Carbohydrate Research, 29 (1973) 181–191
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COMPOSITION AND DISTRIBUTION OF CARBOHYDRATE CHAINS IN GLYCOPROTEINS OF HUMAN ERYTHROCYTE MEMBRANE*

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(Received January 29th, 1973; accepted February 9th, 1973)

ABSTRACT

The M-, N-, and MN-glycoproteins obtained from human erythrocytes by phenol–water extraction were purified by gel filtration and digested with Pronase and trypsin. The products of degradation were fractionated by gel filtration on Sephadex G-25 and DEAE-Sephadex A-50 and the fractions were examined by poly(acrylamide)-gel electrophoresis in the presence of dodecyl sodium sulfate, analyzed for carbohydrate and amino acid contents, and tested for M and N blood-group activity. From the results, it is suggested that the glycoprotein chains are composed of a hydrophobic moiety devoid of carbohydrate chains and a hydrophilic moiety containing carbohydrate chains of different compositions, irregularly distributed along the protein chains and linked to L-asparagine, L-serine, or L-threonine residues. The M and N activity typical for the undegraded glycoproteins, and the "basic" or "precursor-type" N activity, were found in different glycopeptide fractions.

INTRODUCTION

Glycoproteins obtained from human erythrocyte stroma and showing blood-group M and N activities are degraded by proteolytic enzymes to (*a*) an insoluble or readily precipitable fraction that contains a low proportion of carbohydrate residues, plus (*b*) a glycopeptide fraction that contains a higher proportion of carbohydrate residues than does the original glycoprotein[1–4]. The biological activity is lessened by proteolytic degradation[2,3]. Two types of carbohydrate chain, having different compositions, are present: one is composed of *N*-acetylneuraminic acid, D-galactose, and 2-acetamido-2-deoxy-D-galactose (*N*-acetyl-D-galactosamine) in the ratios of 2:1:1; it is alkali-labile, and is linked through an L-serine or L-threonine residue to the protein

*This is publication No. 602 of the Robert W. Lovett Memorial Group for the Study of Diseases Causing Deformities, Harvard Medical School at the Massachusetts General Hospital, Boston, Massachusetts. This work was supported by a research grant from the National Institute of Arthritis and Metabolic Diseases (AM-03564), National Institutes of Health, U. S. Public Health Service.
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backbone[5,6] (Type I). The other kind of chain contains D-galactose, D-mannose, L-fucose, 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine), and a small proportion of *N*-acetylneuraminic acid; it is alkali-stable, and is probably linked to the protein backbone through a 2-acetamido-1-*N*-(L-aspart-4-oyl)-2-deoxy-β-D-glucopyranosylamine bond[7,8] (Type II). The present report describes the fractionation and examination of the products of proteolysis of M and N blood-group glycoproteins, in order to ascertain the relative distribution of the two types of chain along the protein backbone.

EXPERIMENTAL

General methods and material. — The glycoproteins were prepared by phenol–water extraction[9] of erythrocytes of human blood having blood-group O activity, and were purified by gel-filtration on Bio-Gel P-300 in 50 mM pyridine acetate buffer at pH 5.5. Pronase was obtained from Sigma Chemical Co. (St. Louis, Mo.) and trypsin from Worthington Biochemical Corp. (Freehold, N. J.). The amino acid analysis was performed with a Beckman Model 117 automatic amino acid analyzer.

The blood-group activity of the glycopeptides was determined by the inhibition of hemagglutination[2], with rabbit immune anti-M and anti-N sera obtained from Ortho Diagnostics (Raritan, N. J.).

