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Intestinal Glycoproteins of Germfree Rats. III. Characterization of a Water-soluble Glycoprotein Fraction*

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A water-soluble glycoprotein fraction was obtained by fractionation of crude intestinal or fecal extracts from germfree rats. This procedure involved proteolytic digestion with pronase, precipitation of the acidic glycoprotein with cetyltrimethylammonium bromide (CTAB), and chromatography on DEAE-Sephadex and on Sepharose 4 B. The purified fractions obtained from intestinal and fecal material, respectively, were very similar in chemical composition and properties. The mucin-type glycoprotein contained 23 % of galactose, 8.6 % of fucose, 18.6 % of *N*-acetylgalactosamine, 18.2 % of *N*-acetylglucosamine, 13.5 % of sialic acid, 1.3 % of sulphate groups, and *ca.* 15 % of amino acids of which threonine, serine, and proline were the predominant ones. The results of gel electrophoresis and of sedimentation equilibrium indicated some polydispersity, consistent with previous findings for mucin glycoproteins. Incubation of the glycoprotein with a neuraminidase from *Clostridium perfringens* removed 57 % of the sialic acid, and an α-(1→2)-L-fucosidase isolated from the same bacterium released 71 % of the fucose residues. The glycoprotein was shown to possess blood-group A and H activity but was virtually free of B activity.

The epithelial surface of the mammalian intestinal tract is protected by a flowing layer of mucus continuously produced by secretory cells of the intestinal mucosa. This viscous layer not only serves as a mechanical protective barrier, but also contributes to maintain a relatively constant pH and ion concentration in the environment of the tender microvilli.

The intestinal mucin secretions are extensively degraded by the microflora of the digestive tract,[1,2] a fact which complicates the isolation and the study of the native product. Furthermore, mucin-containing extracts from mucosal scrapings and especially from intestinal contents are contaminated with other high-molecular-weight components. But, despite the difficulty in obtaining pure and undegraded mucin preparations, it has been demonstrated that mammalian intestinal mucins are composed of glycoproteins [3,4] that contain, like other glycoproteins obtained from mammalian mucins, carbohydrate residues linked by an *N*-acetylgalactosamine residue to serine and threonine residues in the polypeptide chain.[5]

By the use of animals devoid of any microflora in the digestive tract it is possible to obtain the intestinal glycoprotein in a yield better than that obtained from conventional animals. In addition, feeding the animals an entirely synthetic diet [6] consisting only of low-molecular-weight substances reduces the amount of exogenous macromolecules in the intestinal contents, *e.g.* from food components, to an insignificant level. Under germfree conditions one might expect the intestinal mucin to pass essentially unaltered through the digestive tract, which results in an excretory fecal mucin of a composition similar to the intestinal one. The present report describes the purification and characterization of a water-soluble, intestinal glycoprotein fraction and a corresponding fecal glycoprotein fraction, obtained from germfree rats fed a chemically defined diet.

* Part II. See Ref. 7.

RESULTS

The successive steps of the purification procedure are given in Fig. 1. The water-soluble, non-dialysable extract initially obtained,[6] still contained lipid material that was removed by exhaustive extraction with chloroform-methanol mixtures. This treatment was found to facilitate the subsequent fractionation steps. Incubation with pronase resulted in the degradation of contaminating protein, whereas the mucin appeared unaffected by the proteolysis since no retardation of the major carbohydrate-containing material was observed by gel chromatography on Sepharose 4 B, as compared with the non-proteolysed material. Addition of cetyltrimethylammonium bromide (CTAB) to a solution of the pronase-treated, non-dialysable fraction precipitated a large proportion of the mucin glycoprotein, leaving less acidic material in solution. This step removed most of the pentose-containing glycan which is thought to stem from previously given diet.[7] After dissociation of the methanol-soluble, CTAB-glycoprotein complex, the recovered material was subjected to chromatography on DEAE-Sephadex A 25 and, finally, on Sepharose 4 B (Figs. 2 and 3).

