and mucosa were determined at different sites in the intestinal tract in rats in four nutritional states: (*a*) normal diet of rat cubes; (*b*) normal diet of rat cubes followed by starvation for 24 hr.; (*c*) 'essential fatty acid'-deficiency; (*d*) fat-free diet supplemented by linseed oil.

2. The arachidonic acid and docosahexaenoic acid concentrations were 50 % higher in mucosa than in muscle.

3. Compared with the supplemented animals the 'essential fatty acid'-deficient rats contained 1 % of linoleic acid, 10 % of arachidonic acid, 8 % of docosapentaenoic acid and 20 % of docosahexaenoic acid. They also contained eicosadienoic acid and docosatetraenoic acid, which were not detected in starved or fed rats.

4. Both 'essential fatty acid'-deficient and supplemented rats had 50 % more palmitoleic acid than rats on a normal diet of rat cubes.

5. Essential fatty acids were lost more readily from the colon in deficient animals and replaced to a lesser extent in the supplemented animals.

6. The 'essential fatty acid'-deficient and supplemented animals have 35 % less fat in the intestine than the normal and starved rats.

7. Long-chain aldehydes were also estimated and found to be concentrated in the colon muscle.

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The Neutral Carbohydrate of Bovine Nasal Cartilage

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Besides chondroitin sulphate there exists in cartilage other carbohydrate material that contains hexose and seems to be far less negatively charged. A fraction containing such material was separated by Glegg, Eidinger & Leblond (1954) from an alkaline extract of cartilage by first precipitating the chondroitin sulphate with ethanol (67 %, v/v) and then precipitating the carbohydrate containing the neutral hexose by further addition of ethanol (to 84 %, v/v). So little is known about the hexose-containing carbohydrate that other approaches to its isolation and characterization are desirable. Extraction of bovine nasal cartilage with water in a high-speed homogenizer gives a water-soluble product that contains a large part, but not all, of the chondroitin sulphate of the cartilage in a form combined with protein which was called chondromucoprotein (Malawista & Schubert, 1958). Since

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this product contains little hexose, the hexose-containing carbohydrate seemed to be retained mainly in the cartilage residue left after extraction with water. Such cartilage residue is the starting material used in the present study, which aimed at isolating the fractions containing hexose by methods less drastic, if possible, than alkaline extraction.

EXPERIMENTAL AND RESULTS

The cartilage residue used as the starting material in the present work is the water-insoluble product left after homogenization of whole dried bovine nasal cartilage in water by the use of the VirTis-45 homogenizer, described by Malawista & Schubert (1958). The water-soluble chondromucoprotein separated by this method accounts for nearly a third of the original cartilage weight. The insoluble cartilage residue when dried accounts for the other two-thirds and still contains chondroitin sulphate attached to protein. This cartilage residue can be rendered soluble in water either by treatment with collagenase or by autoclaving with water. From solutions prepared in either way no suitable method for separation of the neutral-carbohydrate fraction was found. However, two methods of rendering the cartilage residue water-soluble were worked out, which are of particular interest because with each a part of its neutral carbohydrate fraction became dialysable, and in each the dialysable carbohydrate was found to be still closely associated with peptide material.

Method 1. Dry cartilage residue (10·0 g.) in water (100 ml.) was autoclaved at 120° for 1 hr. To the solution, which was almost clear, were added $NaHCO_3$ (200 mg.) and crystalline trypsin (125 mg.), and the solution (about pH 7·1) was incubated at 37° for 3 hr. With some toluene added as preservative it was dialysed in a cellophan bag at 0° against three portions of water (1400 ml. each), for 1 day each. The combined diffusate (over 4 l.) was evaporated *in vacuo* to small volume, acetic acid (0·5 ml.) was added and evaporation continued to dryness, ethanol and benzene finally being used to remove all water. The residue, weight 2·1 g., is called fraction III.

The solution remaining in the bag contained a considerable amount of chondroitin sulphate that had not been removed in the initial extraction of the whole cartilage. This chondroitin sulphate was precipitated by the addition of hexamminecobaltic chloride (1·2 g.) and the flocculent precipitate was removed by centrifuging. It was washed with water, ethanol and ether and dried *in vacuo*. It is called fraction I and amounts to 2·6 g.

The supernatant solution left after precipitation of fraction I was stirred with Dowex 50 (potassium form) resin to remove the excess of hexamminecobaltic cations and the solution was then evaporated *in vacuo* to small volume (5 ml.). The non-dialysable fraction containing the neutral carbohydrate was precipitated by the addition of ethanol (250 ml.), and the flocculent precipitate was centrifuged, washed with ethanol and ether and dried *in vacuo*. Its weight was 4·0 g.; it is called fraction II.

In this method for the solubilization of the cartilage residue there is one step that might be considered rather drastic, namely the autoclaving. Since part of the neutral carbohydrate was found to be dialysable it became important to avoid this step, since it seemed possible that the dialysable fraction was a result of a degradation occurring then. To this end an enzymic method of solubilizing the cartilage residue was used.

