Immunochemical Studies on Blood Groups. XXXIV. Structures of Some Oligosaccharides Produced by Alkaline Degradation of Blood Group A, B, and H Substances*

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ABSTRACT: The degradation of A, B, and H blood group substances by alkaline borohydride has yielded a series of reduced fucose-containing oligosaccharides, isolated from the dialyzable fraction by charcoal column and preparative paper chromatography. Further purification on a second charcoal column and in some cases on Dowex 1 (OH^-) and Biogel P2 columns was usually necessary. Among the smaller reduced oligosaccharides isolated was 2-*O*-α-L-fucopyranosyl-D-galactitol, characterized as its crystalline acetate derivative. Pairs of fucose-containing oligosaccharides of related structure in which the terminal reduced residue (R) is unsaturated were isolated from all three blood group substances. The reduced residue is probably identical with a low-molecular weight fragment which has been shown to be hex-3-ene-1,2,5,6-tetrol. The structure of these oligosaccharides may be considered to be based on the pair from H substance. These are a reduced tetrasaccharide of structure: α-L-fucopyranosyl-(1→2)-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-R (I) and a reduced pentasaccharide (II) of the same basic structure but with a second fucose attached to the *N*-acetylglucosamine residue. The corresponding monofuco- and difucooligosaccharides from A substance have a terminal *N*-acetyl-D-galactosamine residue linked α(1→3) to the galactose residue of structures I and II and those from B substance have a D-galactose residue similarly linked. From A substance two oligosaccharides were isolated which are terminated at the reduced end by galactitol. Unlike the other oligosaccharides, one of these has the β-D-galactopyranosyl-(1→3)-2-acetamido-2-deoxy-D-glucopyranose linkage typical of the other type of determinant structure considered to be present in these substances. The structure of the oligosaccharides was determined by partial acid hydrolysis, periodate oxidation, and methylation analysis. The methyl ethers derived from the methylated oligosaccharides were identified by thin layer and gas–liquid partition chromatography. The mechanism whereby these oligosaccharides are formed is discussed. The first step is considered to be scission of alkali-labile bonds linking the carbohydrate chains to the serine and threonine of a peptide backbone followed by degradation of the chains by a peeling reaction from their reducing ends until alkali-stable linkages are reached. Each galactose residue from which the unsaturated tetraol is formed is thought to represent a branch point in the chain. The mechanism is based on degradation studies of polysaccharides by alkali alone and the exact role and influence of borohydride remains to be evaluated.

Soluble blood group substances (for review, see Kabat, 1956; Morgan, 1960; Watkins, 1964) are considered to be glycopeptides in which oligosaccharide chains are attached glycosidically to serine and threonine of a peptide backbone (Anderson *et al.*, 1964; Kabat *et al.*, 1965). By partial acid hydrolysis di- and trisaccharides have been isolated with structures suggesting that the oligosaccharide chains in A, B, H, and Le^a (Lewis) substances have certain similarities and that at least two determinants are present in each (Rege *et al.*, 1963). Thus, two A-active trisaccharides have been isolated (Cheese and Morgan, 1961; Schiffman *et al.* 1961, 1962) which represent partially the two types of antigenic determinants (1)

α-D-GalNAc[1]-(1→3)-β-D-Gal-(1→3)-GNAc

and (2)

α-D-GalNAc-(1→3)-β-D-Gal-(1→4)-GNAc

Similarly from B substances, Painter *et al.* (1963) isolated (1)

α-D-Gal-(1→3)-β-D-Gal-(1→3)-GNAc

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[1] Abbreviations used: GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GNAc, 2-acetamido-2-deoxy-D-glucopyranose; Gal, D-galactopyranose; Fuc, L-fucopyranose; tlc, thin layer chromatography; glpc, gas–liquid partition chromatography; DMF, dimethylformamide; R_L, $R_{Lactose}$; TMS, tetramethylsilane.

and (2)

α-D-Gal-(1→3)-β-D-Gal-(1→4)-GNAc

In all four substances the chains are further substituted with L-fucose residues.

Since partial acid hydrolysis yields mainly oligosaccharides lacking fucose, alkaline hydrolysis has recently been employed for the isolation of fucose-containing oligosaccharides. Sodium hydroxide in the presence of sodium borohydride has been used in this laboratory (Schiffman *et al.*, 1964a,b) and some fragments obtained by this method from A and H substances have been purified and their structures determined (Lloyd and Kabat, 1964). Among the oligosaccharides isolated were a weakly A-active reduced trisaccharide, a highly A-active reduced pentasaccharide, and 2-*O*-α-L-fucopyranosyl-D-galactitol.

Morgan *et al.* (1964) described the use of triethylamine as an alkaline reagent and have recently reported the isolation of active trisaccharides from H (Rege *et al.*, 1964a) and Lewis (Rege *et al.*, 1964b) and active tetrasaccharides from A and B substances (Painter *et al.*, 1965).

We now report a fuller characterization of the oligosaccharides described earlier (Lloyd and Kabat, 1964) and the isolation and structure of other oligosaccharides from A, B, and H substances. Oligosaccharides containing two fucose residues have been obtained for the first time. Activity data are given in the following paper.

Materials

Blood Group Substances. These were from human ovarian cyst fluids: A substances, MSS (4.0 g) from human serous and MSM (4.0 g) from human mucinous fluids; B substance, Beach (3 g); H substance, JS (4.0 g); and A and H substance (10 g) from hog gastric mucin. See Schiffman *et al.* (1964a) for more detailed information.

Sugars. 2,3,4-Tri-*O*-methyl-L-fucose, 4,6-di-*O*-methyl-D-galactose, and fucosidolactose were gifts from Professor R. Kuhn. 3,4-Di-*O*-methyl-D-galactose was provided by Professor B. Lindberg, 2,4,6-tri-*O*-methyl-D-galactose by Professor E. Hirst, and 2,3,4-tri-*O*-methyl-D-galactose and 2,3-di-*O*-methyl-D-galactose by Dr. G. Springer.

Methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside was prepared by methylation (see Methods) of methyl α-D-glucoside. 3,4,6-Tri-*O*-methyl-D-galactose was prepared as follows: methylation of methyl 2-*O*-methanesulfonyl-β-D-galactoside, mp 149–150° (D. A. Rees, unpublished results), gave the 3,4,6-tri-*O*-methyl derivative (mp 136–137°, $[\alpha]_D$ −25.3°). Demethanesulfonylation with $LiAlH_4$ in boiling tetrahydrofuran (sodium amalgam was ineffective) followed by acid hydrolysis of the methyl glycoside gave 3,4,6-tri-*O*-methyl-D-galactose. The product was purified by charcoal column chromatography and was identical by paper chromatography and glpc with a sample prepared by methylation and hydrolysis of fucosidolactose (Kuhn *et al.*, 1958a).

Methods

Analytical Methods. Hexosamine, *N*-acetylhexosamine, reducing sugar, and methylpentose were determined as described by Kabat (1961) and nitrogen was determined by a modified ninhydrin method as described by Schiffman *et al.* (1964a). Galactose was determined by the orcinol method (Winzler, 1955) and corrected for fucose present (Nolan and Smith, 1963). In oligosaccharides containing an unsaturated residue (see below) galactose was determined after bromination—aqueous bromine solution (0.25%) was added to a known amount of sample, excess bromine was removed by aeration, the solution was evaporated to dryness, and the residue was dissolved in a known volume of water for analysis. The bromination eliminated almost completely the contribution of the unsaturated group to the galactose value.

Periodate Oxidation. The microtitration method described by Schiffman *et al.* (1962) was used except that formic acid was determined by adding an excess of 10^{-2} N NaOH solution to the oxidized sample and back titrating after 20 min with 10^{-2} N HCl using methyl red indicator. Formaldehyde was determined by the chromotropic acid method (*cf.* Kabat, 1961).