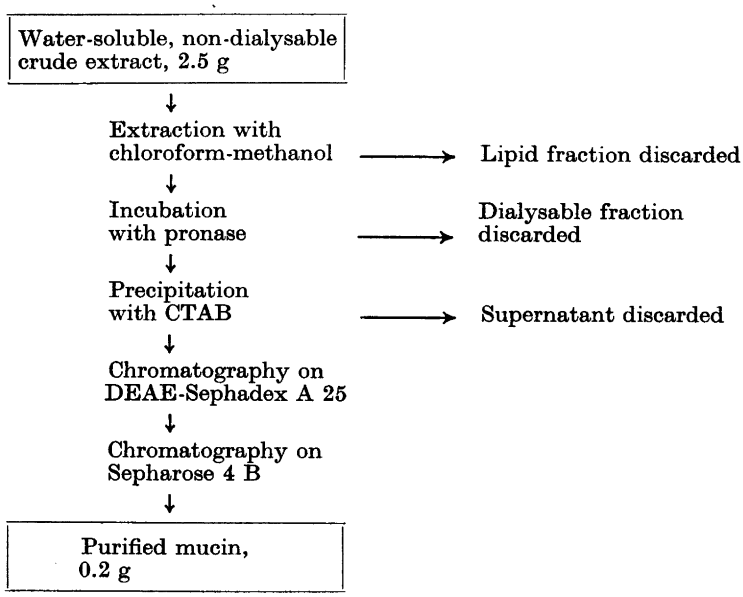

Fig. 1. Schematic diagram of the purification of germfree rat intestinal mucin.

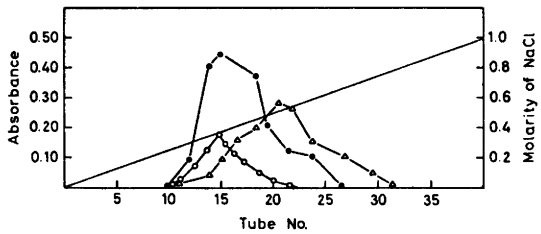

Fig. 2. Chromatography on DEAE-Sephadex A 25 of the material precipitated by CTAB. Effluent fractions were analysed for neutral sugar (●), sialic acid(○), and protein (△). Fractions 11 to 19 were pooled, concentrated, and subjected to gel chromatography on Sepharose 4 B. Experimental details are given in the text.

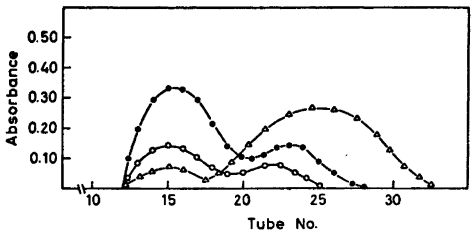

Fig. 3. Chromatography on Sepharose 4 B of fractions 11 to 19 from the DEAE-Sephadex column (Fig. 2). The effluent was analysed for neutral sugar (●), sialic acid (○), and protein (△). Fractions 13 to 17 were pooled, dialysed and lyophilized. Experimental details are given in the text.

The front peak eluted from the Sepharose 4 B column (Fractions 13 – 17) contained the purified glycoprotein; its carbohydrate composition and sulphate content are given in Table 1 and its amino acid composition in Table 2. The second peak obtained from the Sepharose column (Fractions 20 – 25) contained, in addition to the sugars present in the first peak (Table 1), mannose *ca.* 1 %, and smaller amounts of arabinose and xylose. The supernatant solution left after the precipitation with CTAB also contained glycoprotein material that could be recovered by chromatography on Sepharose 4 B, the glycoprotein fraction appearing in the front peak. It had a carbohydrate and amino acid composition similar to that of the purified glycoprotein, but was less acidic and contained some mannose, arabinose, and xylose (Table 3).

The infrared spectrum of the purified glycoprotein showed absorption bands at 3400 cm^{-1} (broad, O – H stretching), at 1640 and 1540

Table 1. Carbohydrate and sulphate content of purified glycoprotein from intestinal and fecal extract.

Residues	Intestinal glycoprotein			Fecal glycoprotein		
	%	molar ratio	μmol/100 mg	%	molar ratio	μmol/100 mg
Galactose	23.0	2.9	128	22.6	2.8	126
Fucose	8.6	1.2	53	8.8	1.2	54
N-Acetylgalactosamine	18.8	1.9	85	19.0	1.9	86
N-Acetylglucosamine	18.2	1.9	82	18.2	1.8	82
N-Acetylneuraminic acid	6.3	1.0	20	6.6	1.0	21
N-Glycolylneuraminic acid	7.2		22	7.2		22
Sulphate	1.3	0.3	13	1.4	0.3	14

Table 2. Amino acid composition of purified glycoprotein from intestinal and fecal extract.