Gel electrophoresis. — Poly(acrylamide)-gel electrophoresis was performed in the presence of dodecyl sodium sulfate as described by Weber and Osborn[10], except that ethanethiol was not added. A mixture containing a solution (50 μl) of the glycopeptide (4 mg/ml) in 10 mM disodium hydrogen phosphate–potassium dihydrogen phosphate buffer containing 1% of dodecyl sodium sulfate (pH 7.1), glycerol (2 drops), buffer (150 μl), and 0.05% Bromophenol Blue (6 μl) was deposited on the gel (2.2 ml), and a current of ~7.5 mA was applied for 4 h. A sample of human α_1-acid glycoprotein was applied as a standard. The gels were stained at room temperature with the periodic acid–Schiff reagent according to the method of Zacharius *et al.*[11] (except that the 12.5% solution of trichloroacetic acid contained 50% of ethanol in order to avoid the elution of glycopeptides), or with a 0.2% solution of Coomassie Brilliant Blue containing 10% of acetic acid and 50% of methanol. The excess of stain was removed with a solution containing 5% of acetic acid and 25% of methanol.

Carbohydrate determination. — The sugar components were determined by gas–liquid chromatography after methanolysis, according to a modification[12] of the procedure of Clamp *et al.*[13], and by the following colorimetric methods: neutral sugars by the phenol–sulfuric acid reagent[14]; 2-amino-2-deoxy-D-galactose (D-galactosamine) and 2-amino-2-deoxy-D-glucose (D-glucosamine) by treatment with 2,4-pentanedione at low and high temperatures according to a modification[7] of the method of Ludowieg and Benmaman[15], after hydrolysis in 3M hydrochloric acid for 2 h at 110°; and sialic acid by the resorcinol test[16].

Digestion with trypsin. — A 2% solution of M-glycoprotein containing 0.02% of trypsin was incubated under toluene for 2 h at 37°. The flocculent, light precipitate

(MT_{ppt}) was centrifuged off at 30,000–40,000 *g*, and washed twice with water, and an aqueous suspension of the precipitate was lyophilized. Trichloroacetic acid was added to the supernatant liquor until a concentration of 5% thereof was reached, and the precipitate was centrifuged off and discarded. The carbohydrate contents of the starting material and of the precipitate (MT_{ppt}) and the yield of the precipitate are reported in Table I. The supernatant liquor was extracted three times with ether (to remove the trichloroacetic acid) and concentrated *in vacuo* to 10–15 ml in a rotary evaporator at a bath temperature of 40°. The concentrated solution was used for the fractionation on Sephadex G-25.

TABLE I

CARBOHYDRATE COMPOSITION OF M-, N-, AND MN-GLYCOPROTEINS AND OF THEIR PRODUCTS OF DEGRADATION WITH TRYPSIN AND PRONASE, AND YIELD OF THE PRODUCTS OF DEGRADATION

Substances	*Yield (g)*[a]	*Carbohydrate components (%)*[b]						
		Gal	*Glc*	*Man*	*Fuc*	*GNAc*	*GalNAc*	*NANA*
Starting materials								
M-Glycoprotein		9.0	0.1	1.4	0.5	2.4	4.8	19.2
N-Glycoprotein		11.0	0.2	1.6	0.5	3.1	5.5	19.2
MN-Glycoprotein		10.2	0.5	1.5	0.5	2.8	5.1	19.3
Degradation, with trypsin, of M-glycoprotein								
Precipitate:								
MT_{ppt}	0.19	3.8	0.3	0.2	0	3.0	0.9	1.7
Sephadex G-25 fraction:								
MT_{vv}	0.45	14.2	0	1.9	0 8	3.2	8.6	31.4
DEAE-Sephadex A-50 fractions:								
MI-I	0.25	14 5	0	2 0	0.9	4.7	10.4	29.8
MI-II	0.09	10.7	0	0.3	0.2	0.9	5.9	29.2
Degradation, with Pronase, of M-, N-, and MN-glycoprotein								
Precipitate:								
MP_{ppt}	0.11	4.4	0 5	0.5	0.5	4 2	0	1.4
NP_{ppt}	0.13	4.9	1.7	0.5	0.4	4.5	0	1.2
MN_{ppt}	0.10	7.2	0.7	0.3	0.5	5.0	0	2 6
Sephadex G-25 fractions:								
MP_A	0.35	17.4	0.1	3.3	0.9	5.1	9.6	29.2
NP_A	0.35	19.1	0.1	3.1	0.8	6.6	10.9	39.8
MNP_A	0.31	19.2	0.1	2.7	1.2	6.2	10.3	31.6
MP_B	0.08	9.5	0.8	0.5	0	0	7.2	31.3
NP_B	0.06	14 3	0.6	0.9	0	0	7.5	38.3
MNP_B	0.10	13.7	0.4	0.9	0.7	0	9.5	32.5
DEAE-Sephadex A-50 fractions:								
MNP_A-I	0.04	17.9	0.6	9.1	1.3	17.5	5.3	21.8
MNP_A-II	0.20	16.6	0	1.2	0.7	4.6	10.6	33.2