Chromatography. Paper chromatography was carried out by the descending technique with S and S589 green label paper. The following solvent systems were used: 1-butanol–pyridine–water, 6:4:3 (solvent 1); 1-butanol–pyridine–water, 6:1:1 (solvent 2), 1-butanol–acetic acid–water, 4:1:5 (solvent 3) for separating galactose and galactitol, 1-butanol–ethanol–water, 4:1:5 (solvent 4) for methylated sugars. Alkaline silver reagent (Trevelyan *et al.*, 1950; *cf.* Kabat, 1961) was generally used for detection of sugars.

Thin layer chromatography (tlc) was carried out on silica gel G (Stahl, 1963) by elution with benzene–ethanol (20:5). Plates were developed twice. Sugars were detected with alkaline silver or by spraying with 50% sulfuric acid and heating at 110° for 30 min. Tlc was used preparatively by streaking a solution of the mixture (*ca.* 2 mg) across a 20-in. plate; the plate was then developed and the zones were located by covering the center of the plate with another glass plate, 2 cm narrower in width, before spraying with alkaline silver. The bands were removed and eluted with chloroform followed by methanol. Charcoal column chromatography was performed on Darco G-60–Celite (1:1) (Whistler and Durso, 1950) with a two-step ethanol gradient (Schiffman *et al.*, 1962).

The method of chromatography on Dowex 1 (OH^-) resin columns used by Austin *et al.* (1963) for the separation of methyl glycosides has been extended to the chromatography of nonreducing oligosaccharides. A column (30 × 1.8 cm) of Dowex 1 X 2 (200–400 mesh) was converted to the OH^- form. The sample (up to 50 mg) was applied and the column eluted with CO_2–free water. Fractions were collected and the various oligosaccharides were eluted as sharp peaks after 400–600 ml had been collected.

Biogel P2 columns were prepared as described by the

TABLE I: Relative Retention Times on Gas–Liquid Partition Chromatography of Methyl Glycosides and Methyl *O*-(Trimethylsilyl)glycosides.

	Methyl Glycosides[a]		Methyl *O*-(TMS)-glycosides[b]	
Sugars[c]	Column a (200°)	Column b (200°)	Column a (185°)	Column b (160°)
2,3-Me_2Gal	1.80 (s),[d] 2.98 (m)		0.63 (w), 0.76 (s), 0.87 (m)	0.79
2,4-Me_2Gal	2.66 (m), 3.10 (s)		0.94	0.99 (s), 1.02 (m)
4,6-Me_2Gal	1.90 (s), 2.60 (m)		0.88 (s), 1.07 (m)	0.83 (s), 1.16 (m)
3,4-Me_2Gal	3.10 (s), 3.80 (m)		0.98	1.33 (s), 1.67 (m)
2,3,4-Me_3Fuc	0.55	0.55	0.53	0.76
3,4,6-Me_3Gal	1.58 (s), 2.00 (m)	2.43 (s), 3.63 (m)	0.97 (s), 1.07 (m)	0.99 (s), 1.19 (m)
2,4,6-Me_3Gal	1.52 (m), 1.68 (s)	2.40 (m), 2.65 (s)	0.70 (s), 0.79 (m)	0.65 (s), 0.75 (m)
2,3,4-Me_3Gal	1.89 (m), 2.06 (s)	3.75	1.25	1.42
2,3,4,6-Me_4Gal	1.19	1.14	1.18 (sh), 1.25 (s)	1.61

[a] Times relative to methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucopyranoside. [b] Times relative to methyl 2,3,4,6-tetra-*O*-(trimethylsilyl)-α-D-glucopyranoside. [c] Abbreviations used: 2,3-Me_2Gal, 2,3-di-*O*-methyl-D-galactose; 2,4-Me_2Gal, 2,4-di-*O*-methyl-D-galactose; 4,6-Me_2Gal, 4,6-di-*O*-methyl-D-galactose; 2,3,4-Me_3Fuc, 2,3,4-tri-*O*-methyl-L-fucose; 3,4,6-Me_3Gal, 3,4,6-tri-*O*-methyl-D-galactose; 2,4,6-Me_3Gal, 2,4,6-tri-*O*-methyl-D-galactose; 2,3,4-Me_3Gal, 2,3,4-tri-*O*-methyl-D-galactose; 2,3,4,6-Me_4Gal, 2,3,4,6-tetra-*O*-methyl-D-galactose. [d] Relative intensities of peaks are indicated by s = strong, m = medium, sh = shoulder, and w = weak. Multiple peaks are found in most cases because equilibrium mixtures of methyl glycosides were used.

manufacturers (Bio-Rad Laboratories). Samples (10–50 mg) were chromatographed on 65–70 × 1.9 cm columns by elution with water. It was found to be important that small (1–1.2 ml) fractions were collected. Oligosaccharides were eluted in approximately the reverse order to their molecular weights; the branched trisaccharide (AR_L 0.96a) was eluted at 110–115 ml, the B pentasaccharide at 90–98 ml, and the B hexasaccharide at 60–65 ml.

Methylation. Methylations were carried out with methyl iodide in dimethylformamide with BaO (Kuhn *et al.*, 1958b) or Ag_2O (Kuhn *et al.*, 1955). For the methylation of oligosaccharides these two methods were used in succession: oligosaccharide (2–3 mg) was dissolved in dry DMF (100 μl) to which 50 μl of methyl iodide and Ag_2O (150 mg) were added, and the tube was sealed. After standing at 37° for about 6 hr, the mixture was shaken overnight. The precipitate was centrifuged off and washed with chloroform (three 0.5-ml portions). The combined solutions were evaporated to dryness at 50°. The residue was remethylated as above but using 100 mg of BaO as catalyst. After evaporation of the chloroform solution to dryness the residue was redissolved in chloroform (1 ml) and washed with water (three 1-ml portions). Evaporation of the chloroform solution yielded the methylated product. Hydrolysis of the methylated oligosaccharides in 2 N H_2SO_4 for 2–3 hr gave the constituent methylated monosaccharides. Their methyl glycosides were prepared by heating with 2.5% methanolic HCl in a sealed tube at 100° for 4–6 hr. After neutralization (Ag_2CO_3) the solutions were evaporated at room temperature to prevent loss of volatile trimethylfucosides and tetramethylgalactosides.

The completeness of methylation could not be checked by infrared analysis because of the –NH absorption at about the same wavelength as the –OH band. However no products of partial methylation were detected by thin layer and gas–liquid partition chromatography after two methylations, although they could be after the first methylation step. This shows that a high degree of methylation was obtained. Also applications of this methylation procedure to lactose and fucosylgalactitol gave products showing no –OH band in their infrared analysis spectrum and giving only the expected products in hydrolysis.

Gas–Liquid Partition Chromatography (glc). A Perkin-Elmer 800 gas chromatograph was used. The separation of methylated sugars as their methyl glycosides was carried out on (a) polyphenyl ether (6 rings) on silanized Celite (10%) at 200°, and (b) butane-1,4-diol succinate polyester on Celite (15%) at 200°, essentially as described by Aspinall (1963). The columns were 6 ft × 0.25 in. and the elution was with nitrogen at 30 ml/min. The elution times are given (Table I) relative to methyl 2,3,4,6-tetra-*O*-methyl-α-D-glucoside (6.2 min on column a and 4.2 min on column b).

The methylated methyl glycosides were also converted to their *O*-(trimethylsilyl) ethers and run on column a at 185° and column b at 160° (*cf.* Lee and Ballou, 1965). This method was more satisfactory for the di-*O*-methylglycosides. The elution times (Table I) are compared to methyl 2,3,4,6-tetra-*O*-(trimethylsilyl)-α-D-glucoside (9.2 min on column a and 10 min on column b). Table I gives elution times for the methyl sugars pertinent to this study and for some other methyl sugars which were available to us.

TABLE II: Yields, Specific Optical Rotations, and Analytical Properties of Isolated Oligosaccharides.

