5. There was evidence that the omission of L-cystine resulted in a high proportion of adult mosquitoes failing to emerge.

6. According to the level of phenylalanine or tyrosine in the medium various shades of pigmentation could be produced in the mosquito larvae. The adults emerging even from wholly unpigmented larvae were normally pigmented and there appeared to be little relation between pigmentation and growth or survival. A number of compounds were found capable of producing intermediate degrees of pigmentation in the mosquito larva.

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The Chemistry of Connective Tissues

1. THE STATE OF COMBINATION OF CHONDROITIN SULPHATE IN CARTILAGE

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In recent years evidence has been presented by several groups of workers that chondroitin sulphate exists in tissues such as cartilage in a very highly polymerized condition. The early workers in the field regarded chondroitin sulphate as an oligosaccharide of quite low molecular weight, and Levene (1925) formulated the substance as a non-reducing tetrasaccharide composed of two residues of glucuronic acid and two residues of a sulphate of *N*-acetylglucosamine. Perhaps the main reason for the failure of the early workers to recognize the highly polymerized character of the polysaccharide lay in the fact that strongly alkaline reagents were invariably used to effect the initial extraction from the tissue, and it has since been shown that the polysaccharide is rapidly degraded in the presence of strong alkali, particularly at temperatures above 0°.

The quantitative extraction of chondroitin sulphate from cartilage remains a matter of considerable difficulty, and dilute NaOH is still frequently employed. This method was investigated by Jorpes (1929), who showed that a concentration of NaOH as high as 0·5 N was necessary to extract the acid polysaccharide from cartilage at 0°. Meyer & Smyth (1937) suggested the use of strong solutions of calcium, strontium or barium chlorides, and obtained good yields of chondroitin sulphate by the extraction of dried cartilage powder with 10% (w/v) $CaCl_2$ solution at neutral reaction.

Blix & Snellman (1945) used $CaCl_2$ extraction and were careful to avoid acids or alkalis during the subsequent separation of the polysaccharide from the protein moiety of the extracted mucoid. Prepared under careful conditions, the chondroitin sulphate was shown to have a molecular weight of the order of 260,000 and a long-chain structure was suggested. In dilute alkali, the molecule was depolymerized at room temperature to a rather definite point, at which the products formed were still of colloidal size, although giving little double refraction of flow. The molecular weight of chondroitin sulphate extracted from cartilage by dilute alkali was estimated to be of the order of 40,000–50,000.

Meyer & Odier (1946) prepared chondroitin sulphate by extraction of hog nasal cartilage in 0·5 N-NaOH at 5°. Their preparation was probably similar to the alkali-degraded polysaccharide of Blix & Snellman (1945), and from viscosity measurements calculated on the basis of an unbranched chain its molecular weight was estimated at 10,000–15,000. The reducing power of the polysaccharide towards NaIO at pH 10·5 and towards HIO_4 at pH 4·2–4·4 was investigated, and from the results obtained it was concluded that the residues of glucuronic acid and *N*-acetylglucosamine-6-sulphate are arranged to form an unbranched chain molecule. This conclusion is in opposition to the earlier view of Bray, Gregory & Stacey (1944) who methylated a degraded chondroitin that had been obtained by acid hydrolysis of a chondroitin sulphate prepared with alkali. From isolation of the products of hydrolysis of the methylated oligosaccharide it was concluded that chondroitin sulphate possesses a structure of the branched-chain type.

The chondroitin sulphate of hyaline cartilage is associated with the semi-transparent, apparently structureless, ground substance that constitutes the bulk of the tissue and gives the cartilage its characteristic physical properties. The cartilage cells occupy cavities in this ground substance and appear to be isolated from one another. In spite of its apparent homogeneity, histological studies show that the intercellular ground substance is not amorphous since after digestion with trypsin or by use of the various silver impregnation methods, collagenous fibrils are discernible which form a dense felt-work running in all directions or gathered together in oriented bundles. From its histochemical reactions the collagen of the ground substance of cartilage is considered to be similar to that occurring in loose connective tissue. When cartilage is extracted with alkalis or with solutions of inorganic salts, part of the material passes into solution and constitutes the so-called 'chondromucoid'; the residue is mainly collagen but retains more or less mucoid according to the efficiency of the agent used for extraction. The chondromucoid contains protein and gives precipitates on addition of acetic acid which usually take the form of clear firm gels.

The nature of the protein moiety of chondromucoid and its relation to the chondroitin sulphate and the collagen structures of the tissue itself have received relatively little attention. Bungenberg de Jong & Dekker (1935) studied the interaction of chondroitin sulphate with proteins and showed the presence of complexes which were regarded as 'coacervates'. Meyer, Palmer & Smyth (1937), on the other hand, looked upon the interaction as a salt formation and showed that in the presence of acetic acid true salts are formed in stoicheiometric proportions by the union of the basic groups of the protein with the acid groups of the polysaccharide. It was shown that in the presence of 2% (w/v) acetic acid, the amount of chondroitin sulphate combined by a protein corresponded to the amount of basic amino-acids contained in the protein, and further, the chondroitin sulphate-binding power of the protein was in agreement with the acid-binding power found by studies of dye-protein complexes. In the salts studied the chondroitin sulphate was found to react with the protein as a dibasic acid; both the sulphuric acid group and the carboxyl group of the uronic acid residue take part in salt formation. Meyer *et al.* (1937) were of the opinion that chondroitin sulphate occurs in nature in salt-like complexes which are present as structurally organized elements of sheet or fibre form, and pointed out that analysis of ground cartilage showed the ratio of hexosamine N to total N to be 0·046, a figure that compares well with the corresponding ratio 0·056 for the gelatin salt of chondroitin sulphate. This close correlation led to the view that the major portion of the cartilage is a protein salt of chondroitin sulphate.