[a]Weight of starting materials: 1.0 g. [b]Abbreviations: Gal, galactose; Glc, glucose; Man, mannose; Fuc, fucose; GNAc, *N*-acetylglucosamine; GalNAc, *N*-acetylgalactosamine; and NANA, *N*-acetylneuraminic acid.

Digestion, with Pronase, of M-, N-, and MN-glycoproteins. — The pH of a 2% solution of glycoprotein (M-, N-, and MN-glycoprotein, respectively; 1.0 g) mM in calcium acetate and containing 0.02% of Pronase, was adjusted to 7.5 with solid sodium hydrogen carbonate, and the solution was incubated under toluene at 37°. After 24 h, the concentration of Pronase was increased to 0.03%, the pH was readjusted to 7.5, and the incubation was continued for 24–48 h. The heavy, dense precipitate formed during the digestion was centrifuged off at 18,000 *g*, was washed twice with water, and lyophilized (MP_{ppt}, NP_{ppt}, and MN_{ppt}, for M-, N-, and MN-glycoprotein, respectively). The carbohydrate contents of the starting materials and precipitates, and the yields of the precipitates, are reported in Table I and the amino acid contents in Table II. The supernatant liquors (MP_{sup}, NP_{sup}, and MNP_{sup}) were treated with trichloroacetic acid, and then fractionated on Sephadex G-25 as described for the supernatant liquor from the trypsin degradation.

TABLE II

AMINO ACID COMPOSITION OF M-GLYCOPROTEIN AND OF ITS PRODUCTS OF DEGRADATION WITH PRONASE[a]

Amino acids	*Substances*			
	M-Glycoprotein	MP_{ppt}	MP_A	MP_B
Asp	1.89	1.08	1.35	2.16
Thr	2.98	3 08	3.56	3.98
Ser	3 18	3.46	4.51	2.50
Glu	2 62	2.60	1.66	2.22
Pro	1.79	1.42	0.79	4.91
Gly	1.54	5.47	0.55	0.41
Ala	1 69	2.34	0 66	2 39
Val	2 64	2.95	1.70	0.67
Ile	1.71	6 85	0.63	0.74
Leu	1.76	4 41	0.02	0 59
Tyr	0.52	0.80	0.30	0.85
Phe	0.58	1.64	0	0
Lys	1.28	0.36	0.98	0.58
His	1.11	0.79	1.03	0 04
Arg	1 58	2.01	0 30	3.09

[a]Expressed in μmoles/10 mg of glycoprotein.

Digestion, with Pronase, of the products of trypsin degradation of M-glycoprotein (MT_{ppt}, MT-I, *and* MT-II). — The glycopeptide fragments obtained by trypsin digestion before and after fractionation on Sephadex G-25 (MT_{ppt}, *MT*-I, and *MT*-II, see following paragraphs) were digested with Pronase for 24 h at 37°, as described for the M-, N-, and MN-glycoproteins, to give the fractions MT_{ppt}-P, *MT*-IP, and *MT*-IIP. These fractions were compared by poly(acrylamide)-gel electrophoresis (see following paragraphs) with the fractions (MP_A and MP_B) obtained by direct Pronase digestion of M-glycoprotein.

Fractionation of glycopeptides obtained by trypsin or Pronase digestion of M-, N-, and MN-glycoproteins on Sephadex G-25. — The concentrated solution obtained by removal of the precipitates and trichloroacetic acid treatment of the trypsin or Pronase digests of ~900 mg of glycoprotein was fractionated on a column of Sephadex G-25 (bed volume, 550 ml) in water. Fractions (4 ml each) were collected every 10 min, and aliquots (0.05 ml) were used for colorimetric determinations with the phenol–sulfuric acid, resorcinol, and ninhydrin reagents. The fractionation of the digest of M-glycoprotein with trypsin gave only one fraction in the void volume (MT_{vv}) (see Fig. 1).