Method 2. Dry cartilage residue (10·0 g.) in aqueous H_2SO_4 (85 mN; 100 ml.) gave a mixture with pH 2·6. To this was added crystalline pepsin (125 mg.) and the mixture was incubated at 37°, with stirring, for 2·5 hr. In this step most of the cartilage becomes soluble and susceptible to the action of trypsin. To the mixture was added $NaHCO_3$ (1·5 g.), bringing the pH to about 7, and crystalline trypsin (125 mg.). The mixture was again incubated with stirring at 40° for 2·5 hr. At the end of this time the mixture had become an almost clear solution and was then treated exactly as the solution obtained by method 1 after the trypsin treatment. As in that method, three fractions were obtained, in the following yields: I, 2·5 g.; II, 3·7 g.; III, 3·6 g. The yields of fractions I and II are almost the same by the two methods. Fraction III appears to be much larger by method 2 (3·6 g.) than by method 1 (2·1 g.) but this is only because all the salts collect in fraction III. By method 1 these amount to 0·2 g.; and by method 2 they amount to 1·4 g., so the corrected yields of fraction III are: by method 1, 1·9 g.; by method 2, 2·2 g. If the enzymes are in fraction II, they will amount to 10 mg. in method 1 and 20 mg. in method 2, or 2 and 5% of the yield of fraction II. The figures are not corrected for these enzymes; the amount of estimated enzyme protein that escapes self-digestion is a calculated figure.

Another experiment was performed in which 10 g. of dry cartilage residue was placed in aqueous H_2SO_4 (85 mN; 100 ml.), which gave a mixture of pH 2·6. Crystalline pepsin (125 mg.) was added, and the mixture was incubated at 37°, with stirring, for 2·5 hr. Most of the cartilage became soluble and was dialysed against distilled water as in methods 1 and 2. No organic material diffused through the bag.

Analytical data on each of the fractions made by both methods 1 and 2 are summarized in Table 1. Hexosamine was determined by the Schloss (1951) modification of the Elson–Morgan method, nitrogen by the Kjeldahl method, protein by a biuret method with serum albumin as standard (Lowry, Rosebrough, Farr & Randall, 1951), neutral sugars by an anthrone method with galactose as standard (Trevelyan & Harrison, 1952), neuraminic acid by the method of Warren (1959) and hydroxyproline by the method of Hutterer & Singer (1960). For fraction I galactosamine was used as the standard; for fractions II and III glucosamine was used as the standard. Uronic acid was determined by the carbazole method of Dische (1947). This method is subject to high error in the presence of large amounts of protein. Therefore no importance is attached to low absolute values of uronic acid (as 0·2 in fraction II and 0·3 in fraction III).

Fraction I was first isolated as hexamminecobaltic chondroitin sulphate. For identification it was converted into a barium salt by dissolving a sample (1·3 g.) in KCl solution (0·15 M; 130 ml.) and treating twice with Dowex 50 (1·2 g.) in the potassium form. The yellow colour of the solution was completely removed. Barium chloride (1·2 g.) was added to the solution and the chondroitin sulphate was precipitated as its barium salt by addition of ethanol (120 ml.). The yield was 0·9 g. and analytical data are included in Table 1.

Table 1. *Analytical data for fractions from cartilage residue treated by method* 1 (*heat and trypsin*) *or method* 2 (*pepsin and trypsin*)

See Experimental and Results section for details of the fractionation and the significance of the numbers I, II and III. Results for fraction III have been corrected for the salt content. Those for fraction I are for the barium salt of the material. Figures for fraction II have not been corrected for enzyme content. —, Not determined.

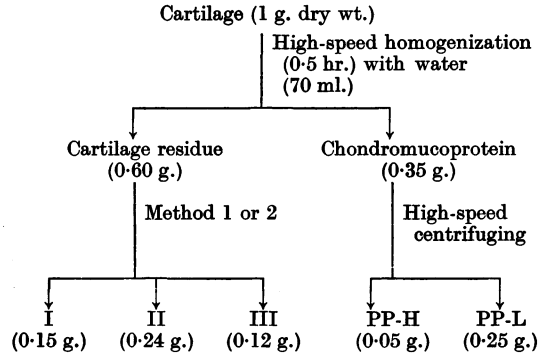
	Fraction I		Fraction II		Fraction III	
Method	1	2	1	2	1	2
Yield (g./g. of cartilage residue)	0·261	0·250	0·401	0·372	0·188	0·220
N (%)	2·3	2·4	12·5	12·2	13·4	17·7
Hexosamine (%)	28·0	29·0	1·8	2·9	2·9	3·0
Reducing sugar (%)	—	—	10·4	10·2	3·4	3·6
Hydroxyproline (%)	0·0	0·0	6·8	6·7	4·1	8·1
Hexuronic acid (%)	—	22·2	0·2	0·2	0·3	0·3
Neuraminic acid (%)	—	—	0·9	1·2	0·2	0·2
Protein (%)	—	—	70·0	70·0	58·0	68·0
$[\alpha]_D$	—	−24·7°	—	—	—	—
Periodate reduced:						
m-moles/100 mg. of sample	—	0·02	—	0·34	—	0·28
m-moles/100 mg. of carbohydrate in sample	—	0·02	—	2·36	—	4·15
Wt. of sample (mg.) assumed to contain 100 mg. of carbohydrate		100		700		1500

The presence of material in cartilage giving a positive reaction after the application of periodate–Schiff staining technique has often been observed in histochemical studies of aging and pathological cartilage. That this material is not chondroitin sulphate has seemed probable because of the very slow reduction of periodate by chondroitin sulphate and the short time (10 min.) of exposure to periodate used in the histochemical test. In this connexion a comparison of amounts of periodate reduced by each of the three fractions isolated in the present work seemed of interest.

