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The Isolation and Characterization of a Glycoprotein from Human Thoracic Aorta

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1. A glycoprotein extracted by cold alkali from the walls of human aorta was purified by chromatography on DEAE-cellulose. 2. The compound was electrophoretically homogeneous and essentially so by chromatography on DEAE-cellulose. Ultracentrifugal examination revealed two components, and it is suggested that the faster-sedimenting component represents an aggregated form of the glycoprotein. 3. Glycoprotein preparations contained approx. 8% of carbohydrate. Digestion with Pronase yielded a glycopeptide fraction containing all the carbohydrate of the glycoprotein. The glycopeptide, of molecular weight about 7800, contained sialic acid, galactose, mannose, fucose and hexosamine in the approximate molar proportions 5:10:5:2:11. Sialic acid was terminal with respect to the polysaccharide chains. 4. Both elastase and elastomucoproteinases exhibited proteolytic activity towards the glycoprotein. Studies by other investigators have led to the conclusion that elastomucoproteinases attack protein–carbohydrate complexes occurring in intimate association with elastin in aorta and other tissues, and it is suggested that the glycoprotein may be identified with one of these compounds.

The presence of glycoproteins in human aorta is indicated by a number of observations. Thus histological studies (Gillman, Hathorn & Penn, 1957; Bertelsen & Jensen, 1960; Gotte, 1952) have revealed periodate–Schiff-positive staining material in sections of human aorta that is located in close association with the elastic membranes of the media. The positive reaction is attributed to the presence of glycoproteins (cf. Leblond, Glegg & Eidinger, 1957; Pearse, 1960) and more specifically to the presence of sialic acid, located in a terminal position in these proteins (Jeanloz, 1958; Montreuil & Biserte, 1959). The presence of sialic acid in human aorta has been shown by Murata & Kirk (1962). Bertelsen & Jensen (1960) state that the periodate–Schiff reaction is first detectable in subjects at about 20 years of age and then continues to increase in intensity from this age.

Enzymic studies have also indicated a close association of glycoproteins with elastin. A number of workers (for a review see Loeven, 1965*a*) have studied enzymes of the elastase complex that act synergistically with elastase on elastin preparations and are believed to digest glycoprotein(s) still present in such preparations and existing, within the native elastic tissue, in intimate association with the elastic structures, both surrounding and penetrating the elastic fibres. Loeven (1965*b*) has reported that there are at least three such enzymes known as elastomucoproteinases or elastomucases.

Some of the glycoproteins occurring in connective tissues, including human aorta, can be readily extracted with neutral or slightly alkaline buffers (Boas, 1955; Kao, Noble & Boucek, 1956; Bowes, Elliott & Moss, 1957; Humphrey, Neuberger & Perkins, 1957), and such extracts, at least with skin, have been shown to contain glycoproteins identical with plasma glycoproteins (Humphrey *et al.* 1957; Adelmann, Marquardt & Kühn, 1966). In this context, Enselme, Frey & Henry (1961) have reported that saline extracts of human aorta reveal an electrophoretic pattern very similar to that of plasma. However, Berenson *et al.* (1966) isolated a water-soluble glycoprotein fraction from human aorta that, by polyacrylamidegel electrophoresis, was shown to contain several components, some of which, it is concluded from immunological studies, are distinct from plasma glycoproteins.

It is also evident that other glycoproteins occur

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in connective tissue that are not so readily extracted and require more drastic procedures such as the use of urea or solvents of high pH for solubilization (Kao *et al.* 1956; Bowes *et al.* 1957; Anderson, 1961; Kao, Hitt, Dawson & McGavack, 1962; Robert, Parlebas, Oudea, Zweibaum & Robert, 1965). Enselme *et al.* (1961) have shown that hexosamine-containing proteins are dissolved from saline-extracted aorta with alkali. Robert, Robert, Moczar & Moczar (1968*b*) have described the extraction of 'structural' glycoproteins from human aorta and other tissues by means of urea.

A similar situation has also been observed by the present authors. Preliminary studies (cf. Barnes, 1964) indicated that elastin prepared from human thoracic aorta contained significant quantities of sialic acid, hexose, hexosamine and tryptophan, indicating the probable presence of small amounts of glycoprotein. It was found that, after removal from aorta of the more soluble proteins with 1% sodium chloride, further sialic acid-containing components could be partially extracted with cold dilute alkali. The isolation and characterization of a glycoprotein from such an extract is described in this paper and its behaviour towards elastomucoproteinases has also been examined.

A preliminary account of this work has already been given (Barnes, 1965).

MATERIALS

Aortae. Samples of human thoracic aortae were collected at post-mortem by Dr R. A. A. Coombs and his staff at the Department of Pathology, University of Cambridge. Samples were kept at −20° until required.

N-*Acetylneuraminic acid.* This was prepared from egg-white essentially as described by Feeney, Rhodes & Anderson (1960) and crystallized from water–methanol–ether (Martensson, Raal & Svennerholm, 1958).

DEAE-cellulose. Whatman DEAE-cellulose (DE50) was employed in these studies. The cellulose was first freed of soluble u.v.-absorbing materials by the procedure described by O'Donnell & Thompson (1960).

Enzymes. A freeze-dried sample of Pronase-P, a proteolytic enzyme from *Streptomyces griseus*, was kindly provided by Dr M. Nomoto (cf. Nomoto & Narahashi, 1959, 1960). Neuraminidase was a solution of *Vibrio comma* (formerly *Vibrio cholerae*) neuraminidase supplied by Burroughs Wellcome and Co., Beckenham, Kent. Two elastomucoproteinase preparations were generously given by Dr W. A. Loeven and were designated mucase I (8% of protein) and mucase II (80% of protein). The elastase preparation (twice-crystallized) was supplied by Worthington Biochemical Corp., Freehold, N.J., U.S.A.

METHODS

Analytical methods

Moisture was determined by drying to constant weight over P_2O_5 at 70° under reduced pressure. Ash was determined as sulphate. The micro-Kjeldahl method was used for the determination of total N. Amide N was determined by means of the micro-Kjeldahl apparatus, after hydrolysis in 2N-HCl for 3hr. at 100° (Bowes & Moss, 1962). Amino acid analysis was performed with the Technicon auto-analyser. Samples for analysis were first hydrolysed by refluxing under N_2 in constant-boiling HCl for 48hr.

Hexose. Total hexose was determined colorimetrically with the anthrone reagent (Yemm & Willis, 1954), with D-galactose as a standard. Correction was made for non-specific absorption due to the presence of protein by measuring the absorption occurring in the absence of anthrone.

Hexoses were identified by paper chromatography essentially as described by Partridge & Elsden (1961*a*). The sample to be examined was first hydrolysed in 1N-H_2SO_4 for 1hr. at 100° in a sealed tube [the adequacy of these conditions was established by prior kinetic studies, employing the Nelson (1944) method].

The galactose:mannose ratio was ascertained by eluting the galactose and mannose spots with water (location of the spots was established by spraying marker strips on either side of the chromatogram) and assaying the eluate with the anthrone reagent. Adjacent regions of the chromatogram were eluted with water to correct for the blank value of the paper.