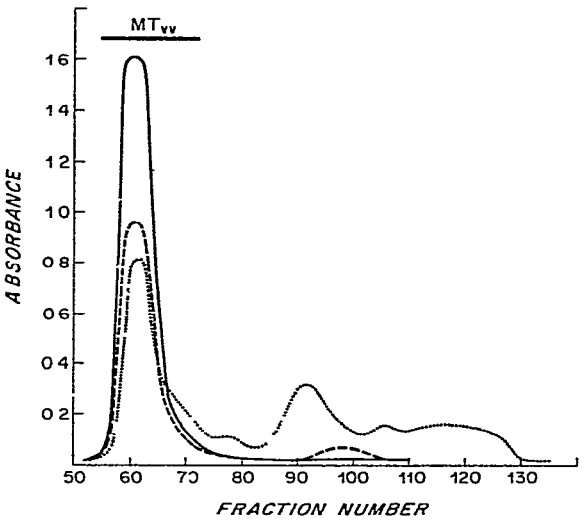

Fig. 1. Fractionation, on Sephadex G-25, of the soluble products of the digestion of M-glycoprotein (about 900 mg) with trypsin. [Bed volume of the column, 550 ml. Fractions (4 ml) were collected at 10-min intervals. Aliquots (0.05 ml) were analyzed for carbohydrates by the phenol–sulfuric acid method (———, absorbance at 490 nm), for sialic acid by the resorcinol method (— — —, absorbance at 580 nm), and for peptides by the ninhydrin method (· · ·, absorbance at 510 nm).]

Fractionation of the digests of M-, N-, and MN-glycoproteins with Pronase gave two fractions partially excluded (MP_A and MP_B, NP_A and NP_B, MN_A and MN_B, respectively). The fractionation of the digest of MN-glycoprotein is illustrated in Fig. 2. The yields and carbohydrate compositions are reported in Table I, and the amino acid composition of MP_A and MP_B in Table II.

Further fractionation on DEAE-Sephadex A-50, of the glycopeptides obtained by trypsin or Pronase digestion. — An aliquot (100 mg) of the materials obtained in the void volume of the Sephadex G-25 fractionations (MT_{vv}, MP_A, NP_A, and MNP_A, respectively) was further fractionated on a column of *O*-(2-diethylaminoethyl)-Sephadex A-50 (2.5 g) that had been equilibrated with a 20mM disodium hydrogen phosphate–potassium dihydrogen phosphate buffer (pH 7.0). Fractions (2 or 2.5 ml) were collected at 10–15 min intervals. After 20 fractions had been obtained, a linear gradient of sodium chloride was applied by using equal proportions of the buffer

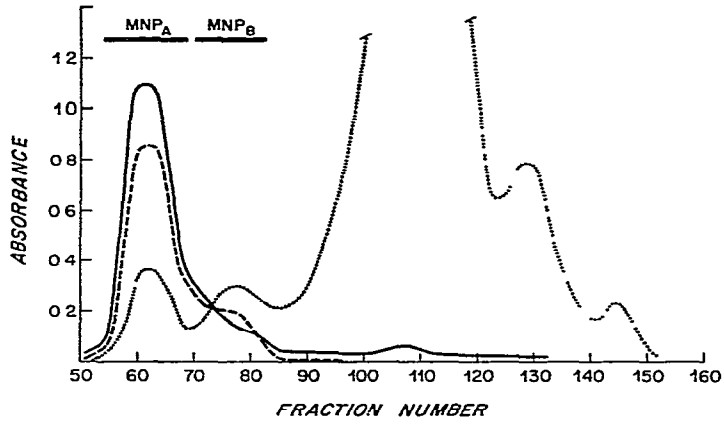

Fig. 2. Fractionation, on Sephadex G-25, of the soluble products of the digestion of MN-glycoprotein (about 900 mg) with Pronase. [Bed volume of the column, 550 ml. Fractions (4 ml) were collected at 10-min intervals. Aliquots (0.05 ml) were analyzed for carbohydrates by the phenol–sulfuric acid method (———, absorbance at 490 nm), for sialic acid by the resorcinol method (— — —, absorbance at 480 nm), and for peptides by the ninhydrin method (· · ·, absorbance at 510 nm).]

