Immunochemical Studies on Blood Groups. XXXVIII. Structures and Activities of Oligosaccharides Produced by Alkaline Degradation of Blood-Group Lewis^a Substance. Proposed Structure of the Carbohydrate Chains of Human Blood-Group A, B, H, Le^a, and Le^b Substances*

Kenneth O. Lloyd, Elvin A. Kabat, and Eduardo Licerio

ABSTRACT: Treatment of blood-group Le^a substance with NaOD–$NaBD_4$ gave reduced oligosaccharides by peeling of the carbohydrate chains from their reducing ends after they had been released from the protein portion by alkaline elimination. Three reduced monosaccharides were identified: 3-hexenetetrol, galactitol, and 2-acetamido-2-deoxy-D-galactitol. Two reduced disaccharides containing D-galactose and either 2-acetamido-2-deoxy-D-glucitol or 2-acetamido-2-deoxy-D-galactitol were partially characterized. One trisaccharide, β-D-Gal-(1→4)-β-D-GNAc-(1→6?)-3-hexenetetrol, has been isolated from the alkaline degradation of other blood-group substances while the other, β-D-Gal-(1→4)-β-D-GNAc-(1→6)-hexane-1,2,4,5,6-pentol, contains a reduced residue not previously identified. Only two monofucosyl oligosaccharides were isolated: β-D-Gal-(1→3)-[α-L-Fuc-(1→4)]-β-D-GNAc-(1→3 or 4)-D-galactitol (I) and β-D-Gal-(1→4)[α-L-Fuc-(1→3)]-β-D-GNAc-(1→6?)-3-hexenetetrol (II); these two oligosaccharides have type 1 and type 2 chains, respectively. Their structures were determined by analysis, methylation, partial acid hydrolysis, and periodate oxidation studies. Oligosaccharide I was highly Le^a active in inhibition of hemagglutination and precipitation whereas II had very little inhibitory activity; this difference in the activities of the two types of determinants is in contrast to the A and H oligosaccharides previously isolated in which both chains were found to have approximately equal activities. A reduced tetrasaccharide, β-D-Gal-(1→3 or 4)-β-D-GNAc-(1→6)-[β-D-Gal-(1→3)]-2-acetamido-2-deoxy-D-galactitol, is thought to be derived from the linkage region. In the original blood-group substance the chains would be linked through the 2-acetamido-2-deoxy-D-galactose of this structure to serine and threonine in the polypeptide portion. A branched oligosaccharide, β-D-Gal-(1→3)-β-D-GNAc-[β-D-Gal-(1→4)-β-D-GNAc]-D-galactitol, was isolated; it is of particular significance since it contains both type 1 and type 2 chains linked to the same galactitol residue. The branching in this oligosaccharide was established by demonstrating that tetra-O-methyl-D-galactose was the only galactose methyl ether occurring after methylation and hydrolysis and by showing that both D-galactosyl residues could be removed by β-galactosidase.

The structure of this oligosaccharide accounts for many features of the alkaline degradation of blood-group substances. Based on this key structure, the oligosaccharides previously isolated from blood-group substances may be assembled into a megalosaccharide representing the structure of the majority of the carbohydrate chains found in blood-group A, B, H, Le^a, and Le^b substances.

Blood-group Lewis^a[1] substances are found in the secretions of most individuals who are nonsecretors of A, B, or H substances. Available evidence suggests that they are glycoproteins related in structure to the blood-group A, B, H, and Lewis^b substances (for reviews, see Watkins (1966) and Kabat (1956)). The antigenic determinant mainly responsible for Le^a specificity has been shown to be

α-L-Fuc*p*-1
↓
4
β-D-Gal*p*-(1→3)-D-GNAc*p*-

by inhibition studies using oligosaccharides from milk containing this structure (Watkins and Morgan, 1962) and later by the actual isolation of an active oligosaccharide from human Le^a substance (Rege *et al.*, 1964b).