Hexosamine. Total hexosamine was determined by the colorimetric method of Elson & Morgan (1933) as modified by Blix (1948), with D-glucosamine hydrochloride as a standard. Samples were first hydrolysed in 4N-HCl in sealed tubes at 100° for 16hr. (Partridge & Davis, 1958). Recovery of glucosamine added to test samples before hydrolysis indicated negligible loss during hydrolysis. Results are expressed as percentages of the free base present in the test samples.

Glucosamine and galactosamine were determined individually after separation by ion-exchange chromatography on Amberlite IR-120 and analysis of the column eluate by the Elson & Morgan (1933) procedure. The sample was first hydrolysed under the conditions just described and a portion of the hydrolysate applied directly to the column (Partridge & Elsden, 1961*b*).

Sialic acid. Sialic acid was determined by the colorimetric procedure of Warren (1959), after hydrolysis with 0·1N-H_2SO_4 for 1hr. at 80°. Crystalline *N*-acetylneuraminic acid was employed as a standard. A standard sample was hydrolysed at the same time as the test samples to correct for any losses occurring during hydrolysis.

Methylpentose. The procedure of Dische & Shettles (Dische, 1955) was employed, with L-fucose as a standard. Allowance was made for the occurrence of non-specific absorption due to the presence of protein in the test sample by measuring the absorption occurring in the absence of cysteine.

A qualitative examination of methylpentoses was made by means of the paper-chromatographic procedure described for the examination of hexoses (with the same hydrolysate).

Uronic acid. Uronic acid was determined by the carbazole reaction described by Dische (1947), with D-glucurone as a standard. Correction was made for non-specific absorption due to the presence of protein by measuring the absorption before and after the addition of carbazole.

Pentose. Total pentose was assayed by the orcinol reaction of Bial as modified by Albaum & Umbreit (1947), with D-ribose as a standard. A correction was made for the

presence of hexose in the test sample by examining the absorption obtained with a sample of galactose that was equal in amount to the hexose present in the test sample.

Ester sulphate. The turbidimetric method of Dodgson (Dodgson, 1961; Dodgson & Price, 1962) was employed, after hydrolysis in 1N-HCl for 5hr. at 105–110° in sealed tubes. Correction was made for the absorption due to the presence of protein by measuring the absorption obtained in the absence of $BaCl_2$. K_2SO_4 was used as a standard.

Lipid. The method of Rapport & Alonzo (1955) for the measurement of carboxylic acid ester groups was used. Methyl stearate was used as a standard.

Ninhydrin colorimetric assay of amino groups. The method employed was essentially that described by Yemm & Cocking (1955). Results are expressed as the number of μmoles of leucine equivalent to the extinction of the test sample.

Electrophoretic methods

Tiselius moving-boundary electrophoresis. Electrophoretic examination was carried out in the apparatus of Tiselius (1937) with the Philpot–Svensson optical arrangement (Svensson, 1939, 1946). The bath temperature was approx. 1°. Samples to be examined were thoroughly dialysed at 1° against the appropriate buffer.

Low-voltage paper electrophoresis. Samples were subjected to low-voltage paper electrophoresis on Whatman no. 3 paper, in 1% (w/v) $(NH_4)_2CO_3$, pH8·9, in the Shandon vertical-electrophoresis tank. The paper was soaked in the buffer, excess of buffer removed by blotting and the sample then applied to the origin as two separate streaks, one in each half of the paper. Electrophoresis was at 200v for 5–6hr. After electrophoresis the paper was dried at 100° and then cut into two halves, one half stained with Alcian Blue [1% (w/v) in 90% (v/v) acetic acid] to locate glycosaminoglycan sulphates and the other half stained with Bromophenol Blue [1% (w/v) in 90% (v/v) ethanol saturated with $HgCl_2$] to detect protein.

Starch-gel electrophoresis. This was kindly performed by Dr R. K. Scopes by using a discontinuous buffer system in a vertical apparatus as described by Scopes (1964). Electrophoresis was carried out at 400v (approx. 22v/cm.) and 13–18mA at 1° for 3½hr. After electrophoresis, the gels were stained with 1% (w/v) Naphthalene Black 10B+2% (w/v) Nigrosine in methanol–acetic acid–water (5:1:4, by vol.).

Ion-exchange chromatography on DEAE-cellulose

Chromatography was performed at 1°. The sample to be chromatographed was equilibrated by dialysis against 5mM-tris–HCl buffer, pH7·0, containing 1mM-EDTA (referred to below as standard tris buffer) and then applied to a column (1·7cm. × 17cm.) of DEAE-cellulose previously equilibrated with the same standard tris buffer. The column was then eluted with a KCl gradient (0–1M) achieved by use of a constant-volume mixing chamber (volume approx. 350ml.) containing the standard tris buffer and connected to a reservoir containing 2M-KCl in the same tris buffer.

The eluate was collected in 5ml. fractions and the extinction at 276mμ (in 1cm. cuvettes) of each fraction was recorded. The course of the gradient was determined by examining the refractive index of a selected number of fractions.

When an alkaline extract (concentrated extract; cf. Scheme 1) of aortae was applied to the column, the recovery, as judged by u.v. absorption at 276mμ, was generally only about 50–60%. Elution of the column with 0·1N-NaOH effected the recovery of a further 10%, and on allowing the column to stand with 0·1N-NaOH overnight (at 1°) and then eluting with alkali for a second time a further 5% was recovered. The poor recovery of material applied was thought to be due to irreversible adsorption of material of very high molecular weight.

Enzymic methods

Neuraminidase. A 2ml. volume of a 0·5% solution of the glycoprotein preparation (preparation 1) in 0·05M-tris–maleate buffer, pH6·5, containing 0·9% NaCl and 0·01M-$CaCl_2$, was incubated at 37° with 0·5ml. of enzyme solution. Samples (0·2ml.) were removed at appropriate time-intervals and examined for free sialic acid by the Warren (1959) method.

Pronase. A 95mg. sample of the glycoprotein preparation (water-insoluble fraction of preparation 2), dissolved in 0·05M-tris–maleate buffer, pH7, containing 5% (v/v) ethanol and 15mM-$CaCl_2$, was incubated at 37° with 2mg. of Pronase. After 24hr. another 2mg. of enzyme was added and incubation allowed to continue for a further 24hr.

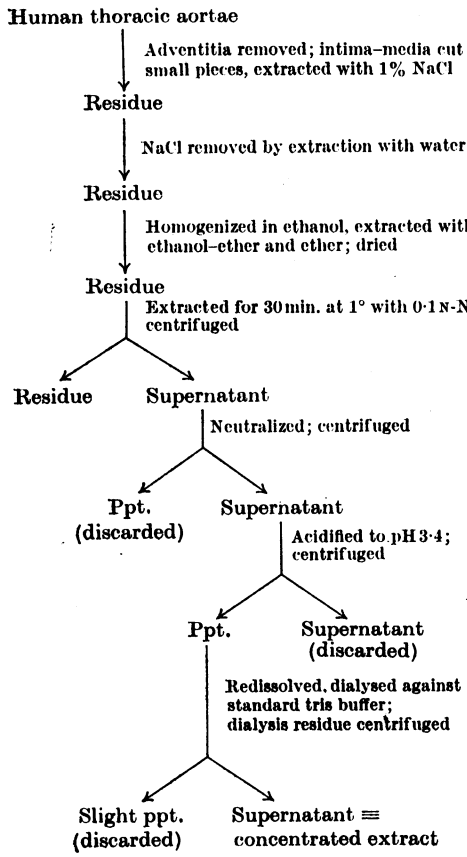
Elastase and elastomucoproteinases. The rupture of peptide bonds was measured by titration with NaOH in the Radiometer automatic titrator. The glycoprotein preparation (water-insoluble fraction of preparation 2) was dissolved in 0·1N-NaOH, the solution immediately adjusted to pH8·7 with HCl and, after addition of the appropriate enzyme, the uptake of alkali required to maintain the pH at 8·7 was recorded. During the incubation, the cell was kept at 37° and N_2 was bubbled through the incubation mixture to exclude CO_2.

