ISOLATION OF A TETRASACCHARIDE COMMON TO MM, NN AND MN ANTIGENS

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SUMMARY: A tetrasaccharide consisting of 2 moles sialic acid, 1 mole galactose, and 1 mole N-acetylgalactosaminol was isolated from MM, NN, and MN blood group substances subsequent to degradation with alkaline sodium borohydride. Upon removal of sialic acid with bacterial or viral sialidases, a disaccharide, galactosyl-N-acetylgalactosaminol, was obtained. Periodate oxidation studies suggested that the two sialic acid residues are linked to galactose and N-acetylgalactosaminol, respectively. A tentative structure is proposed.

The capacity of MM, NN, and MN blood group antigens to inhibit hemagglutination by myxoviruses necessitates the presence on these glycoproteins of distinct antigenic regions, as well as common receptor sites for the virus. Several investigators (1-3) have reported that a comparison of the gross chemical composition and physical behavior of the antigens revealed few differences. Furthermore, the predominant carbohydrate to protein linkage in the three substances was found to be glycosidic, probably involving N-acetylgalactosamine, serine and threonine (1). Since the integrity of the carbohydrate prosthetic groups is essential for both activities, structure studies of these moieties might reveal the requirements for the various specificities. The lability of the carbohydrate-protein linkage to alkali and alkaline borohydride (1), permits a facile release of oligosaccharides from the protein.

This communication presents evidence for the presence of a tetrasaccharide common to the three antigens.

MATERIALS AND METHODS

1. Preparation of Oligosaccharides. Degradation of the glycoproteins with alkaline sodium borohydride was carried out as described previously (1). The reaction time was extended to 144 hours to insure maximal cleavage of labile protein-carbohydrate bonds. Partial purification of the released oligosaccharides was accomplished as shown in Figure I.

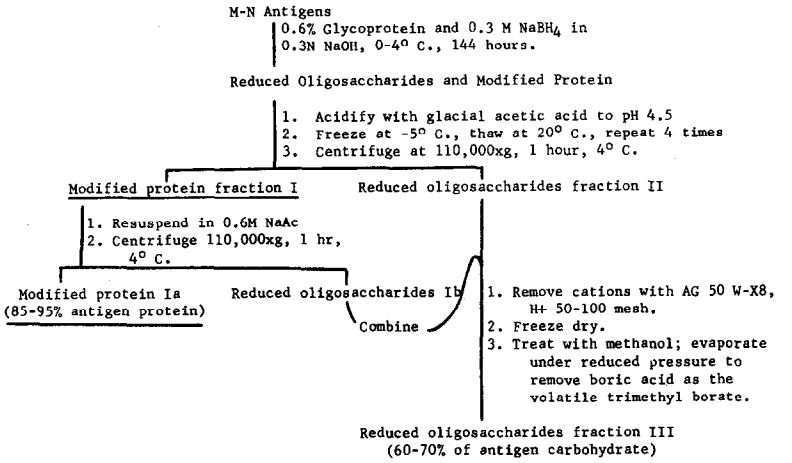

Fig. 1. Partial purification of oligosaccharides of MM, NN, and MN antigens.

Further purification of reduced oligosaccharides fraction III was accomplished by sequential gel filtration on Bio-Gel P-100 and Sephadex G-15, and by preparative high voltage electrophoresis on Whatman 3MM paper in pyridine-acetic acid-water (1:10:289 by vol.), pH 3.65, 2,000 volts, 100 mA, 80 mins.

2. Synthetic and Analytical Techniques. N-acetylgalactosaminol and N-acetylglucosaminol were prepared from the respective N-acetyl hexosamines (4). Xylosamine hydrochloride was synthesized from N-acetylglucosamine according to Wolfrom, et al. (5). Xylosaminol was prepared by reducing the pentosamine with sodium borohydride (4). Dl-Serinol was purchased from Sigma Chemical Co.

Hexosamines, hexosaminols, pentosaminols, and serinol were determined with the Technicon amino acid analyzer as described in the legend to Figure 5. Galactose was determined by the orcinol-sulfuric acid colorimetric method (6), and by gas-liquid chromatography (GLC) as the alditol hexa-acetate (7). Sialic acid was quantitated according to Warren (8) after mild acid hydrolysis (0.07N H_2SO_4, 35 minutes, 80° C.)