Oligosaccharide	Yield (mg)	$[\alpha]_{5461}$ (deg)	Unsatn[a] Test	% Compn						Molar Ratios							
				N	Fuc	Gal	Gal[b]	Hex	NAc-Hex	N/Fuc	N/Hex	Gal/Fuc	Gal[b]/Fuc	Hex/Fuc	NAc-Hex/Hex	Gal[b]/Hex	Gal[b]/N
MSS ADR_{IM5} 1.0[c]	7.1	−44.6	+	2.18	26.7	20.2	16.3	23.6	20.9	0.96	1.19	0.69	0.58	0.81	0.72	0.69	0.58
Calcd				2.79	32.3	17.9	17.9	35.6		1.0	1.0	0.5	0.5	1.0	0.65	0.5	0.5
Beach BR_{IM5} 1.2	23.7	−118	+	1.25	33.2	39.3	38.4	14.2	18.6	0.48	1.13	1.17	1.05	0.42	1.05	2.7	2.4
Calcd				1.45	34.0	37.2	37.2	18.5	22.9	0.5	1.0	1.0	1.0	0.5	1.0	2.0	2.0
JS HR_{IM5} 2.5	29.7	−130	+	1.5	39.2	37.7	29.6	22.0	24.7	0.45	1.13	1.14	0.67	0.51	0.95	1.3	1.5
Calcd				1.74	40.8	22.4	22.4	22.3	27.5	0.5	1.0	0.5	0.5	0.5	1.0	1.0	1.0
MSS ADR_{IM5} 2.5a[c]	6.4	+26.4	−	2.5	14.6	14.9		27.7	24.3	1.99	1.15	0.94		1.74	0.70	0.54	0.47
MSS ADR_{IM5} 2.5b[c]	8.2	+26.4	−	2.6	14.3	15.6		26.8	23.4	2.09	1.22	0.99		1.71	0.70	0.58	0.47
Calcd				3.13	18.3	20.1	20.1	40.1		2.0	1.0	1.0	1.0	2.0	0.65	0.5	0.5
MSS AR_L 0.52	8.0	+28.7	+	2.8	17.0	19.0		32.0	24.0	1.9	1.1	1.0		1.70	0.66	0.61	0.53
MSS ADR_L 0.52	97.0	+33.5	+	3.4	18.0	29.2	21.2	35.0	28.6	2.2	1.2	1.48	1.0	1.77	0.66	0.84	0.67
MSM AR_L 0.52	110	+27.0	+	3.4	19.0	25.3	21.7	37.0	29.0	2.0	1.17	1.18	1.04	1.75	0.64	0.59	0.58
Hog AR_L 0.52	45.5	+25.8	+	3.5	18.3	23.8	21.7	36.0	30.2	2.1	1.2	1.17	1.1	1.85	0.66	0.76	0.53
Calcd				3.3	19.2	20.9	20.9	41.5		2.0	1.0	1.0	1.0	2.0	0.65	0.5	0.5
Beach BR_L 0.44	10.0	−8	+	1.58	20.0	44.7	43.5	18.3	23.0	1.02	1.10	2.16	1.99	0.89	1.01	2.35	2.13
Calcd				1.71	20.0	43.9	43.9	21.8	26.9	1.0	1.0	2.0	2.0	1.0	1.0	2.0	2.0
JS HR_L 0.75	28.0	−116	+	2.0	23.8	43.0	32.5	24.8	28.7	0.97	1.0	1.65	1.3	0.96	0.95	1.34	1.33
Hog HR_L 0.75	19.4	−111	+	2.0	24.5	40.0	32.0	28.1	29.1	0.98	0.92	1.48	1.2	1.05	0.92	1.30	1.35
Calcd				2.14	25.0	27.6	27.6	27.4	34.1	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
MSS ADR_L 0.96a	18.7	+17.1	−	2.27	30.6	0	0	28.9	13.1	0.87	1.01			0.86	0.37		
MSM AR_L 0.96a	5.08	+65.3	−	2.22	28.6	0	0	25.2	12.3	0.91	1.12			0.81	0.39		
Calcd				2.64	30.4	0	0	33.9		1.0	1.0			1.0	0.31		
MSM AR_L 0.96b	3.0		+	2.6	2.3	24.7		19.2	25.7		1.70				1.08	1.28	0.74
JS HR_L 0.96b	8.3	−7.2	+	2.4	1.5	41.8	32.0	26.5	30.7		1.17				0.93	1.20	1.02
Calcd				2.74	0	35.1	35.1	35.1	43.2		1.0				1.0	1.0	1.0

[a] $KMnO_4$ test. [b] Corrected for orcinol color given by unsaturated residue by bromination before analysis. [c] The per cent composition values of these fractions are low although the molar ratios are close to the expected values. Because of the small amounts isolated, inert material from the columns which usually does not contribute significantly makes up a larger proportion of the total weight.

Experimental Section and Results

Isolation and Purification of Oligosaccharides. Purified blood group substances (see Materials) were hydrolyzed in 0.2 M sodium hydroxide containing 1% sodium borohydride at room temperature for 7 days. A sample of human A substance (MSS, 3 g) was also treated with NaOD and $NaBD_4$ in deuterated water (*cf.* Kabat *et al.*, 1965). Fragments isolated from this reaction are designated as AD. The hydrolysis products were separated by dialysis as described by Schiffman *et al.* (1964a).

The products from human A and H substances were fractionated by preparative paper chromatography using solvent 1 for oligosaccharides with $R_{Lactose}$ (R_L) <1 and solvent 2 for those with greater R_F. Fractions were rechromatographed until only a single component could be detected by paper chromatography. The R_F for the various fractions is given in the nomenclature used to identify the oligosaccharides.[2] Two fractions from human B substances of corresponding degree of purification were also available (Schiffman *et al.*, 1964a; fractions B_2 and B_3). The oligosaccharides usually contained excess nitrogen (over hexosamine) at this stage and were further purified by charcoal column chromatography. This step gave MSM AR_L 0.52 (16%),[3] MSS AR_L 0.52 (15%), MSS ADR_L 0.52 (15%), hog AR_L 0.52 (13%), MSS ADR_{IM5} 2.5a (13%), MSS ADR_{IM5} 2.5b (15%), the trisaccharides (12%), and the disaccharides (7–10%) described earlier (Lloyd and Kabat, 1964) as analytically pure products. The dialyzable material from hog A + H substance was fractionated on a large charcoal column before the preparative paper step. Fractions, which after final purification by charcoal chromatography did not give satisfactory analytical values, were further purified in either of two ways: (a) chromatography on Dowex 1 (OH) columns; fractions JS HR_{IM5} 2.5, JS HR_L 0.75, Hog HR_L 0.75, and MSS ADR_{IM5} 1.0 were obtained pure by this method; and (b) chromatography on Biogel P2 columns. This was used in the final step of the purification of Beach BR_{IM5} 1.2, Beach BR_{IM5} 0.44, MSM AR_L 0.96a, and MSS ADR_L 0.96a.

The yields of the various oligosaccharides together with chromatographic data and their optical rotation are given in Table II. In addition 57 mg of *N*-acetylgalactosaminitol was isolated from Beach B substance. This fraction crystallized to give 32 mg of product with mp and mmp 172–173°. The compound had $[\alpha]_{5461}$ −48.4° and *N*-acetylgalactosaminitol has $[\alpha]_{5461}$ −50.5°. The weights of crude and purified product represent 44 and 25%, respectively, of the total *N*-acetylgalactosamine present in Beach B substance.

Determination of Constituent Sugars of the Oligosaccharides. Analytical data (Table II) showed the reducing sugars present. These were confirmed by chromatographic examination (solvent 1) of the sugars present after hydrolysis in 2 N sulfuric acid for 2 hr at 100°. The hydrolysates were also examined for galactitol by chromatography in solvent 3.

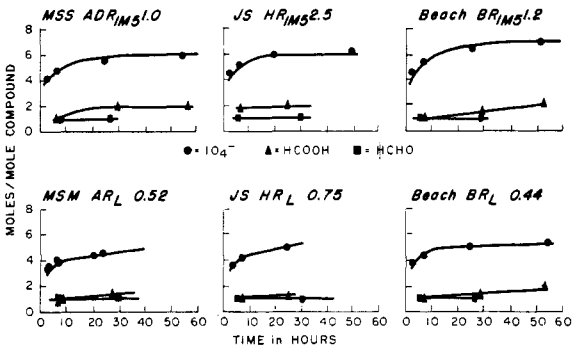

FIGURE 1: Curves for periodate uptake and formic acid and formaldehyde formed on periodate oxidation of fucose-containing oligosaccharides.

