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Purification and Characterization of a Glycoprotein from the Intimal Region of Porcine Aorta†

Premanand V. Wagh† and Bruce I. Roberts‡

ABSTRACT: A relatively simple procedure was devised for the isolation and purification of a glycoprotein from the intimal region (150 μ in thickness). This procedure included the extraction of the tissue with buffer at neutral pH and fractionation of the extract with ammonium sulfate followed by column chromatography on diethylaminoethylcellulose. A highly purified glycoprotein obtained by above methods appeared to be homogeneous by ultracentrifugation and polyacrylamide gel electrophoresis. The $s_{25,w}$ value was found to be 4.86 S. The weight-average molecular weight of the glycoprotein determined by sedimentation equilibrium method was 71,000. The glycoprotein did not contain hexuronic acid nor sulfate. The amino acid and carbohydrate composition of the glycoprotein was determined. The carbohydrate moiety consisted of 1 mole each of fucose and sialic acid, 2 moles of mannose, 3 moles each of glucose and galactose, and 4 moles of *N*-acetylhexosamine per molecule of the glycoprotein. Due to the presence of equimolar amounts of glucose and galactose and the absence of hydroxylysine in the molecule, it is suggested that this glycoprotein is unique in its characteristics and hence is of a new type.

Normally, intima may be defined as that layer extending from the endothelium to the internal elastic lamellae of the arteries. It has been recognized that the histochemical, ultrastructural and biochemical properties of the intima are distinctly different from those of the rest of the arterial wall (Lazzarini-Robertson, 1963).

The occurrence of glycoproteins in the mammalian arterial wall has been demonstrated histologically (Bertelsen, 1963), chemically by the presence of sialic acid and of those neutral sugars known to be unassociated with mucopolysaccharides (Murata and Kirk, 1962; Seng *et al.*, 1965), and by direct isolation of these macromolecules from this tissue (Radhakrishnamurthy *et al.*, 1964; Barnes and Partridge, 1968; Moczar and Robert, 1970). In these studies the entire aortic

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‡ This research constitutes a part of the dissertation to be submitted for the Ph.D. in Biochemistry, University of Arkansas, Little Rock, Ark.