This suggestion is not borne out by the experiments of Blix (1940), who submitted an aqueous extract of cartilage (nasal septum of ox) to electrophoresis in the apparatus of Tiselius (1937) at hydrogen-ion concentrations within the physiological range. Two components only appeared, the faster being almost pure chondroitin sulphate while the slower was a protein containing only 0·02% hexosamine. No evidence of combination at a higher pH than the isoelectric point of the protein was reported, and the result appears to support the earlier view of Mörner (1889) that in cartilage the acid polysaccharide is at least partly present as alkali salt and is, therefore, not fully combined with protein. It should be pointed out, however, that only a very small proportion of the chondroitin sulphate is extractable from fresh cartilage by water under the conditions used by Blix, and the extracted mucoid may not be in the same form as the material remaining in the tissue.

The intention of the work described here was to make a preliminary study of the chemical structure of the intact tissue, and in embarking on this problem it was considered that a possible line of approach would lie in the application of a process of successive mild degradation of one or other of the components of the tissue, followed by an examination of the breakdown products that resulted. The process of degradation of collagen fibres as they occur in tendon or hide has already received much attention, particularly by workers interested in the mechanical properties of leather, and for this reason the first approach has been a study of the liberation of chondromucoid under conditions that would be expected to give rise to thermal degradation of the collagen structures present in the tissue.

The work of Lloyd & Garrod (1946) has shown that collagen fibres of tendons (from the tails of rats) rapidly contract to about one third of their original length when heated in water to 60–70°, and that in the contracted state the fibres show a new property of rubber-like elastic extension. Lloyd and her colleagues found that formamide, strong solutions of the phenols and anhydrous formic acid are very effective in reducing the temperature at which thermal contraction takes place. Strongly acid (pH 2) or alkaline (pH 12) solutions and concentrated solutions of salts are also effective. Among the salts the effect of the lyotropic series is evident and, in addition, Ca^{++} is stated to be particularly effective in causing shortening in length at low temperatures.

Since published data on the thermal contraction of collagen fibres were insufficient to afford a direct comparison with the methods devised for the extraction of cartilage, a number of experiments were carried out during the course of this work to determine the effect on rat-tail tendon of the temperature and concentration conditions used in the extraction experiments, and these are reported in the experimental section.

It is probable that the chondroitin sulphate liberated under the rather mild conditions that give rise to thermal shrinking of collagen fibres remains in a condition closely approaching its state in the tissue itself and, with this in mind, it was considered that a study of the physical and chemical properties of the extracted mucoid would be of value. Evidence of complex formation between the undegraded polysaccharide and the protein of the mucoid under conditions of pH and salt concentration in the physiological range is of particular interest, and for this reason a preliminary electrophoretic study of the material extracted by the new method has been undertaken.

EXPERIMENTAL

Analytical methods

A modification (Lugg, 1938) of the peroxide fusion method was used for the estimation of total S. Ester-bound S was determined as SO_4-S after hydrolysis with 5 N-HCl for 20 hr. at 100°, using the gravimetric procedure described by Lugg (1938). The micro-Kjeldahl method was used for the determination of N. Moisture was determined by drying to constant weight over P_2O_5 at 80° under reduced pressure, and in all cases ash was weighed as sulphate.

All analyses quoted in the text are corrected for ash and water unless it is otherwise stated.

Preparation of the cartilage powder

Fresh cartilage from septum nasi of cattle was freed from adhering connective tissue and finely chopped with a scalpel. The pieces were introduced at once into acetone, and after standing for some time in two or three changes of solvent they were dried in air at 70° and ground in a hammer mill through a sieve with circular apertures 0·56 mm. in diameter.

Extraction of cartilage powder with water at room temperature

Although little mucoid could be extracted from fresh cartilage by water at room temperature, even after the tissue had been finely minced, a considerable proportion of the mucoid became extractable after the tissue had been dehydrated in acetone and ground to a powder. Portions of the powdered cartilage (N, 12·4; ester S, 1·2; P, 0·06; ash, 5·6; moisture, 5·6 %) were extracted with distilled water by shaking gently for 2–3 hr. at room temperature. One extraction was sufficient to remove the bulk of the extractable material, but in order to exhaust the residue, two further similar treatments were given. The chondromucoid was precipitated from the aqueous solution by addition of ethanol (3 vol.) after adding a few ml. of saturated sodium acetate solution to bring the concentration of the salt to about 0·5 % (w/v). The precipitate was washed first with 80 % (v/v) ethanol and then thoroughly dehydrated with anhydrous ethanol before drying. Prepared in this way the mucoid was a fine white powder having N, 9·2–11·5; ester S, 3·2–3·3; P, 0·04 %; and dissolved in water to give a clear viscous solution. Results obtained from a number of experiments showed that 30–35 % of the acid-hydrolyzable S present in the original tissue was extractable by distilled water at 18–23°.