Partial Acid Hydrolysis. By hydrolysis of 200 μg in 200 μl of dilute HCl solution (pH 2.5) for 1.5 hr followed by chromatography in solvent 2 many of the constituent disaccharides could be recognized. In particular β-D-Gal-(1→3)-GNAc and β-D-Gal-(1→4)-GNAc could be distinguished. In oligosaccharides containing the unsaturated tetraol R, both free R and the disaccharide GNAc-R were detected. In the case of the two AR_{IM5} 2.5 oligosaccharides the galactosyl-*N*-acetylglucosamine linkage was determined after oxidation of the oligosaccharides by periodate. These oligosaccharides would give GNAc-galactitol disaccharides on hydrolysis which have approximately the same R_F as the galactosyl-*N*-acetylglucosamine disaccharides in paper chromatography. By oxidation of the galactitol residue this complication was eliminated. Results are summarized in Table III.

Periodate Oxidation Studies. The curves for the periodate oxidation of some of the oligosaccharide are shown in Figure 1. The data are calculated on a weight basis but except for ADR_{IM5} 1.0, the same values are obtained when they are calculated on the basis of moles of fucose or galactose. ADR_{IM5} 1.0 which contains some inert material consumes 6.2 moles of IO_4^-, and liberates 2.2 moles of HCOOH and 1.1 moles of HCHO/mole of galactose (after 30 hr). These values are essentially the same as those given in Figure 1. In the data previously reported (Lloyd and Kabat, 1964), the figures for formic acid release determined iodometrically were often low. These determinations were repeated using the acidimetric method described above and values close to theoretical were obtained. Recently, Belcher *et al.* (1965) also found that determination of formic acid by the iodometric method gives low results and that an acidimetric method is superior for this determination on a microscale. Compounds containing the terminal unsaturated tetraol were

[2] The oligosaccharides are named according to their source and their R_L (R relative to lactose) or their R_{IM5} (R relative to isomaltopentaose) in solvent 1, *e.g.*, MSS AR_L 0.52 is an oligosaccharide from MSS A substance which has an R_L of 0.52 in solvent 1.

[3] The concentration of ethanol at which the compound was eluted from the column is given after each fraction.

TABLE III: Disaccharides Identified after Partial Acid Hydrolysis of Oligosaccharides and Sugar Methyl Ethers Identified by Gas–Liquid Partition Chromatography after Hydrolysis of Methylated Oligosaccharides.

Oligosaccharide	Disaccharides Identified after Partial Acid Hydrolysis	Sugar Methyl Ethers Identified by Gas–Liquid Partition Chromatography
MSS ADR_{IM5} 1.0	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	2,3,4-Me_3Fuc[a] and 4,6-Me_2Gal
MSS ADR_{IM5} 2.5a	β-D-Gal-(1→3)-GNAc[b]	2,3,4-Me_3Fuc and 4,6-Me_2Gal
MSS ADR_{IM5} 2.5b	β-D-Gal-(1→4)-GNAc[b]	2,3,4-Me_3Fuc and 4,6-Me_2Gal
MSS AR_L 0.52	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	2,3,4-Me_3Fuc and 4,6-Me_2Gal
Hog AR_L 0.52	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	2,3,4-Me_3Fuc and 4,6-Me_2Gal
Beach BR_{IM5} 1.2	β-D-Gal-(1→4)-GNAc, β-D-GNAc-R, and α-D-Gal-(1→3)-Gal	2,3,4-Me_3Fuc, 2,3,4,6-Me_4Gal, and 4,6-Me_2Gal
Beach BR_L 0.44	β-D-Gal-(1→4)-GNAc, β-D-GNAc-R, and α-D-Gal-(1→3)-Gal	2,3,4-Me_3Fuc, 2,3,4,6-Me_4Gal, and 4,6-Me_2Gal
JS HR_{IM5} 2.5	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	2,3,4-Me_3Fuc and 3,4,6-Me_3Gal
JS HR_L 0.75	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	2,3,4-Me_3Fuc and 3,4,6-Me_3Gal
Hog HR_L 0.75	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	2,3,4-Me_3Fuc and 3,4,6-Me_3Gal
MSM AR_L 0.96b	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	
JS HR_L 0.96b	β-D-Gal-(1→4)-GNAc and β-D-GNAc-R	

[a] See Table I for abbreviations used. [b] Determined after periodate oxidation of oligosaccharides.

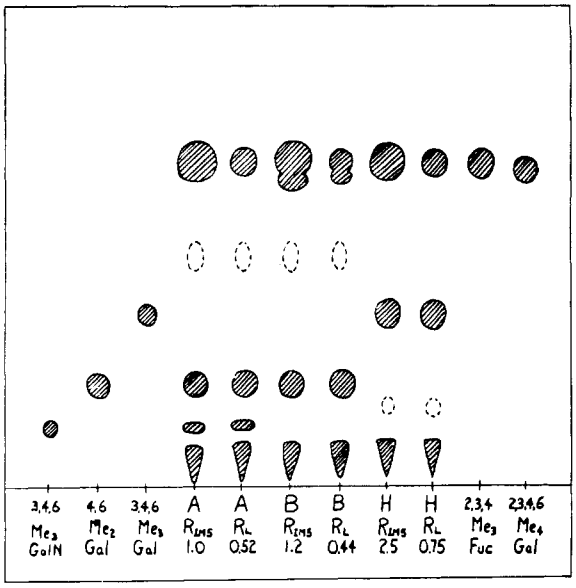

FIGURE 2: Thin layer chromatography of hydrolysates of methylated oligosaccharides. See footnote 1 and Table I for abbreviations used; also 3,4,6-Me_3GalN, 3,4,6-tri-*O*-methylgalactosamine.

overoxidized fairly rapidly and this effect is particularly noticeable in compounds AR_L 0.52 and HR_L 0.75.

Methylation Analysis. Following methylation of oligosaccharides the products were hydrolyzed and examined by thin layer chromatography. This gave a quick indication of the fucose and galactose methyl ethers present (Figure 2). The hydrolysate of AR_L 0.96a was examined on two plates, one of which showed three spots when developed with H_2SO_4 spray. The other was sprayed with periodate–benzidine (Viscontini *et al.*, 1955) and only one component could be detected. Comparison with the other plate showed that this compound, which must have at least two vicinal hydroxyl groups, is a methylated galactitol.

The identification of the fucose and galactose methyl ethers made by tlc was confirmed by gas chromatography. The sugars were chromatographed on two different columns, first as their methyl glycosides and then as the trimethylsilyl ethers of the methyl glycosides. The elution times were compared with the times for the known methyl ethers. In all cases the observed values were within 3% (and in most cases within 2%) of the values given in Table I. Although the identity of the methyl sugars with authentic samples has been demonstrated, the possibility that the three unstudied di- and trimethylgalactoses (other than those derived from furanose sugars) may give identical results cannot be completely ruled out. However in conjunction with the values given by Aspinall (1963) for two of these derivatives it is clear that, with the possible exception of 3,6-dimethylgalactose which has not been studied, all the di- and trimethylgalactoses under consideration can be distinguished on these two columns as their methyl glycosides. In most cases the mixture of methyl sugars was separated by preparative tlc before preparation of the methyl glycosides. Small amounts of volatile material were eluted from the silica gel, and a blank determination was necessary. It was also possible to prepare the methyl glycosides of the hydrolysate mixture directly since the presence of amino sugars and their TMS derivatives did not interfere with gas chromatography of the fucose and galactose glycosides. The methyl ethers of fucose and galactose identified are shown in Table III.