Amino acid	Intestinal glycoprotein		Fecal glycoprotein	
	%	μmol/100 mg	%	μmol/100 mg
Aspartic acid	0.60	4.6	0.67	5.1
Threonine	6.82	57.0	6.73	56.3
Serine	2.20	21.0	2.18	20.8
Glutamic acid	0.73	5.0	0.82	5.6
Proline	2.43	21.1	2.31	20.2
Glycine	0.32	4.3	0.40	5.3
Alanine	0.31	3.5	0.37	4.1
Valine	0.72	6.2	0.75	6.4
Isoleucine	0.45	3.5	0.51	3.9
Leucine	0.12	0.9	0.17	1.3
Lysine	0.23	1.6	0.19	1.3

Table 3. Carbohydrate and amino acid composition of the glycoprotein fraction soluble in the presence of CTAB; the material was eluted in the front fraction by chromatography on Sepharose 4 B.

Carbohydrate		Amino acid	
Residues	%	Residues	%
Galactose	28.6	Aspartic acid	0.70
Mannose	0.3	Threonine	5.45
Fucose	11.8	Serine	2.30
Arabinose	0.4	Glutamic acid	0.51
Xylose	0.9	Proline	2.20
N-Acetylgalactosamine	19.7	Glycine	0.25
N-Acetylglucosamine	19.6	Alanine	0.60
Sialic acid	5.0	Valine	0.23
Sulphate	0.0	Isoleucine	0.21
		Leucine	0.19
		Lysine	0.32

cm^{-1} (acetamido group), at 1240 cm^{-1} (S=O stretching in sulphate group), and at 820 cm^{-1} (C–O–S vibration), indicative of primary alcohol sulphate ester groups.

Gel electrophoresis of the purified glycoprotein in 2 % polyacrylamide – 0.5 % agarose or in 1 % agarose, either in the presence or the absence of sodium dodecyl sulphate, resulted in a single, broad band. At a polyacrylamide concentration of 7.5 %, the whole glycoprotein sample remained at the sample-running gel interphase, whereas a more porous gel containing 5 % polyacrylamide allowed a partial penetration of the glycoprotein into the running gel. The glycoprotein gave a strong reaction for carbohydrate with the periodic acid-Schiff reagent; it could also be detected with Toluidine Blue, owing to its content of sialic acid and sulphate residues, but no bands were revealed with Coomassie Brilliant Blue or with Amido Black under standard conditions for protein staining; this would preclude any significant protein contamination. The failure of carbohydrate-rich glycoproteins to react with protein-stain reagents in polyacrylamide and agarose gels has been observed previously.[8] Treatment of the glycoprotein with dithioerythritol in a 0.05 M Tris chloride buffer of pH 7.8 and subsequent Sepharose gel chromatography in the same buffer, with or without 7 M urea, gave no change in the usual chromatographic elution pattern. Thus, the possibility of polypeptide chains linked by disulphide bridges seems to be ruled out; this is consistent with the absence of cysteine residues in the glycoprotein. The molecular weight was estimated by determination of the sedimentation equilibrium. After the sample had been for 24 h in the ultracentrifuge, an almost straight line was obtained when log c was plotted against r^2; the extrapolation of the curve indicates a molecular weight of 550 000 dalton. After a 48 h run, the curve showed a sharp deflection towards the bottom of the cell, corresponding to a M. W. value in the 1.5 to 2 million range; however, this part of the curve is bound to indicate inaccurate molecular weights. The lower M. W.-limit value calculated after the 48 h run was 390 000. A glycoprotein sample not treated with pronase was subjected to the same procedure, and the curves obtained for the proteolysed and non-proteolysed material were very similar, both in 24 and 48 h ultracentrifuge runs, revealing no essential difference in molecular weight values.