Effect of a short heat treatment on the extraction of cartilage powder

Cartilage powder that had been thoroughly extracted with water at room temperature yielded a further amount of chondromucoid after a short heat treatment in water at 60–80°. Portions of the water-extracted powder (1·55 g.) yielded 0·273 g. of chondromucoid after heating with distilled water at 80° for 10 min., followed by shaking gently for 2 hr. at room temperature. In a subsequent series of experiments it was found that a preparation of cartilage powder that had already yielded 35 % of its content of ester S by extraction with cold water yielded a further 30–35 % of the ester S originally present in the tissue, on treatment with distilled water at 60° for 30 min. The chondromucoid extracted after heat treatment was similar in composition to that extracted by cold water having N, 10·7–12·4; ester S, 3·1–3·7 %. The material remaining insoluble still contained 33–36 % of its original content of ester S, having N, 14·7–15·0; ester S, 0·6–0·63 %.

Extraction with calcium chloride solution

Extraction with a 10 % (w/v) aqueous solution of $CaCl_2$ was found to be more effective after an initial short period of gentle heating. A sample of cartilage

powder that had already been exhaustively extracted with cold water was warmed to 45° for 30 min. with 10 % (w/v) $CaCl_2$ solution (pH 7·49), followed by cooling to room temperature and shaking for 3 hr. The extracted chondromucoid had N, 8·2; ester S, 3·8 % and represented 28 % of the ester S originally present in the fresh cartilage. The substance after recovery from the $CaCl_2$ solution was not entirely soluble in distilled water or in dilute salt solutions, and gave a small amount of gelatinous residue. After removal of insoluble material by centrifugation, the aqueous solution was clear and viscous. Since extraction with neutral $CaCl_2$ solution under these conditions was incomplete, the extraction was repeated with a solution of $CaCl_2$ (10 % w/v) that had been adjusted to pH 11 by addition of $Ca(OH)_2$ followed by filtration. The mixture was heated to 37° for 30 min., and in this case a total extraction of 90 % of the ester S was obtained, but the product (N, 11·7; ester S, 3·6 %) did not give highly viscous aqueous solutions and appeared to be more readily soluble in water than the mucoid extracted at neutrality. The residue from the cartilage after a further extraction with the alkaline $CaCl_2$ reagent had N, 16·5; ester S, 0·15; total S, 0·56 %, and since all but a small residue (6·3 %) was converted to gelatin on heating with water at 120°, the substance was considered to be almost wholly collagen.

Extraction with aqueous solutions of formamide

Freshly prepared solutions of anhydrous formamide (m.p. −2°) were used for these experiments. Neither 10 % (v/v) nor 30 % (v/v) solutions were effective in extracting further mucoid from water-extracted cartilage powder at temperatures up to 37°. However, on warming to 45° for 30 min. a further 30 % of the ester S originally present in the tissue was extracted by 30 % (v/v) formamide solution, and the product after recovery by precipitation with ethanol (N, 10·0; ester S, 3·3 %) was similar in analytical composition to the product of extraction with hot water or calcium chloride solutions. The mucoid extracted with 30 % (v/v) formamide, redissolved in water to give clear viscous solutions.

Thermal contraction of tendons from the tail of the rat

The procedure adopted was a modification of that of Lloyd & Garrod (1946). The tendons were carefully extracted from rat tails that had been stored for 2 weeks at 0°, and any tendon that had been stretched or damaged in the process of removing it from the tail was rejected. To both ends of each tendon a small lead shot was attached by inserting the end of the tendon into a deep cut in the shot, made by a razor blade, and then gently closing the cleft by means of a pair of forceps.

Table 1. *Thermal contraction of rat-tail tendon*

(The length of the tendon at the time indicated is expressed as percentage of the initial length.)

Temp.	2 min.	5 min.	10 min.	20 min.	60 min.
		Solvent: distilled water			
55°	100	100	100	100	100
60	49	64	95	97	100
65	47	93	100	103	110
70	33	104	108	112	116
		Solvent: 10 % (w/v) $CaCl_2$ (pH 6·05)			
37°	100	100	100	98	92
45	—	60	53	90	120
50	43	68	88	100	106
		Solvent: 10 % (w/v) $CaCl_2$ (pH 11·2)			
40°	—	86	27	30	116
45	—	27	27	44	111
50	23	23	36	84	118
		Solvent: anhydrous formamide			
35°	—	95	67	67	95
40	—	67	69	100	102
45	—	61	100	110	112
		Solvent: 30 % (v/v) aqueous formamide			
30°	100	100	100	97	35
40	67	76	95	102	106
45	57	57	66	74	77
		Solvent: 10 % (v/v) aqueous formamide			
40°	—	87	27	30	110
45	—	27	27	44	87
50	23	23	36	84	118

The tendon was suspended in a narrow graduated cylinder which contained the solvent, the upper lead shot being gripped in a clip while the lower one hung free. The weight of the lower shot was chosen to be just sufficient to sink the tendon and hold it at full length. The graduations on the cylinder were used as an arbitrary unit for measuring the changes in length of the tendon, and for temperature control the cylinder was placed in a water bath fitted with a stirrer.

The results of a number of experiments are given in Table 1, which shows the changes in length with time at various temperatures and in various solvents. In all experiments, little change in length occurred below a certain limiting temperature; above this temperature, however, rapid contraction took place and this was followed by a slow increase in length so that the final length of the tendon was often greater than the initial value. At this stage the tendon had lost much of its tensile strength and had become partially gelatinous. In the table, only those experiments carried out near the limiting temperature are reported. It should be noted that there was some variation in the behaviour of individual tendons, even among those extracted from the same rat's tail, but the values recorded are typical of the results obtained from many experiments.

