

MUCOPROTEINS OF OX TRACHEAL CARTILAGE

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PREVIOUS investigations (Glegg, Eidinger and Leblond, 1954*a*) have revealed the presence of two alkali-soluble fractions in mammalian connective tissues, *viz.* lung, tendon, ligamentum nuchae, tracheal cartilage and bone matrix. The first fraction consisted of the acidic mucopolysaccharides, probably including the chondroitin sulphates, hyaluronic acid and keratosulphate. The second fraction contained no hexuronic acids but had a high proportion of aldoses which were identified by paper chromatography. All tissues showed the presence of galactose, mannose and fucose in the second fraction and in some, glucose also was identified. Using a periodic acid-Schiff (PAS) spot-test, the second fraction showed a strongly positive reaction, while the first fraction was non-reactive. No attempt was made to characterize the two fractions according to homogeneity, purity or chemical composition.

The present work is concerned with the electrophoretic separation and the chemical characterization of mucosubstances of ox tracheal cartilage.

EXPERIMENTAL METHODS AND RESULTS

Extraction procedure.—The fresh ox tracheal tissue (1600 g.) was minced, and extracted with 2000 ml. of 2 per cent potassium hydroxide at 4°, with intermittent shaking, for 3 days. The mixture was filtered through gauze and the liquid was centrifuged at 2500 r.p.m. This procedure was repeated twice and the combined centrifuged extracts were brought to pH 4 with glacial acetic acid. A precipitate (I) separated immediately, and, after standing for several hours at 4°, it was collected by centrifugation. Addition of 2 volumes of ethanol containing 1 per cent potassium acetate and glacial acetic acid (1 per cent) to the above supernatant gave a second precipitate (II) which, after standing for 24 hr. at 4°, was collected by centrifugation. Ethanol was added to the supernatant to give a final concentration of 85 per cent and a further precipitate (III) was collected after 24 hr. at 4°. Fraction I was washed with slightly acidified water (pH 5) and all 3 fractions washed and dried with absolute ethanol and ether.

Analysis of carbohydrate constituents.—Hydrolysis of samples from each fraction was carried out using a cationic ion-exchange resin (Zeo-Karb 225) in water at 100° for 48 hr., after the method of Glegg, Eidinger and Leblond (1954*b*). Aldoses in the hydrolysate were identified by paper chromatography on Whatman No. 1 paper using a water-saturated mixture of butanol/pyridine (4 : 1) as solvent (Chargaff, Levine and Green, 1948). Each chromatogram was run 3 times successively with ascending elution (Jeanes, Wise and Dimmler, 1951) before spraying with aniline hydrogen oxalate reagent (Horrocks and Manning, 1949). Amino-sugars retained by the Zeo-Karb resin used in the hydrolysis, were eluted with 0.5 N-HCl; these eluates were evaporated to dryness over sodium hydroxide *in vacuo*, and amino-sugars identified by the method of Stoffyn and Jeanloz (1954).

Analyses for N, S, and ash were carried out by Drs. Weiler and Strauss, Oxford. The results are shown in Table I.

Paper electrophoresis.—The 3 original fractions were examined by paper electrophoresis using Whatman No. 1 paper with a potential gradient of 2 v./cm. and a current of 2 mA applied for a period of 16 hr. Chondroitin sulphate and other metachromatic substances were revealed by staining the papers with 0.1 per cent toluidine blue in 85 per cent ethanol; after washing

the papers with 85 per cent ethanol, further treatment with 0.05 per cent bromophenol blue in 85 per cent ethanol showed the presence of protein components. Separate electrophoretic papers were stained with a periodic acid-Schiff technique described by Laurell (1955). Paper electrophoretic examination of the fractions, carried out in both 0.05 M-sodium barbitone buffer (pH 8.6), and 0.2 M-sodium acetate buffer (pH 5.0), gave the results shown in Fig. 1. Fraction I was almost insoluble in the acetate buffer (pH 5.0) and gave one faint metachromatic spot. The main component of fraction II in both buffers had the same mobility as chondroitin sulphate.