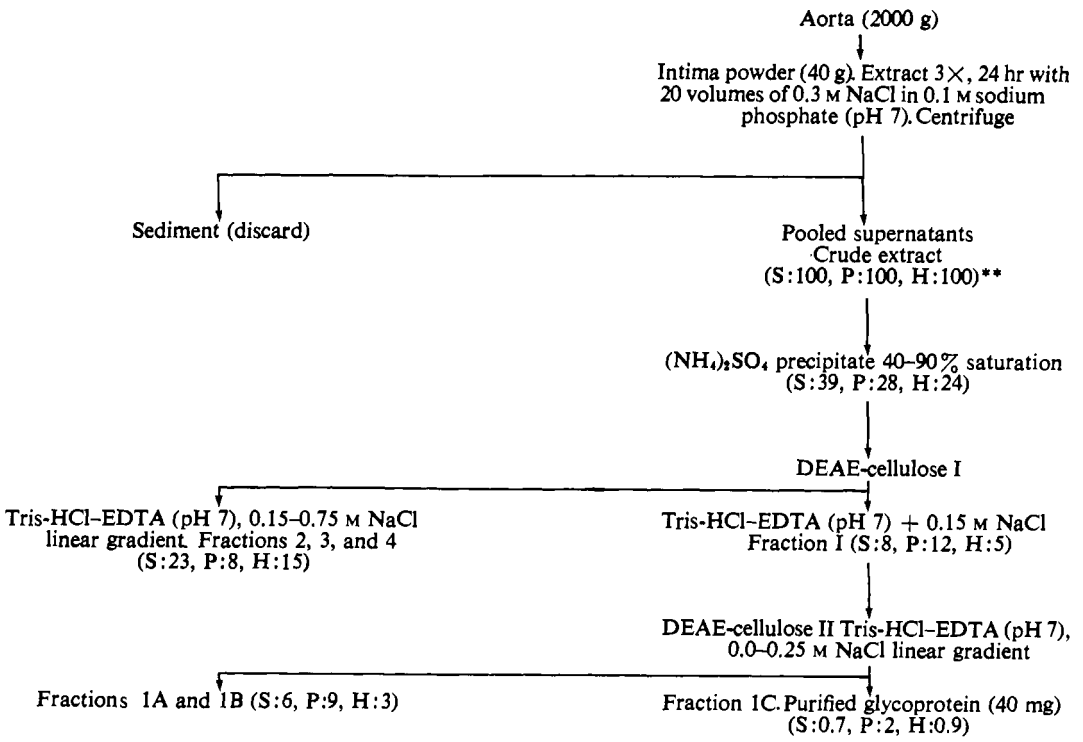

FIGURE 1: The flow sheet for extraction and purification of porcine intimal glycoprotein. **Values represent percentage recoveries relative to those in the crude extract. S = sialic acid; P = protein and H = hexose.

wall was used for the isolation of the glycoprotein. Also, the methods employed during isolation and purification were harsher than generally employed in protein purification (Barnes and Partridge, 1968; Moczar and Robert, 1970).

One of the shortcomings in the isolation of a purified glycoprotein from the intimal region has been to obtain a separation of the internal vascular coat with reasonably uniform thickness from the rest of the aortic wall with least manipulations. Several methods described to date include separation of intima and media with the aid of forceps, scalpel, and magnifying glass or by prying the two tissue layers apart following repeated freezing and thawing in Dry Ice. Often the method used for the separation has not been stated beyond the comment that intima and media were separated as much as possible.

In this communication, we present data on the isolation, purification, and characterization of a glycoprotein from porcine intima. For the purpose of clarity, throughout this paper we have called our preparation of intima as that layer of the thoracic aorta extending from the endothelium toward the media with a thickness of 150 μ.

Experimental Section

Materials. All chemicals were reagent grade. DEAE-cellulose (Cellex-D, 0.7 mequiv/g) and Coomassie Brilliant Blue R-250 dye were purchased from Bio-Rad.

Preparation of Porcine Intimal Powder. Thoracic aortae from freshly killed pigs (about 5 months of age) were packed in ice and brought to the laboratory. They were dissected free from adventitia and the adhering tissue material. The hollow cylindrical blood vessels so obtained were cut longitudinally along the line of intercostal arterial openings. Blood and debris on the intimal surface were removed by rinsing the tissue briefly in distilled water. The specimens were clamped to a rubber mat (approximately 2 in. in thickness) with the entire intimal surface exposed free to the operator. They were stretched under tension with additional clamps to provide a uniform flat surface. Removal of the intimal layer was accomplished using an electric Dermatome (Brown Model 901, Zimmer Manufacturing Co., Warsaw, Ind.).[1] The intimal layer was easily stripped off with a micrometer setting of 10, which corresponded to a cutting depth of 148 ● 37 μ (mean ± standard deviation, 100 observations) as determined by microscopic examination. The sheets of intima were immediately frozen in powdered Dry Ice and ground in a Waring blender. The pulverized material along with the Dry Ice was held in a freezer until all CO_2 had sublimed. The tissue powder was then spread on an aluminum foil and dried at room temperature *in vacuo* in a Virtis biodryer. The dried powder was further ground with liquid nitrogen and was held at −20° until used.

Extraction of Glycoproteins from Intima Powder. The flowsheet of the extraction procedure is shown in Figure 1. In a typical run approximately 40 g of intima powder was processed. Unless stated otherwise, all operations during isolation of the glycoprotein were performed at 4°. Intima powder was extracted three times each for a period of 24 hr with 20 volumes of extraction buffer (0.1 M phosphate buffer containing 0.3 M NaCl, pH 7.0) with constant stirring. At the end of each extraction period, the tissue suspension was centrifuged at 1500*g* for 30 min. The supernatant was decanted and stored. The tissue pellet was then resuspended in 20 volumes of extraction buffer and stirring was resumed. After the final ex-

[1] This instrument employs a blade with rapid cyclic-lateral movement to cut thin tissue sheets on a flat surface and mostly used in surgery by plastic surgeons. The desired cutting depth is achieved by adjusting the settings on the micrometer which control the blade angle with precision. Further details on the use of the Dermatome and histological data will be presented elsewhere.