solution and the same buffer solution that was 0.5M in sodium chloride. The fractions were tested (on aliquots) with the phenol–sulfuric acid reagent. The material obtained by elution, from the DEAE-Sephadex columns, of the trypsin digest of the M-glycoprotein was further fractionated on Sephadex G-25 (MT_{vv}) (see Fig. 3) and thus arbitrarily separated into Fractions *MT*-I and *MT*-II. The fractionation, on DEAE-

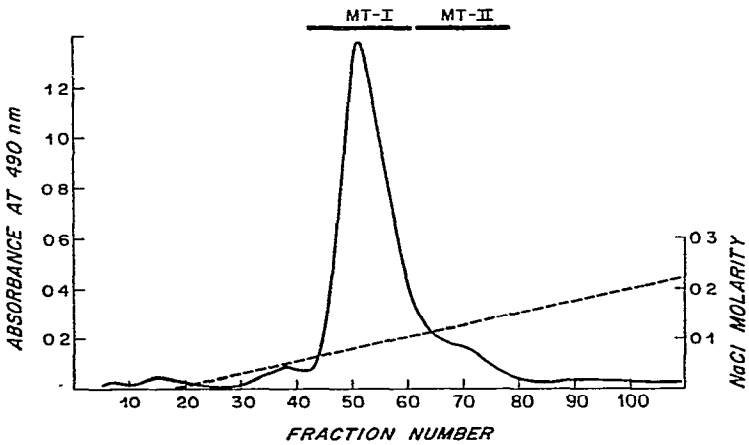

Fig. 3. Fractionation, on DEAE-Sephadex A-50, of the glycopeptides (MT_{vv}) obtained by digestion of M-glycoprotein with trypsin, followed by fractionation on Sephadex G-25. [MT_{vv} (100 mg) was adsorbed on a column of DEAE-Sephadex A-50 (2.5 g), equilibrated with 0.02M phosphate buffer at pH 7.0, and eluted with the same buffer solution. Fractions (2 ml) were collected at 10-min intervals; after collection of 20 fractions, a linear gradient of sodium chloride was applied by mixing 300 ml of phosphate buffer with 300 ml of phosphate buffer that was 0.5M in sodium chloride; the phenol–sulfuric acid test was performed on aliquots (0.1 ml).]

Sephadex, of the material obtained after Pronase digestion of the M-, N-, and MN-glycoproteins, followed by fractionation on Sephadex G-25 (MP_A, NP_A, and MNP_A, respectively) is illustrated for MNP_A in Fig. 4; it gave two fractions (M-, N-) MNP_A-I and (M-, N-) MNP_A-II, respectively. The fractions were desalted by filtration on Bio-Gel P-6, and lyophilized. The carbohydrate content and yields for *MT*-I, *MT*-II, MNP_A-I, and MNP_A-II are reported in Table I.