Incubation of the glycoprotein with a *Clostridium perfringens* neuraminidase decreased the sialic acid content from 13.5 to 5.8 %; repeated treatment with the same enzyme failed to liberate any additional sialic acid. Paper chromatographic examination of the sialic acid released showed the presence of approximately equal proportions of *N*-acetyl- and *N*-glycolyl-neuraminic acid. Incubation with trypsin or renewed incubation with pronase of the neuraminidase-treated glycoprotein was followed by chromatography on Sepharose 6 B. No change of the

chromatographic elution curve was observed after proteolysis.

Treatment of the glycoprotein with a *Clostridium perfringens* α(1→2)-L-fucosidase and subsequent fractionation of the digest on Sepharose 6 B yielded a glycoprotein fraction having a fucose content of 2.5 %, a reduction from the value of 8.6 % observed for the original compound. The low-molecular-weight fraction eluted from the Sepharose column contained fucose and a minute proportion of galactose, as shown by GLC.

The glycoprotein was tested for blood-group activity by the hemagglutination-inhibition technique. At a concentration of 1 mg/ml, the glycoprotein inhibited the agglutination of human red A cells by human anti-A serum and the agglutination of human red O cells by human anti-H serum or by *Ulex europeus* H-lectin. Thus A- and H-blood-group activity was clearly demonstrated. On the other hand, the mucin had almost no inhibitory effect in the human red B cell – anti-B cell system.

DISCUSSION

Preparation and characterization of glycoproteins presents a major problem in view of the known microheterogeneity of these polymers.[9] All mucin-type glycoproteins studied so far appear to possess microheterogeneity, both with respect to molecular weight and charge, and the purification of these compounds leads to fractions comprising closely related but mostly nonidentical molecules.

The purified glycoprotein obtained (*ca.* 8 %) is only a part of the total mucin content of the original crude extract, namely the final product of the particular fractionation scheme employed. The use of a different purification procedure would probably have given fractions with compositions different from those observed. Like other mucin-type glycoproteins studied,[5,8,10] the germfree-rat intestinal glycoprotein was clearly polydisperse. It gave a broad band on gel electrophoresis and a broad peak on Sepharose 4 B gel chromatography (Fig. 3). The result of the 48 h sedimentation equilibrium experiment indicated a polydispersity greater than expected. However, aggregation may occur to some extent during prolonged periods of centrifugation and it is difficult to ascertain the effect of molecular aggregation in evaluating the polydispersity.[11]

The present study demonstrates a close similarity between the purified glycoprotein preparations of the intestinal and the fecal extracts. No difference could be observed in the chromatographic elution curves from columns of DEAE-Sephadex or Sepharose, and identical bands were obtained on gel electrophoresis. The carbohydrate and amino acid composition of the two products were strikingly similar (Table 1 and 2). These data suggest that the soluble or brush border-bound enzymes which may degrade or modify the glycoprotein during its passage through the intestinal tract had no effect, in agreement with the observation that the glycoprotein is resistant to trypsin and pronase, even after partial removal of sialic acid residues. In addition to the content of carbohydrate and protein the purified glycoprotein fractions invariably contained *ca.* 1 % of sulphate groups. Since uronic acid residues were absent, contamination with sulphate-containing polysaccharides was precluded. The infrared spectrum of the glycoprotein showed absorption bands characteristic of sulphate ester groups. The presence of a band at 820 cm^{-1} and the absence of the band at 850 cm^{-1} suggested [12] that the sulphate groups were most probably located at C-6 of the galactose or hexosamine residues. Bella and Kim [5] have recently suggested that nearly 40 % of the sulphate groups present in an acidic glycoprotein of the small intestine of the rat were *N*-linked, since they were released by mild acid hydrolysis. When the glycoprotein of the germfree rat was analysed for *N*-sulphate groups by the nitrous acid procedure [13] no such groups could be detected, in agreement with a similar observation made on a glycoprotein of dog gastric mucosa by Pamer *et al.*[14]

Repeated neuraminidase treatment released only 57 % of the total sialic acid present in the glycoprotein. The resistance to neuraminidase may possibly be due to *O*-acetyl groups at C-4 of the sialic acid residues, since Schauer and Faillard [15] have shown that the *N*-acetyl-4-*O*-acetylneuraminic acid groups of horse submaxillary glycoprotein were not split off by *Clostridium perfringens* neuraminidase. In contrast, the authors found that *N*-acetyl-7-*O*-acetyl-, *N*-acetyl-8-*O*-acetyl-, and *N*-acetyl-7,8-

di-*O*-acetylneuraminic acid residues of bovine submaxillary glycoprotein were all released by the same enzyme.