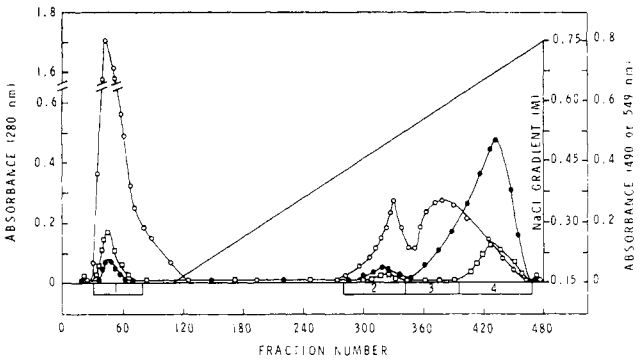

FIGURE 2: Chromatography of ammonium sulfate fraction on DEAE -cellulose (column I). The ammonium sulfate fraction (50 ml) was chromatographed on a column (4 × 40 cm). The elution was initiated with 1.1 liters of standard buffer (see Experimental Procedure) containing 0.15 M NaCl followed by a continuous linear ionic gradient set up between 1.85 l. of standard buffer containing 0.15 and 0.75 M NaCl in the same buffer. Fractions of 10 ml were collected. The flow rate was 60 ml/hr. (○) Absorbance at 280 nm; (●) absorbance at 490 nm/0.5 ml, pheno-sulfuric acid test for carbohydrate; (□) absorbance at 549 nm/1.0 ml, thiobarbituric acid test for sialic acid. Numbered areas under peaks represent fractions which were pooled.

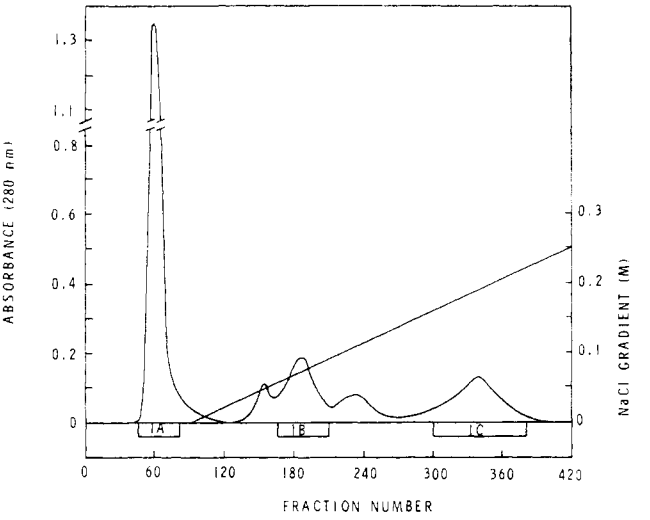

FIGURE 3: Chromatography of fraction 1 on DEAE-cellulose (column II). Fraction 1 (100 ml in standard buffer, see Experimental Procedure) was chromatographed on a column (4 × 55 cm). The column was eluted with 720 ml of standard buffer and the elution was continued with a continuous linear ionic gradient between 1.32 l. of standard buffer and 0.25 M NaCl in the same buffer. Fractions of 8 ml were collected. The flow rate was 60 ml/hr. Numbered areas under peaks represent fractions which were pooled.

traction, the supernatants were combined, centrifuged at 2000*g* for 1 hr, and filtered through glass wool. The resulting clear solution is referred to as the crude extract.

Ammonium Sulfate Precipitation. To the above crude extract (without dialysis), 24.3 g of solid ammonium sulfate/100 ml of the extract were added under continuous stirring, to bring the mixture to 40% ammonium sulfate saturation. The solution was stirred for 16 hr and then centrifuged at 10,000*g* for 20 min. The precipitate was discarded. The level of ammonium sulfate saturation in the supernatant was raised to 90% by addition of 37.5 g of solid ammonium sulfate/100 ml of the supernatant. The solution was stirred for 16 hr and was centrifuged at 10,000*g* for 60 min. The precipitate was taken up in 150 ml of 0.005 M Tris-HCl buffer containing 0.001 M EDTA (pH 7.0) (hereafter called the standard buffer), and was exhaustively dialyzed against the same buffer. The dialyzed material was lyophilized, redissolved in 50 ml of the standard buffer and redialyzed against the same buffer exhaustively. A small amount of insoluble material appearing in the dialysis bag was removed by centrifugation and the supernatant was designated ammonium sulfate fraction.