This is now confirmed in the present study by the isolation from Le^a substance treated with NaOH–$NaBH_4$ of an active monofucosyl oligosaccharide of related structure; this oligosaccharide has a type 1 chain (Rege

* From the Departments of Microbiology and Neurology, College of Physicians and Surgeons, Columbia University, and the Neurological Institute, Presbyterian Hospital, New York, New York. *Received April 29, 1968.* Aided by grants from the National Science Foundation (GB-3675), the Office of Naval Research (Nonr-266(13)), and the General Research Support Grant of the U. S. Public Health Service.

[1] Abbreviations used that are not listed in *Biochemistry* 5, 1445 (1966), are: Le^a, Lewis^a; Le^b, Lewis^b; DMF, dimethylformamide; NANA, *N*-acetylneuraminic acid; R_L, $R_{lactose}$.

et al., 1963) (*i.e.*, containing a β-D-Gal-(1→3)-D-GNAc sequence). Also isolated was a similar oligosaccharide but with a type 2 (β-D-Gal-(1→4)-D-GNAc) structure which in contrast is largely inactive as an inhibitor. We also report the isolation of a number of other oligosaccharides with important implications for the structure of blood-group glycoproteins including a branched oligosaccharide and an oligosaccharide probably derived from the region involved in linkage to the peptide backbone. A preliminary account of this work has appeared (Lloyd *et al.*, 1968). With these oligosaccharides it has become possible to propose an over-all structure of the carbohydrate portion of blood-group A, B, H, Le^a, and Le^b substances.

Materials

Sugar methyl ethers used as reference compounds in gas–liquid partition chromatography have been described (Lloyd *et al.*, 1966a). 3-Deoxy-D-glucose was a gift from Dr. I. J. Goldstein; it was reduced to its alcohol with sodium borohydride. Milk oligosaccharides were a gift from Professor R. Kuhn. β-D-GNAc-(1→3)-[β-D-GNAc-(1→6)]-D-Gal was kindly provided by Dr. Z. Yosizawa; reduction with sodium borohydride gave the alcohol.

Methods

Analytical Methods. Colorimetric methods for the analysis of nitrogen, hexosamine, *N*-acetylhexosamine, fucose, and galactose have been described previously (Kabat, 1961; Lloyd *et al.*, 1966a). Galactosamine was determined by the method of Ludowieg and Benmaman (1967).

Oligosaccharides were also analyzed after hydrolysis by gas–liquid partition chromatography of the constituent sugars as their trimethylsilyl derivatives (*cf.* Bolton *et al.*, 1965). Oligosaccharide (about 0.5 mg) was hydrolyzed for 2 hr in 2 N HCl at 100° with 30 μg of erythritol as an internal standard. The solution was neutralized with Ag_2CO_3 and a small excess was added. The mixture was cooled in ice–water and acetic anhydride (25 μl) was added to acetylate free amino groups. After 15 min in the cold and 1–2 hr at room temperature, the supernatant was removed and evaporated to dryness. Trimethylsilyl derivatives were prepared by adding 25 μl of reagent (1 ml of pyridine, 0.2 ml of hexamethyldisilazane, and 0.1 ml of trimethylchlorosilane) as described by Sweeley *et al.* (1963). The mixture was warmed to 50° to ensure complete reaction; small amounts of residual silver salts did not interfere with the preparation of the derivatives. Trimethylsilyl derivatives of a standard mixture containing known amounts of erythritol, fucose, galactose, galactitol, and *N*-acetylglucosamine were also prepared. The trimethylsilyl derivatives of these five substances could be completely separated on an SE-30 (10%) on Diatoport S column; the temperature of the column was held at 150° for 5 min and then raised at 4°/min to 240°. Areas of the peaks were calculated using a disc integrator and the ratio of each to the area of the internal standard was determined. By comparison with the standard sugar mixture the amount of each sugar present was calculated.

Quantitative oxidation by periodate was carried out as described previously (Lloyd *et al.*, 1966a).

