Some Studies on Mucosubstances of Bovine Cortical Bone

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The presence in cortical bone of mucosubstances containing neutral sugars such as galactose, mannose, fucose and hexosamines has been recognized for some time (see review by Eastoe, 1956). More recent studies have shown that the carbohydrate-containing material from alkaline extracts may be separated into several different fractions (Glegg & Eidinger, 1955; Dische, Danilczenko & Zelmenis, 1958). Carbohydrates have also been shown to be present in the residue after water-soluble substances and collagen have been removed (King & Boyce, 1959). Lately the presence of sialic acid has also been noted, particularly in gelatins derived from bone (Courts, 1959; Leach, 1960) and also in untreated bone (Courts, 1960).

The conditions used in previous investigations, however, may be expected to lead to extensive degradation of organic constituents, particularly mucoproteins, and few attempts have been made to establish the homogeneity of isolated material. The present work, therefore, is concerned with the extraction and separation of such substances under mild conditions, and with the characterization of the isolated constituents. Some of these results have already been reported in brief (Herring & Kent, 1961; Kent & Herring, 1962).

MATERIALS AND METHODS

Bones. Long bones, which had been stored in a cold room for not more than a week, were obtained from cattle aged about 3 years. Epiphyseal and metaphyseal bone was removed with a band-saw, and the cortical bone, after removal of the marrow and extraneous tissue, was cut into pieces measuring 5–8 cm. × 1·5–2·5 cm. These were stored in ethanol at 4° until required.

Preparation of powdered bone. The bone was ground to a fine powder by using a milling machine (Jowsey, 1955; Sheldon-Peters & Vaughan, 1956) with xylene as a cooling fluid which kept the temperature below 25°. After collection, the bone powder was washed with xylene, ethanol and ether, dried at room temperature and stored at 4°.

Extraction. A solution (pH 7·9) of the tri- and tetra-sodium salts of EDTA was prepared by dissolving the di-sodium salt of EDTA (400 g.) and sodium hydroxide (50 g.) in water (1 l.). The bone powder (250 g.) was stirred with the EDTA solution (1 l.) at 4° for 48 hr. The mixture was centrifuged (3000 rev./min.) at 5° for 30 min. and the supernatant extract removed. The extraction procedure was repeated four times and the insoluble decalcified bone powder washed free of EDTA with water. The extracts and washings were dialysed in cellophan against running tap water for 48 hr. and adjusted to pH 4·5–5·0 with acetic acid. The precipitate (fraction I) was collected by centrifuging, washed with 0·1% acetic acid and retained for reprecipitation. To the supernatant was added 2 vol. of ethanol containing 1% (w/v) of potassium acetate and 1% (v/v) of acetic acid (referred to below as 'buffered ethanol'). After at least 24 hr. at 4°, the precipitate (fraction II) was collected by centrifuging and further buffered ethanol was added to the supernatant to bring the final concentration of ethanol to 85% (v/v). A precipitate (fraction III) formed slowly on standing at 4° and after at least 48 hr. was collected by centrifuging. Each of the fractions (I, II and III) was shaken with EDTA solution and the mixture centrifuged at 105 400 *g* for 30 min. at 5°. The clear supernatants were dialysed in cellophan against distilled water (100 vol., four times) and the fractions reprecipitated according to the above scheme. The three fractions were finally reprecipitated under the same conditions after redissolving in 0·1 M-phosphate buffer, pH 8·0. About 30% of fraction I initially obtained was insoluble when re-extracted with EDTA solution and was also insoluble in 0·1 M-phosphate buffer, pH 8·0. This is referred to as 'fraction *R*'. The precipitates were washed and dried with ethanol and ether.

Moving-boundary electrophoresis. Electrophoresis was carried out at 4° in a Hilger Tiselius electrophoresis apparatus by using 0·1 M-sodium diethylbarbiturate–HCl buffer, pH 8, or 0·1 M-phosphate containing NaCl (0·1 M), pH 7 or 8. A current of 7–10 mA was used and photographs were taken of the schlieren pattern every 10 or 20 min. An estimation of the relative proportions of the electrophoretic components was made by determining the area under each peak in the schlieren pattern.

Isolation of one component was carried out by using a cell of 11·5 ml. capacity with 0·1 M-diethylbarbiturate buffer, pH 8·0. By compensation over a period of 6 hr., the slowest-moving component (I*A*) of fraction I was retained in the top cell of the descending limb. After removal from the cell compartment, the solution was filtered through fine sintered glass, dialysed against distilled water and finally precipitated with 2 vol. of ethanol. The precipitate was collected, after 24 hr. at 4°, and washed and dried with ethanol and ether. Seven separations of fraction I*A* were carried out in this way, giving a total yield of 65 mg.

Analysis by paper electrophoresis. The apparatus was the type with free horizontal strips, as described by White, Beaven & Ellis (1956). Strips of Whatman no. 1 filter paper (11 cm. × 38 cm.) were soaked in buffer, blotted and allowed to equilibrate in the apparatus for about 1 hr. A solution of the substance being examined was prepared in a Wintrobe haematocrit tube which was centrifuged at 2500 rev./min. for 30 min. and the clear supernatant applied as a spot

0·5–1·0 cm. in diameter by using a capillary tube; three spots about 3 cm. apart were applied to each paper. A voltage of 70–150 v across the electrodes was used, giving a current of 2–4 ma per strip, and adequate migration of the components was obtained after 16–26 hr. After drying the strips at room temperature or for 20 min. at 110°, separate strips were stained with the following: (*a*) light green (Smith, 1960); (*b*) toluidine blue (Muir, 1958) or alcian blue (Heremans, Vaerman & Heremans, 1959); (*c*) periodic acid–Schiff staining technique (Laurell, 1956).