Correlation of the conditions resulting in extraction of chondromucoid with those giving rise to thermal contraction in collagen fibres

In Table 2 the results of a number of extraction experiments are given. The sample of cartilage powder used had not been previously extracted with water at room temperature so that the yields given (expressed as percentage ester S extracted from the original tissue) are total yields. It seems clear that a short period of heating materially assists the release of chondromucoid from the remaining constituents of the tissue, either in aqueous solution or in solutions of formamide or salts. Further, the action of $CaCl_2$ or formamide solutions is not due entirely to an enhanced solubility of the chondromucoid in these reagents, since the substance is readily soluble in cold water, and can in fact be extracted in good yield by distilled water alone after a short heat treatment. Solutions of the active reagents have, however, a marked effect in reducing the temperature of the heat treatment necessary to effect extraction. Comparison of Table 2 with Table 1 shows the marked correlation between the reaction conditions necessary to extract chondromucoid in good yield with those giving rise to thermal contraction of free collagen fibres. It should be pointed out, however, that, except in the experiments with $CaCl_2$ solution at pH 11, complete extraction was not obtained, and in this one case there is reason to suppose that the mucoid itself suffered degradation since the product gave solutions that were considerably less viscous than those obtained from chondromucoid extracted with less alkaline media. Although there were considerable variations in the N and S contents of the extracted mucoids in individual experiments, the figures given in Table 2, which show the range obtained over a number of duplicate experiments, indicate little systematic variation and show that the product extracted under the varying conditions had substantially the same analytical composition.

Isolation of the protein component

Investigations on mucopolysaccharides of bacterial origin have shown that the solubility of the polysaccharide moiety of the complexes in strong solutions of phenol is in some cases less than that of the protein (cf. Morgan & Partridge, 1941). In the case of chondromucoid it was found that at 37° considerable amounts of protein may be extracted from the

Table 2. *Effect of heat treatment on the extraction of cartilage powder in various solvents*

Treatment	Ester S extracted (%)	Chondromucoid	
		Ester S (%)	N (%)
Water at 20°	30–35	3·15–3·3	9·2–11·5
Water at 60°, 30 min.	58	3·1	12·4
Water at 80°, 10 min.	65–70	3·5–3·7	10·7–11·0
$CaCl_2$ (pH 7·5) 45°, 30 min.	60	3·5–3·8	8·2–9·7
$CaCl_2$ (pH 7·5) 55°, 30 min.	65	3·5–3·6	9·3
$CaCl_2$ (pH 11) 37°, 30 min.	95–98	3·6–3·8	11·7
Formamide 30% (v/v) 37°, 30 min.	44	—	—
Formamide 30% (v/v) 45°, 30 min.	63	3·3	10·0

dry powder by 90% (w/w) aqueous phenol. The mucoid (2 g.; N, 9·41; ester S, 2·33; ash, 9·3; moisture, 6·5%) was allowed to stand at 37° with 90% (w/w) phenol solution (150 ml.) for 24 hr., the mixture being occasionally stirred. The residue was removed by centrifugation and the clear solution treated with ethanol (3 vol.). The heavy precipitate, after collecting and washing with ethanol in the centrifuge, was dissolved in water at 60° and again precipitated with ethanol. After washing with ethanol and ether the substance was dried at room temperature over P_2O_5.

Prepared in this way, the substance had 'N, 17·53; ester S, 0·13; total S, 0·44% and was thus essentially protein in character. The residue from phenol extraction had 'N, 7·96% (uncorrected for ash and water) and was extracted with further quantities of 90% phenol solution. The yield of protein from the first extract was 0·2 g., but from successive extracts the amount of protein yielded diminished rapidly and after four extractions the N content of the residue remained constant at 7·26% (uncorrected). It was clear, therefore, that although part of the protein contained in the 'undegraded' mucoid was freely extracted by phenol solution, a further part of it was not available for extraction, and in subsequent experiments it was not found possible to reduce the N content of the mucoid below about 7% by repeated extraction with phenol. The protein was not soluble in cold water or cold dilute alkalis or acids, but dissolved readily in water at 50–60° giving clear viscous solutions. On cooling, the solutions set to a firm gel.

Viscosity of the extracts

The dry preparations of chondromucoid were usually not completely soluble in water or buffer solutions, and solubility slowly decreased on storage in the dry state. Solutions of the chondromucoid, on the other hand, were fairly stable, but it was observed from time to time that the viscous solutions slowly became more opalescent on standing at 0° and sometimes gave rise to a fine precipitate.

These effects caused difficulty in making up solutions of the mucoid for viscosity determinations or for electrophoresis experiments and for this reason fractionation with ethanol was eliminated and the extracts obtained in solutions of electrolytes were first dialyzed against distilled water for 48 hr. followed by dialysis against the appropriate buffer solution. Concentration was then measured refractometrically by making use of a value for the refractive increment of the mucoid in the buffer solution. Since the composition of the mucoid was rather variable, particularly as regards ash and N content, there was some uncertainty about the value of the refractive increment to be adopted, but from a number of experimental determinations on mucoid extracted from the cartilage powder in phosphate-NaCl buffer of pH 6·90 at 60° an average value of $1·60 \times 10^{-3}$ (1% (w/v) at 25·2°) was adopted for all the refractometric estimations.