DEAE-cellulose Chromatography. A column of DEAE-cellulose (4 × 40 cm, column I) was equilibrated with the standard buffer. All of the ammonium sulfate fraction (approximately 50 ml) from the preceding step was applied to the column. The elution of the column was initiated with the standard buffer containing 0.15 M NaCl (1.1 l.) followed by a continuous linear gradient between 0.15 and 0.75 M NaCl in the standard buffer. Fractions of 10 ml were collected. The elution diagram is shown in Figure 2 and indicates various fractions pooled.

The fraction between 300 and 800 ml was dialyzed extensively against distilled water. A small amount of precipitate which occurred upon dialysis was centrifuged off. The supernatant was lyophilized and dissolved in 100 ml of standard buffer. This fraction, designated 1, was applied to a second DEAE-cellulose column (4.0 × 55 cm, column II) preequilibrated with the standard buffer. The column was eluted with standard buffer (720 ml) followed by a continuous linear gradient between 0.0 and 0.25 M NaCl in the standard buffer. Fractions of 8 ml were collected. The elution diagram is shown in Figure 3. The fraction between 2.40 and 3.04 l. was designated 1C and was found to be the purified glycoprotein. This fraction was extensively dialyzed against water and lyophilized and is the subject of present communication.

Disc Electrophoresis. Analytical polyacrylamide gel electrophoresis was performed on the purified glycoprotein preparation at pH 8.4 (Davis, 1964). Gels were stained and destained by the method of Catterall and Pedersen (1971) using Coomassie Brilliant Blue R-250 dye.

Ultracentrifugation. Both sedimentation velocity and sedimentation equilibrium measurements were performed in a Beckman Spinco Model E analytical ultracentrifuge. A 4.9-mg/ml solution of purified glycoprotein in 0.1 M NaCl was used for sedimentation velocity run at 59,780 rpm (25°). The weight-average molecular weight was determined by sedimentation equilibrium method of Yphantis (1964) using Rayleigh interference optics. The fringes were measured employing a Nikon microcomparator. A 0.4-mg/ml solution of glycoprotein in 0.1 M NaCl was used. The centrifugation proceeded for 24 hr at 25,980 rpm at 15.3°. The speed was then reduced to 21,740 rpm and the temperature lowered to 11°. Equilibrium was attained after an additional 16 hr.

Analytical Methods. Effluents from columns were monitored for protein content by measuring absorbance at 280 nm (Warburg and Christian, 1942). Total protein content in pooled samples during purification was determined by the method of Lowry *et al.* (1951). Total hexose content was estimated by the phenol–sulfuric acid method (Dubois *et al.*, 1956) employing galactose as standard. Sialic acid was determined by the procedure of Aminoff (1961) after hydrolysis of the samples in 0.1 N HCl at 80° for 1 hr. Hexosamine was quantitated using the method of Gatt and Berman (1966) employing galactosamine as the standard and values were reported as *N*-acetylhexosamine. For hexosamine determination, the samples were hydrolyzed in 2 N HCl at 100° for 16 hr. Hexuronic acid was estimated by the carbazole method according

to Bitter and Muir (1962). Sulfate content was measured by the method of Antonopoulous (1962).

Quantitation of individual monosaccharides by gas–liquid chromatography was accomplished after derivatizing the neutral sugars to their corresponding alditol acetates. Samples of purified glycoprotein weighing approximately 5 mg was hydrolyzed for 48 hr in a 40% (w/v) suspension of Dowex 50-X2 resin in 0.02 N HCl. Arabinose was added as an internal standard. All other steps were essentially similar to those described by Lehnhardt and Winzler (1968). A Barber Colman (Series 5000) gas–liquid chromatograph equipped with a hydrogen flame ionization detector was used for the quantitation of the alditol acetates. The procedure of Neidermeir (1971) was followed since this method completely resolved glucose and galactose.