Chromatography. For paper chromatography the following solvent systems were used: 1-butanol–pyridine–water (6:1:1), solvent 1; 1-butanol–pyridine–water (6:4:3), solvent 2; and 1-butanol–acetic acid–water (4:1:5), solvent 3.

Methods for charcoal column chromatography and gel filtration on Bio-Gel P2 columns have been described (Lloyd *et al.*, 1966a).

N-Acetylglucosaminitol and *N*-acetylgalactosaminitol were distinguished by gas–liquid partition chromatography of their trimethylsilyl derivatives on a neopentyl glycol sebacate column (10% on Chromosorb W). The separation of the compounds was not complete and the identification was always confirmed by showing that the unknown cochromatographed with the appropriate authentic *N*-acetylhexosaminitol but gave two peaks when the other *N*-acetylhexosaminitol was added. The temperature of the column was programmed to rise from 165 to 215° at 4°/min and then held at 215° until all the compounds had been eluted. Galactose was eluted first, followed by *N*-acetylglucosaminitol and *N*-acetylgalactosaminitol; free *N*-acetylhexosamines were eluted last. Oligosaccharides were examined for the presence of the hexosaminitols by hydrolysis and re-*N*-acetylation (see Analytical Methods section).

Acid Hydrolysis of Oligosaccharides. Sugars were identified by complete hydrolysis of the oligosaccharide in 2 N HCl for 2 hr at 100° followed by neutralization with Ag_2CO_3 and paper chromatography in solvent 2. Solvent 3 was used for the separation of galactose from galactitol. By hydrolysis of the oligosaccharides in dilute HCl (pH 2.5) for 1.5 hr followed by paper chromatography in solvent 1 many of the constituent disaccharides could be recognized. In particular β-D-Gal-(1→3)-D-GNAc (R_{Gal} 0.50), β-D-Gal-(1→4)-D-GNAc (R_{Gal} 0.43), and β-D-Gal-(1→6)-D-GNAc (R_{Gal} 0.33) could be distinguished.

Methylation. Oligosaccharides (2–3 mg) were methylated with (a) methyl iodide and Ag_2O in DMF followed by (b) methyl iodide and BaO in DMF as described by Lloyd *et al.* (1966a). After hydrolysis, methylated amino sugars were separated from the neutral methyl ethers on a Dowex 50 (H^+) column (Lloyd *et al.*, 1967). The neutral methyl sugars were identified by gas chromatography of their methyl glycosides, essentially as described by Aspinall (1963) (*cf.* Lloyd *et al.*, 1966a). The monomethyl amino sugars were identified by conversion into the corresponding *O*-methylarabinose derivatives by treatment with ninhydrin (Lloyd *et al.*, 1967). The possibility of partial *N*-methylation of the hexosamine residues (see Rao *et al.*, 1966) is not a serious limitation of this method as Bhavanandan and Meyer (1967) have shown that *N*-methylglucosamine is largely converted into arabinose (about 60%) by oxidation with ninhydrin.

Immunochemical Methods. Quantitative precipitin studies (Kabat, 1961) were carried out on a microscale (1–4 μg of N) and the nitrogen was determined as de-