Viscosity determinations were carried out at 25·4° in an Ostwald viscosimeter having a capillary length of about 9 cm. and a flow-time for water of 31·2 sec. As would be expected the relative viscosity (η_r) of the mucoid in salt solution was much lower than that given by the same preparation in distilled water; for instance, mucoid that had been extracted by distilled water at 60° gave (after dialysis against changes of distilled water) a value for η_r in water of 3·16 for a 0·25% solution, while after dialysis against phosphate-NaCl buffer of pH 6·97 (μ, phosphate, 0·02; μ, NaCl, 0·18) the solution had η_r 1·53 at the same concentration. The viscosity of the mucoid, both in water and salt solutions, showed variations from preparation to preparation even under carefully controlled conditions of extraction, but the values for the viscosity of individual preparations at different concentrations lay on smooth curves. The values for log η_r for different dilutions of most of the preparations could be fitted by straight lines, and Fig. 1 shows log η_r plotted against concentration for a number of the preparations.

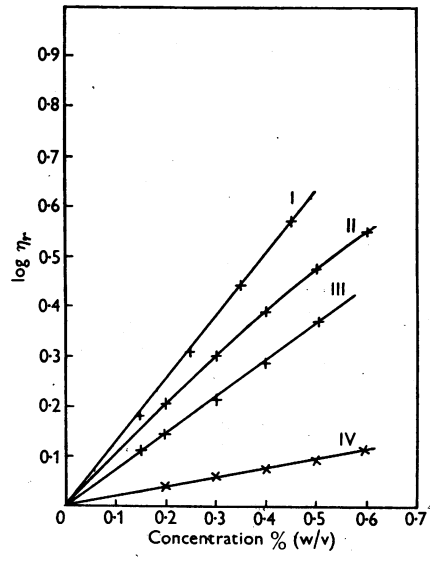

Fig. 1. Log η_r for extracts of cartilage, viscosity determinations carried out in phosphate-NaCl buffer of pH 6·90; μ, phosphate, 0·02; μ, NaCl, 0·20. I, H_2O at 70°; II, $CaCl_2$ (pH 7·4) at 55°; III, Phosphate-NaCl at 60°; IV, $CaCl_2$ (pH 11·0) at 37°.

If the equation of the log η_r-concentration curve is taken as

$$\log \eta_r = Kc,$$

where K is a constant and c is the concentration of the solute, then, at infinite dilution (provided natural logs are used), k = the 'intrinsic viscosity' $[\eta]$, where

$$[\eta] = \lim_{c \to 0} \frac{(\eta_r - 1)}{(c)}$$ (Kraemer & Lansing, 1935).

According to Staudinger (1940) the value of intrinsic viscosity is proportional to particle weight even in the case of heteropolar substances, provided that the particles are linear macromolecules and charge effects are suppressed by carrying out the viscosity determinations in solutions containing a sufficient concentration of electrolyte. However, the solutions of mucoid contain more than one molecular species and, as will appear later, in dilute solutions containing electrolytes chondromucoid may be looked upon as a complex in equilibrium with its dissociation products. Under these conditions it is probable that the significance of $[\eta]$ as a measure of particle length is qualitative only, but since $[\eta]$ for the extracts made under alkaline conditions is of a lower order compared with the values given by the mucoid extracted at neutral reaction, it is clear that in the former case considerable degradation has occurred.

It is implicit in the theory of Staudinger that the effect of molecular association on viscosity depends upon the relative position of the molecules associating together. If compact bundles are formed, the viscosity is supposed not to be affected, while, if two similar molecules associate end to end, the value for $[\eta]$ is twice as great as in the absence of association. The linearity of the log η_r curves is therefore of especial interest since it indicates that, in the presence of electrolytes, the degree of end to end association is not significantly increased with increasing concentration.

Effect of heat treatment on the viscosity of chondromucoid

An extract of chondromucoid was prepared in phosphate-NaCl buffer of pH 6·9 (μ, phosphate, 0·02; μ, NaCl, 0·18) and was dialyzed against several changes of the same buffer. Samples of the solution (10 ml.; *c.* 0·481 % (w/v)) were sealed in glass tubes and heated for varying periods of time, one series being heated at 100° and another at 80°. At both temperatures the viscosity of the solutions fell very rapidly in the first 5 min. of heating, the initial rapid fall being followed by a further slow reduction in viscosity over a period of some hours. The initial viscosity of the solution was η_r 3·12 at 25·4° and after heating 5 min. at 80° the value fell to η_r 1·69, after which, on heating a further 10 hr. at 80°, the value obtained was η_r 1·35. Since, as will appear later, the solution of mucoid contained free gelatin, the slow continuous fall in viscosity which followed the first dramatic change may have been due to the degradation of this protein (cf. Ames, 1947).

Electrophoresis experiments

The experiments were carried out at 3·2° in the apparatus of Tiselius (1937) using the optical arrangement of Philpot as modified by Svensson (1939, 1946). In order to suppress boundary anomalies and to reduce the effects of viscosity the experiments were carried out with solutions containing high concentrations of salt and low concentrations of colloid. Phosphate-NaCl, glycine-NaCl and acetate-NaCl buffers were used, the solutions having ionic strength 0·02 with respect to the buffer ions and ionic strength 0·18 with respect to NaCl. The preparations of mucoid and protein were dialyzed against the appropriate buffer for 3–4 days until equilibrium was reached, the concentration of the colloid was then measured refractometrically and the solutions were adjusted to 0·25–0·5 % (w/v) by dilution with the buffer. The conductivities of the buffer and mucoid solution were measured at 0° and the mean of the two values was used in conjunction with the milliampere readings in order to determine a mean value for the potential gradient at the boundaries. Since conductivity was measured at 0° the values for mobility given in the tables refer to 0° rather than to the temperature at which the experiment was carried out. The pH of the buffer solutions was measured by means of a hydrogen electrode at 0°.