The course of the reaction was also examined by measuring the release of amino groups with ninhydrin. A solution of the glycoprotein (water-insoluble fraction of preparation 2) in borate buffer, pH8·7 (Loeven, 1960), was incubated at 37° in the presence of the appropriate enzyme and samples were removed at fixed time-intervals for assay with ninhydrin.

Extraction of aortae

The extraction procedure is outlined in Scheme 1. All operations were performed at 1°.

Samples of human thoracic aortae, kept frozen at −20° until an appropriate number had been collected, were thawed and the adventitia removed by hand. The combined intima–media preparations were then minced in 1% (w/v) NaCl and the minced tissue was extracted by gently rocking with approx. 20vol. of 1% NaCl in a mechanical shaker. Several extractions each of approx. 2hr. duration were employed. After each extraction, the suspension was filtered through butter-muslin and the residue suspended in fresh 1% NaCl (a small volume of toluene was incorporated to prevent bacterial contamination). This preliminary extraction with saline removed any blood remaining in the tissue and also extracted from the ground substance the more readily soluble glycoproteins (cf. Enselme *et al.* 1961).



Scheme 1. Extraction of human thoracic aortae with cold dilute NaOH: derivation of the crude concentrated extract.

The NaCl was removed by several washes in distilled water and the residue then homogenized in ethanol, in a Waring Blendor, for two 5 min. periods. After homogenization the tissue was thoroughly extracted with a large volume of ethanol, 50% (v/v) ethanol–ether and finally ether. At the completion of the extraction residual ether was removed on a warm-water bath and the residue was then finally dried *in vacuo* over conc. H_2SO_4.

The dried defatted tissue was then stirred with 0·1 N-NaOH (50 ml./g.) for 30 min., after which time the suspension was centrifuged at 15 000*g* for 2 min. The supernatant was immediately neutralized to approx. pH 7·5 and then allowed to stand for a period. A slight precipitate was removed by centrifugation. The supernatant solution was then acidified to approx. pH 3·4. Precipitation in the form of an opalescence commenced at about pH 5·5 and increased in intensity until about pH 3·4. The optimum value for this final pH was not always easy to establish, and occasionally it was necessary to lower the pH still further (to just below pH 3) to obtain the maximum precipitation. Usually 15–25% of the total u.v.-absorbing material (measured in 1 cm. silica cells at 276 mμ) remained unprecipitated. The precipitate was collected by centrifugation, washed in distilled water and then suspended in the standard tris buffer. To allow dissolution the pH was raised to approx. 8, when slow dissolution occurred, and the pH was finally raised to approx. 10 to allow maximum dissolution before thorough dialysis against the tris buffer (such a procedure was generally employed when dissolution of either purified glycoprotein preparations or crude glycoprotein extracts was required). The dialysis residue was then centrifuged at 1° to remove undissolved matter, which usually represented approx. 20% of the total u.v.-absorbing material (at 276 mμ) precipitated at pH 3·4. The supernatant solution is referred to below as the concentrated extract.

Isolation of the glycoprotein by large-scale preparative chromatography on DEAE-cellulose

All operations were carried out at 1°. The concentrated extract derived from the 30 min. alkaline extraction (see Scheme 1) was dialysed against standard tris buffer containing 0·3 M-KCl. It was then applied to a large column of DEAE-cellulose previously equilibrated with the same buffer. The column was then developed with the same buffer and those fractions of the eluate containing the u.v. absorption (measured in 1 cm. cells at 276 mμ) unretained by the column were pooled and the material was recovered by dialysis and freeze-drying.

Two preparations of the glycoprotein were isolated by this procedure, and specific experimental details relating to each preparation are given below.

RESULTS

Sialic acid content in relation to age. Preliminary experiments were carried out to assess the variability to be expected with aorta samples from subjects in different age groups. For this purpose aortae were collected in groups as in Table 1 and extracted with 1% sodium chloride and ethanol–ether. The dried residues, after equilibration with the atmosphere to constant weight, were examined for moisture, ash and sialic acid. A correction for the presence of deoxyribose was made according to the procedure given by Warren (1959). The correction was about 20% for the first age group, 5–10% for the two groups 1–20 years and 21–40 years, and zero for the last age group. The results, presented in Table 1, revealed an increase in sialic acid content with increasing age, and this was assumed to indicate an increase in the content of glycoprotein with increasing age. Ash values reflected the very marked calcification that occurs in the later decades of life.

Extraction with 0·1 N-sodium hydroxide. Preliminary serial extractions with 0·1 N-sodium hydroxide at 1° were carried out with the dry defatted aorta powder. Extraction times and amount of material extracted (as estimated by u.v.-absorption) are shown in Table 2. Extraction during the first 30 min. period was rapid, but a total time of about 16 hr. was required to bring the process to near completion. With the exception of

Table 1. *Changes with age in sialic acid content of human thoracic aorta*

Aortae were first extracted with 1% NaCl and ethanol–ether. Details are given in the text. Values for sialic acid are corrected for deoxyribose.

Age group	Total no. of aortae	Moisture (%)	Ash (% dry wt.)	Sialic acid (% ash-free dry wt.)
Under 1 year (mean 3 weeks)	18	11·9	1·54, 1·53 (mean 1·54)	0·25, 0·28 (mean 0·27)
1–20 years (mean 12 years)	14	10·4	0·8, 0·64 (mean 0·72)	0·38, 0·39 (mean 0·39)
21–40 years (mean 29 years)	24	11·5	1·16, 1·34 (mean 1·25)	0·46, 0·5 (mean 0·48)
Over 50 years (mean 73 years)	35	12·2	8·4, 8·37 (mean 8·39)	0·59

the decrease in the final age group, increasing amounts of material were extracted with increasing age. The initial extracts of the three later age groups showed a typical protein absorption curve with a maximum of 276 mμ. In the first age group, the absorption maximum occurred at 260 mμ, and subsequent chromatographic analysis confirmed that nucleic acid was the major u.v.-absorbing component.

Ion-exchange chromatography on DEAE-cellulose. A preliminary batch of aortae from subjects over 50 years of age was extracted with cold dilute alkali (2 hr.), and a sample of the concentrated extract (cf. Scheme 1) containing about 50 mg. of protein was examined by chromatography on a column (1·7 cm. × 17 cm.) of DEAE-cellulose as described in the Methods section. Samples (1 ml.) from fractions of the eluate were assayed for hexose and uronic acid (Fig. 1).

The uronic acid-containing acidic glycosaminoglycans, identified by the carbazole reaction, appeared as two distinct peaks, the first associated with an appreciable amount of protein (peak C) and the second with little or no protein. This double uronic acid peak was a consistent feature of chromatograms of extracts of aorta.