Enzymatic cleavage of sialic acid was accomplished with Vibrio cholera

sialidase obtained from General Biochemicals, and with influenza virus vaccine polyvalent types A and B, purchased from Merck, Sharp and Dohme Co. Neuraminlactose (NL), isolated from cow colostrum in our own laboratory, served as a reference substrate.

Periodate oxidation was carried out in the dark at 20° C using 10 mM aqueous sodium metaperiodate in fourfold molar excess of theoretical uptake. Reduction of periodate was measured spectrophotometrically at 222.5 mμ (9) and titrimetrically according to Fleury and Lange (10). Formaldehyde was determined by the chromotropic acid procedure (11) using galactose as a standard. Glyceraldehyde and threose were quantitated by GLC (7). Also, glyceraldehyde was assayed enzymatically with glycerol dehydrogenase from Aerobacter aerogenes (12), after reduction with $NaBH_4$.

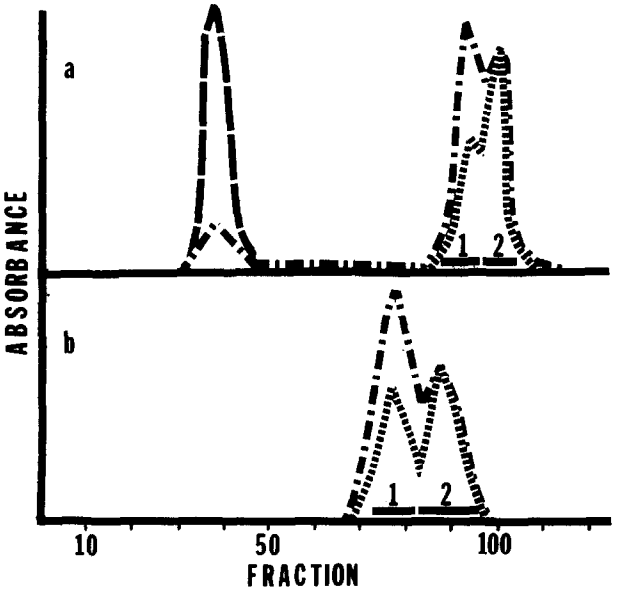

Fig. 2a Gel filtration of oligosaccharide fraction III on a 45 x 4.5 cm Bio-Gel P-100 column using H_2O as eluant. 6.3 ml fractions were collected.

Fig. 2b Pooled fraction 1 (from 2a above) was loaded on a 105 x 3 cm Sephadex G-15 column and eluted with H_2O. 3 ml fractions were collected.

All fractions were analyzed for: protein, ▬▬ OD 280 mμ; NANA, ▪ ▬ ▪ ▬ ▪ OD 549 mμ (8); and 3-deoxyhexitols, ▪▪▪▪▪▪▪▪▪▪▪ OD 532 mμ (8).

RESULTS AND DISCUSSION

Exclusion chromatography of oligosaccharide fraction III, derived from type MM, NN, and MN glycoproteins (Fig. 1), on Bio-Gel P-100 (Fig. 2a) resulted in the complete separation of soluble antigen protein from the oligosaccharides and indicated that the latter were heterogeneous. When pooled fraction-1 was rechromatographed on Sephadex G-15, a major peak, G-15-1 (Fig. 2b), emerged and contained 80% of the sialic acid placed on the column. Quantitative analysis of G-15-1 revealed the presence of sialic acid (NANA), galactose, galactosaminol and small quantities of galactitol.

High-voltage electrophoresis (HVE) of G-15-1 revealed four spots: A,B,C and D (Fig. 3). The areas corresponding to these spots were eluted from the paper with 10% ethanol in water and the eluates were lyophilized. Area D contained 70% of the sialic acid recovered in the eluates. Purity of this preparation was confirmed by repassage of Sephadex G-25 and G-15 and by HVE.