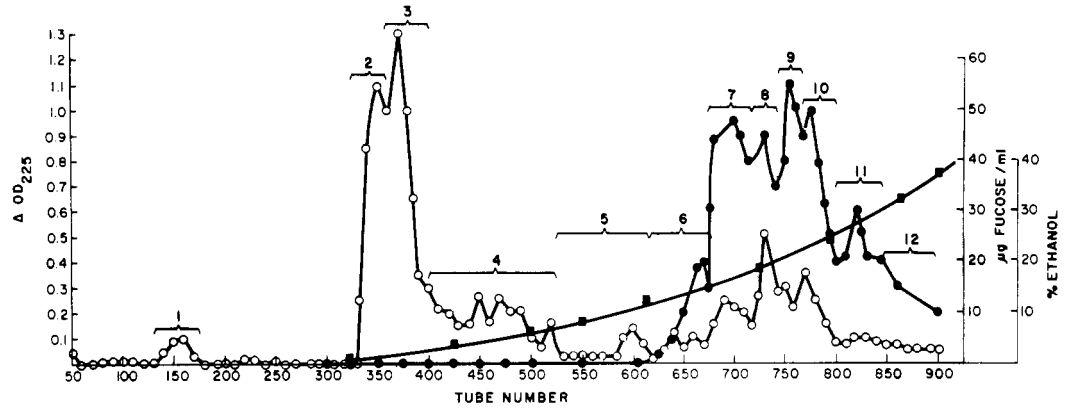

FIGURE 1: Fractionation on charcoal–Celite column of dialysate (1.05 g) from treatment of N-1 with NaOD–$NaBD_4$. (O——O) Periodate consumption ΔOD_{225}; (●——●) fucose; (■——■) ethanol concentration.

TABLE I: Analytical and Activity Data on Le^a Cyst Substance N-1 and Products of Its Alkaline Degradation.

Fraction	Per Cent Composition						Le^a Activity
	N	Fuc	Gal	Hex	NAcHex	$GalNH_2$	Min Amt for Inhibn (μg/ml)
N-1-1	4.2	8.5	32.3	31.5	30.8	9.0	0.7
N-1-2	5.0	8.4	32.3	32.5	32.0	nd[a]	2
N-1-3	5.5	6.0	28.6	28.6	28.3	10.7	0.6
Alkaline degradation							
Dialyzable	3.2	4.5	25.7	23.3	23.8	0.3	48
Nondialyzable	5.6	9.1	30.8	28.8	28.6	1.4	4

[a] nd = not determined.

scribed by Schiffman *et al.* (1964). Inhibition of precipitation was studied using a goat anti-Le^a serum (Marcus and Grollman, 1966), kindly provided by Dr. D. Marcus Albert Einstein College of Medicine, New York, N. Y. Inhibition of the cross-reaction between horse anti-Pn XIV H635 (Kabat, 1962b) and Le^a substance was also studied. Hemagglutination inhibition assays for Le^a activity were carried out using a Takatsy microtitrator on ficin-treated O Le^{a+} Le^{b-} cells (Haber and Rosenfield, 1957) with a human anti-Le^a serum (23727) provided by Dr. R. E. Rosenfield, Mount Sinai Hospital, New York, N. Y.

Experimental Section and Results

Degradation of Blood-Group Le^a Substance and Purification of Oligosaccharides

Isolation and Activity of Blood-Group Le^a Substance. Le^a active cyst material (N-1) from a Nigerian patient (kindly provided by Miss Ada E. Bezer) was digested with pepsin and precipitated with ethanol. A portion (8.6 g) was fractionated in phenol–water with ethanol (*cf.* Kabat, 1956). The following fractions were used: fraction N-1-1 was isolated by two precipitations with 10% ethanol (2.47 g), N-1-2 was precipitated with 20% ethanol from the second precipitation with 10% ethanol (0.119 g), and N-1-3 was purified by two precipitations with 20% ethanol from the supernatant of N-1-1 (1.40g). Their analytical properties and activities are given in Table I.

Treatment of Le^a Substance with NaOD–$NaBD_4$. The degradation was carried out with deuterated reagents with the aim of isolating labeled products and because these conditions had been found to give less destruction of sugars than did NaOH–$NaBH_4$ (Kabat *et al.*, 1965).

N-1-1, N-1-2, and N-1-3 were combined (3.01 g) and treated with 0.2 M NaOD and 1% $NaBD_4$ in D_2O at room temperature for 7 days as described by Kabat *et al.* (1965) (*cf.* Schiffman *et al.*, 1964). After exhaustive dialysis 1.38 g (47%) of the product was nondialyzable while 1.1 g (37%) was isolated in the dialysate. Analytical and activity data for these products are shown in Table I.