The major protein peak (peak B), equivalent to 35–40% of the total u.v. absorption applied to the column, contained no uronic acid but an appreciable amount of hexose. The subsequent identification of sialic acid and hexosamine in this peak confirmed the presence of glycoprotein.

The pattern of peaks preceding the major peak has shown some variation from experiment to experiment. In this chromatogram two peaks (A and A′) were found and together represented approx. 3–4% of the total u.v. absorption applied to the column. Frequently only one peak has been observed preceding the main peak.

The last peak eluted (peak D) was often smaller or was entirely absent. The u.v. absorption maximum was at 260 mμ, in contrast with the maximum at 276 mμ in the other peaks.

Rechromatography of the B peak. The eluate containing peak B was concentrated at 1° by ultrafiltration through collodion thimbles (Membranfiltergesellschaft, Göttingen, Germany) under a vacuum of 500 mm. Hg, dialysed against the standard tris buffer and rechromatographed. A minor peak (A) was observed preceding the main peak B, but peaks subsequent to peak B in the original chromatogram were absent. There was approx. 95% recovery of the u.v. absorption applied to the column (cf. Fig. 2).

Chromatographic behaviour of the extracts from different age groups. The concentrated extracts derived from the initial 30 min. alkaline extraction (cf. Scheme 1) of each of the age groups referred to in Tables 1 and 2 were examined by DEAE-cellulose chromatography. The results were very similar, with the same essential features shown in Fig. 1, except that peak D was the major component in the first age group and absent from the fourth age group.

As judged by u.v. absorption, increasing amounts of glycoprotein (peak B) were extracted with increasing age until the final age group (cf. Table 3). In the latter case, despite the increase in sialic acid content, the amount of glycoprotein extracted was decreased and it is thought this might be associated with the marked degree of calcification that occurs in this group.

Isolation of the glycoprotein fraction from a 30 min. alkaline extract. Two preparations of the glycoprotein fraction were obtained by large-scale preparative chromatography on DEAE-cellulose as outlined in Scheme 2. Preparation 1 was obtained as follows.

Aortae, collected from subjects over 50 years of age and extracted with cold saline and organic solvents (residue weight, 30 g.), were extracted with cold 0·1 N-sodium hydroxide for 30 min., and the concentrated extract (cf. Scheme 1), containing approx. 1·5 g. of protein, was applied to a large column (5 cm. × 43 cm.) of DEAE-cellulose under the conditions described in the Methods section.

Table 2. *Distribution of u.v.-absorbing material in successive alkaline extracts of human thoracic aortae*

Aortae, after prior extraction with 1% NaCl and ethanol–ether, were extracted at 1° with 0·1N-NaOH for the periods indicated. The data refer to the supernatants obtained after neutralization of the extracts and centrifugation. Details are given in the text.

		First extract (30min.)			Second extract (1½hr.)			Third extract (16hr.)		
Age group	Dry wt. of tissue (g.)	Vol. (ml.)	E_{276}	Vol. (ml.)× E_{276}/g. dry wt. of tissue	Vol. (ml.)	E_{276}	Vol. (ml.)× E_{276}/g. dry wt. of tissue	Vol. (ml.)	E_{276}	Vol. (ml.)× E_{276}/g. dry wt. of tissue
Under 1 year	1·0	43	2·30	98	47	0·82	38·5	48	0·47	23
1–20 years	9·0	393	2·60	114	458	1·03	52·4	438	0·77	37·5
21–40 years	3·0	126	3·37	140	154	0·98	50·0	150	0·78	38
	22·3	925	3·30	137						
Over 50 years	3·0	131	2·10	92						
	21·4	955	2·13	95						
	21·4	968	2·14	97	152	0·71	36·0	149	0·64	32
	21·4	965	2·0	90						
	21·4	965	1·96	88						
	17·4	1010	1·42	89						

These conditions were selected from a consideration of the chromatographic behaviour of the concentrated extract on DEAE-cellulose on gradient elution (see Fig. 1) and were designed to eliminate the glycosaminoglycans and other components eluted at a salt concentration greater than that required for the elution of the glycoprotein fraction (peak B). The u.v.-absorbing material unretained by the column, representing about one-third of the total u.v. absorption applied, was recovered by dialysis (during the course of which the dialysis residue became opalescent) and freeze-drying.

The material (500mg.) referred to below as preparation 1 was redissolved and fractionated on a column (1·7cm. × 17cm.) of DEAE-cellulose, as described in the Methods section. The results (Fig. 3) indicated that the large-scale chromatography described above had allowed a complete removal of those components eluted after peak B. The latter represented approx. 90% and the preceding minor A peak approx. 7% of the total u.v. absorption applied to the column. Two cuts of the eluate (Fig. 3), one to include material preceding the main peak and the other to include the trailing edge of the main peak, were dialysed against the standard tris buffer and rechromatographed under the same conditions. The results (Fig. 4) again confirmed the very close relationship between peaks A and B.

Preparation of water-soluble and water-insoluble fractions of the glycoprotein (preparation 2). The concentrated extract from the three later age groups referred to in Tables 1–3 were combined and the glycoprotein was isolated (as preparation 2) by large-scale preparative chromatography on a DEAE-cellulose column (7·5cm. × 60cm.) under the conditions described in the Methods section. Fractions of the eluate containing u.v. absorption were combined and the solution was dialysed against water. Towards completion of dialysis a precipitate separated. It was collected by centrifugation, washed once with water and then suspended in water and freeze-dried. The dry product (1·6g.) is referred to below as the water-insoluble fraction of preparation 2 and the supernatant solution after freeze-drying (700mg.) as the water-soluble fraction.

Both preparations were examined by DEAE-cellulose chromatography (Figs. 5 and 6). The water-insoluble fraction chromatographed essentially as a single peak (peak B), but the water-soluble fraction revealed, in addition to the major B peak representing 70% of the total u.v. absorption applied to the column, two small peaks, one unretained by the column (peak O) and the other immediately preceding the main peak (peak A). The material of each of these three peaks was recovered by dialysis and freeze-drying and found to have a similar sialic acid content (approx. 2%).

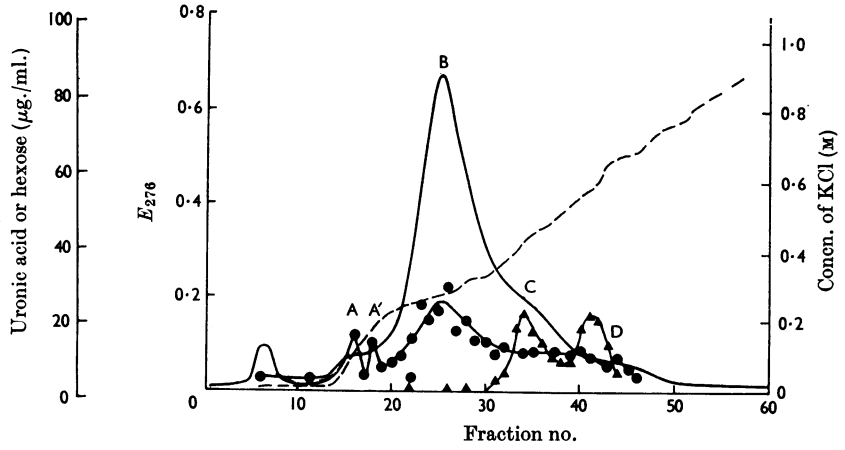


Fig. 1. Chromatography on DEAE-cellulose of the concentrated extract (cf. Scheme 1) derived from an alkaline extraction (0·1 N-NaOH at 1° for 2 hr.) of human thoracic aortae (from subjects over 50 years of age). A 5 ml. sample ($E^{1cm.}_{276}$ 11·0) was applied to the column (1·7 cm. × 17 cm.). Elution was by means of a KCl gradient (0–1 M) in standard tris buffer. Chromatography was at 1°. Fractions of volume 5 ml. were collected. ——, $E^{1cm.}_{276}$; ----, gradient (molarity of KCl); ▲, uronic acid (carbazole reaction); ●, hexose (anthrone reaction).

