratio of galactosamine to glucosamine in the original complex was 6:1. After alkaline hydrolysis this ratio in the protein moiety was 1:5 in the head fraction and 1:3 in the tail fraction and nearly one-third of the original glucosamine was recovered with the protein. If keratosulphate were present as an independent complex originally this must be much less readily hydrolysed by alkali than is the chondroitin sulphate–protein complex. It would therefore be retained as a highly charged anion and would easily be separable from protein both by electrophoresis and by ion-exchange chromatography. This was not found; in the ion-exchange chromatogram there was wide separation between chondroitin sulphate and protein, but the glucosamine appeared with the protein peak and its concentration was not very different in head and tail fractions.

A conclusive decision on this point is not easy to obtain in a study of the present kind owing to the difficulty in separating large anionic protein–polysaccharide complexes, which may form heterogeneous aggregates. A more revealing approach to the problem may lie in an attempt to isolate the protein moiety by a method which does not give rise to extensive degradation, and work is now in progress on these lines.

SUMMARY

1. The dissociation of the chondroitin sulphate–protein complex of hyaline cartilage in alkaline solution was studied by zone electrophoresis in a column stabilized by a density gradient.

2. The complex was stable in a sodium hydroxide–sucrose solution of pH 10·8 but was irreversibly dissociated by treatment with 0·5N-sodium hydroxide for 24 hr. at 25°. It was concluded that linkage between chondroitin sulphate and protein is most probably covalent.

3. The protein fraction released by alkaline treatment was examined by chromatography with a carboxylic acid ion-exchange resin (IRC-50).

4. The protein was heterogeneous and appeared as a close group of poorly resolved peaks. Head and tail fractions were analysed separately and found to have the same qualitative composition, but the head fraction contained 9·6 % of carbohydrate and the tail fraction 5·4 %. It was concluded that the fractions were derived from a single carbohydrate-containing protein which had been degraded by the action of alkali.

5. The carbohydrate moiety of the protein was rich in glucosamine and galactose, but neutral sugars other than galactose were present in trace amount only. The core protein may thus contain keratosulphate.

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