Separation of proteins by using an ion-exchange resin. A carboxylic cation-exchange resin [Amberlite CG-50 (chromatographic grade; type II; 200 mesh)] was used to remove protein from more acidic substances. In some experiments adsorption of protein was carried out by the addition of resin to a solution of the fraction at pH 5 by the method of Partridge & Davis (1958); in other experiments the resin was used in the form of a column. In the latter case the procedure developed by Boardman & Partridge (1955) was employed. For analytical experiments columns (20 cm. × 1 cm.) of purified (Partridge & Davis, 1958) resin were prepared and washed overnight with 2 l. of 0·2 M-sodium phosphate buffer, pH 5·2. Before addition of the protein mixture the level of the washing buffer was allowed to descend to 1 mm. from the surface of the resin, the sample (20 mg.) dissolved in 2 ml. of the phosphate buffer, pH 5·2, then being added. This was followed by a washing with 20 ml. of the buffer. The column tube was then filled with buffer and the resin eluted with 50 ml., after which the buffer was changed to 0·2 M-sodium phosphate buffer of higher pH at a flow rate of 40 ml./hr. Fractions (10 ml.) were collected.

The amount of protein in eluted fractions was determined colorimetrically by the modified biuret method of Lowry, Rosebrough, Farr & Randall (1951), gelatin (British Drug Houses Ltd.) being used as the standard. The elution was also followed by measurement of ultraviolet absorption at 240 mμ which, though less specific, gave about twice the sensitivity compared with the absorption at 280 mμ; none of the eluted fractions possessed an ultraviolet-absorption maximum at 280 mμ. Carbohydrate was determined in alternate tubes by the anthrone method of Yemm & Willis (1954), with galactose as a standard. The amount of protein in a sample of the solution applied to the column was determined so that the recovery of protein could be calculated. The fractions corresponding to the individual peaks in the elution pattern were pooled and concentrated by dialysis against polyethylene glycol (Carbowax 20M; made by Union Carbide Ltd.) by the method described by Kohn (1959). The pooled eluates were placed in dialysis tubes which were laid in conical flasks and sprinkled with Carbowax (10 g. to 100 ml. of solution). These were left for 16–20 hr. at 4° to achieve a concentration of 50–100-fold. The dialysis tubes were removed, rinsed with distilled water and the contents with washings (0·1 M-sodium phosphate buffer, pH 8·0) filtered through sintered glass. The solutions were dialysed against several changes of distilled water and the fractions precipitated with 4 or more vol. of ethanol containing potassium acetate (1%, w/v).

On a preparative scale, 60–80 mg. of a fraction was dissolved in 5 ml. of 0·2 M-sodium phosphate buffer, pH 5·2, and applied to a column (2 cm. × 15 cm.). Since there appeared to be a satisfactory correlation between ultraviolet absorption and protein content, the elution of peaks from the column was followed by measuring the absorption at 240 mμ for each tube. The concentration and precipitation of the eluted fractions was carried out as described above.

Separation of acidic substances with cetylpyridinium chloride. The substances which were eluted from Amberlite CG-50 at pH 5·2 were precipitated by cetylpyridinium chloride in the presence of Celite as described by Korn (1959). Each precipitate was collected on a sintered-glass filter, washed with distilled water (50 ml.) and treated successively with 0·3 M-, 1·2 M- and 2·1 M-sodium chloride (50 ml. of each) to release, respectively, hyaluronic acid, chondroitin sulphates and heparin (Schiller & Dorfman, 1960). An excess of 1 M-potassium thiocyanate was added to each extract to precipitate cetylpyridinium ions as the insoluble thiocyanate. After filtration through Celite, the solutions were dialysed and concentrated, and buffered ethanol (5 vol.) was added. The mixtures were kept for 24 hr. at 4°, and the precipitates were then collected by centrifuging. Each fraction was redissolved in distilled water (5–10 ml.), and the precipitation with cetylpyridinium chloride and extraction procedure were repeated. The fractions were finally reprecipitated by dissolving in 4 M-potassium acetate, filtering through fine sintered glass and precipitating with 5 vol. of ethanol.

Hydrolysis for examination of monosaccharides. Except where otherwise stated, hydrolysis was carried out with a cation-exchange resin in the acid form by the method of Glegg, Eidinger & Leblond (1954), who used it for general hydrolysis of extracted polysaccharide material before identification of the monosaccharides by paper chromatography. We found this procedure convenient in this respect, particularly as there was usually insufficient material for the determination of the optimum hydrolysis conditions, and because it facilitated the isolation of the amino sugars from the hydrolysate. The fraction (5–20 mg.) was heated with 20 times its weight of resin [Zeo-Karb 225 (H^+ form; 2% cross-linking; < 200 mesh)] and 2 ml. of distilled water in a sealed tube at 100° for 48 hr. The hydrolysates were separated from the resin by filtration through sintered glass and amino sugars were eluted from the resin with 6 ml. of 0·5 N-HCl. When known amounts of sugars were treated with resin under the same conditions, recoveries were 96–97% for galactose, 92–93% for fucose and 93–95% for glucosamine.

Removal of salts from hydrolysates. The method of Hughes & Whelan (1958) was used with slight modifications. A mixture of 250 mg. of activated carbon (grade Ultrasorb S.C. 120/240; British Carbo-Norit Union Ltd.) and 250 mg. of Celite 535 was sandwiched between two layers 2–3 mm. thick of Celite 535 in 10 cm. × 1 cm. microfilters, each layer being well tamped down after being moistened. Each column was washed with 40 ml. of distilled water and the hydrolysates were applied with a Pasteur pipette. The solutions were allowed to drip through the columns under gravity and salts were eluted with 8 ml. of washings. Monosaccharides were eluted with 10 ml. of aq. 5% (v/v) ethanol and these eluates were evaporated to dryness under reduced pressure in the presence of phosphorus pentoxide. The recovery of 300 μg. samples of galactose under the above conditions was 96–97%.