Amino acid analyses were performed with a Beckman 120B analyzer equipped with an accelerated system (Spackman *et al.*, 1958). Samples of 1–1.5 mg were hydrolyzed in 1 ml of 6 N HCl at 110° for 24, 48, and 72 hr. After removal of acid in a biodryer, the residue was dissolved in 2 ml of sodium citrate buffer (0.2 M Na) (pH 2.2). Aliquots of 0.5 ml were directly applied to each column.

Half-cystine content was determined independently as cysteic acid after performic acid oxidation (Moore, 1963). Tryptophan was estimated spectrophotometrically by the method of Goodwin and Morton (1946).

Partial specific volume of the glycoprotein was calculated from the amino acid and carbohydrate composition (McMeekin and Marshall, 1952; Gibbons, 1966).

Results

Preparation of Intima. The intimal layer separated with the aid of a Dermatome was approximately 150 μ in thickness. As judged histologically, the preparations contained elastic fibers indicating that a portion of the inner media was also in association with intima. The thickness of the porcine aorta (excluding adventitia) was found to be 1.25 mm. Therefore, our intimal preparations represent 12% of the total thickness of intima plus media.[2]

Extraction and Purification of Intimal Glycoprotein. A flowchart illustrating the procedure for extraction and purification of the glycoprotein is shown in Figure 1. The dry weight of the intimal powder represented 2% of the wet weight of fresh aorta and had the following composition: sialic acid, 2.8 μg; hexose, 9.8 μg; and hexosamine, 10 μg per mg of intima powder.

The analyses of the carbohydrate constituents in the crude extract obtained from three sequential extractions revealed that 20, 30, and 25% of the sialic acid, hexose and hexosamine, respectively, were extracted from the total amount of each constituent present in the intima. The amount of protein extractable was 54 μg of protein/mg of intima powder. The values given in parentheses in Figure 1 indicate percentage recoveries relative to 100% of each material in the crude extract and represent averages from three different experiments which were quite reproducible.

Ammonium sulfate fractionation of the crude extract resulted in a recovery of 40% sialic acid, 30% of protein, and 20% of hexose occurring in the crude extract. Although this fractionation procedure resulted in low recoveries of

[2] The measurements of the thickness of our intima preparations and histological evaluations were kindly performed for us by Dr. L. M. Krista, Department of Anatomy, Auburn University, Ala.

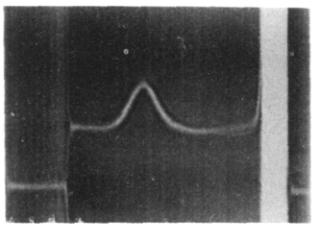

FIGURE 4: Schlieren sedimentation pattern of purified glycoprotein. A solution of glycoprotein in 0.1 M NaCl at a concentration of 4.9 mg/ml was used. This photograph was taken after 64 min at a speed of 59,780 rpm and 25°.

of carbohydrate constituents, the ratio of sialic acid to protein was increased about 1.5-fold. It was apparent that a large amount of glycoproteins was present in 0–40% ammonium sulfate fraction. However, we included this step in our attempts to obtain an enriched sialoprotein.

Chromatography of the ammonium sulfate fraction on DEAE-cellulose column I resulted in the isolation of four fractions designated as 1, 2, 3, and 4 in Figure 2. Fraction 1 contained about 25% of sialic acid and 40% of the protein applied to the column. This fraction was free of hexuronic acid and sulfate, indicating the absence of mucopolysaccharides. We have noted that eliminating the ammonium sulfate fractionation step and application of the crude extract directly to the DEAE-cellulose column results in a significant amount of hexuronic acid in fraction 1. Interestingly, mucopolysaccharides and other glycoprotein material remaining in ammonium sulfate fraction (most likely to be more acidic than fraction 1) were tightly bound to the column and were only eluted at high ionic strength. These fractions designated 2, 3, and 4 have been stored for future study.