Preparative electrophoresis.—For analytical electrophoretic examination in the Tiselius moving boundary apparatus, fraction I was reprecipitated 5 times from 0.1 N-KOH by adding glacial acetic acid to pH 4. The reprecipitated fraction was dissolved in buffer solution and then dialysed for 48 hr. in cellophane at 4° against the same buffer. In each of 2 experiments using 0.1 M-glycine-NaOH buffer (pH 9.2) and 0.1 M-sodium barbitone-HCl buffer (pH 7.4), two components were observed; a slow-moving (*Ia*) and a fast-moving (*Ib*). The proportion of these constituents was calculated from the areas under the curves of the Schlieren patterns and was of the order of 9 : 1 respectively. A preparative separation was effected using a large-scale Tiselius apparatus, the U-tube consisting of cells with a cross-section of 7.5 sq. cm. and a capacity of 200 ml.; the electrode vessels each held 5 l. of buffer. A 1 per cent solution of fraction I in 200 ml. of 0.1 M-glycine-NaOH buffer (pH 9.2) was dialysed against 12 l. of buffer at 4° for 48 hr. and then subjected to electrophoresis for 5 days at 1–2° using a current of 20 mA. The movement of the boundaries was followed by direct observation and isolation of the fast and slow components was achieved by the insertion of long, finely-drawn, Pasteur pipettes into the areas between the boundaries; the solutions were withdrawn using gentle suction. The 2 fractions (*Ia* and *Ib*) were dialysed against distilled water, precipitated with ethanol, washed and dried with ethanol and ether. The slow-moving component (*Ia*) was re-examined electrophoretically after being redissolved in, and dialysed against, the above glycine buffer and was found to give a single peak with mobility unchanged. The 2 fractions, hydrolysed with 4 N-HCl for 4 hr., had the composition showed in Table II.

DISCUSSION

The analytical and electrophoretic results indicate that the major fraction (II) is composed mainly of chondroitin sulphate (cf. Meyer, Davidson, Linker and Hoffman, 1956), and is only weakly PAS positive. Fraction I shows some similarity to the “chondromucoid” obtained from bull trachea and whale nasal septum (Hisamura, 1938). Using a kaolin adsorption procedure “chondromucoid”

TABLE I.—*Composition of Alkali-soluble Fractions of Ox trachea*

Fraction	Per cent yield (from moist tissue)	* Per cent N	* Per cent S	* Per cent Ash	Per cent total carbo-hydrate (Anthrone reaction)†	[α] _D ²¹ (in 0.1 N NaOH)	Monosaccharides present	P.A.S. spot test‡
I	1.7	10.01	1.35	0.43	6.36	-50.4	Fucose Mannose Galactose Glucosamine Galactosamine	+++
II	5.5	4.01	4.58	13.1	15.9	-21.7	Glucuronic acid Galactosamine	+
III	0.18	7.11	6.10	2.59	10.2	-28.8	Fucose Mannose Galactose Glucosamine Galactosamine	+++

* Samples dried at room temperature.

† Windrum, Kent and Eastoe, 1954.

‡ Hotchkiss, 1948.

TABLE II.—*Composition of Electrophoretically Separated Tracheal Constituents*

	Nitrogen* (per cent)	Amino-sugar† (per cent)	Uronic acid‡ (per cent)
Ia	14.7	2.3	0.45
Ib	5.63	12.9	14.0

* Chibnall, Rees and Williams, 1943.

† Johnston, Ogston and Stanier, 1951.

‡ Dische, 1947.

was separated by Hisamura into chondroitin sulphate and a mucoprotein (11.87 per cent N) containing both neutral sugar (7.33 per cent) and amino-sugar (8.21 per cent). Fraction Ia produced by our preparative electrophoretic separation,

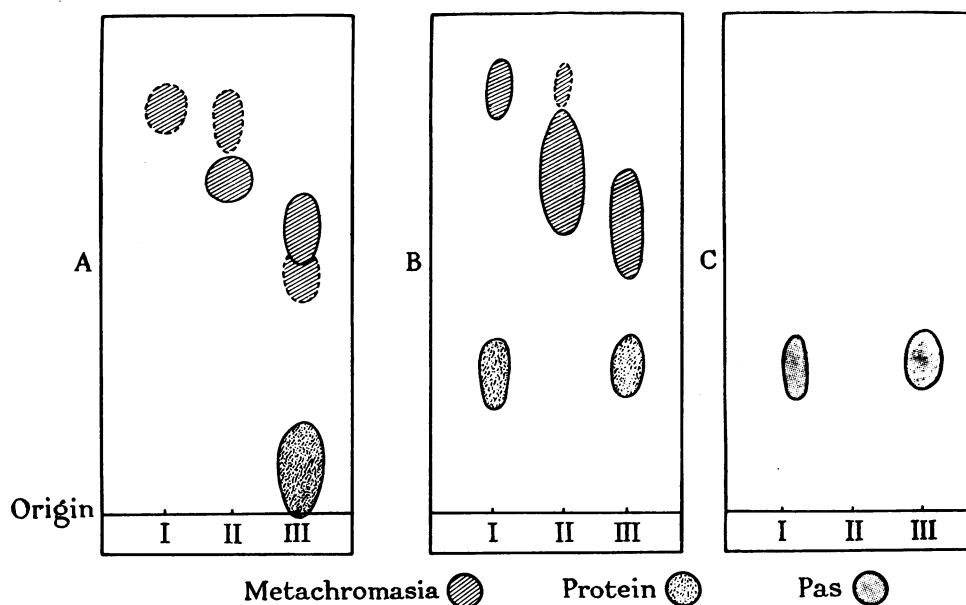


FIG.—Separation of fractions I, II and III by paper electrophoresis. Migration of anionic tracheal mucoprotein fractions on Whatman No. 1 paper after 16 hr. at potential gradient of 2 v./cm. A run in 0.2 M acetate buffer. B and C run in 0.05 M barbital buffer.