One incubation of the glycoprotein with a specific α-(1→2)-L-fucosidase [16] removed 71 % of the total fucose content. Studies on hog submaxillary [17] and gastric [18] glycoproteins have established that an α-L-fucopyranosyl residue is linked to C-2 of a β-D-galactose residue. Since a terminal *O*-α-L-fucopyranosyl-(1→2)-β-D-galactose moiety is a prerequisite for blood-group H activity, this disaccharide unit probably constitutes the nonreducing end group of, at least, some of the carbohydrate chains in the intestinal glycoprotein of the germfree rat. Further, the serological demonstration of blood-group A activity of the glycoprotein suggests also the presence of chains terminated with the sequence *O*-2-acetamido-2-deoxy-α-D-galactopyranosyl-(1→3)-[*O*-α-L-fucopyranosyl-(1→2)]-β-D-galactose.

EXPERIMENTAL

Prior to analysis, samples were dried in the presence of phosphorus pentaoxide *in vacuo;* solutions were concentrated under reduced pressure with an outside bath temperature of 35 – 40°.

Analytical methods. Quantitative carbohydrate analysis was performed by gas-liquid chromatography (GLC) on a Perkin-Elmer 900 gas chromatograph. After methanolysis the methyl glycosides were converted into the per-(trimethyl)-silyl ethers and separated on a column (150 × 0.3 cm) packed with 0.1 % OV-17 on glass beads (No. GLC 110, 120/140 mesh), as described by Reinhold.[19] Methanolysis of the mucin sample (1 mg) with *myo*-inositol (40 μg) as the internal standard was effected with 1 M hydrogen chloride in methanol (1 ml) at 85° for 20 h. For simultaneous estimation of *N*-acetyl- and *N*-glycolylneuraminic acid, methanolysis was performed with 0.5 M hydrogen chloride in methanol at 65° for 1 h. Sialic acid, free or glycosidically bound, was also estimated by the periodate-resorcinol method of Jourdian *et al.*[20] with the following modification: After the oxidation step, 0.2 ml of 0.1 M sodium arsenite was added and the solution kept for 2 – 3 min at room temperature until the brown colour had disappeared. Then the resorcinol reagent was added and the assay completed as described. The original procedure [20] led to coloured blanks and unsatisfactory reproducibility; however, conversion of the excess of periodate into iodate prior to the hydrochloric acid-resorcinol treatment resulted in colourless blanks and reproducible results.

Amino acids were estimated by GLC of the derived *N*-trifluoroacetyl 1-butyl esters on a column of 0.325 % EGA on Chromosorb W;[21] 1-butyl stearate was used as the internal standard. The glycoprotein sample (2 mg) was hydrolysed with 6 M hydrochloric acid (1 ml) at 110° for 18 – 20 h under nitrogen. After evaporation of the hydrolysate under a stream of nitrogen, the residue was dissolved in water and the insoluble material centrifuged off. The clear supernatant was concentrated to dryness, and the amino acid hydrochlorides converted into the *N*-trifluoroacetyl 1-butyl esters.[21]

Total sulphate groups were determined by the barium chloroanilate method of Spencer,[22] and *N*-sulphate groups by the method of Lagunoff and Warren.[13]

The elution of columns of DEAE-Sephadex and Sepharose was monitored by the phenol-sulphuric acid test,[23] by the method of Lowry *et al.*,[24] and by the periodate-resorcinol test [20] for neutral sugars, protein, and sialic acid, respectively.

Gel electrophoresis of glycoprotein samples (0.1 mg) was performed in 0.09 M Tris-borate buffer, pH 8.2, in 0.5 % agarose – 2 % polyacrylamide or in 1 % agarose (Agarose for electrophoresis, General Biochemicals), as described by Holden *et al.*,[8] some experiments being done in gels containing 0.1 % sodium dodecyl sulphate. In addition gels were stained with 0.2 % Toluidine Blue in 0.1 M acetic acid.[25]

Infrared spectra were recorded for glycoprotein samples (1.5 – 2 mg) in potassium bromide discs with a Perkin-Elmer Model 237 spectrophotometer.