Fractionation of Dialyzable Products. In general, the fractionation followed the procedure already found to be effective for the corresponding A, B, and H oligosaccharides (Lloyd *et al.*, 1966a). Fractions were purified until the products met the criteria of purity described previously (Lloyd *et al.*, 1966a).

The dialyzable material (1.05 g) was first fractionated by chromatography on a charcoal (175 g)–Celite (75 g)

TABLE II: Fractions from Charcoal Column of Dialyzable Product (1.05 g).

Charcoal Column Fraction	Yield (mg)	Components Identified
1	32.7	3-Hexenetetrols
2	37.6	Galactitol
3	79.7	Galactitol
4	88.4	*N*-Acetylgalactosaminitol, Lewis R_{Gal} 1.54, Lewis R_{Gal} 1.62
5	59.0	Lewis R_{Gal} 0.83, Lewis R_{Gal} 0.71
6	50.7	Lewis R_L 0.28
7	83.8	
8	90.2	Lewis R_L 0.96, Lewis R_L 0.71a,
9	192.8	Lewis R_L 0.71b
10	107.0	Lewis R_L 0.41, Lewis R_L 0.17
11	86.9	
12	51.6	
>12	47.4	

column (Whistler and Durso, 1950) eluted with an ethanol gradient. The elution curve is shown in Figure 1; the tubes were pooled as shown. Yields of the fractions and substances present are given in Table II. Compounds were isolated as follows.

FRACTION 1 was purified by gel filtration through a Bio-Gel P2 column. The product (5.6 mg) was eluted as a single sharp peak and gave a single spot on paper chromatography in solvents 1 (R_{Gal} 3.5) and 2 (R_{Gal} 1.6).

FRACTIONS 2 AND 3 on passing through a Bio-Gel P2 column gave identical crystalline products. Recrystallization from methanol–water gave 80.2 mg, mp 188–189°.

FRACTION 4 was shown to contain a number of components by paper chromatography in solvent 2. Preparative paper chromatography in the same solvent gave the following. (i) Lewis R_{Gal} 1.29 (33.6 mg) was further purified by gel filtration through a Bio-Gel P2 column (23.8 mg). The product crystallized and was recrystallized from methanol to give 14 mg of product, mp 172–173°. (ii) Lewis R_{Gal} 1.06 (13.6 mg) was identical with the compound from fractions 2 and 3. (iii) Lewis R_{Gal} 1.54 (9.9 mg) was purified by chromatography through a Bio-Gel P2 column (7.7 mg). (iv) Lewis R_{Gal} 1.62 (17.1 mg) was also purified on a Bio-Gel P2 column (16.7 mg).

FRACTION 5 was shown, by paper chromatography in solvent 2, to have two major components. Preparative paper chromatography gave the following. (i) Lewis R_{Gal} 0.71 (9.3 mg) which was finally purified by passing through a Bio-Gel P2 column (5.2 mg). (ii) Lewis R_{Gal} 0.83 (12.6 mg) was also chromatographed on a Bio-Gel P2 column (7.1 mg).

FRACTION 6. Paper chromatography in solvent 2 showed the presence of only a single component (R_L 0.28). On gel filtration through a Bio-Gel P2 column the compound was eluted as a single peak to give 11.1 mg of a fucose-containing oligosaccharide (Lewis R_L 0.28).

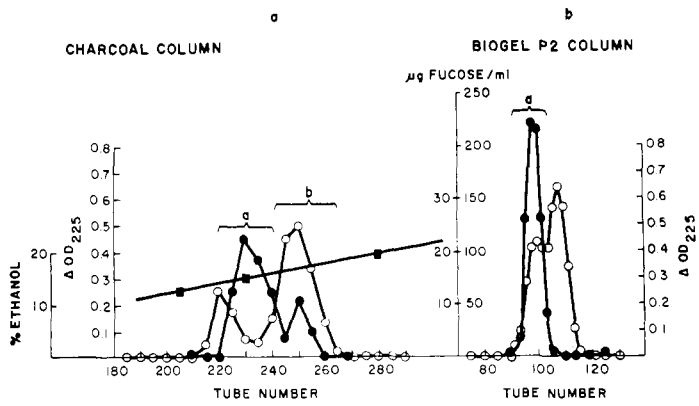

FIGURE 2: (a) Fractionation of Lewis R_L 0.71 on a charcoal–Celite column into fraction a (I) and fraction b (II). (b) Further purification of fraction a on Bio-Gel P2 column. Symbols as in Figure 1.