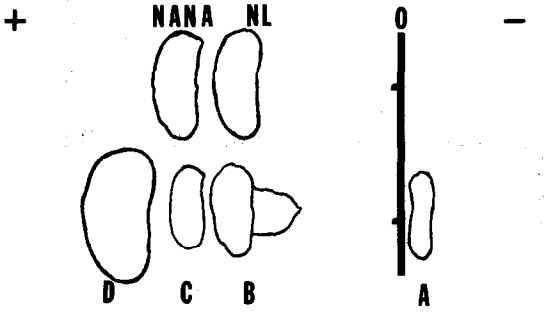

Fig. 3. High-voltage electrophoresis of G-15-1. Control strips were stained with IO_4^- $KMnO_4$ (15).

HVE fraction D contained sialic acid, galactose and N-acetylgalactosaminol in a molar ratio of 2:1:1 (Table I). The presence of approximately one mole of N-acetylgalactosaminol per mg oligosaccharide is consistent with a tetrasaccharide having an approximate molecular weight of 1,000.

Sialic acid was completely released with viral as well as bacterial sialidases (Fig. 4). Interestingly, the tetrasaccharide was a better substrate

TABLE I

COMPOSITION OF HVE FRACTION D FROM MM, NN AND MN ANTIGENS

Type	N-Ac-Gal-ol*		Sialic Acid		Galactose	
	%	μmole/mg	%	μmole/mg	%	μmole/mg
MM	21.2	0.95	60.8	1.07	17.8	0.99
NN	20.2	0.91	60.0	1.94	17.7	0.95
MN	21.2	0.95	61.3	1.98	17.5	0.98

* N-acetylgalactosaminol, determined as the free base.

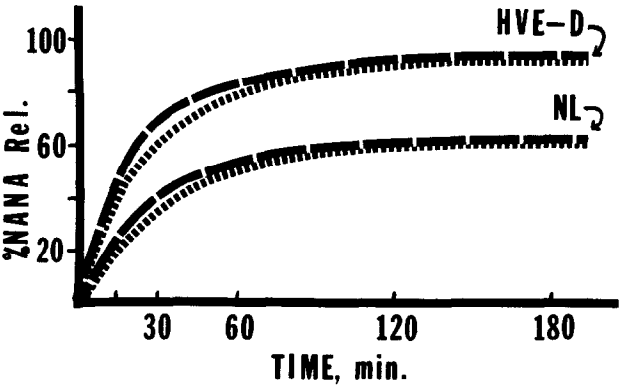

Fig. 4. Release of sialic acid (NANA) from HVE fraction D and neuraminlactose (NL).

Samples containing 600-700 μg NANA were placed in solution with 1.8 ml phosphate-buffered NaCl solution (PBS) pH 7.0 containing .001 M Ca^{++}. 0.2 ml enzyme solution was added and the mixture was incubated at 37° C.

▪▪▪▪▪▪▪▪▪▪▪▪ Vibrio cholera sialidase (1 mg/ml PBS)
▬▬▬ Influenza vaccine (full strength)

than neuraminlactose. When the sialidase digestion mixture was passed through a column containing Ag 1-x4 (acetate form), a neutral disaccharide fraction, Ag-D-1, was obtained consisting of equimolar quantities of galactose and N-acetylgalactosaminol.

HVE fraction D reduced 6 moles of periodate (Table II), after which analysis indicated that all constituent monosaccharides were oxidized and 2 moles formaldehyde were produced. Unexpectedly, neither glyceraldehyde nor threose were detected as oxidation products, but a new amino sugar alcohol, Compound

TABLE II

PERIODATE OXIDATION OF HVE FRACTION D AND AG-D-1

	Time (hr)	IO_4^-*	HCHO*	Glycerol*
HVE Fraction D	1	5.80	1.96	
	2	5.88		
	4	6.02	2.08	0.08
	7	6.08		
	20	6.17	2.04	0.20
AG-D-1	1	3.20	0.96	
	2	3.51	1.03	0.72
	18	3.75	0.98	
	23	3.86	1.09	0.68

* expressed as μmoles/μmole N-acetylgalactosaminol.