Incubation with neuraminidase (Type V, Sigma, purified from *Clostridium perfringens*) was performed by adding 0.02 units of the enzyme to the sample (20 mg) in 0.05 M sodium acetate buffer, (5 ml) pH 5.4, and keeping the digest at 35° for 24 h under toluene. The same amount of enzyme was added and the incubation continued for 24 h. The digest was then concentrated to 1 ml, applied to a column (1.2 × 45 cm) of Sepharose 6 B and eluted with 0.01 M pyridine – acetate buffer, pH 4.8, 2 ml fractions being collected. The glycoprotein fraction was eluted in the front peak and isolated by lyophilization; the following low-molecular-weight fraction obtained was deionized by passage through a column of Dowex 50 (H^+), and the acidic eluate concentrated at room temperature. The resulting syrup was analysed by paper chromatography in the solvent system 1-butanol-1-propanol-0.1 M hydrochloric acid, 1:2:1 (v/v), and the spots were stained with the thiobarbituric acid spray reagent.[26]

Incubation with α-L-fucosidase purified from *Clostridium perfringens* was kindly performed by Dr. D. Aminoff according to the conditions of Aminoff and Furukawa.[16] The lyophilized enzyme digest (2 mg) was dissolved in 0.01 M pyridine-acetate buffer, pH 4.8, (0.3 ml), applied on a Sepharose 6 B column (1.2 × 29 cm) and

eluted with the same buffer, fractions of 1.5 ml being collected. The glycoprotein-containing front peak was dialysed and lyophilized. The material of the final, phenol-sulphuric acid-positive peak was deionized on columns of Dowex 50 (H^+) and Dowex 1 formate. The carbohydrate composition of the two products was analysed by GLC.

Incubation with trypsin (Type I, Sigma, 2 × crystallized) was performed by dissolving the sample (10 mg) in 0.05 M Tris-chloride buffer containing 0.01 M calcium chloride, pH 7.9, (2 ml) and incubating with the enzyme (0.5 mg) at 35° for 8 h. The digest was immersed briefly in a boiling water bath, applied to a Sepharose 6 B column (1.2 × 45 cm) and eluted with 0.025 M Tris-chloride buffer, pH 7.3, 2 ml fractions being collected.

For the determination of blood group activity the glycoprotein was serologically typed by its ability at a concentration of 1 mg/ml, to inhibit the agglutination of human red cells in the presence of the appropriate antiserum. In addition to human anti-H serum, *Ulex europeus* H-lectin was used as anti-H substance.

The molecular weight determination was performed by sedimentation equilibrium. The sample (4 mg), dissolved in 1 ml of 0.1 M acetate buffer, pH 5.7, was dialysed against the same buffer at 4° for 18 h. The solution was analysed by low-speed equilibrium at 3000 rpm for 48 h at 20° in a Spinco Model E analytical ultracentrifuge. Optical registration was done by a photoelectric scanner at 280 nm. The partial specific volume ($\bar{V}$, 0.64) was calculated from the carbohydrate and amino acid composition of the glycoprotein sample.

Preparation of purified glycoprotein fraction. The crude, nondialysable intestinal or fecal extract was isolated as described previously.[6] The extract (2.5 g) was stirred at room temperature for 24 h with chloroform – methanol, 2:1 (v/v), and then 1:2 (v/v), 300 ml each time. After filtration the remaining material (2.4 g) was dissolved in 0.05 M Tris – chloride buffer, pH 7.9 (300 ml) containing 0.01 M calcium chloride, and incubated with pronase (B grade, Calbiochem.) (25 mg) at 35° for 24 h under toluene. The same amount of pronase was again added and the incubation continued for a further 24 h. The resulting proteolysate was dialysed exhaustively against distilled water, concentrated, and lyophilized to yield 1.8 g. The pronase-resistant material (1.8 g) was dissolved in water (360 ml) to which an aqueous 1 % solution of CTAB (180 ml) was added slowly with stirring. The precipitate was allowed to settle for 4 h at 4°, and was then centrifuged off and dissolved in methanol (400 ml) by stirring at room temperature overnight. A small amount of undissolved dark material was centrifuged off and discarded. To dissociate the CTAB-glycoprotein complex, a solution of equimolar proportions of sodium acetate trihydrate (5.0 g) and acetic acid (2.2 g) in 50 ml of methanol was added with stirring to the clear supernatant.