Paper chromatography of monosaccharides. The dry salt-free hydrolysates were dissolved in sufficient distilled water to give about 30 μg. of monosaccharides/10 μl. and this

quantity was applied on as small a spot as possible on strips of Whatman no. 1 filter paper that had been previously washed with 10% (v/v) acetic acid followed by distilled water and then dried. The best separation of the monosaccharides was obtained with a water-saturated mixture of butan-1-ol and pyridine (4:1, v/v) (Chargaff, Levine & Green, 1948), chromatograms being developed three times successively with ascending elution. Other solvents used were the organic phase of ethyl acetate–pyridine–water (5:2:7, by vol.) (McFarren, Brand & Rutkowski, 1951) and butan-1-ol–ethanol–water (4:1:1, by vol.) (Stoffyn & Jeanloz, 1954). Reducing substances were identified by using alkaline silver nitrate (Trevelyan, Procter & Harrison, 1950), the ethanolic sodium hydroxide being used as either a spray or a bath (Sharon & Jeanloz, 1960). After the colour had fully developed, the chromatograms were rinsed with distilled water and placed in a bath of 20% (w/v) sodium thiosulphate for 20 min. This was followed by washing with running tap water for 1 hr. and the chromatograms were dried at 100°.

The sialic acid liberated by hydrolysis with 0·01 N-H_2SO_4 at 80° for 1 hr. was examined by paper chromatography by using two different solvents: butan-1-ol–propan-1-ol–0·1 N-HCl (1:2:1, by vol.) and butan-1-ol–pyridine–water 6:4:3, by vol.) (Gottschalk, 1960). After being dried, the chromatograms were sprayed with a thiobarbituric acid reagent (Warren, 1960).

Analytical methods. Samples for analysis were dried to constant weight *in vacuo* over phosphorus pentoxide at room temperature. Nitrogen, ash and sulphur determinations were carried out by A. Bernhardt, Mikroanalytisches Laboratorium in Max-Planck Institut für Kohlenforschung, Mülheim.

Colorimetric and ultraviolet-absorption determinations were carried out with the Unicam SP. 500 spectrophotometer (quartz cells), and pH measurements with the Cambridge pH-meter.

Determination of hexose and methylpentose. The anthrone method (Yemm & Willis, 1954) was used as a semi-quantitative estimation of 'carbohydrate' (i.e. hexose plus methylpentose). By using a 5 min. heating period the ratios of the extinction coefficients to that of glucose were 0·77, 0·74 and 1·01 for galactose, mannose and fucose respectively. Under these conditions, glucosamine gave no detectable reaction, and glucuronic acid and ribose gave 4% and 22% respectively of the absorption obtained with galactose.

Hexose was determined by the Dische 'primary' cysteine reaction (Dische, Shettles & Osnos, 1949), and methylpentose by the method of Dische & Shettles (1948).

Amino sugar. The method described by Kraan & Muir (1957) was used to determine hexosamine after resin or acid hydrolysis. The following modification (Johnston, Ogston & Stanier, 1951) was incorporated: before addition of the Ehlich's reagent, the tubes were allowed to equilibrate at 37°, and after the addition kept at this temperature for 1 hr. before measurement of the extinction at 535 mμ. Glucosamine was used as the standard.

Glucosamine and galactosamine were separated and determined by the method of Gardell (1953). A column (0·6 cm. × 37 cm.) of Dowex 50 (WX 8; 250–400 mesh) was employed with a flow rate of 2 ml./hr.

Uronic acid. The carbazole method (Dische, 1947) was used to determine the uronic acid content of unhydrolysed samples, with glucuronic acid as a standard.

Sialic acid. Sialic acid was determined by the thiobarbituric acid method of Warren (1959) on samples hydrolysed with 0·1 N-H_2SO_4 at 80° for 1 hr., with *N*-acetylneuraminic acid as the standard.

Hydroxyproline. The method of Neuman & Logan (1950) as modified by Hutterer & Singer (1960) was employed to measure the hydroxyproline liberated by 6 N-HCl at 100° for 24 hr.

Periodic acid–Schiff reaction. The spot-test method of Hotchkiss (1948) was used to demonstrate qualitatively the periodic acid–Schiff reactivity of the fractions.

Sulphate. Sulphate determinations were made by the method of Belcher, Bhasin, Shah & West (1958).

Amino acids. The separation and determination of amino acids was carried out by the method of Moore & Stein (1951).

RESULTS

The analyses of the four fractions obtained from the EDTA extracts are shown in Table 1. Fraction *R*, which contained galactose, glucose, mannose and fucose, was insoluble in 0·1 M-phosphate buffers, pH 7·0 and 8·0, and in 0·2 M-acetate buffer, pH 5·0, so it was not possible to carry out electrophoretic studies on it. The residual bone powder after extraction was fully demineralized (the calcium content was 0·14%) and contained 17·2% of N, 10·5% of hydroxyproline and 0·6% of carbohydrate.

Electrophoretic studies. Moving-boundary electrophoresis in 0·1 M-diethylbarbiturate buffer, pH 8·0, and paper electrophoresis with 0·1 M-phosphate

Table 1. *Analysis of fractions from ethylenediaminetetra-acetate extracts of powdered bone*

Experimental details and description of the fractions are given in the text. The yield is expressed as weight of dry fraction extracted from 250 g. of bone powder. Nitrogen, sulphur and carbohydrate values are given for the dry ash-free fraction.