Identification of α-L-Fucopyranosyl-(1→2)-D-galactitol. The disaccharide containing fucose and galactitol (Lloyd and Kabat, 1964) was acetylated by treating a sample (Hog HR_{gal} 0.70, 5 mg) with acetic anhydride (50 μl) in dry pyridine (50 μl). After standing at room temperature for 1 day the solution was added to ice water and the mixture was extracted with chloroform. Evaporation of the chloroform solution gave a syrup which crystallized on standing in the cold. On recrystallization from ethanol–water 3 mg of product (mp 60–62°) was obtained. An authentic sample of α-L-fucopyranosyl-(1→2)-D-galactitol was prepared from fucosidolactose by alkaline hydrolysis (as described by Kuhn *et al.*, 1958a) and reduction of the product (α-fucosyl-(1→2)-galactose) with sodium borohydride. Acetylation gave a crystalline product (mp 60–62°) and a mixture melting point with the acetate of the compound from hog mucin was not depressed. Their infrared spectra (KBr disk) were also identical. No talitol was detectable by chromatography in solvent 2 after hydrolysis of the fucosylgalactitol.

Isolation of 3-Hexene-1,2,5,6-tetrol. A compound eluted from charcoal with water and having a high R_F on paper chromatography (R_{gal} 3.5 in solvent 2) was finally purified on a Biogel P2 column. The syrupy product (15 mg) contained almost no nitrogen (0.5%). The compound quickly decolorized permanganate and bromine solutions, showing that it was unsaturated. This was confirmed by its infrared spectrum (in KBr pellet) which had a strong band at 1640 cm^{-1}. It consumed 3.5 moles of periodate and liberated 1.83 moles of formaldehyde and 0.5 mole of formic acid in 5 hr. The formaldehyde figure indicates that the compound has two primary hydroxyl groups with vicinal hydroxyls and thus a double bond between C-3 and C-4. This was confirmed by ozonolysis: a small sample was treated with excess ozone in methanol at −50° and the ozonide reduced with hydrogen with palladium–charcoal catalyst. Examination of the reduced product by paper chromatography in solvents 1 and 2 showed an elongated spot with the same R_F as glyceraldehyde. After reduction with sodium borohydride only glycerol could be identified chromatographically in solvent 4 (glycerol is clearly separated from ethylene glycol and erythritol or threitol in this solvent). These results show the compound is a 3-hexene-1,2,5,6-tetrol. An authentic sample of *trans*-3-hexene-*erythro*-1,2,5,6-tetrol was provided by Dr. E. F. L. J. Anet [this compound, mp 114°, was originally described by Anet (1965) as being the D-*threo* isomer (E. F. L. J. Anet, personal communication)]. It had the same R_F as the tetrol described above and behaved similarly on periodate oxidation, although their infrared spectra were different. However, the infrared spectrum of sample from blood group substance was identical with the spectrum (also provided by Dr. Anet) of *trans*-3-hexene-D-*threo*-1,2,5,6-tetrol. An authentic sample of the D-*threo* isomer (provided by Dr. R. S. Tipson; Tipson and Cohen, 1966) melted at 64–65° and had $[\alpha]_{5461}$ −32°. Our syrupy sample crystallized on standing and gave mp 60–65° and mmp 59–62° and $[\alpha]_{5461}$ −21°.

Identification of the 3-Hexene-1,2,5,6-tetrol in Oligosaccharides. Some of the oligosaccharides also decolorized permanganate and bromine (Table II). Acid hydrolysis of these compounds did not give galactitol and after hydrolysis in 2 N HCl for 2 hr no reduced residue corresponding to the reducing end could be detected by alkaline silver. However after mild hydrolysis (pH 2.5 for 1 hr) a component having the same R_F as the unsaturated tetraol described above could be detected by paper chromatography in solvents 1 and 2. Treatment of the unsaturated tetraol (R) with 0.1 M HCl at 100° for 6 hours resulted in the formation of a compound which did not reduce alkaline silver, thus accounting for the failure to detect R by 2 N acid hydrolysis and chromatography. The compound formed by the action of acid had a strong absorption in the ultraviolet (λ_{max} 270 mμ) and treatment of 3-hexene-*erythro*-1,2,5,6-tetrol gave a product with the same ultraviolet spectrum.

Discussion

Isolation and Purification of Oligosaccharides. The action of sodium hydroxide and sodium borohydride on blood group substances results in a very complex mixture of products and examination of a paper chromatogram of the dialysates reveals a series of compounds ranging from monosaccharides through oligosaccharides to higher molecular weight material not moving from the origin (Schiffman *et al.*, 1964a). The fractionation of this complex mixture, even with the new chromatographic methods now available, is a difficult task and pure samples of only the smaller oligosaccharides (hexasaccharides and smaller) have been isolated. The yields of oligosaccharides reported in Table II are only minimum yields since the fractionation methods were developed during their isolation, loss of material necessarily occurring. A fractionation sequence considered to give optimum yields of pure oligosaccharides is the following. The dialyzable product is first fractionated on a large charcoal column to give a rough separation into fractions each containing two or three oligosaccharides. A major advantage of the initial charcoal column fractionation is that nonfucose-containing low molecular weight reduced mono- and disaccharides and some amino acid containing materials are eluted before any fucose-containing oligosaccharides. The latter fractions are then purified by preparative paper chromatography and compounds from different, but adjacent charcoal fractions, with the same R_F values, are combined. These products, although chromatographically pure on paper, usually have a high nitrogen content and are further purified by charcoal column chromatography and rechromatographed if necessary to give a single peak. The products can then be finally purified by chromatography on either Dowex 1 (OH^-) ion-exchange columns or by gel filtration on Biogel P2.

The purity of the isolated oligosaccharides is based on many criteria, some of which are inherent in the purification procedures. The fragments give one spot on paper chromatography (in two solvents for the faster

moving oligosaccharides). They give single peaks on column chromatography on at least one and in most cases two different types of columns. The peaks are symmetrical when analyzed for both periodate uptake and fucose content. Complete hydrolysis and paper chromatography showed the presence of only those sugars indicated by analysis and no traces of other sugars. Analysis of the oligosaccharides for N, methylpentose, galactose, hexosamine, and *N*-acetylhexosamine showed that in most cases the sugars are present in molar stoichiometric amounts within the precision of the analytical methods. The most serious exception is AR_{IM5} 1.0 of which only 7 mg were isolated. In this fraction the molar ratios are only approximately stoichiometric and it contains some inert, nonsugar material. Fractions AR_{IM5} 2.5a and AR_{IM5} 2.5b also contain a little inert material. We have also measured the optical rotation of the oligosaccharides so that different samples and products from different sources (human and hog) may be compared as an additional check on purity.

Although the oligosaccharides are considered to be homogeneous with respect to their sugar chains, no claim is made at this stage that they are necessarily homogeneous with respect to isomers of the unsaturated residue R. Thus at least two isomers of this residue, D-*threo* and -*erythro*, are possible and recently MSS ADR_L 0.52 has been separated by chromatography on Biogel P-2 into two components having identical analytical composition and periodate oxidation curves.

Structure of Oligosaccharides. Since the importance of alkaline degradation lies in the ability to produce fucose-containing oligosaccharides, the early isolation of a reduced disaccharide containing only fucose and galactitol was of interest and helped in the elucidation of the structure of the higher fucose-containing oligosaccharides. On the basis of analyses and periodate oxidation studies it was suggested (Lloyd and Kabat, 1964) that the structure of this disaccharide was α-L-fucopyranosyl-(1→2)-D-galactitol. This structure has been confirmed by the preparation of a crystalline acetate and comparison with an authentic sample prepared from Kuhn's fucosidolactose.