Fraction 1 from DEAE-cellulose column I was used for further purification by chromatography on DEAE-cellulose column II. The elution diagram is shown in Figure 3. Five distinct protein peaks were observed. Sialic acid was detected in fractions designated 1A, 1B, and 1C. Fractions 1A and 1B appeared to be heterogeneous. The yield of fraction 1C, the purified glycoprotein, was 40 mg/40g of intima powder.

Criteria of Homogeneity and Determination of Molecular Weight. Sedimentation velocity studies indicated a high degree of homogeneity of the preparation (Figure 4). The value for the apparent sedimentation coefficient, $s_{25,w}$ was found to be 4.86 S. This value corresponds to a molecular weight of about 70,000 for globular proteins (Sober, 1968). The molecular weight of the purified glycoprotein was assessed by sedimentation equilibrium. A linear dependence of ln of fringe displacement and γ^2 was found. The weight-average molecular weight of 71,000 was calculated using a least-squares fit of the experimental points. A value of 0.733 for partial specific volume calculated from amino acid and carbohydrate composition of the glycoprotein was used. The purified glycoprotein moved in polyacrylamide gel as a single component (Figure 5).

Composition of Glycoprotein. The amino acid and carbohydrate composition of the purified glycoprotein is shown in Table I. The sum of the values in the first column, representing the amino acid and carbohydrate content on a weight percentage basis, show a recovery of 100.98%. This yield of almost 100% is a gross check on the reliability of the experimental results. The molecule exhibits a large preponderence of acidic over the basic groups which is consistent with its behavior on DEAE-cellulose. The glycoprotein did not con-

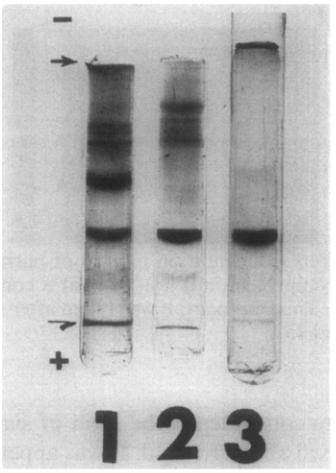

FIGURE 5: Disc electrophoresis on polyacrylamide gel of the crude extract, fraction 1, and the purified glycoprotein. Sample size approximately 25–35 μg of protein. (1) Crude extract; (2) fraction 1 (from DEAE-cellulose column I); (3) purified glycoprotein (fraction 1C from DEAE-cellulose column II); →, junction of the spacer and separation gels; —, dye marker.

tain hydroxyproline nor hydroxylysine. The carbohydrate portion of the molecule contained one residue each of fucose and sialic acid, two residues of mannose, three each of galactose and glucose, and four of *N*-acetylhexosamine per molecule of purified glycoprotein. It was assumed that lower value of fucose was due to hydrolytic loss. However, it always occurred as a measurable peak on the chromatogram.

Discussion

Interest in the knowledge of structure and function of intimal layer of aortic wall may be mainly due to its unique location where the intimal surface exposed to continuous hemodynamic insult is subject to alterations so as to initiate a number of pathological events such as atherosclerosis, dissecting aneurysms, etc. For such biochemical studies, therefore, it is essential to acquire intimal tissue nearest to the endothelium which is uniform in thickness. The studies described herein indicate that this goal has been attained. It was estimated that the isolated intimal tissue was histologically uniform in thickness and represented 12% of the total thickness of the aortic wall excluding adventitia.

Unless the stability of a tissue glycoprotein relative to the conditions by which it is isolated is known, it is customary that mild conditions for extraction and purification should be employed. Radhakrishnamurthy *et al.* (1964) employing mild procedures have isolated a purified glycoprotein from bovine aorta. Barnes and Partridge (1968) also have described a glycoprotein preparation from human aorta. Their conditions appear to be somewhat drastic since extraction of the glycoprotein was carried out in 0.1 N NaOH. Moczar and Robert (1970), on the other hand, have attempted the isolation of glycoproteins from the media of the thoracic aorta. The glycoprotein preparations which they have designated as water-soluble and urea-soluble structrual glycoproteins were derived after treatment of the tissue material with 2.7% trichloroacetic acid at 90° up to 30 min. Such a treatment undoubtedly must have destroyed considerable sialic acid and several other characteristics of the native molecule. Thus far, isolation and purification of a glycoprotein from defined intimal region and employing mild procedures have never been reported.