FRACTIONS 7–11 were shown by paper chromatography to contain the same components in varying proportions. The mixtures were fractionated by preparative paper chromatography in solvent 2 into the following oligosaccharides. (i) Lewis R_L 0.96 (137 mg); this fraction rechromatographed as a single peak on a charcoal–Celite column. A portion of the product crystallized (28.9 mg) and had a melting point of about 178°. Recrystallization was not successful as the product was very hygroscopic. The remaining portion was purified by gel filtration through a Bio-Gel P2 column (55.9 mg). (ii) Lewis R_L 0.71 (97.4 mg) was rechromatographed on a charcoal–Celite column by elution with an ethanol gradient. The elution diagram is shown in Figure 2a and the tubes were pooled into fractions I and II as shown. Fraction I was further purified by gel filtration through a Bio-Gel P2 column and gave two components (Figure 2b); rechromatography of fraction a on a Bio-Gel P2 column gave a homogeneous product, Lewis R_L 0.71a (8.2 mg). Fraction II was passed through a Bio-Gel P2 column and 43.8 mg (Lewis R_L 0.71b) was isolated from a single sharp peak. (iii) Lewis R_L 0.41 (25.5 mg) was adsorbed on a charcoal–Celite column. An ethanol gradient was applied and the fraction was eluted as a single peak at 20% ethanol. Gel filtration through a Bio-Gel P2 column gave 12.2 mg of this oligosaccharide. (iv) Lewis R_L 0.17 (19.4 mg) was shown to be homogeneous by paper chromatography and was finally purified by chromatography on a Bio-Gel P2 column.

Determination of Structures of Oligosaccharides

Reduced Monosaccharides. The chromatographic mobility of the compound from fraction 1 (R_{Gal} 3.5 in solvent 1) suggested that it was the 3-hexenetetrol previously isolated from the products of alkaline degradation of human A, H, and hog (A + H) substances (Lloyd *et al.*, 1966a). The compound decolorized bromine and potassium permanganate solutions. Its infrared spectrum was characteristic of a mixture of D-*threo*- and -*erythro*-*trans*-3-hexenetetrols (the asymmetric D-*threo* isomer has a band at 1640 cm^{-1} due to the C=C group (*cf.* Tipson and Cohen, 1965; Anet, 1965; Lloyd *et al.*, 1966a).

TABLE III: Analytical Properties of Isolated Oligosaccharides.

Oligosaccharide	Yield (mg)	Unsatn Test[a]	Per Cent Composition					Mole Ratios						NAcHex/ Hex[c]
			N	Fuc	Gal	Hex[b]	NAc-Hex[b]	N	Fuc	Gal	Hex[b]	NAcHex[b]	Galactitol	
Lewis R_L 0.96	28.9	+	2.9	2.5	38.2[d]	32.1	42.7	1.03		1.00	0.85	0.91		1.07
Calcd			2.7	0	35.1	34.8	43.2	1.0		1.0	1.0	1.0		1.0
Lewis R_L 0.71a	8.0	+	2.2	19.8	24.4[d]	27.2	27.5	1.17	0.90	1.00	1.11	0.93		0.85
Calcd			2.1	25.0	27.6	27.4	31.1	1.0	1.0	1.0	1.0	1.0		1.0
Lewis R_L 0.71b	43.8	−	2.4	2.6	34.7	29.4	31.8	0.96		1.00	0.91	0.86		0.96
Calcd			2.6	0	34.0	33.8	41.5	1.0		1.0	1.0	1.0		1.0
Lewis R_L 0.41	12.0	−	3.2	0.5	42.5	23.5	25.5	1.96		2.00	1.10	0.98		0.88
Calcd			3.7	0	48.0	23.9	29.5	2.0		2.0	1.0	1.0		1.0
Lewis R_L 0.28	11.0	−	2.0	20.0	19.2	18.6	22.7	1.25	1.11 (1.0)[e]	1.00 (1.00)	0.98	0.98 (0.90)	(0.85)	1.00
Calcd			2.0	23.7	26.0	25.9	32.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Lewis R_L 0.17	17.4	−	3.2	4.5	44.8	32.6	38.0	1.82		2.00 (2.00)	1.50	1.40 (1.67)	(0.97)	0.93
Calcd			3.1	0	40.0	39.2	48.5	2.0		2.0	2.0	2.0	1.0	1.0