Fraction	Yield (g.)	Yield (% of bone)	N (%)	S (%)	Carbohydrate* (%)	Uronic acid (%)	Ash	Periodic acid–Schiff spot test
R	1·649	0·710	17·3	0·9	0·9	—	0·7	+
I	1·181	0·508	14·1	2·7	2·3	2·3	0·7	+ + +
II	0·304	0·131	13·3	2·1	4·5	1·0	14·4	+ + + +
III	0·243	0·105	13·7	0·9	6·1	—	9·3	+ + + +

* Equal parts of galactose, mannose, glucose as standard.

buffer, pH 8·0, showed the presence of four main components which are here designated *A*, *B*, *C* and *D* in order of increasing mobility (Fig. 1). The approximate amounts of the four components present in each fraction were calculated from the area in the moving-boundary schlieren patterns (Table 2).

The staining reactions of these components after paper electrophoresis gave some indication of the type of substance present. These were as follows: (1) The *D* components stained with alcian blue and metachromatically with toluidine blue. (2) The *C* components stained with toluidine blue, alcian blue and periodic acid–Schiff reagents, and very faintly with light green. (3) The *B* components stained with light green and periodic acid–Schiff reagents, and very faintly with toluidine blue. (4) The *A* components stained with light green, and very faintly with periodic acid–Schiff reagents and toluidine blue.

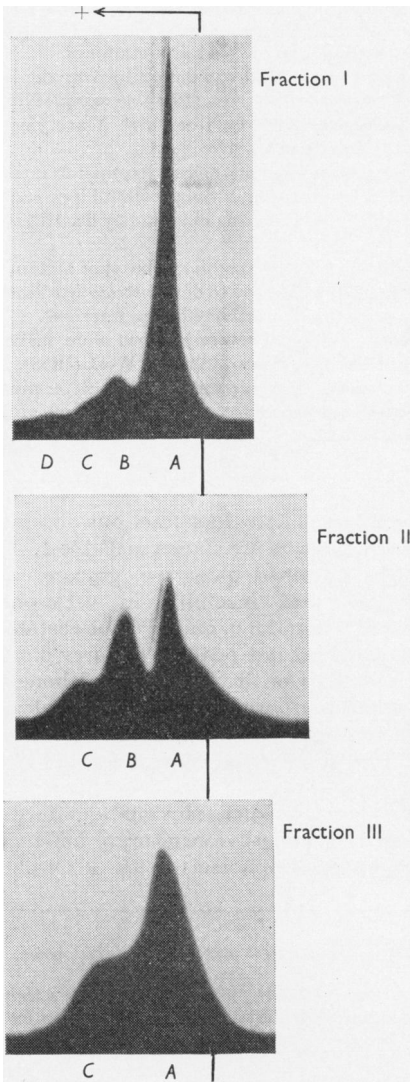


Fig. 1. Moving-boundary electrophoresis (ascending limb) of the EDTA-extracted fractions (I, II and III) in 0·1 M-sodium diethylbarbiturate–HCl buffer, pH 8·0. Experimental details and description of the fractions are given in the text. Fraction I: after 45 min.; fractions II and III: after 40 min.

Electrophoretic separation of an A *component.* Details of the procedure used are described in the Materials and Methods section. The isolated fraction I*A*, when re-examined by moving-boundary electrophoresis under the same conditions used for separation, was shown to consist of a single component within the limits of detection by this method. This was also confirmed by paper electrophoresis in 0·1 M-phosphate buffer, pH 8·0. The composition of this fraction (I*A*) is shown in Table 3.

Isolation of the B *component of fraction I* (*Scheme* 1). A sample of fraction I (460 mg.) was shaken for 1 hr. with 50 ml. of 0·2 M-potassium acetate buffer, pH 5·0. The mixture was centrifuged at 3000 rev./min. and the supernatant removed. This procedure was repeated twice on the insoluble residue with 25 ml. of buffer. To the combined supernatants from these extractions 2 vol. of ethanol was added and the mixture kept at 4° for 24 hr. A precipitate formed which was collected by centrifuging. This and the insoluble residue were washed and dried with ethanol and ether. The acetate-soluble fraction (291 mg.; 63 % of fraction I) and the insoluble residue (163 mg.; 35 % of fraction I) were examined by paper electrophoresis which showed that the former contained most of the *A*, *C* and *D* components, whereas the latter showed a marked concentration of the *B* component. To purify the acetate-insoluble fraction further it was dissolved in 0·1 M-phosphate buffer, pH 8·0, and dialysed in cellophan against 0·1 M-acetate buffer, pH 5·0.

Table 2. *Percentage of each electrophoretic component in fractions I, II and III*

Experimental details and description of the fractions and components are given in the text.

	Component			
Fraction	*A* (slowest) (%)	*B* (%)	*C* (%)	*D* (fastest) (%)
I	76	13	7	4
II	41	41	18	
III	72	—	28	—

A fine precipitate formed which, after 24 hr. at 4°, was collected by centrifuging (14 830 *g*) at 5°, and then was redissolved in the same phosphate buffer, pH 8·0, and was reprecipitated by dialysis against acetate buffer, pH 5·0. The resulting amber-coloured viscous product was washed with 0·1 M-acetate buffer, pH 5·0, and finally washed and dried with ethanol and ether (yield: 42 mg.; 9 % of fraction I). Examination by paper electrophoresis showed a single component *B* staining both with light green and in the periodic acid–Schiff reaction. The composition of this fraction (I*B*) is shown in Table 3.