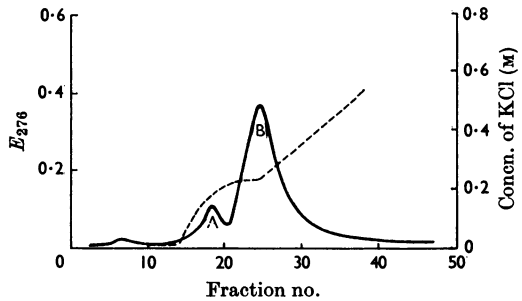


Fig. 2. Rechromatography of peak B on DEAE-cellulose. Fractions 23–28 inclusive of the chromatogram shown in Fig. 1 were combined, concentrated and re-equilibrated with the standard tris buffer. A sample (6·1 ml.; $E^{1cm.}_{276}$ 2·16) was then chromatographed under the conditions described for Fig. 1. The A peak represents approx. 15% and the B peak approx. 80% of the total u.v. absorption applied to the column. ——, $E^{1cm.}_{276}$; ----, gradient (molarity of KCl).

Composition of glycoprotein preparations. Analytical data for the water-insoluble and water-soluble fractions of preparation 2 are given in Table 4. Corresponding data for preparation 1 (which was not fractionated into water-soluble and water-insoluble fractions) are also included. Though there was variation in the total carbohydrate present, the proportions of the individual carbohydrate constituents in all three preparations were similar. Paper chromatography after acid hydrolysis of preparation 1 and the water-insoluble fraction (of preparation 2) showed the presence of galactose, mannose and fucose. The galactose: mannose ratio was approx. 2:1 in both cases. The two preparations both contained glucosamine and galactosamine, the latter amino sugar representing 10–15% of the total hexosamine.

The amino acid analysis of the water-insoluble fraction (of preparation 2) is given in Table 5. The recovery of both total weight and total nitrogen was only about 90% and this may be explained by destruction of amino acids during hydrolysis in the presence of carbohydrate. Similar low recovery of weight and nitrogen from analysis of plasma glycoproteins is not uncommon (cf. Putnam, 1960; Gottschalk, 1963).

The amino acid analysis of the B peak of the water-soluble fraction (of preparation 2), isolated by gradient-elution chromatography on DEAE-cellulose, showed a very close resemblance to that given in Table 5.

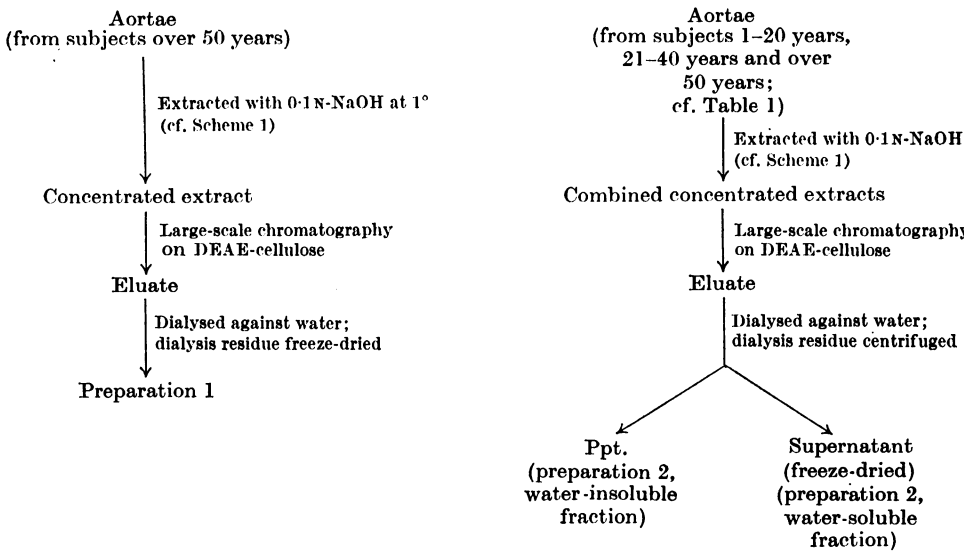
Electrophoresis experiments. Tiselius electrophoresis of preparation 1 and the water-insoluble fraction (of preparation 2) revealed only a single peak. Electrophoretic mobilities (in the ascending limb) were as follows: preparation 1 at pH 7, $2·6 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$; at pH 8, $5·5 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$; preparation 2 (water-insoluble fraction) at pH 7, $2·4 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$.

Tiselius electrophoresis at pH 7 of the concentrated extract before purification revealed three peaks of increasing mobility (2·8, 5·6 and $8·4 \times 10^{-5}$ cm.^{2} v^{-1} sec.$^{-1}$) and decreasing magnitude.

Table 3. *Variation in amount of glycoprotein (B peak) extracted with increasing age*

The contribution of the glycoprotein (peak B) to the total extinction of the extract was assessed by chromatographic analysis of the extract on DEAE-cellulose as described in the text.

Age group	Dry wt. of tissue (g.)	Concentrated extract: Vol. (ml.)	Concentrated extract: Total E_{276}	Concentrated extract: E_{276} attributable to peak B	Vol. (ml.) × E_{276} of peak B/g. dry wt. of tissue
Under 1 year	1	10	6·7	1·4	14·0
1–20 years	9	80	9·0	3·3	29·6
21–40 years	3	40	8·0	2·9	38·2
Over 50 years	3	25	8·0	3·2	26·7



Scheme 2. Derivation of preparations 1 and 2.

These are presumed to correspond to the B peak, C peak and second uronic acid peak respectively, observed by chromatography of the concentrated extract on DEAE-cellulose (see Fig. 1).

Paper electrophoresis (of preparation 1) also revealed only a single protein band. No band due to acidic glycosaminoglycans was detectable with Alcian Blue. Electrophoresis of the concentrated extract before purification revealed, in addition to the glycoprotein band, two glycosaminoglycan zones, both of greater mobility, the slower of which was associated with an additional protein band.

Starch-gel electrophoresis of both the water-soluble and water-insoluble fractions (of preparation 2) revealed a single diffuse zone close to the origin, the mobility of the zone from the water-soluble preparation being slightly greater.