I, was obtained (Fig. 5a). Fraction AG-D-1 reduced 4 moles of periodate, yielding 1.1 moles formaldehyde, 0.7 moles glycerol and a new amino sugar alcohol (Fig. 5b), as obtained for the tetrasaccharide. The oxidation of N-acetylgalactosaminol apparently resulted in Compound I whose elution pattern upon ion exchange chromatography did not correspond to any of a mixture of amino sugars (Fig. 5c); its position between xylosaminol and serinol suggested a probable 4-carbon fragment.

Isolation of disaccharide AG-D-1 from HVE fraction D revealed that galactose is linked to N-acetylgalactosaminol, and that NANA is terminal. It is unlikely that the two NANA residues are linked to each other for two reasons: they are readily released with sialidases at pH 7.0, and their removal increased the uptake of periodate by two moles.

HVE fraction D NANA probably reduced 4 moles periodate and released 2 moles formaldehyde (13), while galactose and N-acetylgalactosaminol reduced 1 mole periodate each. On the other hand, AG-D-1 reduced 4 moles periodate and yielded 1 mole formaldehyde, suggesting that the C_6-hydroxyl of N-acetylgalactosaminol is no longer substituted. Thus, if NANA is bound to the C_6-hydroxyl, galactose must be linked to the hydroxyl on either C_3 or C_4 of the hexosamine. Substitution on the C_4-hydroxyl may be negated, since it would

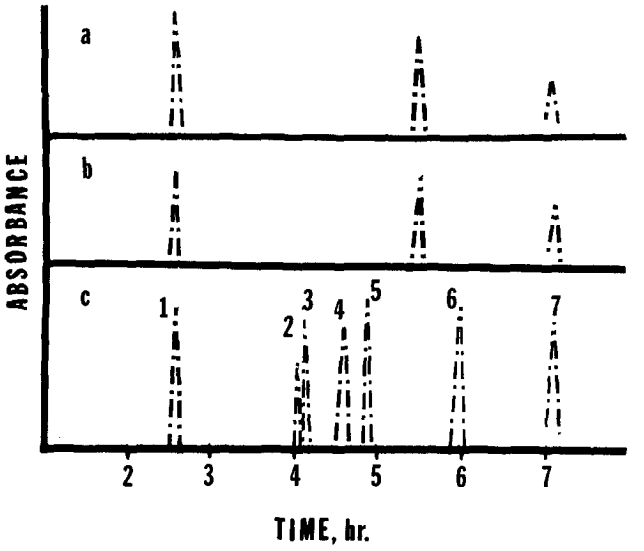

Fig. 5. Ion exchange chromatography of hexosamines and amino sugar alcohols.

The procedure used here is a modification of the method of Piez and Morris (16). A 4-chamber autograd was utilized with citrate buffers, pH 3.8 in chamber 1, and pH 5.0 in chambers 2, 3 and 4; each chamber contained 75 ml buffer. Both buffers were made 0.12M in borate with addition of boric acid prior to final adjustment of pH.

a) HVE-fraction D (350 μg) was oxidized with IO_4 (10 μmoles), 16 hours, 20° C. The mixture was treated with 12 μmoles $NaAsO_2$, 10 mins, and 2 mg $NaBH_4$, 30 mins. 0.2 μmoles norleucine was added as internal standard; the mixture was made 2N with conc. HCl, hydrolyzed (1 hr, 105° C), lyophilized, and analyzed for amino sugars. Compound I eluted at 5.7 hrs.

b) AG-D-1 was oxidized, then analyzed as in a) above.

c) Elution pattern for a standard mixture of amino compounds: 1) Norleucine; 2) Galactosaminol; 3) Glucosamine; 4) Galactosamine; 5) Xylosamine; 6) Serinol; and, 7) Ammonia.

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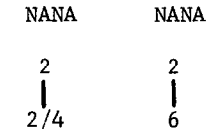
afford protection from oxidation. The second residue of sialic acid is probably linked to galactose, but the position of linkage is in doubt, for the absence of threose or glyceraldehyde is puzzling. Substitution at the C_6-hydroxyl is unlikely, for such a linkage is not easily cleaved with sialidases at pH 7.0 (14). As a result, C_2 and C_4-hydroxyls remain as the most likely sites, both of which agree well with periodate uptake data. The above results suggested the following structure:



Gal, 1——3 NAcgal-ol

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