A branched trisaccharide from A substance was earlier suggested (Lloyd and Kabat, 1964) to have the structure AR_L 0.96a as shown in Figure 3. This structure was suggested on the basis of periodate and immunochemical data, which did not however definitely exclude the possibility that the galactitol was substituted on C-4 and C-6 rather than on C-2 and C-3. This ambiguity has been resolved by the methylation of AR_L 0.96a. In addition to 2,3,4-tri-*O*-methylfucose and methylated galactosamine the hydrolysate also contained a tetramethylgalactitol which could be oxidized with periodate. This alcohol, therefore, has a pair of vicinal hydroxyl groups and must be 1,4,5,6-tetra-*O*-methylgalactitol required by the structure AR_L 0.96a and not the 1,2,3,5-tetra-*O*-methylgalactitol expected from the alternate structure. Two samples of AR_L 0.96a, one from MSS and the other from MSM blood group substances, have been isolated. Although both seemed to have the same structure as determined by periodate and methylation studies, their optical rotations were different (Table II). This difference was possibly explained when complete acid hydrolysis and chromatography in solvent 2 showed that MSM AR_L 0.96a contained a small amount of talitol in addition to galactitol. MSS had only a minute trace of talitol and is therefore the purer sample. This talitol-containing isomer had probably been formed by an epimerization reaction due to the alkaline degradation conditions. It is pertinent that Kuhn *et al.* (1958a) produced fucosyltalose by the action of alkali on fucosidolactose.

The majority of the larger oligosaccharides are terminated by a reduced, unsaturated residue R. A disaccharide with this residue has been described earlier (Lloyd and Kabat, 1964) and it has the structure β-D-GNAc→R, the linkage being assigned on the basis of its low optical rotation. This reduced disaccharide was recognized in the partial hydrolysates of many of the larger oligosaccharides. Also two samples of a trisaccharide containing this residue have been isolated (MSM AR_L 0.96b and JS AR_L 0.96b, Table II). This oligosaccharide was isolated from both human A and H substances and partial acid hydrolysis (Table III) has shown it to have the structure β-D-Gal-(1→4)-β-D-GNAc-R.

On the basis of periodate oxidation studies a partial structure had earlier (Lloyd and Kabat, 1964) been suggested for the highly A-active pentasaccharide AR_L 0.52 (then called A_3). These studies did not definitely determine the position of the fucose residue in the molecule. The identification of 2,3,4-tri-*O*-methylfucose and 4,6-di-*O*-methylgalactose after methylation and hydrolysis showed the fucose is attached to the galactose residue and not to *N*-acetylglucosamine, an alternate possibility. The linkage is probably α by analogy with the α-fucosylgalactitol disaccharide isolated. Identification by partial acid hydrolysis of the internal galactosyl-*N*-acetylglucosamine linkage as β-(1→4) established the structure shown in Figure 3. This oligosaccharide was isolated from both human A and hog (A + H) substances.

A reduced pentasaccharide (Beach B R_L 0.44) from B substance has the same general structure as AR_L 0.52 except that galactose replaces *N*-acetylgalactosamine as the terminal nonreducing sugar. Partial acid hydrolysis gave the sequence of sugars and showed that this oligosaccharide also has the β-D-Gal-(1→4)-GNAc linkage. The fucose was again shown to be terminal and to occupy a branched position on C-2 of the internal galactose residue by identification of 2,3,4-tri-*O*-methylfucose and 4,6-di-*O*-methylgalactose by methylation analysis. The presence of 2,3,4,6-tetra-*O*-methylgalactose in the methylation hydrolysate showed that the oligosaccharide has a terminal galactose residue. Periodate oxidation data (Figure 1; Calcd: 5 moles of periodate consumed with the liberation of 1 mole of HCHO and 2 moles of HCOOH) confirmed the structure for BR_L 0.44 shown in Figure 3.

The high serologic activity (see following paper) of these two oligosaccharides confirms earlier evidence

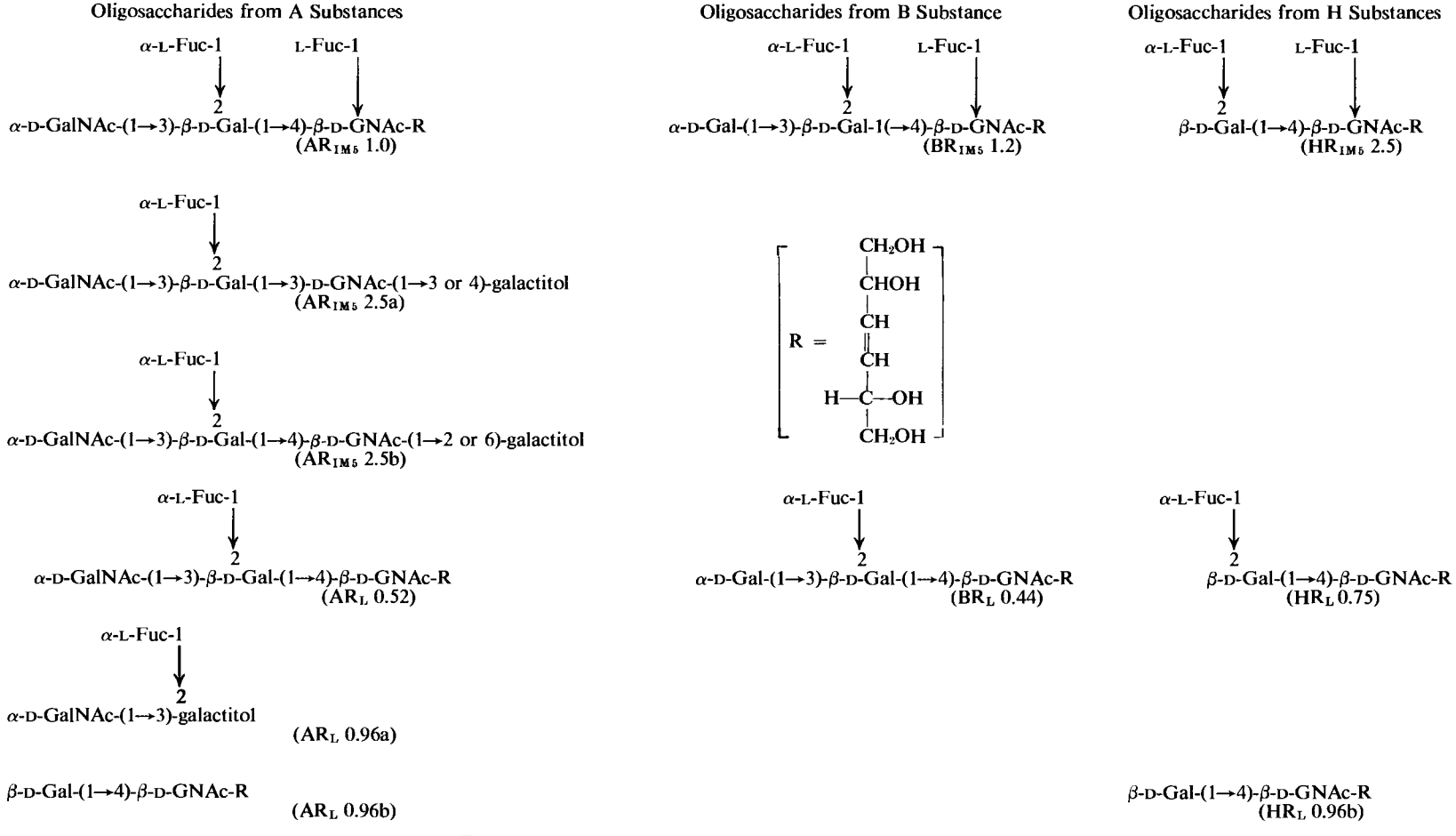

FIGURE 3: Partial structures of oligosaccharides from A, B, and H substances. Abbreviations used: Fuc, L-fucopyranose; Gal, D-galactopyranose; GalNAc, 2-acetamido-2-deoxy-D-galactopyranose; GNAc, 2-acetamido-2-deoxy-D-glucopyranose.

that terminal nonreducing *N*-acetylgalactosamine and galactose play important roles in the determinants of A and B substances, respectively. In H specificity L-fucose assumes a dominant role and in agreement with this the H-active oligosaccharide HR_L 0.75 contains only fucose, galactose, and *N*-acetylglucosamine. The terminal position of the fucose residue and its substitution on the C-2 position of the galactose was demonstrated by methylation studies. Thus after methylation and hydrolysis of JS and hog HR_L 0.75 both 2,3,4-tri-*O*-methylfucose and 3,4,6-tri-*O*-methylgalactose were identified. Partial acid hydrolysis showed that β-D-Gal-(1→4)-GNAc is again present. These conclusions were confirmed by periodate oxidation studies (Figure 1; Calcd: 4 moles of periodate consumed with 1 mole of HCHO and 1 mole of HCOOH liberated) and the destruction of galactose and fucose by the oxidation. This oligosaccharide was also isolated from both human and hog blood group H substances. The structure of HR_L 0.75 (Figure 3) is basic to the A and B oligosaccharides discussed earlier (*cf.* Rege *et al.*, 1964a; Painter *et al.*, 1965). All three oligosaccharides also have the fucosyl-(1→2)-galactose linkage indicated by the isolation of the reduced form of this disaccharide. α-L-Fucosyl-(1→2)-galactose has been isolated by triethylamine hydrolysis (Rege *et al.*, 1964a), in agreement with these findings.