The weighed sample of each fraction was dissolved in 5 ml. of acetate buffer (0·1 M, pH 4·5) and at zero time 5 ml. of KIO_4 solution (0·10 M) was added. An appropriate blank consisting of the acetate buffer and KIO_4 solution was also prepared. Samples (2 ml.) were withdrawn at various times and to each sample was added KI solution (1 ml.; 20%, w/v) and concentrated HCl (1 ml.). The iodine liberated in each sample was then titrated with standardized thiosulphate solution (0·05 M). Fraction I, as expected for chondroitin sulphate, showed a slow and progressive utilization of periodate (Einbinder & Schubert, 1951), which did not reach a plateau in 20 hr. Fractions II and III showed a rapid utilization of periodate, which reached a plateau in 1 hr. and did not change in the next 20 hr. Figures in Table 1 are given for the amount of periodate consumed in 1 hr. They are calculated in two ways: as m-moles of periodate consumed for 100 mg. of sample, and as m-moles of periodate consumed for a weight of sample containing 100 mg. of total carbohydrate. For the latter calculation fraction II is considered to contain 14%, and fraction III to contain 7%, of carbohydrate.

DISCUSSION

One point of interest is that the cartilage residue still yields 25% of its weight as chondroitin sulphate, or 15% of the weight of the original whole cartilage. This amount is the same by both methods used. Optical rotation of this chondroitin sulphate is the same as that of the fractions previously isolated as PP-H and PP-L from the chondromucoprotein (Gerber, Franklin & Schubert, 1960), and so it appears to be chondroitin sulphate A. That so large a fraction of the chondroitin sulphate of whole cartilage resists extraction by high-speed homogenization in a large volume of water and is isolated after complete destruction of the integrity of the collagen fibres by either of the two methods described in the present work must be of some significance for the structure of the native cartilage as a tissue. The situation is shown in a diagram (Scheme 1), which summarizes yields obtainable from 1 g. of dry bovine nasal cartilage. This shows that the total amount of protein compounds containing chondroitin sulphate in the original carti-



Scheme 1

lage is about 50 % of the cartilage dry weight. This is in agreement with the weights of products extracted from cartilage by long homogenization time and large amounts of water (Malawista & Schubert, 1958), though such products contain larger amounts of collagen.

The second main point is that, with treatment by either method, 20 % of the weight of the cartilage residue has been rendered dialysable (fraction III), and this fraction contains about 15 % of the hexosamine of the cartilage residue. This indicates that in the cartilage residue as well as in the original cartilage the neutral carbohydrate exists in part at least as oligosaccharides attached to an insoluble component of the matrix. The essential step in generating the dialysable fragments that contain these oligosaccharides is the treatment with trypsin. The autoclaving procedure (the first step of method 1) renders the cartilage residue soluble, but no detectable dialysable products are formed. Similarly, treatment of the cartilage residue with pepsin (the first step of method 2) gives an essentially clear solution, but again no dialysable products are formed. Only after treatment of the clear solution produced by the first step of either method 1 or 2 with trypsin does dialysable material containing oligosaccharide appear. Treatment of the original cartilage residue directly with trypsin does not render it soluble, and produces no dialysable products. It seems likely that formation of the dialysable oligosaccharides is not an immediate result of the more drastic steps of method 1 (autoclaving) or of method 2 (low pH).

A final point of interest is the behaviour of the three fractions with respect to periodate consumption. When compared on the basis of equal weights of sample, II and III reduce over ten times the amount of periodate that is reduced by I (chondroitin sulphate). When it is further considered that II and III contain only about 14 and 7 % respectively of carbohydrate, and the periodate consumed is calculated per 100 mg. of carbohydrate, then II and III consume over a hundred times the periodate consumed by I.

SUMMARY

1. The residue of bovine nasal cartilage left after extraction of the compounds of chondroitin sulphate and protein (earlier called chondromucoprotein) still contains about 30 % of the hexosamine of the original cartilage.

2. This cartilage residue has been brought into solution by two methods which seem not to have affected the integrity of the carbohydrate components.

3. A fractionation of the material in this solution has yielded three fractions: I, 25 % of the cartilage residue weight as crude chondroitin sulphate; II, 38 % as non-dialysable products other than chondroitin sulphate; III, 20 % as dialysable products. These three fractions contain respectively 60, 20 and 15 % of the hexosamine of the cartilage residue.

4. A comparison of the amounts of periodate reduced by each of these three fractions shows that II and III reduce ten or a hundred times as much periodate as I, depending on whether equal sample weights are used or samples containing equal weights of carbohydrate.

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