TABLE I: Amino Acid and Carbohydrate Composition of Purified Porcine Intimal Glycoprotein.

Component	Residues/Glycoprotein		Nearest Integral Residue
	Per 100 g	Per Mole[a]	
Amino acids			
Lysine	8.61 ± 0.09	47.7	48
Histidine	2.76 ± 0.13	14.3	14
Arginine	4.80 ± 0.44	21.8	22
Aspartic acid	9.62 ± 0.28	59.3	59
Threonine	3.79[b]	26.6	27
Serine	3.43[b]	22.9	23
Glutamic acid	14.65 ± 2.08	80.5	81
Proline	4.80 ± 0.18	35.1	35
Glycine	2.52 ± 0.26	31.3	31
Alanine	4.92 ± 0.23	49.0	49
Half-cystine	5.01[c]	34.5	35
Valine	4.71[d]	33.7	34
Methionine	0.55 ± 0.01	2.9	3
Isoleucine	3.75 ± 0.11	23.5	24
Leucine	11.75 ± 0.20	73.7	74
Tyrosine	5.20 ± 0.19	22.6	23
Phenylalanine	5.69 ± 0.27	27.4	27
Tryptophan	1.36[e]	5.2	5
Carbohydrates			
Fucose	0.09 ± 0.01	0.4	1
Mannose	0.37 ± 0.02	1.6	2
Galactose	0.56 ± 0.02	2.5	3
Glucose	0.59 ± 0.02	2.6	3
N-Acetylhexosamine	1.05[f]	3.6	4
N-Acetylneuraminic acid	0.40	0.97	1
Total	100.98	623.7	628

[a] Based on a molecular weight of 71,000. [b] Extrapolation to zero time. [c] Single determination as cysteic acid by performic acid oxidation. [d] 48-hr hydrolysis. [e] Spectrophotometric determination relative to tyrosine. [f] Determined as galactosamine.

The data accrued from our studies reveal that purification of the glycoprotein from the crude extract can be accomplished in four steps. By our procedure, one can readily separate the bulk of the glycoprotein from mucopolysaccharides. Although the intention of our communication was to provide information on the highly purified preparation, we have realized that the intimal tissue containes several more glycoproteins.

The carbohydrate composition of the glycoprotein from porcine intima appeared to be unique in view of the fact that it contained equimolar amounts of glucose and galactose. One of the well-known examples wherein such a disaccharide exists is collagen. We cannot assign this to any collagen impurities because we have ascertained that the purified glycoprotein did not contain hydroxyproline nor hydroxylysine. Glucose, therefore, is an integral part of intimal glycoprotein. This observation is consistent with the findings of Radhakrishnamurthy and Berenson (1966) who have presented evidence for a glycopeptide isolated from bovine aorta glyco-

protein containing glucose and galactose. Preliminary studies regarding the nature of the carbohydrate–peptide linkage suggest that the linkage is stable to alkali and may be therefore N-glycosidic or of some other type which is not susceptible to β-elimination.

Berenson *et al.* (1966) isolated a water-soluble glycoprotein fraction from human aorta. This fraction upon polyacrylamide gel electrophoresis was resolved into several components, some of which, it was concluded from immunological observations, to be distinct from plasma glycoproteins. Thus far, the occurrence of a glycoprotein in circulating plasma with similar composition as that of our preparation has not been reported. It is therefore likely that the intimal glycoprotein reported here may be synthesized *de novo*. Richard *et al.* (1972) have reported the presence of a mannosyl transferase in the microsomal fraction isolated from bovine intimal preparations. Indeed, if the glycoproteins of the type we have isolated are synthesized in the intima, a specific glycosyl transferase system in the intimal layer must exist.

Acknowledgements

We express our thanks to Drs. Raymond C. Read and Harold J. White for providing us facilities and their encouragement during this work. We are indebted to Dr. Charles A. Nelson for his assistance and advice in ultracentrifugation studies and to Dr. LaVerne M. Krista for counseling us on our tissue preparations. We also thank Mrs. Diane Butler for secretarial assistance.

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