These three oligosaccharides are terminated, at what was originally the reducing end, by the unsaturated residue R. However two A-active pentasaccharides have been isolated which have structures similar to AR_L 0.52 but which are terminated by galactitol. After hydrolysis of the methylated oligosaccharides both 2,3,4-tri-*O*-methylfucose and 4,6-di-*O*-methylgalactose were identified, illustrating this similarity. AR_{IM5} 2.5b has the β-D-Gal-(1→4)-GNAc linkage present in the other three oligosaccharides but AR_{IM5} 2.5a has the alternative β-D-Gal-(1→3)-GNAc linkage. The oxidation of these two oligosaccharides by periodate was studied to determine the position of substitution on the galactitol residues. Since these two fractions contain some inert, noncarbohydrate material (see Table II) the results were calculated both on a weight basis and on the basis of moles of fucose. AR_{IM5} 2.5a reduced 5.8 moles of periodate and liberated 1.75 moles of HCHO and 2.7 moles of HCOOH/mole of compound in 30 hr (or 7.2 moles, 2.18 moles, and 3.38 moles, respectively/mole of fucose). There was some overoxidation. AR_{IM5} 2.5b consumed 5 moles of periodate and liberated 1 mole of HCHO and 2 moles of HCOOH/mole of compound in 30 hr, with little further oxidation (or 6 moles, 1.2 moles, and 2.5 moles, respectively/mole of fucose). Although these compounds are not completely pure the large difference in the amount of formaldehyde release from the two compounds shows that in AR_{IM5} 2.5a the linkage to galactitol is to C-3 or C-4 and in AR_{IM5} 2.5b to C-2 or C-6. The difference is well beyond that which could result from contamination. Partial structures for AR_{IM5} 2.5a and AR_{IM5} 2.5b are shown in Figure 3.

Oligosaccharides AR_{IM5} 1.0, BR_{IM5} 1.2, and HR_{IM5} 2.5 are the first active oligosaccharides having two fucose residues which have been isolated from blood group substances. Periodate oxidation studies (Figure 1) suggest that the extra fucose is unsubstituted and occurs as a single sugar side chain since each of the oligosaccharides consumes 2 moles of periodate and liberates 1 mole of formic acid more than the corresponding monofucooligosaccharide. This was confirmed by methylation studies. 2,3,4-Tri-*O*-methylfucose but no di- or monomethylfucoses could be detected and the tri-*O*-methylfucose spot on tlc was visually estimated to be about twice the size of the trimethylfucose spot from the monofucooligosaccharides (Figure 2). All three oligosaccharides are unsaturated and partial acid hydrolysis (Table III) shows that all have the sequence: β-D-Gal-(1→4)-GNAc-R as a part of their structure.

The reduced pentasaccharide from H substance (HR_{IM5} 2.5) is the simplest of these oligosaccharides and except for the extra fucose residue it has the same structure as the tetrasaccharide HR_L 0.75. After methylation and hydrolysis 2,3,4-tri-*O*-methylfucose and 3,4,6-tri-*O*-methylgalactose were detected (Table III). Thus one of the fucose residues is again terminal and substituted on the C-2 position of galactose. The galactose residue cannot be further substituted and periodate oxidation releases 1 mole of formaldehyde from the unsaturated residue R, so that if it was originally present as a galactopyranose it is also not substituted by the second fucose residue. Therefore, the *N*-acetylglucosamine residue is the site of attachment of the other fucose to the oligosaccharide chain (Figure 3). Similarly the structure of BR_{IM5} 1.2 is based on the pentasaccharide BR_L 0.44 with an additional fucose attached to the *N*-acetylglucosamine residue. 2,3,4,6-Tetra-*O*-methylgalactose, 2,3,4-tri-*O*-methylfucose, and 4,6-di-*O*-methylgalactose were detected in the hydrolysate of the methylated oligosaccharide, again showing that one of the fucose residues is linked to the C-2 of the penultimate galactose and, therefore, that only *N*-acetylglucosamine remains as the site of attachment of the second fucose residue (Figure 3). After methylation and hydrolysis of the reduced hexasaccharide from A substance both 2,3,4-tri-*O*-methylfucose and 4,6-di-*O*-methylgalactose were detected (Table III). The unlikely possibility that the second fucose is linked to the terminal *N*-acetylgalactosamine, rather than the *N*-acetylglucosamine as in the other two oligosaccharides, can be discounted since in the methylation hydrolysate a component having the same R_F as 3,4,6-tri-*O*-methylgalactosamine was detected by tlc (Figure 2). AR_{IM5} 1.0, therefore, has the partial structure shown in Figure 3.

Many of the oligosaccharides described are terminated, at what was originally the reducing end, by the unsaturated residue R, and a monomeric unsaturated sugar alcohol was isolated as a product of the degradation reaction. Since mild acid hydrolysis of the unsaturated oligosaccharides gave a component having the same R_F as this alcohol it seemed likely that it had the same structure as the residues with which these

oligosaccharides were terminated. The monomeric alcohol formed two moles of formaldehyde on periodate oxidation, and identification of glycerol following ozonolysis and reduction showed that it was a 3-hexene-1,2,5,6-tetraol. It was identical, by paper chromatography, gas chromatography of the tetra-*O*-methyl and tetra-*O*-(trimethylsilyl) ethers, and periodate oxidation with *trans*-3-hexene-*erythro*-1,2,5,6-tetrol (prepared by Anet (1965) from 3-deoxy-2-*O*-methyl-β-D-*erythro*-hexofuranos-2-ene). Its infrared spectrum however was different from this compound but almost identical with the spectrum of *trans*-3-hexene-D-*threo*-1,2,5,6-tetrol (prepared by Tipson and Cohen (1966) from 3,4-dimethanesulfonate or 3,4-di-*p*-toluenesulfonate of 1,2,5,6-di-*O*-isopropylidene-D-mannitol). Although the spectrum indicates the presence of only one isomer, melting point and optical rotation data show that up to 33% of the *erythro* isomer might be present, although a part of the low rotation could be attributable to small amounts of inert material from the column. The formation of the unsaturated tetrol from galactose with retention of configuration would give only the *erythro* isomer; therefore, the isolation of the D-*threo* shows that the degradation proceeds through a 2-keto intermediate which is reduced by borohydride.

In the oligosaccharides terminated by R the position on the tetrol to which the rest of the chain is attached has not been determined exactly. The formation of 1 mole of formaldehyde on periodate oxidation (see Figure 2) shows that it must be attached either to C-2 or to C-6. This unsaturated residue also gave some color in the orcinol reaction (15% of galactose value) but bromination before analysis largely eliminated its contribution to the color reaction.