The material that remained soluble after dialysis against acetate buffer, pH 5·0, was precipitated with ethanol (2 vol.) and weighed (yield: 107 mg.; 23 % of fraction I). On paper electrophoresis this appeared to consist of *A* and *B* components.

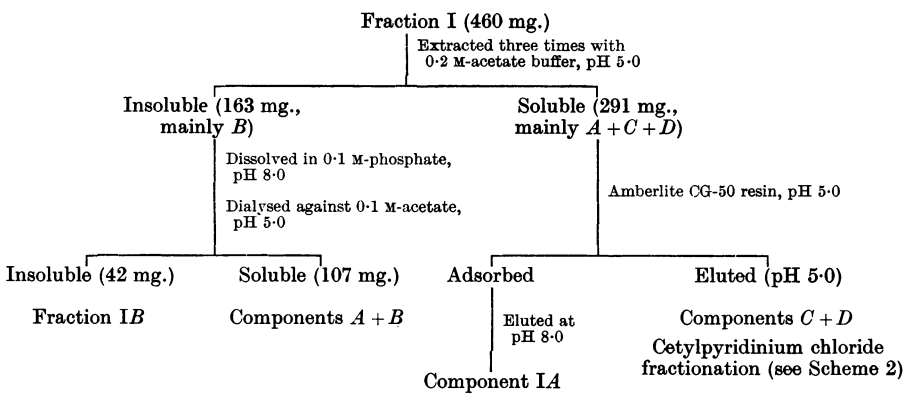
Fractionation with a cation-exchange resin. Amberlite CG-50 cation-exchange resin at pH 5·0 was found to adsorb the *A* and *B* protein components in 0·2 M-acetate buffer, pH 5·0, or in 0·2 M-phosphate buffer, pH 5·2, of fractions II, III and the acetate-soluble material of fraction I. A typical separation is shown in Fig. 2 with a solution of fraction II in 0·2 M-phosphate buffer, pH 5·2. The eluate at this pH (peaks 1 plus 2) indicated the presence of protein, hexoses and uronic acid, and examination by paper electrophoresis showed that only the *C* and *D* components were eluted under these conditions. These were precipitated from peaks 1 plus 2, after concentration, by treatment with buffered ethanol (5 vol.), and were subsequently separated by fractionation with cetylpyridinium chloride (Scheme 2).

Separation of A *and* B *components.* These proteins that were adsorbed on the resin at pH 5·2 could be eluted together by using 0·2 M-phosphate buffer, pH 8·0. If, however, elution commenced with 0·2 M-phosphate buffer, pH 6·0, a substantial separation of the *B* protein (peak 3) from the *A* protein (peak 4) could be effected (Fig. 2). Fraction III differed slightly from fraction II in its elution behaviour, the peak material eluted by the pH 6·0 buffer having the properties of an *A* protein.

The composition of the *A* and *B* proteins, isolated by the various methods described above, is shown in Table 3.

Cetylpyridinium chloride precipitation and fractionation. The material from fractions I and II which had been eluted from Amberlite CG-50 resin at pH 5·2 consisted, as far as could be ascertained by paper electrophoresis, entirely of the *C* and *D* components. After a further treatment with Amberlite CG-50, this fraction (138 mg.) was dissolved in distilled water (10 ml.) and dialysed in cellophan against distilled water (2 l.; four times) for 48 hr. The solution was then treated with excess of 2 % (w/v) cetylpyridinium chloride with sufficient Celite to adsorb all the precipitate formed. The precipitate was collected on a sintered-glass funnel and elution of mucopolysaccharides carried out as described in the Materials and Methods section.

Three fractions were obtained (Scheme 2). The first (fraction C_s), presumably not precipitated by cetylpyridinium chloride under these conditions, was removed from the Celite by the initial washing with distilled water. The second (fraction *C*) was eluted with 0·3 M-sodium chloride, and the third (fraction *D*) eluted with 1·2 M-sodium chloride. Fraction *C* required two further reprecipitations before no trace of the *D* component could be detected after paper electrophoresis. The separated *C* and *D* fractions moved as single components on electrophoresis in 0·1 M-phosphate buffer, pH 8·0, and in 0·1 M-acetate buffer, pH 5·0. The *D* fraction had a mobility slightly less than that of a sample of chondroitin sulphate; the C_s fraction moved slightly slower than the *C* fraction. The final yields



Scheme 1. Separation of the components of fraction I.

for the three fractions were: C_s, 8·6 mg.; C, 27·4 mg.; D, 61·7 mg. Their compositions are shown in Table 4.

Ultracentrifugal analysis of fraction C. Sedimentation properties were investigated (by Dr A. R. Peacocke) in a Spinco model E analytical ultracentrifuge with a solution of fraction C at a concentration of 0·8 mg./ml. in 0·2 M-sodium chloride. The component moved as a single boundary and had a molecular weight of about 30 000.

Examination of fraction C *by moving-boundary electrophoresis.* Fraction C behaved as a single homogeneous component when electrophoresis was carried out in 0·1 M-acetate buffer, pH 5·0, in 0·1 M-diethylbarbiturate buffer, pH 7·0, and in 0·12 M-diethylbarbiturate buffer, pH 8·6.