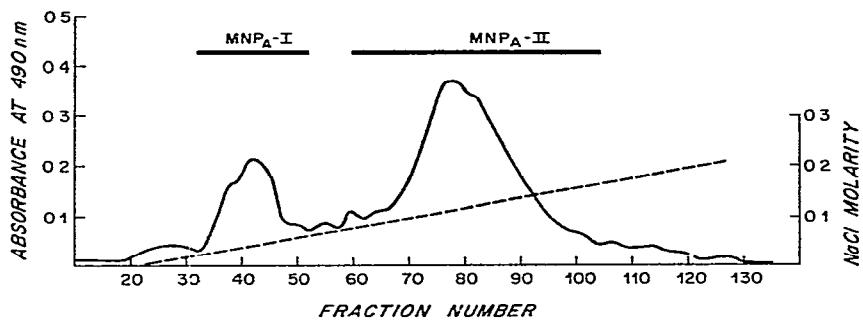

Fig. 4. Fractionation, on DEAE-Sephadex A-50, of the glycoprotein (MNP_A) obtained by Pronase digestion of MN-glycoprotein, followed by fractionation on Sephadex G-25. [MNP_A (100 mg) was adsorbed on a column of DEAE-Sephadex A-50 (2.5 g), equilibrated with 0.02M phosphate buffer at pH 7.0, and eluted with the same buffer solution. Fractions (2 ml) were collected at 10-min intervals; after collection of 20 fractions, a linear gradient of sodium chloride was applied by mixing 300 ml of phosphate buffer with 300 ml of phosphate buffer that was 0.5M in sodium chloride; the phenol–sulfuric acid test was performed on aliquots (0.1 ml).]

Fractionation of the material obtained by trypsin digestion of M-glycoprotein on Bio-Gel P-100 gave results similar to those obtained by fractionations on DEAE-Sephadex A-50, and the carbohydrate compositions correspond to each other.

RESULTS

Degradation, with trypsin and Pronase, of glycoproteins having blood-group M (M-glycoprotein), N (N-glycoprotein), and MN (MN-glycoprotein) activity showed the formation of a light, difficultly sedimentable fragment with the first enzyme, whereas the second enzyme gave a heavy precipitate that was readily centrifuged off. The yield of the precipitate obtained by Pronase digestion was almost identical for the three glycoproteins (11–14%). Precipitates obtained by both enzymic degradations showed a low content of carbohydrate, with a very small proportion of sialic acid and no (or only traces of) galactosamine. The main carbohydrate components were galactose and glucosamine, the other carbohydrates (sialic acid, fucose, mannose, and glucose) being present in trace proportions only (see Table I). The amino acid analysis of one of the precipitates (MP_{ppt}) showed a large proportion of hydrophobic acids (see Table II).

Fractionation on Sephadex G-25 (see Fig. 1) of the supernatant liquor from the trypsin degradation of M-glycoprotein gave one main peak of material eluted in the

void volume (MT_{vv}), with only a small proportion of peptides of low molecular weight and amino acids. Fractionation of this material on DEAE-Sephadex (see Fig. 3) gave a sharp, main peak (*MT*-I) and a "tail" fraction (*MT*-II). The carbohydrate content of these two fractions (see Table I) indicated the presence of both types (I and II) of chain in the first fraction, and a preponderance of Type I chains in the second fraction. Further degradation of these two fractions with Pronase gave two fractions (*MT*-IP and *MT*-IIP) that were shown, by disc-gel electrophoresis, to be similar to the fractions obtained directly by Pronase digestion of the M-glycoprotein (MP_A and MP_B).

Fractionation, on Sephadex G-25, of the supernatant liquor from the Pronase digestion of the M-, N-, and MN-glycoproteins gave a main peak in the void volume (MNP_A, illustrated for MN-glycoprotein in Fig. 2) also, and, in addition, a slightly retarded peak (MNP_B), besides a large proportion of peptides of low molecular weight and amino acids. The yields of the first fraction (MP_A, NP_A, and MNP_A) were 37, 38, and 32%, respectively, whereas those of the second fraction varied from 7 to 10% (see Table I). A low content of mannose and the absence of glucosamine indicated that Fraction MNP_B contained mainly Type II chains, whereas Fraction MNP_A contained both types of chain, as shown by its composition (which was similar to that of the starting material). Further fractionation of Fraction MNP_A on DEAE-Sephadex A-50 gave, however, in the ratio of 1:4, two distinct Fractions, MNP_A-I and MNP_A-II (see Fig. 4), which showed very different contents of carbohydrate (see Table I). In relation to the second fraction, the first fraction contains about 8 times as much mannose and 4 times as much glucosamine, and 2/3rds the proportion of sialic acid; this indicates a large preponderance of Type II chains in Fraction MNP_A-I and of Type I chains in Fraction MNP_A-II.