appears to be similar to Hisamura's mucoprotein. The composition of Ib, however, suggests that it is a complex formed from protein and chondroitin sulphate. The occurrence of such a complex in bovine nasal and tracheal cartilage has already been reported (Shatton and Schubert, 1954; Webber and Bayley, 1956; Partridge and Davies, 1958; Malavista and Schubert, 1958) and their results indicate that Ia closely resembles the non-collagenous protein moiety which has been found (Partridge and Davies, 1958) to be liberated from the chondroitin sulphate protein complex by alkaline hydrolysis. A strong PAS reaction was shown by both fractions I and III; paper electrophoresis (Fig. 1) showed this staining reaction to be localized in the slow-moving protein-staining components. It seems probable, therefore, that some or all of the monosaccharides demonstrated by paper chromatography in fractions I and III, are combined with these proteins. This would also apply to the electrophoretically separated fraction Ia if it is assumed

to be identical with the slower moving component of I noticed in paper electrophoresis. Fractions I and III show similarities with regard to their PAS/protein staining fractions but differ markedly in their solubilities in water. The insolubility of I, compared to the solubility of III may be related to the degradation of the protein by the extraction procedure. Such a degradation may have occurred at one stage of the extraction procedure used by Glegg *et al.* (1954a), and would explain why they did not find a water-insoluble fraction; the aldoses were found all to occur in the fraction requiring 85 per cent ethanol for precipitation. Whether both soluble and insoluble forms of the protein occur normally requires further investigation.

These methods are now being applied to the isolation of mucoproteins from bone and preliminary experiments indicate the presence of similar PAS reactive fractions.

SUMMARY

Three fractions have been obtained by alkaline extraction of tracheal cartilage. One of these (II) consists mainly of chondroitin sulphate. The other fractions (I and III) contain fucose, mannose, galactose and amino-sugars and on paper electrophoresis showed components which contained protein and were PAS positive. A preparative electrophoretic separation was carried out on fraction I, the main component of which proved to be a mucoprotein which is considered to contain neutral sugars and therefore to be responsible for the strong PAS reaction.

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REFERENCES

- CHARGAFF, E., LEVINE, G. AND GREEN, C.—(1948) *J. biol. Chem.*, **175**, 67.
 CHIBNALL, A. C., REES, M. W. AND WILLIAMS, E. F.—(1943) *Biochem. J.*, **37**, 354.
 DISCHE, Z.—(1947) *J. biol. Chem.*, **167**, 189.
 GLEGG, R. E., EIDINGER, D. AND LEBLOND, C. P.—(1954a) *Science*, **120**, 839.—(1954b) *Anal. Chem.*, **26**, 1365.
 HISAMURA, H.—(1938) *Tokyo J. Biochem.*, **28**, 217.
 HORROCKS, R. H. AND MANNING, G. B.—(1949) *Lancet*, **256**, 1042.
 HOTCHKISS, R. D.—(1948) *Arch. Biochem.*, **16**, 131.
 JEANES, A., WISE, C. S. AND DIMMLER, R. J.—(1951) *Anal. Chem.*, **23**, 415.
 JOHNSTON, J. P., OGSTON, A. G. AND STANIER, J. E.—(1951) *Analyst*, **76**, 88.
 LAURELL, H.—(1955) 'Paper Electrophoresis'. London (Ciba) (J. & A. Churchill Ltd.).
 MALAVISTA, I. AND SCHUBERT, M.—(1958) *J. biol. Chem.*, **230**, 535.
 MEYER, K., DAVIDSON, E., LINKER, A. AND HOFFMANN, P.—(1956) *Biochem. biophys. acta*, **21**, 506.
 PARTRIDGE, S. M. AND DAVIES, H. F.—(1958) *Biochem. J.*, **68**, 298.
 SHATTON, J. AND SCHUBERT, M.—(1954) *J. biol. Chem.*, **211**, 565.
 STOFFYIN, P. J. AND JEANLOZ, R. M.—(1954) *Arch. Biochem. Biophys.*, **52**, 373.
 WEBBER, R. V. AND BAYLEY, S. T.—(1956) *Can. J. Biochem. Physiol.*, **34**, 993.
 WINDRUM, G. M., KENT, P. W. AND EASTOE, J. E.—(1954) *Brit. J. exp. Path.*, **36**, 49.