Ultracentrifugal analysis. Solutions (approx. 1%) of the water-soluble and water-insoluble fractions (of preparation 2) were equilibrated with phosphate buffer, pH 7·8 and I 0·2, by dialysis and then examined in the Spinco model E ultracentrifuge. Each preparation revealed two peaks both of very considerable polydispersity. The two peaks of one preparation appeared to be identical in mobility with the two peaks of the other preparation. However, the relative amounts of the individual peaks were different in the two preparations. Thus with the water-soluble fraction the slower peak ($S_{20,w}$ 3·5s) was dominant, whereas with the water-insoluble fraction the more rapidly sedimenting material gave the major peak ($S_{20,w}$ 10·5s).

Release of sialic acid by neuraminidase. After 24hr. incubation 83% of the total sialic acid, as

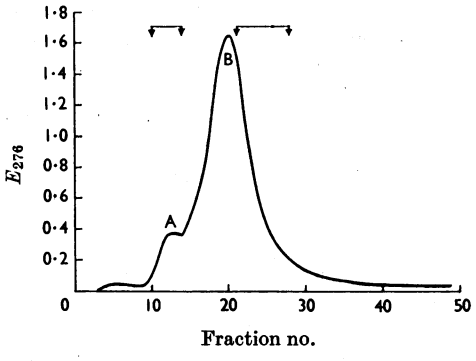


Fig. 3. Chromatography of a 9ml. sample ($E^{1cm.}_{276}$ 7·9) of glycoprotein preparation 1 on DEAE-cellulose. Conditions of chromatography were as given in Fig. 1. The A peak represents approx. 7% and the B peak approx. 90% of the total u.v. absorption applied to the column. Two cuts of the eluate, as indicated in the diagram, were rechromatographed (cf. Fig. 4).

determined by hydrolysis with dilute acid, was released in a free state from the glycoprotein (preparation 1) by neuraminidase, indicating a terminal location for the sialic acid residues.

Digestion with Pronase. A sample (95mg.) of the water-insoluble fraction of the purified glycoprotein (preparation 2) was digested with Pronase and the incubation mixture was dialysed against water (4 × 20ml.). Measurement of the u.v. absorption at 276mμ indicated that approx. 80% of the original total was present in the diffusate, and the ninhydrin reaction indicated approx. 50% hydrolysis of peptide bonds. The combined diffusates contained no neutral sugars.

The contents of the dialysis tubing, after completion of dialysis, were centrifuged to remove a slight precipitate that appeared during the incubation with Pronase. The supernatant (14ml.) contained approx. 12% of the original u.v. absorption.

The hexose and sialic acid contents of the non-diffusible material were examined and found to be close to the values expected if it was assumed that all the carbohydrate remained in the dialysis residue. The dialysis residue was freeze-dried and the product dissolved in 0·5ml. of 1% sodium chloride. This solution was applied to a column (0·9cm. × 107cm.) of Sephadex G-50 previously equilibrated with 1% sodium chloride. The column was developed with the same salt solution and the eluate collected in 2·7ml. fractions. The u.v. absorption at 276mμ of each tube was recorded and a 0·5ml. sample from each alternate tube was examined with the anthrone reagent.

All of the carbohydrate appeared as a single well-defined peak, unassociated with any peak of u.v.

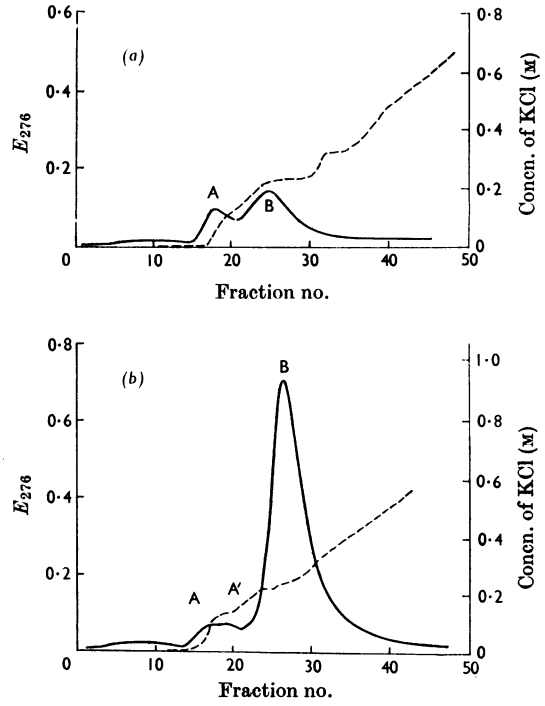


Fig. 4. (*a*) Rechromatography of peak A on DEAE-cellulose. Fractions 10–14 inclusive of the chromatogram of Fig. 3 were rechromatographed after dialysis against the standard tris buffer. Recovery of the u.v. absorption applied to the column was 100%. Peak A represented approx. 25% of the total u.v. absorption. Conditions of chromatography were as given in Fig. 1. (*b*) Rechromatography of the trailing edge of peak B (Fig. 3) on DEAE-cellulose. Tubes 21–28 inclusive of the chromatogram of Fig. 3 were rechromatographed after dialysis against the standard tris buffer. Recovery of u.v. absorption applied to the column was 100%. The minor peaks (A and A') represented approx. 9% of the total u.v. absorption. Conditions of chromatography were as given in Fig. 1. ——, $E^{1cm.}_{276}$; ----, gradient (molarity of KCl).

absorption. The carbohydrate peak was slightly retarded and this suggested a molecular weight close to 7000 (the limits of exclusion for the grade of Sephadex used are given as 7000–10000). Tubes corresponding to the central portion of the carbohydrate peak were pooled and dialysed against water. All of the sialic acid originally present in the sample applied to the column was in the hexose peak.

The dialysis residue (14·2ml.) was freeze-dried and the product dissolved in 0·5ml. of water. The solution was rechromatographed on the same Sephadex column (previously equilibrated with water), and the hexose peak was collected and rechromatographed once more to ensure complete

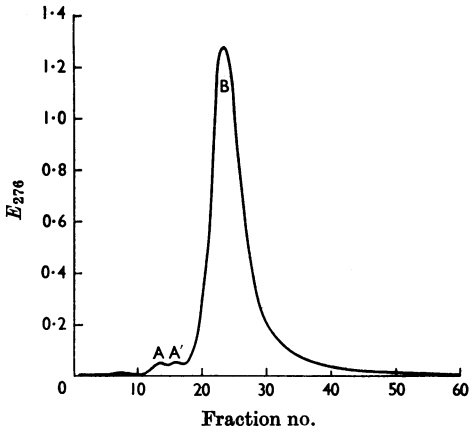


Fig. 5. Chromatography of a 3ml. sample ($E^{1cm.}_{276}$ 15·1) of the water-insoluble fraction of preparation 2 on DEAE-cellulose. Conditions of chromatography were as given in Fig. 1. The major peak (B) represents 95% and the minor preceding peaks (A and A') approx. 3% of the total u.v. absorption applied to the column.