Comparison of the various glycopeptides by disc-gel electrophoresis (see Fig. 5) showed, in the component of high molecular weight of the trypsin digest (MT_{vv}) of M-glycoprotein, the presence of two components (*MT*-I and *MT*-II) incompletely separated by chromatography on DEAE-Sephadex. The main glycopeptide component of the Pronase digest of the M-glycoprotein (MP_A) had a molecular size lower

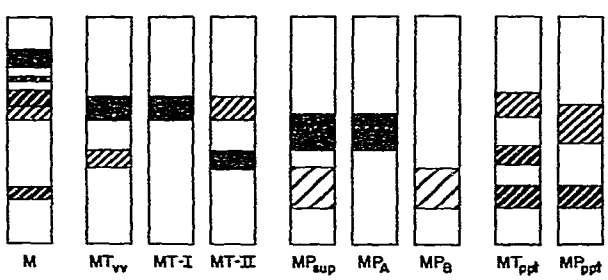

Fig. 5. Electrophoretic patterns, in poly(acrylamide) gel–dodecyl sodium sulfate, of the fractions obtained after digestion of M-glycoprotein with trypsin and Pronase. [The gels were stained with the periodate–Schiff reagent, and the color intensity is given by the depth of shading. The products of proteolytic digestion of M-, N-, and MN-glycoproteins gave no color with Coomassie Brilliant Blue.]

than that of *MT*-I, and was heterogeneous. Fraction MP_B also migrated faster than *MT*-II, and showed little reactivity with the periodate–Schiff reagent, most probably because of losses (during the staining procedure) owing to the low molecular weight. The glycopeptides obtained by trypsin and Pronase digestion (*MT*-IP and *MT*-IIP) gave patterns similar to those obtained directly by Pronase digestion (MP_A and MP_B, respectively). The precipitates obtained by trypsin and Pronase digestion (MT_{ppt} and MP_{ppt}) were heterogeneous. All of the products of proteolytic digestion of the M-, N-, and MN-blood-group active glycoproteins gave a positive reaction with the periodate–Schiff reagent, but they gave no color with Coomassie Brilliant Blue.

Proteolytic digestion of M- and N-glycoproteins is known to lessen the biological activity[2,3]. The glycopeptides that possess the highest molecular weight showed specific, blood-group activity. The Fractions *MT*-I, *NT*-I, MP_A, and NP_A inhibited hemagglutination by anti-M and anti-N sera, respectively, at a concentration of $\sim$1 mg/ml for a sera titer of 1:8, whereas the untreated glycoproteins showed specific inhibition at a concentration of 40–80 μg/ml (see Table III). The precipitate of the Pronase digest (MP_{ppt}) and the glycopeptide fractions (*MT*-II, *NT*-II, MP_B, and NP_B) of lower molecular weight were inactive, or showed a very weak, unspecific inhibition of anti-M and anti-N sera at a concentration of 10 mg/ml. The readily precipitable fractions of the trypsin digest (*MT* and NT_{ppt}) always showed weak N-activity, independent of the specificity of the starting material (see Table III).

TABLE III

M- AND N-BLOOD-GROUP ACTIVITY OF M- AND N-GLYCOPROTEINS AND THEIR PRODUCTS OF DEGRADATION WITH TRYPSIN AND PRONASE[a]

Substances	*Blood-group activity*	
	M	*N*
M-Glycoprotein	128	<1
Trypsin digest		
precipitate (MT_{ppt})	<1	16
supernatant liquor (*MT*-I)	8	<1
Pronase digest		
precipitate (MP_{ppt})	≤1	≤1
supernatant liquor fractionated on Sephadex G-25 and DEAE-Sephadex A-50 (MP_A-II)	8	<1
N-Glycoprotein	<1	64
Trypsin digest		
precipitate (NT_{ppt})	≤1	8
supernatant liquor (*NT*-I)	<1	8
Pronase digest		
precipitate (NP_{ppt})	≤1	<1
supernatant liquor fractionated on Sephadex G-25 and DEAE-Sephadex A-50 (NP_A-II)	<1	8

[a]A maximal dilution of a 1% solution at which 4 agglutinating doses of antiserum were completely inhibited.