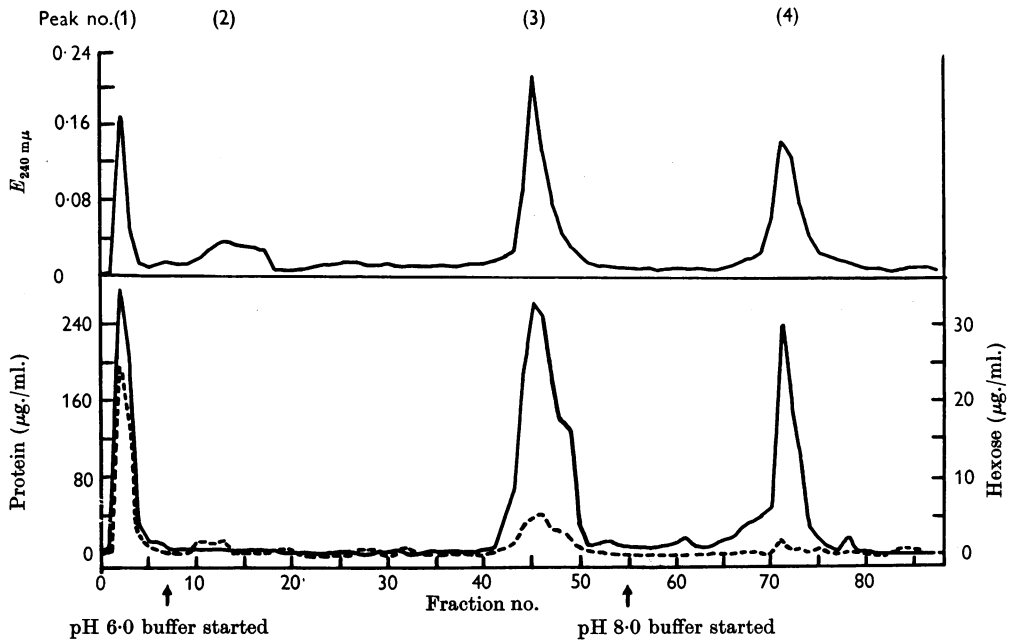


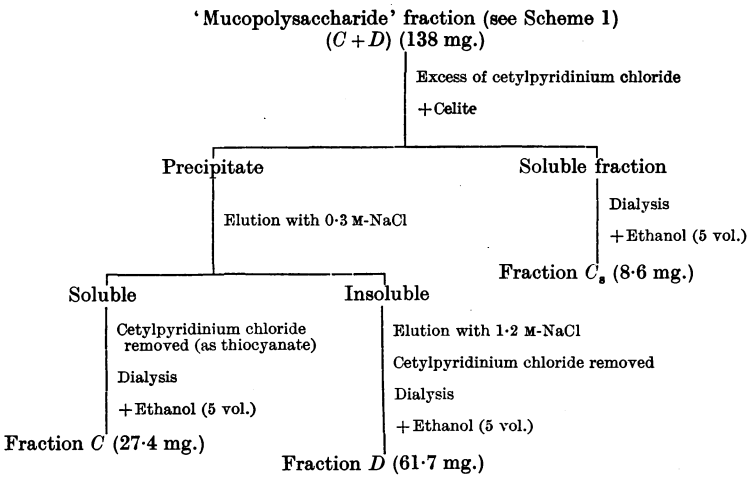
Fig. 2. Separation on Amberlite CG-50 of extracted substances (fraction II) applied in 0·2 M-sodium phosphate buffer, pH 5·2, and eluted with 0·2 M-sodium phosphate buffers at pH 6·0 and pH 8·0 (changes being made at the points indicated). Experimental details and description of the fraction are given in the text. Continuous line (top) represents extinction at 240 mμ; continuous line (bottom) represents protein (μg./ml. by left-hand axis); broken line represents hexose (μg./ml. by right-hand axis).

Table 3. *Composition of isolated fractions moving electrophoretically as* A *or* B *components*

Experimental details and description of the fractions and components are given in the text. Values are expressed as % of dry ash-free fraction. The + signs opposite the neutral sugars indicate the relative intensity of the spots (alkaline-silver nitrate staining) observed on paper chromatograms; results were not obtained for fractions I*A* and II*A*.

Component	*A*			*B*	
Fraction	I*A*	II*A*	III*A*	I*B*	II*B*
Nitrogen (%)	17·1	17·3	17·6	15·5	15·4
Hydroxyproline (%)	12·8	12·1	11·2	2·8	6·9
Hexose* (%)	1·3	1·7	2·3	3·3	4·3
Methylpentose† (%)	0·4	0·4	0·7	0·9	0·9
Amino sugar‡ (%)	0·5	0·6	0·4	1·1	1·3
Sialic acid§ (%)	0·4	0·5	0·9	1·6	2·3
Ash (%)	2·2	1·3	5·3	6·4	5·1
Neutral sugars					
Galactose	.	.	+ + +	+ + +	+ + +
Glucose	.	.	+ +	+ + +	+
Mannose	.	.	+ + +	+ + +	+ + +
Fucose	.	.	+	+	+

* As galactose. † As fucose. ‡ As glucosamine. § As *N*-acetylneuraminic acid.



Scheme 2. Procedure for cetylpyridinium chloride fractionation.

Table 4. *Composition of acidic mucosubstances obtained by cetyl pyridinium chloride fractionation*

Experimental details and description of the fractions are given in the text. Values are expressed as % of fractions dried to constant weight.

Fraction	C_s	C	D
Nitrogen (%)	12·6	11·4	3·5
Sulphate (%)	—	0·2	10·3
Ash (%)	7·1	—	19·3
Hexose* (%)	14·2	13·6	0·3
Methylpentose† (%)	2·4	2·4	0·0
Amino sugar‡ (%)	—	6·4¶	25·4**
Uronic acid§ (%)	0·4	0·7	29·2
Sialic acid‖ (%)	3·1	15·9	0·8

* As galactose.
† As fucose.
‡ As glucosamine.
§ As glucuronic acid.
‖ As *N*-acetylneuraminic acid.
¶ After hydrolysis with 2N-HCl for 4 hr. at 100°.
** After hydrolysis with 4N-HCl for 16 hr. at 100°.