Electrophoresis of mucoid solutions. The results of a series of electrophoresis experiments carried out on the same batch of cartilage extract are given in Table 3. The extract was made at 60° in phosphate-NaCl buffer of pH 6·9 following the procedure already given, and, for the individual mobility determinations, samples of the extract were dialyzed against buffers of the desired pH.

Fig. 2 (*a*) shows the electrophoretic pattern obtained at pH 8·5 in phosphate-NaCl buffer (μ, phosphate, 0·02; μ, NaCl, 0·18). Over a range of pH values from 5·9 to 9·0 the patterns given were essentially similar. In the positive limb of the cell three boundaries were invariably present, and these were denoted *A*, *CP* and *B* in the order of their mobilities. In the negative limb, however, two boundaries only were visible, the mobilities corresponding to fractions *A* and *B*. The *A* peaks were sharp in both limbs, and comparison of the mobilities given for *A* in Table 3 with those found by Blix (1940) showed the *A* component to be chondroitin sulphate. This conclusion was afterwards confirmed by isolation of the component using the large apparatus of Tiselius (1938). The peaks due to substance *B* were sharp in the negative limb, but rather diffuse in the positive limb, and owing to the difficulty in measuring the position of the diffuse peak

the observed average values of mobility for *B* were subject to some variation. The observed values, however, were near to those recorded for the purified

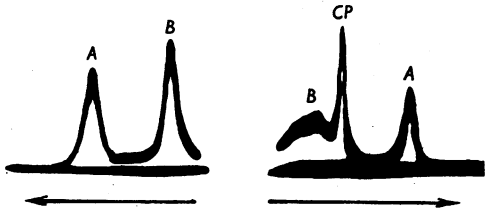

Fig. 2 *a*. Electrophoresis pattern of chondromucoid in phosphate-NaCl buffer at pH 8·5. The preparation was obtained by extracting cartilage powder with phosphate-NaCl solution (pH 6·9) at 60°. Left, negative, descending; right, positive, ascending.

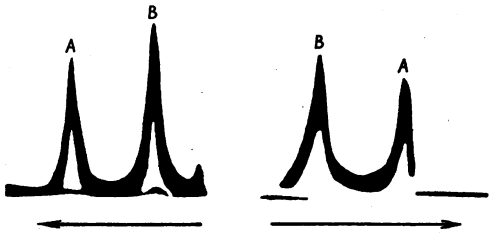

Fig. 2 *b*. Electrophoresis pattern of chondromucoid after heating at 100° for 30 min. in phosphate-NaCl solution of pH 6·88. Left, negative, descending; right, positive, ascending.

protein component of the mucoid (given in Table 4), and for this reason the peaks *B* were identified with the protein. The component *CP* gave a very sharp peak in the positive limb, but within the pH range indicated in Table 3 the boundary was absent in the negative limb. Although the sharpness of the peak facilitated mobility readings, its position varied rather considerably in different preparations and the mobility values recorded in Table 3 show some scatter. At pH 5·25 the peak *CP* showed in both limbs. At this acid reaction the preparations were usually rather opalescent and it was observed that after the current had been passed for some time opalescence was largely confined between the two *CP* boundaries.

The reduction of viscosity on heating the cartilage extracts has already been reported, and Fig. 2 (*b*) shows the electrophoresis pattern obtained with a phosphate-NaCl extract (pH 6·88) of cartilage that had been heated at 100° for 30 min. in the same buffer in which the extract was made. In heated preparations the *CP* peak was invariably absent, and the two components *A* and *B* appeared with symmetrical peaks in both limbs. Table 3 shows the values for mobility of the two components in phosphate-NaCl buffers of pH 6·88 and 5·33. The time and temperature of heat treatment required to eliminate the component *CP* was found in all cases to be sufficient to complete the first rapid stage in the reaction leading to reduction of viscosity of the extracts, whether the heat treatment was carried out in phosphate-NaCl buffers of varying pH or in aqueous acetic acid solution. It thus appears probable that the component *CP* contributes significantly to the viscosity of the unheated solution. This conclusion is borne out by the extreme sharpness of the *CP* peak and also by the opalescent appearance associated with the complex, both of which observations indicate a particle of considerably greater size than either of the other two components in the system.

Table 3. *Electrophoretic pattern of cartilage extract*

Exp. no.	Buffer	pH (0°)	Mobilities (average from both limbs, $u \times 10^5$) Chondroitin sulphate	*CP* component	Protein
		Unheated extract			
25	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	5·25	−10·54	−5·64	−1·06
21	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	5·89	−10·90	−5·8*	−1·94
22	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	6·93	−11·35	−2·34*	−1·36
19	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	7·86	−11·68	−4·32*	−1·31
26	Glycine (μ, 0·02) +NaCl (μ, 0·18)	9·01	−11·76	−4·04*	−1·48
	Extract heated at 100° for 30 min. in phosphate-NaCl (pH 6·88) before dialysis against appropriate buffer				
35	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	6·88	−12·35	—	−1·12
36	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	5·33	−12·4	—	−1·22

* The boundary appeared in the positive limb of the cell, but was absent from the negative limb

Electrophoresis of the protein component. The protein component isolated by extraction of the dry mucoid with 90% (w/w) phenol solution was electrophoretically homogeneous over a pH range 3·6–7·0. The mobility readings in a series of buffers of total ionic strength 0·20 are recorded in Table 4. The boundaries in both limbs of the cell were sharp and symmetrical and the pH-mobility curve obtained by plotting the data in Table 4 was

DISCUSSION

The work described in the experimental section shows that the intercellular matter of hyaline cartilage of beef septum nasi is composed almost exclusively of chondroitin sulphate and proteins of the collagen group, the relationship between these substances having been examined by degradation of the tissue material in a series of controlled steps.