The precipitated glycoprotein was centrifuged off, dissolved in water, dialysed, and recovered by lyophilization to yield 0.9 g. A portion of this material (0.45 g) dissolved in 0.05 M Tris-chloride buffer, pH 7.3, was applied to a column (2.5 × 30 cm) of DEAE-Sephadex A 25 equilibrated with the same buffer. The column was eluted with a linear gradient of sodium chloride in 0.05 M Tris buffer pH 7.3, from 0 to 1.0 M sodium chloride in a total volume of 400 ml. Fractions of 10 ml were collected and aliquots analysed for hexose, protein, and sialic acid. The fractions containing the glycoprotein (Fractions 11 to 19, Fig. 2) were pooled, dialysed, and lyophilized to afford 0.25 g. The combined fractions obtained from two separations on the DEAE-Sephadex column (0.5 g) were dissolved in 0.025 M Tris-chloride buffer, pH 7.3, and the solution was applied to a 2.5 × 80 cm column of Sepharose 4 B, and eluted with the same buffer; 8 ml fractions were collected and analysed as described for the DEAE-Sephadex column. The fractions containing the glycoprotein (Fractions 13 to 17, Fig. 3) were pooled, dialysed, and lyophilized to yield 0.2 g.

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REFERENCES

1. Lindstedt, G., Lindstedt, S. and Gustafsson, B. E. *J. Exp. Med. 121* (1965) 201.
2. Hoskins, L. C. and Zamchek, N. *Gastroenterology 54* (1968) 210.
3. Kent, P. W. and Marsden, J. C. *Biochem. J. 87* (1963) 38 P.
4. Inoue, S. and Yosizawa, Z. *Arch. Biochem. Biophys. 117* (1966) 257.
5. Bella, A. and Kim, Y. S. *Arch. Biochem. Biophys. 150* (1972) 679.
6. Wold, J. K., Khan, R. and Midtvedt, T. *Acta Pathol. Microbiol. Scand. 79 B* (1971) 525.

7. Wold, J. K., Midvedt, T. and Winsnes, R. *Acta Chem. Scand. 27* (1973) 2997.
8. Holden, K. G., Yim, N. C. F., Griggs, L. J. and Weisbach, J. A. *Biochemistry 10* (1971) 3105.
9. Montgomery, R. In Gottschalk, A., Ed., *Glycoproteins*, 2nd Ed., Elsevier, Amsterdam 1972, p. 518.
10. Nemoto, T. and Yosizawa, Z. *Biochim. Biophys. Acta 192* (1969) 37.
11. Gibbons, R. A. In Gottschalk, A., Ed., *Glycoproteins*, 2nd Ed., Elsevier, Amsterdam 1972, p. 31.
12. Lloyd, A. G., Dodgson, K. S., Price, R. G. and Rose, F. A. *Biochim. Biophys. Acta 46* (1961) 108.
13. Lagunoff, D. and Warren, G. *Arch. Biochem. Biophys. 99* (1962) 396.
14. Pamer, T., Glass, G. B. J. and Horowitz, M. I. *Biochemistry 7* (1968) 3821.
15. Schauer, R. and Faillard, H. *Hoppe-Seyler's Z. Physiol. Chem. 349* (1968) 969.
16. Aminoff, D. and Furukawa, K. *J. Biol. Chem. 245* (1970) 1659.
17. Baig, M. M. and Aminoff, D. *J. Biol. Chem. 247* (1972) 6111.
18. Slomiany, B. L. and Meyer, K. *J. Biol. Chem. 248* (1973) 2290.
19. Reinhold, V. N. *Methods Enzymol. 25* (1972) 244.
20. Jourdian, G. W., Dean, L. and Roseman, S. *J. Biol. Chem. 246* (1971) 430.
21. Roach, D. and Gehrke, C. W. *J. Chromatog. 43* (1969) 303.
22. Spencer, B. *Biochem. J. 75* (1960) 435.
23. Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F. *Anal. Chem. 28* (1956) 350.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem. 193* (1951) 265.
25. McDevitt, C. A. and Muir, H. *Anal. Biochem. 44* (1971) 612.
26. Warren, L. *Nature 186* (1960) 237.

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