Separation of amino sugars. There was insufficient fraction *C* available for the determination of the hydrolysis conditions for maximum yield of amino sugar. Instead, the known optimum conditions (2N-hydrochloric acid for 4 hr. at 100°) (Winzler, 1955) for the closely similar substance, orosomucoid, were used. Fraction *D* was hydrolysed with 4N-hydrochloric acid for 16 hr. at 100°, under the conditions used by Partridge & Davis (1958) for maximum yield of amino sugar from chondroitin sulphate. The hydrolysates, after evaporation to dryness under reduced pressure in the presence of sodium hydroxide pellets and phosphorus pentoxide, were dissolved in 0·5 ml. of 0·3N-hydrochloric acid for application to an ion-exchange resin column as described in the Materials and Methods section. The results indicated that, whereas galactosamine was the only amino sugar present in fraction *D*, both glucosamine and galactosamine occurred in fraction *C*. The ratios for these sugars in two separate determinations were 51%:49% of the total amino sugar content.

Other sugars. The aldols present in fraction *C* were obtained by hydrolysis with 2N-sulphuric acid for 8 hr. at 100° (Dische, Sant'Agnese, Pallavicini & Youlos, 1959). The hydrolysate was passed through a short column of De-Acidite FF resin (OH^- form) to remove SO_4^{2-} ions, and then through a similar column of Amberlite IRC-50 buffered to pH 7·0 to adsorb amino sugars (Exley, 1957). The eluate was freed of remaining salts by treatment with activated carbon, concentrated under reduced pressure and submitted to paper chromatography which indicated the presence of galactose, glucose, mannose and fucose. To determine the ratio of the three hexoses, chromatograms containing standards and hydrolysates were stained with alkaline silver nitrate. After the papers had been made translucent with liquid paraffin, the density of the spots was measured with the double-beam recording microdensitometer (Joyce, Loebl and Co. Ltd.) giving molar proportions of 8:1:5 for galactose, glucose and mannose respectively.

The sialic acid, liberated by hydrolysis with 0·01N-sulphuric acid at 80° for 1 hr., had the same R_F as a sample of *N*-acetylneuraminic acid on paper chromatography in two different solvents. No other form of sialic acid could be detected in the hydrolysates under these conditions.

Amino acid composition. The peptide constituents of fraction *C* accounted for about 60% (by total amino acids) of the composition, and chromato-

graphic analysis under the conditions of Moore & Stein (1951) indicated the presence of at least 14 amino acids, including cysteic acid, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, methionine, isoleucine, leucine, tyrosine, phenylalanine, lysine and histidine.

DISCUSSION

Extraction methods. In the study of calcified tissues, extraction methods are hindered by the presence of the hard apatite mineral. In the present work, an attempt to overcome this problem was made by grinding the bone to a finely divided state when it was found that rapid decalcification could be achieved in relatively neutral conditions with the sodium salts of EDTA. [A similar procedure was adopted by Foster & Hackman (1957) in an endeavour to obtain 'native' chitin.] This treatment also brought into solution about 6% of the total organic material of bone, and hexose determinations indicated that about 20% of the total carbohydrate was present in the extracts. Previous studies (Dische *et al.* 1958; King & Boyce, 1959) in which EDTA was used for demineralization also reported the presence of carbohydrate in the decalcifying solution, but this was not examined in any detail.

An additional 5% of the total carbohydrate could be extracted by further treatment of the residual bone powder with potassium carbonate and dilute potassium hydroxide. It was considered advisable, however, to avoid these more alkaline conditions as used in some previous investigations (Hawk & Gies, 1901; Hisamura, 1938; Glegg & Eidinger, 1955), particularly since preliminary studies with dilute alkali extraction (G. M. Herring, unpublished work) indicated a considerable loss of sialic acid-containing constituents. Likewise, it has been shown that the protein–carbohydrate bond of the chondroitin sulphate complex of cartilage is readily broken by alkaline conditions (Muir, 1958; Partridge & Davis, 1958).

Fractionation of the extracts. The preliminary fractionation of the dialysed extracts with ethanol did not produce appreciable separation of the four main electrophoretic components which were shown to be distributed between the fractions in various proportions. On paper electrophoresis, the faster moving components (*C* and *D*) had staining properties of acidic mucosubstances with alcian blue and toluidine blue, whereas the slower-moving components (*A* and *B*) stained with light green, characteristic of proteins. Only the *B* and *C* components, however, showed a strong periodic acid–Schiff reaction.

In attempts to fractionate these mixtures further it was found that only the *A* and *B* components were retained by Amberlite CG-50 resin at pH 5·0 (Partridge & Davis, 1958). The *C* and *D* components were eluted at this pH and were separated by fractionation of their cetylpyridinium salts.

Component C (*sialoprotein*). This acidic mucosubstance formed a cetylpyridinium salt which was insoluble in water but dissolved in 0·3 M-sodium chloride, enabling it to be readily separated from the complex of the *D* component, which was not dissociated under these conditions. The acidic nature of component *C*, shown also by its electrophoretic mobility, was due, at least partly, to the presence of 15·9% of *N*-acetylneuraminic acid which, like the sialic acid of orosomucoid, urinary mucoprotein and bovine submaxillary protein, was removed by hydrolysis with 0·01 N-sulphuric acid. Hexoses made up 13·6% of component *C* which after acid hydrolysis (2 N-sulphuric acid for 8 hr. at 100°) showed the presence on paper chromatography of galactose, glucose and mannose in the molar proportions 8:1:5. The amino sugars in acid hydrolysates were glucosamine and galactosamine in the ratio of 51%:49% of the total (6·4%); a small amount of fucose (2·4%) was also present. In many respects this substance resembles the orosomucoid (α_1-acid glycoprotein) of serum (Table 5), but in a comparison of bovine orosomucoid and bone sialoprotein (Herring, 1963) the amino sugar of the former was found to be predominantly glucosamine, galactosamine being present in only small amounts. This appears to be the first occasion on which a sialoprotein has been isolated in a reasonably homogeneous form from bone, and it is of some interest in relation to studies on sialic acid present in bone and other connective tissues (Courts, 1959; Castellani, Ferri, Bolognani & Graziano, 1960). The qualitative periodic acid–Schiff reaction shown by the spot test and on paper electrophoresis indicates that the sialoprotein may be a contributor to this reaction observed histologically in bone sections.