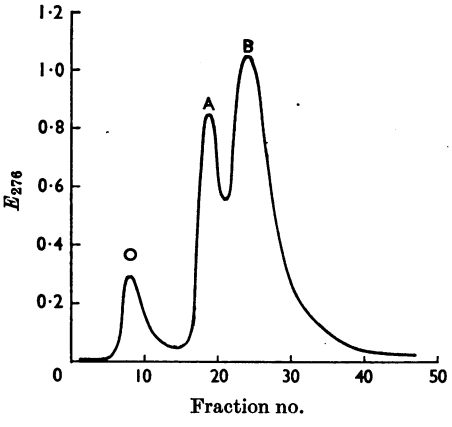


Fig. 6. Chromatography of a 10ml. sample ($E^{1cm.}_{276}$ 6) of the water-soluble fraction of preparation 2 on DEAE-cellulose. Conditions of chromatography were as given in Fig. 1. Peak O represents approx. 10%, peak A 18% and peak B 70% of the total u.v. absorption applied to the column.

removal of the adjacent u.v.-absorbing peaks appearing on the original chromatogram. The tubes containing the carbohydrate peak from the final chromatogram were pooled and the solution was freeze-dried to give a residue of approx. 5mg. A sample was dried at 70° under vacuum and the dried preparation (2·53mg.) dissolved in 5ml. of water.

An estimate of the molecular weight of the glycopeptide was then made by the dinitrophenylation procedure essentially as described by Neuberger & Papkoff (1963). The absorption spectrum of the dinitrophenylated product was that of a typical DNP-glycopeptide (Sanger, 1945) with an absorption maximum at 350mμ. The extinction at 350mμ was 0·137 (concn. of glycopeptide: 0·063mg./ml.), and assuming ϵ is 16000 (Sanger, 1945) it was calculated that the molecular weight of the glycopeptide (assuming 1 DNP-amino acid residue/mol. of glycopeptide) is approx. 7500.

Analytical results obtained with the glycopeptide are reported in Table 6 together with a calculation of the molar composition of the carbohydrate fraction rounded off to the nearest integer. The amino acids present in major amounts were determined independently as follows: aspartic acid, 5·9%; glutamic acid, 3·6%; valine, 3·2%; threonine, 2%; proline, 2%; serine, 1·4%. Several other amino acids were present in trace amounts. The contribution to the total molecular weight of the constituents analysed, calculated on the basis of the tentative molar composition, is 7822. This is in good agreement with the value 7500 estimated by dinitrophenylation of the *N*-terminal amino acid residue. From the carbohydrate content, the minimum molecular weight of the glycoprotein is about 80000.

Action of elastase and elastomucoproteinases. The water-insoluble fraction of preparation 2 was used in these studies. In a preliminary examination, a solution of the glycoprotein preparation (12·9mg.) in borate buffer, pH 8·7, was incubated with mucase I (3mg.) at 37°. After 18hr. the incubation mixture was dialysed against water (4 × 5ml.). The total diffusate contained 45% of the original u.v. absorption, and its ninhydrin reaction indicated 15–20% hydrolysis of peptide bonds. Hexose was absent from the diffusate, and an attack on the carbohydrate moiety was therefore considered unlikely.

The rupture of peptide bonds by both elastase and the two elastomucoproteinase preparations was studied by automatic titration. The reaction was allowed to proceed to near completion as judged by the falling rate of uptake of alkali. At the completion of the incubation the ninhydrin reaction of the mixture was examined. A comparison of the results of the two methods of demonstrating peptide bond hydrolysis is given in Table 7. The course of the reaction with each enzyme was also examined colorimetrically by measuring the increase in the ninhydrin reaction of the incubation mixture. The results are shown in Fig. 7.

DISCUSSION

The main objective of this work was to attempt the isolation of the sialic acid-containing glycoproteins reported to be in close association with the

Table 4. *Analysis of glycoprotein preparations* 1 *and* 2

	Composition (% of dry ash-free preparation)		
	Prep. 1	Water-insoluble fraction of prep. 2	Water-soluble fraction of prep. 2
Sialic acid	2·2, 2·2, 2·3 (mean 2·2)	1·69, 1·70 (mean 1·7)	1·93, 2·04 (mean 1·99)
Hexosamine	3·5, 3·4, 3·2 (mean 3·4)	2·55, 2·64 (mean 2·6)	2·9
Hexose	4·2, 4·0, 3·8 (mean 4·0)	3·20, 3·26 (mean 3·23)	
Uronic acid	Absent	Absent	Absent
Methylpentose		0·4	
Pentose		Absent	
Ester sulphate		Absent	
Lipid		Absent	
$\frac{\text{Hexosamine (\%)}}{\text{Sialic acid (\%)}}$ ratio	1·55	1·53	1·46
$\frac{\text{Hexose (\%)}}{\text{Sialic acid (\%)}}$ ratio	1·81	1·9	

Table 5. *Amino acid analysis of the water-insoluble fraction of preparation* 2

Tryptophan was determined essentially by the method of Horn & Jones (1945) (cf. Barnes, 1964). The total N content of the water-insoluble fraction of preparation 2 was 14·9%.

	Composition (g./100g. of dry ash-free protein)	% of total N
Asp	9·3	6·56
Thr	4·2	3·2
Ser	4·3	3·87
Glu	11·8	7·5
Pro	5·5	4·5
Gly	3·9	4·88
Ala	4·1	4·35
Val	5·2	4·19
CyS	1·0	0·8
Met	2·2	1·4
Ile	4·6	3·27
Leu	7·9	5·66
Tyr	5·6	2·9
Phe	5·0	2·8
Lys	5·5	7·05
His	2·2	4·06
Arg	6·2	13·4
Trp	2·1	1·93
Total	90·6	82·33
Amide N	1·1	7·5
Sialic acid	1·7	0·52
Hexosamine	2·6	1·34
Fucose	0·4	
Hexose	3·2	
Total	99·6	91·7

elastic laminae in the walls of the large blood vessels, particularly the aorta. As mentioned in the introduction the main evidence for the existence of these glycoproteins in the human aorta arose from histological examination with the periodate–Schiff reaction, and from detailed study of the kinetics of the action of enzymes on material from the elastic membranes (reviewed by Hall, 1964; Loeven, 1965*a*).

The isolation procedure adopted in this work was based on the observation that, after a preliminary extraction of aorta with 1% sodium chloride and organic solvents, glycoprotein could be extracted from the residue by cold dilute alkali but not by 1M-potassium chloride, carbonate–bicarbonate buffer, pH 9, or 4% EDTA, pH 7·2. Complete extraction was not attempted, since prolonged exposure to alkaline conditions was considered likely to cause serious degradation of the glycoproteins extracted. The 30min. extraction with cold 0·1N-sodium hydroxide employed in these studies solubilized approx. 30% of the sialic acid in the saline-extracted defatted residue.

The glycoprotein fraction isolated from the alkaline extract was electrophoretically homogeneous and essentially so by chromatography on DEAE-cellulose. The relationship between the main chromatographic component (peak B) and the minor peak(s) preceding it is not entirely certain, but the similar proportion of sialic acid in each and their behaviour on rechromatography

Table 6. *Composition of the glycopeptide fraction obtained by Pronase digestion of the glycoprotein (water-insoluble fraction of preparation 2)*

Constituent (anhydrous residues)	Contribution to total mol.wt.	Composition required (%)	Composition found (%)
N-Acetylneuraminic acid* (5 moles)	1365	20·2	20·2
Galactose (10 moles) } Mannose (5 moles) }	2550	33·4	34
Fucose (2 moles)	324	4·7	4·8
N-Acetylhexosamine (11 moles)	2233	31·6	33·0†
Amino acids	1350	16·5	16·5
Total	7822	106·4	108·5

* It is assumed that the sialic acid is *N*-acetylneuraminic acid.