Table 4. *Mobility of the protein isolated from chondromucoid*

Exp. no.	Buffer	pH	Mobilities ($u \times 10^5$) u +ve	u −ve	Average
28	Glycine (μ, 0·02) +NaCl (μ, 0·18)	3·58	+2·29	+2·59	+2·44
30	Acetate (μ, 0·02) +NaCl (μ, 0·18)	4·29	+1·19	+1·19	+1·19
31	Acetate (μ, 0·02) +NaCl (μ, 0·18)	5·11	−0·31	−0·31	−0·31
32	Acetate (μ, 0·02) +NaCl (μ, 0·18)	5·53	−0·58	−0·47	−0·53
29	Phosphate (μ, 0·02) +NaCl (μ, 0·18)	6·95	−0·85	−0·75	−0·80

generally of the shape found for gelatin (Moyer & Moyer, 1940). The isoelectric point of the protein in acetate-NaCl buffer (μ, acetate, 0·02; μ, NaCl, 0·18) was 4·86. This is close to the value (4·9) found by Moyer & Moyer for the isoelectric point of electrodialyzed commercial gelatin adsorbed on quartz or collodion particles, and since the solubility properties, gel-formation and analytical composition of the protein were similar to those of authentic gelatin, the protein must be regarded as having originated from collagen.

Electrophoresis of gelatin prepared from the collagenous residue remaining after extraction of the cartilage with alkaline $CaCl_2$ *solution.* The gel-forming protein prepared by heating the collagenous residue in an autoclave was similar in analytical composition to authentic gelatin, and showed a single sharp boundary in the electrophoresis apparatus. The mobility readings given by the protein fitted the mobility-pH curve obtained with the samples of gelatin isolated from the chondromucoid (Table 4). It thus appears that, apart from a small insoluble residue (comprising less than 6% of the intact tissue), the ground substance of cartilage is a structure built up from no more than two clearly recognizable chemical individuals, chondroitin sulphate and collagen; the process of successive degradation described in this work may therefore be looked upon as leading to the destruction of a collagen-chondroitin sulphate complex, and ultimately to the complete degradation of the collagen structure to gelatin and the liberation of chondroitin sulphate in a degraded form.

The extreme sensitivity of collagen to increase in temperature led to the view that the collagen structure of cartilage would suffer modification at lower temperatures than does the chondroitin sulphate, and experiment showed that if the temperature of an aqueous suspension of powdered cartilage was raised to a point at which thermal contraction of the collagen would be expected to take place (60–70°) the treatment resulted in the release of a highly viscous, water-soluble mucoid containing chondroitin sulphate in a yield representing some 60% of the amount originally present in the tissue. The use of salt solutions, particularly those of the divalent alkaline earths, had the expected effect in reducing both the temperature of contraction of the collagen of rat-tail tendons and the temperature required to release chondromucoid from cartilage to approximately the same degree.

The analytical composition of the mucoid extracted from cartilage remained remarkably constant over a series of extraction experiments in which a number of solvents and salt solutions were used, provided the lowest effective temperature was employed, but the yield of chondroitin sulphate obtained was usually no more than 60–70% of that present in the tissue. If higher temperatures were used, the mucoid had a lower viscosity and contained an excess of protein. Extraction with 10% calcium chloride solution at pH 11 gave yields of 90–95% of the ester-bound sulphur originally present in the tissue, but here again, the viscosity of the extracted mucoid was low.

The water-soluble mucoid was examined in the electrophoresis apparatus of Tiselius over a considerable pH range and, in addition to the boundaries due to the protein and the acid polysaccharide, a third boundary, due to a complex between the two, was observed over the whole range covered by the experiments. From pH 9·0 to 5·8 the third boundary (designated *CP*) appeared in the positive limb only, but at pH 5·3 it showed its presence in both limbs and at this acid reaction the solution had a distinct opalescence which appeared to be confined between the two *CP* boundaries. The substance responsible for the *CP* boundary was heat labile and a further stage in the process of degradation was carried out by heating the mucoid at 80–100° for 10–30 min. when it no longer showed the presence of the third boundary in the electrophoresis apparatus and the protein and polysaccharide components migrated independently. The disappearance of the *CP* boundary on heating the mucoid was coincident with a considerable fall in the viscosity of the solution, and since the sharpness of the *CP* boundary indicated a particle of considerable length, it seems probable that the complex contributed significantly to the viscosity of the unheated solution. The failure of the component to appear as a distinct boundary in the negative limb at hydrogen-ion concentrations near neutrality may be due to the instability of the complex except in the presence of an excess of chondroitin sulphate. Such mass-action effects are common in antigen-antibody reactions and reveal their presence in zoning phenomena.

A similar position has been revealed by the work of Chargaff, Ziff & Moore (1941), who examined the electrophoretic behaviour of heparin when mixed with serum albumin or dialyzed plasma. A third component was observed (designated by these authors the *C component*), which had a mobility intermediate between that of serum albumin and the acid polysaccharide. In this case, however, the *C component* was more clearly discernible on the descending side than on the ascending side, which usually showed only a broadening of the albumin peak. Some preparations of heparin failed to produce the *C component*, but in this case the heparin peak appearing on the descending side was many times larger than on the ascending side. Chargaff and his colleagues also examined the effect of chondroitin sulphate on human plasma proteins, but no evidence of complex formation was obtained. However, a highly purified specimen of chondroitin sulphate was used and it may have been somewhat degraded in the process of purification.