Table 5. *Comparison of sialoproteins*

	Bone (bovine) sialoprotein	Bovine oroso-mucoid*	Human oroso-mucoid†
Nitrogen (%)	11·4	11·9	11·1
Hexose (%)	13·6	13·9	14·4
Hexosamine (%)	6·4	7·9	10·0
Sialic acid (%)	15·9	10·9	12·5
Fucose (%)	2·4	—	1·5
Uronic acid (%)	0·7	—	—
Tyrosine (%)	1·6	—	4·2
Tryptophan (%)	—	—	1·7
Protein (%)	60·0	67·0	63·0
S_{20} (s)	—	—	3·0

* Weimer & Winzler (1955).

† Bezkorovainy & Winzler (1961) (cf. also Bezkorovainy & Doherty, 1962; Schultze, Heide & Haupt, 1962*a*, *b*).

Component D (*chondroitin sulphate*). This fraction appeared to be identical with the chondroitin sulphate A isolated before by Hisamura (1938), Rogers (1951) and Meyer, Davidson, Linker & Hoffman (1956). The use of proteolytic enzymes (Rogers, 1951; Meyer *et al.* 1956) enables the total amount of the pure acid mucopolysaccharides to be isolated. This important technique, however, results in the loss of most of the peptide chain (Muir, 1958) in complexes such as the chondromucoprotein of cartilage (Malawista & Schubert, 1958; Partridge & Davis, 1958; Muir, 1958). In the present work it was thought that the conditions used might result in the isolation of such a complex, but the nitrogen content, electrophoretic mobility on paper (Malawista & Schubert, 1958) and examination by paper chromatography (Muir, 1958) indicated that the isolated material was not in this form.

Component A. Analysis of the slowest electrophoretic component of fractions I, II and III (Table 3) indicated that it was a collagen-like material. It appears likely to be a degraded rather than a true soluble collagen which is apparently present in only small amounts in cortical bone (Araya, Saito, Nakanishi, Kawanishi, 1961). The amount of collagen extractable by EDTA solutions seems to be dependent to some degree on pH and temperature. Thus, by using a saturated solution of EDTA (sodium salt) at pH 8·5 and at 22° for 48 hr., Dische *et al.* (1958) extracted about 35% of the total protein from cortical bone.

Component B. The properties of fractions I*B* and II*B* suggested that these might be mucoproteins, though neither had been obtained in a homogeneous state free from collagen. Fraction I*B*, unlike fraction II*B*, was insoluble below pH 5·0 and in this respect resembles the mucoprotein of osseomucoid (Hisamura, 1938). A further difference in the two *B* fractions was in their apparent hexose composition (Table 3).

SUMMARY

1. Bone powder, obtained by fragmentation of bovine cortical bone under cooling conditions, on extraction with sodium ethylenediaminetetraacetate (pH 7–8) gives four soluble products.

2. The extracted substances fractionated electrophoretically and by ion-exchange chromatography consist of a collagen-like fraction, a mucoprotein fraction, chondroitin sulphate and a sialoprotein.

3. The sialoprotein is characterized by a high content of *N*-acetylneuraminic acid (15·9%) and hexoses (galactose, glucose and mannose) (13·6%), with fucose (2·4%) and amino sugars (made up of approximately equimolar proportions of glucosamine and galactosamine) (6·4%). The material contained traces only of uronic acid.

4. In general properties, the sialoprotein resembles the orosomucoid of serum, but is distinguished from it at least by its content of galactosamine.

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Blood Lipids

3. PLASMA LIPIDS OF THE COW DURING PREGNANCY AND LACTATION*

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Maynard, Harrison & McCay (1931) found that, after parturition in cows, there was a rapid increase in the plasma concentrations of total fatty acids, phospholipid fatty acids and total cholesterol; this was followed by a gradual decline throughout the remainder of the period of lactation. Schaible (1932) found that average plasma concentrations of total fatty acids and lipid P were considerably greater in lactating cows than in non-lactating cows or in steers, and similar observations for plasma concentrations of total cholesterol were made by Lennon & Mixner (1957). These workers also confirmed the findings of Long, Hibbs & Gilmore (1953) that plasma concentrations of total cholesterol at parturition were significantly lower than those observed before and after calving. Riis (1959) reported observations that corroborated those of Maynard *et al.* (1931), and also found that the changes in the concentration of total cholesterol were not accompanied by any significant alteration in the relative proportions of free and esterified cholesterol.

Our continuing interest in the role of plasma lipids in lactation led us to study these changes in more detail, with particular reference to the fatty acid composition of the major classes of plasma lipid in blood samples obtained at intervals during pregnancy and lactation.

* Part 2: Lough & Garton (1957).

EXPERIMENTAL

Animals and diet. Five Ayrshire heifers, aged 2–3 years, were used in this study; three (animals A, B and C) were pregnant for the first time and two (animals D and E) had been served but failed to conceive. The pregnant animals were stall-fed to appetite on grass silage from about