DISCUSSION

The differences in the carbohydrate and amino acid composition of the fractions obtained by proteolytic digestion of erythrocyte glycoproteins indicate the high asymmetry of the chemical structure of these glycoproteins. This asymmetry has been reported[2–4], and is in agreement with the current concept of membrane structure: the hydrophobic portion of the glycoprotein is anchored in a lipid bilayer, or spans the membrane[17], whereas the hydrophilic portion, rich in carbohydrates, is exposed on the external surface of the erythrocyte. This chemical asymmetry of the molecule explains the strong tendency of isolated glycoproteins to aggregate in aqueous solution[18].

Human erythrocytes contain one main type of glycoproteins that show the same pattern in dodecyl sodium sulfate–poly(acrylamide)-gel electrophoresis, regardless of the method used for isolation[19]. On examination by dodecyl sodium sulfate–poly-(acrylamide)-gel electrophoresis (see Fig. 5, M, and ref. 19), the glycoproteins isolated are usually found to be not completely homogeneous, but it has not yet been established whether the additional fractions, which react less intensively with the periodate–Schiff reagent, are products of autolysis of the main glycoprotein, or whether they are minor glycoprotein components of the erythrocyte. These fractions, which have lower molecular weights, include a lipid component that is the fastest-moving on electrophoresis; they seem to form, with the main glycoprotein, strong micelles that can be separated only by the action of detergents.

The insoluble fractions obtained by proteolytic digestion of the glycoproteins are readily dispersed in a solution of dodecyl sodium sulfate, and they were shown by electrophoretic examination (see Fig. 5; MT_{ppt} and MP_{ppt}) to be heterogeneous. This heterogeneity, the low content of carbohydrate components, and the high proportion of hydrophobic amino acids suggest that these insoluble fractions constitute the hydrophobic center of the micelles, and that they contain that part of the glycoprotein that is embedded in the erythrocyte membrane. This hydrophobic portion of the glycoprotein is more strongly aggregated than the original glycoprotein, as shown by the insolubility, in water, of the precipitates obtained by digestion with Pronase.

Most of the carbohydrate chains of the degraded glycoproteins are found in the soluble glycopeptides. A more heterogeneous glycopeptide mixture is formed by extensive proteolysis with Pronase than by limited degradation with trypsin; this observation suggests that the heterogeneity of the glycopeptides is a consequence of the internal heterogeneity of each glycoprotein molecule and not of a heterogeneity as between individual glycoprotein molecules: each glycopeptide fraction represents a different fragment of the glycoprotein molecule, which has a heterogeneous distribution of oligosaccharide chains along the protein backbone. Some fragments of the glycoprotein chain contain only Type I or Type II carbohydrate chains, whereas others contain both types of chain, but in distinctly different proportions. The existence of both types of chain in the same glycoprotein molecule seems more probable than the existence of Type I and Type II chains in separate glycoprotein molecules,

but some microheterogeneity of the structure and distribution of the oligosaccharide chains cannot be excluded, and some of the glycoprotein molecules may not be identical.

The M and N blood-group specificity probably depends on the structure of the alkali-labile, Type I oligosaccharide chains[7]. The fractions of MP_B and NP_B (which contain, almost exclusively, Type I chains) did not inhibit the hemagglutination, probably because of too low a molecular weight or lack of additional, structural requirements[20]. The specific, blood-group M or N activity was always found in the glycopeptide fractions of higher molecular weight (*MT*-I, *NT*-I, MP_A, and NP_A; see also Table III). The hydrophobic, precipitable fraction from the trypsin digest always showed blood-group N activity, regardless of the serological specificity of the original glycoprotein. Blood-group N activity has been detected in M-erythrocytes and M-glycoproteins after partial degradation (for a review, see ref. 21) and the N-glycoprotein is considered to be a precursor that can be transformed into an M-specific substance by genetic control[22,23]. The results presented here demonstrate that the "precursor-type", blood-group active, N immunodeterminants are situated on a fragment of the glycoprotein molecule different from that where "typical" M or N determinants are situated; this observation may be helpful in the elucidation of the mechanism, as yet unknown, of genetic control of biosynthesis of M- and N-specific structures.

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