† Corrected for loss of acetyl residue during hydrolysis, assuming the hexosamine to be acetylated.

Table 7. *Action of elastase and the elastomucoproteinases on the glycoprotein (water-insoluble fraction of preparation 2)*

The consumption of NaOH during incubation was measured in a Radiometer automatic titrator and the ninhydrin reaction of the incubation mixture was determined at the completion of the incubation. The values given for the ninhydrin reaction are corrected for the ninhydrin value of the whole protein (before digestion) and for the ninhydrin value of the enzyme preparation.

Enzyme	Quantity of substrate (mg.)	Quantity of enzyme (mg.)	Duration of incubation (hr.)	Total NaOH consumed (μmoles)	Ninhydrin reaction (μmoles of leucine equivalent)	μmoles of NaOH/mg. of substrate	μmoles of leucine equivalent/ mg. of substrate
Elastase	12·9	0·13	4·0	10·2	11·7	0·8	0·91
Mucase I	13·3	1·60	9·0	17·3	12·3	1·3	0·92
Mucase II	7·5	1·05	3·5	9·0	8·5	1·2	1·13

might indicate a common origin. The minor component may arise as an artifact of the chromatography, particularly as the elution of the minor peak coincides with an abrupt rise in the salt concentration of the eluate at the commencement of the gradient. Alternatively, it is thought that the two peaks may be related, in some way, to the formation of an aggregated state of the glycoprotein, evidence for the existence of which is discussed below.

Two components were observed by ultracentrifugal analysis in both water-soluble and water-insoluble glycoprotein preparations. In the former the slower-sedimenting component was the greater, in the latter the reverse was true. As both preparations consisted of the same major chromatographic component (peak B) it was considered that the faster ultracentrifugal component represented an aggregated form of the slower component. It is suggested that the precipitation of the insoluble fraction during prolonged dialysis against water arises by aggregation. In the tissue, deposits of glycoprotein in the elastica may be at very high concentration. This would be expected to favour molecular association, and it may be that the formation of large association complexes is responsible for the integrity of the glycoprotein structures in the tissues and for the experimental finding that the structures are very resistant to extraction, even with alkaline buffer solutions that prove good solvents once the material has been dispersed.

The reason for the polydispersity of the two ultracentrifugal components is not clear, but it might possibly reflect slight damage during extraction with sodium hydroxide or limited hydrolysis, before extraction, by enzymes present in the tissue (see Buddecke, 1966).

The glycoprotein isolated in the present study was found to be susceptible to proteolytic attack by both elastase and the two elastomucoproteinase preparations. Loeven (1965*a*) suggests the existence of two protein–carbohydrate complexes containing sialic acid, hexose and hexosamine associated with elastin, one tightly bound and present in both acid-treated and alkali-treated elastin and a second more loosely bound and present only in acid-treated elastin. The latter is readily released from acid-treated elastin by both elastase and the elastomuco-

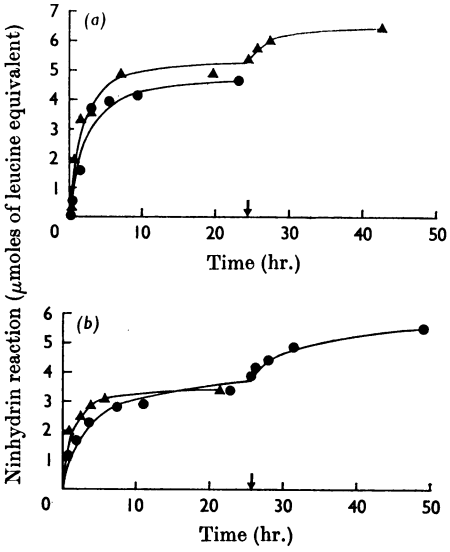


Fig. 7. Reaction of the glycoprotein (water-insoluble fraction of preparation 2) with elastase and elastomucoproteinase, as indicated by measurement of the ninhydrin reaction of the incubation mixture. (*a*) Reaction with elastase: ▲, 3·84mg. of substrate and 0·04mg. of enzyme (a further 0·04mg. of enzyme was introduced at the point indicated by the arrow); ●, 4mg. of substrate and 0·02mg. of enzyme. (*b*) Reaction with mucases I and II: ●, 4·25mg. of substrate and 0·53mg. of mucase I (a further 0·58mg. of enzyme was introduced at the point indicated by the arrow). ▲, 3·65mg. of substrate and 0·48mg. of mucase II.

proteinases, and it is suggested that the glycoprotein isolated in the present study may be identifiable with this proposed protein–carbohydrate compound present in acid-treated elastin. The molar proportions of carbohydrate constituents released from acid-treated elastin by enzymes of the elastase complex as observed by Loeven (1965*a*) are in reasonable accord with this suggestion.

Lansing, Roberts, Ramasarma, Rosenthal & Alex (1951) have reported that the amino acid composition of aortic elastin of elderly subjects shows a greater content of polar amino acids in comparison with that of aortic elastin of young subjects, and these authors suggested that the elastin preparations contain a second protein that increases with age. Gotte, Meneghelli & Castellani (1965) have found a similar situation when comparing elastin prepared from calcified and uncalcified aorta, and have concluded that a second protein is present in the elastin preparations and that this protein is more difficult to extract from calcified tissue. The amino acid composition of the glycoprotein isolated in the present study is compatible with the suggestion that its presence could be responsible for the anomalous composition of elastin prepared from old calcified aorta. It has also been found that, as judged by u.v. absorption, there are increasing amounts of the glycoprotein extractable with increasing age, in accord with the increased content of sialic acid, until the final age group (in which calcification was very marked) where, despite the further increase in sialic acid, there was a decrease in the amount of glycoprotein extracted. It is noteworthy, in relation to the above observations, that FitzPatrick & Hospelhorn (1965) have reported that elastin prepared after repeated collagenase digestion of powdered defatted human aorta revealed, particularly in older subjects, an anomalous amino acid composition and contained sialic acid, hexose and fucose but no uronic acid.

It seems likely that an association of elastin with glycoprotein(s) is a phenomenon common to all elastic tissues. Gotte and colleagues (Gotte, Serafini-Fracassini & Moret, 1963; Moret, Serafini-Fracassini & Gotte, 1964) have, for example, described the extraction from elastin fibres, prepared from bovine ligamentum nuchae by autoclaving, of a protein–polysaccharide component, containing hexose, hexosamine and sialic acid, by means of prolonged treatment of the fibres with 2M-sodium chloride. Much of the work with enzymes of the elastase complex (cf. Loeven, 1965*a*) has related to elastins prepared from bovine ligamentum nuchae or aorta. Robert and colleagues (see Robert, Robert & Moczar, 1968*a*) have described the presence of 'structural' glycoproteins in a number of connective tissues, including elastic tissues. These glycoproteins are believed to be closely associated with the fibrous proteins of the tissue, and can be extracted with 8M-urea after prior extraction of the tissue with 1M-calcium chloride, pH 7·5, and hot trichloroacetic acid. Recently (and since the completion of the present study) the same group of workers (Robert *et al.* 1968*b*) have described the extraction by such means of a glycoprotein from human aorta. Sufficient information to permit a detailed comparison with the glycoprotein isolated in the present work is not yet available.

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