Since the complex formation of heparin with serum albumin is of considerable importance in the anticoagulant activity of the former it was considered of interest to assay the activity of 'undegraded' chondroitin sulphate as an anticoagulant. However, it was found that both the mucoid itself and a preparation of chondroitin sulphate obtained from the mucoid by separation in the large apparatus of Tiselius (1938) had less than 2 % of the activity of a commercial preparation of heparin.

Complex formations between proteins and chondroitin sulphate at reactions more acid than the isoelectric point of the protein have been studied in some detail by Meyer *et al.* (1937), who show that the mucins produced are in fact true salts, and are formed in stoicheiometric proportions. The present demonstration of reversible complex formation at alkaline reactions, however, indicates association of a different type wherein the protein and the polysaccharide are both negatively charged. The character of the bonds involved in a union of the latter type is unknown, but provided the particle size of the components is sufficiently great, polar association is not excluded since, except under very strongly alkaline conditions, the more strongly basic centres of the protein molecule are still charged positively.

It is known that native collagen suffers a loss of ammonia on conversion into gelatin, and there is evidence that in its untreated condition collagen has an isoelectric point higher than that of gelatin prepared from it (cf. Ames, 1944). It is, therefore, probable that conditions in the intact tissue are different from those obtaining in the extracted mucoid. However, since complex formation occurs between chondroitin sulphate and protein in the mucoid at hydrogen-ion concentrations within the physiological range, it seems reasonable to presume that it also occurs in the intact tissue. This assumption provides an answer to a point that has frequently been debated. The early workers, in particular Mörner (1889), held that chondroitin sulphate occurred in the tissue as an alkali metal salt, while Meyer *et al.* (1937) were of the view that the tissue contains a salt between chondroitin sulphate and protein. It now appears that the position lies between the two extremes, and that the strongly acid sulphate groups are most probably held in combination with some of the basic residues of the protein, the net charge of the complex being adjusted mainly by a competition between alkali metal ions and hydrogen ions for carboxylic acid residues in both protein and polysaccharide.

It has not proved possible to secure the liberation of mucoid from the tissue by any treatment sufficiently mild to avoid modifying collagen, and it is therefore clear that the mucoid, particularly as regards its protein moiety, should be looked upon as an artifact. However, the circumstance that chondroitin sulphate retains its capacity of complex formation in the water-soluble system resulting from degradation of the tissue affords some insight into the structure of the tissue itself. If our knowledge

of the chemical behaviour of the polysaccharide is combined with the results of histological investigations the structure may be visualized as a network of collagen fibrils, in some places organized into parallel bundles to form microscopic fibres and in others relatively disorganized and heavily cross-linked by association with chondroitin sulphate. The extraction experiments also suggest that the protein of the mucoid springs from the 'disordered' collagen of the cementing substance rather than from the ordered fibres.

This view of the structure of the tissue is supported by the recent observations of Cohen (1942), who showed that many proteins of plant origin having a molecular weight greater than 10^6 were precipitated by heparin, chondroitin sulphate and hyaluronic acid, and in some cases gave rise to extremely long particles often of paracrystalline form. Large concentrations of the colloidal anion were required to effect the precipitation, but only small quantities of the polysaccharides were carried down by the crystalline precipitates.

These facts invite speculation as to the part played by chondroitin sulphate in the orientation of collagen in developing connective tissue. In tissue cultures and healing wounds the fibres arise from fibroblasts which migrate into the medium or the exudate from the wound, the fibres afterwards becoming oriented into fibre bundles, finally adopting the characteristic form of true collagenous tissue. It may be that the typical structure of collagen in cartilage and connective tissue generally is the result of a process similar in character to the production of the paracrystals observed by Cohen (1942) in which chondroitin sulphate, acting as a multivalent anion, cements together the protein molecules to form fibrous macromolecules and eventually fibre bundles. It is noteworthy that even the most regular white connective tissue, that in tendon, always contains appreciable amounts of mucoid.

SUMMARY

1. The intercellular substance of bovine nasal cartilage is composed almost exclusively of two major components—collagen and chondroitin sulphate.

2. A soluble mucoid may be extracted from dried cartilage powder after a short heat treatment with water at 60–70°.

3. The temperature required to liberate mucoid is reduced in the presence of inorganic salts, alkalis and formamide, and the conditions necessary to secure extraction in good yield are generally those which give rise to thermal contraction of collagen.

4. The mucoid contains chondroitin sulphate and a protein derived from the degradation of collagen. Prepared under the lowest effective temperature conditions, the mucoid behaves in the electrophoresis apparatus of Tiselius as an equilibrium mixture of chondroitin sulphate, protein and a complex formed between the two.

5. The association of chondroitin sulphate with protein in the mucoid occurs over the range pH 5–9 within which both the protein and the acid polysaccharide are negatively charged. The complex formation is of a different type from the salt formation known to occur below pH 4·85.

6. On further heating, the mucoid loses its capacity to form complexes at pH 5–9 and suffers a rapid reduction in viscosity.

7. The part played by such complex formation in the intact tissue is discussed and it is suggested that chondroitin sulphate has an important role in the organization of collagen in developing connective tissue.

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