The Over-All Structure of Blood Group Substances and the Mode of Action of Alkaline Borohydride. Many of the sugar chains in blood group substances are linked by glycosidic bonds to serine and threonine residues in a peptide backbone and these linkages are especially labile to alkali (Anderson *et al.*, 1964; Kabat *et al.*, 1965). The sugar involved in the linkage is probably *N*-acetylgalactosamine (Kabat *et al.*, 1965), as is the case in ovine submaxillary gland glycoprotein (Carubelli *et al.*, 1965) and the mucin from a human breast carcinoma (Adams, 1965). It would seem likely, therefore, that it is this linkage which is first broken under the alkaline conditions used in the degradation. The original purpose of the borohydride (Schiffman *et al.*, 1964a) was to prevent degradation of the liberated oligosaccharides by reduction of the alkali-susceptible reducing ends to stable sugar alcohols. The very wide molecular weight range of the fragments isolated shows that this intention was not fulfilled and that the sugar chains have been degraded, to a greater or lesser extent, by a peeling reaction from the reducing ends. A possible, although less likely, explanation is that each of the oligosaccharides occurs as a unit attached to the peptide backbone.

The commonly accepted mechanism for the alkaline degradation of polysaccharide chains (Whistler and BeMiller, 1958; Ballou, 1954) postulates that the chains are degraded by a peeling reaction starting from the reducing end and continuing, one residue at a time, until a stable linkage is reached. The oligosaccharides isolated in this study are terminated either by galactitol or the unsaturated tetraol R. The galactitol is substituted on C-2 in two of the oligosaccharides (AR_L 0.96a and α-L-fucosyl-(1→2)-galactitol); such a linkage is well known to be stable to alkali (Kuhn *et al.*, 1958a). In the case of the hexene-terminated oligosaccharides also, the sugar chain must be attached to an alkali-stable position on the unsaturated residue. Rege *et al.* (1963) have isolated β-D-Gal-(1→3)-β-D-GNAc-(1→3)-Gal and β-D-Gal-(1→4)-β-D-GNAc-(1→3)-Gal from A, B, and H substances and have suggested that in A substance, *N*-acetylgalactosamine, and in B substance, galactose, are linked to the terminal nonreducing galactose of these trisaccharides. The reducing galactose of these two oligosaccharides is, therefore, the sugar which in the alkaline degradation of the polysaccharide is converted to the hexenetetraol to form oligosaccharides terminated by this residue. The alkaline elimination of alkoxide groups from substituted sugars is known to give rise to unsaturated sugars; *e.g.*, Kenner and Richards (1956) reported the formation of a 2,3-unsaturated sugar by treating 2,3-di-*O*-methyl-D-glucose with alkali and unsaturated intermediates are involved in the formation of saccharinic acids from sugars (Whistler and BeMiller, 1958; Anet, 1964). The formation of an unsaturated oligosaccharide might be explained by a similar mechanism if the internal galactose which formed the unsaturated residue was a branch point in the chain. Thus elimination of an alkali-labile side chain (or chains) could give rise to an unsaturated sugar with the chain of the isolated oligosaccharide still attached to an alkali stable position. Reduction by borohydride would then give oligosaccharides terminated by an unsaturated sugar alcohol. Free hexenetetraol might arise from galactose residues substituted only on alkali-labile positions. Partial acid hydrolysis has given no evidence of such a branch point on galactose in human blood group substances. However, using a different degradation method, hydrazinolysis, the branched trisaccharide β-D-GNAc-(1→3)-[β-D-GNAc-(1→6)-]-Gal has been isolated from hog mucin blood group substances (Yosizawa, 1962). The postulated mechanism is consistent with the structure of this oligosaccharide if the β-D-GNAc-(1→6)-Gal linkage were a portion of the alkali stable chain and would, therefore, be converted to β-D-GNAc-R.

The mechanism would also indicate that each of the isolated fragments must have originated from the nonreducing end of a chain. It follows, therefore, that there must be a wide variety of nonreducing ends since oligosaccharides terminated by fucose, galactose, and *N*-acetylglucosamine and free *N*-acetylgalactosaminitol and galactitol have been isolated (Lloyd and Kabat, 1964), in addition to A and B specific oligosaccharides terminated by *N*-acetylgalactosamine and galactose, respectively. It is unlikely that each of these oligosaccharides represents a separate chain attached to the peptide backbone but rather that a highly branched

structure is present. Indeed this must be the case if there are no other types of linkages to the peptide portion except through serine and threonine, since the moles of oligosaccharides isolated exceeds the moles of these two amino acids in the backbone. Thus in 10 g of hog mucin blood group substance there are 454 μmoles of serine and threonine, whereas 477 μmoles of purified oligosaccharides, representing only 3.1% of the weight of dialyzable fragments, has been isolated. This branched type of structure is inherent in the mechanism postulated for the formation of oligosaccharides terminated by the unsaturated tetraol. The isolation of a considerable yield of *N*-acetylgalactosaminitol (25–45% total GalNAc) from B substance is an unexpected finding. By this mechanism it would have to have been formed from terminal nonreducing *N*-acetylgalactosamine residues which must have been present in positions such that they do not confer A specificity to the blood group B substance. The linkage of the chains to the peptide backbone is thought to be through *N*-acetylgalactosamine to serine and threonine but since there is about twice the molar amount of these amino acids as *N*-acetylgalactosamine in B substance it is not possible to decide whether this hexosamine is present in side chains as well as in the main chains. Further, it may well be that some of the serine and threonine residues are not substituted by *N*-acetylgalactosamine and since about 80% of these hydroxy amino acids are destroyed by the elimination reaction during alkaline hydrolysis (Kabat *et al.*, 1965) it follows that some galactose or *N*-acetylglucosamine may also be involved.

It is interesting that the majority of the oligosaccharides isolated have the β-D-Gal-(1→4)-GNAc sequence typical of only one of the two chains suggested to be present in blood group substances (Rege *et al.*, 1963a). This phenomenon could be accounted for if the β-D-Gal-(1→3)-GNAc containing chains were linked to alkali-labile positions on the internal galactoses of the branch points responsible for the formation of the unsaturated residues as discussed earlier. Rege *et al.* (1963) have isolated β-D-Gal-(1→3)-β-D-GNAc(1→3)-Gal from A, B, H, and Lewis substances in which β-D-Gal-(1→3)-GNAc is linked (1→3) to galactose and would, therefore, be eliminated in such a mechanism. Further, in this present study it has been shown that oligosaccharide AR_{IM5} 2.5a has the sequence: β-D-Gal-(1→3)-β-D-GNAc-(1→3 or 4)-galactitol whereas AR_{IM5} 2.5b has: β-D-Gal-(1→4)-β-D-GNAc-(1→2 or 6)-galactitol. The structure of these two oligosaccharides, therefore, supports the idea that where the chain is of the β-D-Gal(1→3)-GNAc type it is linked to C-3 (or C-4) of the galactose whereas chains of the other type are linked to C-2 or C-6 of the galactose (as they are in chains in which the galactose is degraded to R). Since the C-3 position of *N*-acetylhexosamines is especially labile to alkali, chains of the β-D-Gal-(1→3)-GNAc type would be further degraded and could account for the isolation of β-D-GalNAc(1→3)-[α-L-Fuc-(1→2)]-galactitol and other small oligosaccharides.

During the formation of the two AR_{IM5} 2.5 oligosaccharides the terminal galactose has simply been reduced to galactitol rather than degraded to an unsaturated residue. Although the peeling reaction seems to have occurred faster than the reduction by borohydride there is probably a competition between the two and these two oligosaccharides, present in very small amounts, may represent low yields of fragments which were reduced before elimination occurred. It may be that in chains from which they were derived the galactose which became reduced might have been substituted differently than the galactose in chains which gave rise to unsaturated oligosaccharides.

The proposed mechanism for the hydrolysis of blood group substances by alkaline borohydride assumes that the degradation proceeds by a peeling reaction similar to that proposed by Whistler and BeMiller (1958) for the degradation of polysaccharides by alkali alone. The possibility exists that in the presence of sodium borohydride a different mechanism, which could more easily account for the complex mixture of products, is in operation. Indeed the isolation of *N*-acetylgalactosaminitol from B substance is so unexpected that a mechanism whereby it arose from *N*-acetylgalactosamine residues in the internal portions of the chains rather than from terminal nonreducing ends as required by the mechanism discussed above would be an attractive alternative.

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