[a] Ability to decolorize $KMnO_4$ and Br_2 (Lloyd *et al.*, 1966a). [b] Hexosamine and *N*-acetylhexosamine determinations on oligosaccharides are often low because of difficulties in hydrolyzing amino sugar glycosidic linkages. [c] Using GNAc as standard in the *N*-acetylhexosamine determination; GalNAc gives only 0.3 as much color per mole. [d] Determined after bromination (see Lloyd *et al.*, 1966a). [e] Values in parentheses were obtained by gas–liquid partition chromatography (see Methods section).

The crystalline compound from fractions 2 and 3 was shown to be galactitol both by its chromatographic behavior (R_{Gal} 1.1 in solvent 1 and R_{Gal} 1.06 in solvent 2) and by its melting point (188–189°) and mixture melting point with galactitol (188°).

Lewis R_{Gal} 1.29 from fraction 4 was chromatographically identical with *N*-acetylgalactosaminitol in solvent 1 (R_{Gal} 1.85) and solvent 2 (R_{Gal} 1.29). The crystalline product melted at 172–173° and its mixture melting point with authentic *N*-acetylgalactosaminitol was not depressed.

Compounds Lewis R_{Gal} 1.54 and Lewis R_{Gal} 1.62 have not been completely identified. Lewis R_{Gal} 1.54 contains only 2% N, is oxidized by periodate, does not decolorize Br_2 or $KMnO_4$, and has an ultraviolet absorption band at 225 mμ ($\epsilon^{1\%}_{max}$ 146). Lewis R_{Gal} 1.62 has only 0.9% N, is oxidized by periodate, decolorizes Br_2 and $KMnO_4$, but does not have an absorption peak in the 210–300-mμ region.

Reduced Disaccharides. Only two compounds had the chromatographic behavior on paper and Bio-Gel columns expected of disaccharides. Only small amounts of each were isolated (R_{Gal} 0.83, 7 mg; R_{Gal} 0.71, 5 mg) and they were not fully characterized. Lewis R_{Gal} 0.83 has galactose (27.8%) and *N*-acetylgalactosaminitol. Lewis R_{Gal} 0.71 contains galactose (35.0%) and *N*-acetylglucosaminitol.

No α-L-fucosyl-(1→2)-D-galactitol, which is the major disaccharide produced by the alkaline borohydride degradation of A, H, and probably B substances (Lloyd *et al.*, 1966a), was detected.

Lewis R_L 0.96. Analytical data (Table III) and chromatographic properties suggested that this oligosaccharide is identical with a reduced trisaccharide previously isolated from human A and hog (A + H) substances having the structure shown in Figure 3 (Lloyd *et al.*, 1966a). The oligosaccharide was unsaturated. Analysis showed that glucosamine and galactose were present in approximately equal amounts and they were also identified after hydrolysis by paper chromatography in solvent 2. Quantitative periodate oxidation data (Figure 4), the chromatographic identification of β-D-Gal-(1→4)-D-GNAc by partial hydrolysis with acid, and the identification of only 2,3,4,6-tetra-*O*-methylgalactose after methylation and hydrolysis (Table IV) confirmed the structure of this oligosaccharide. The release of 1 mole of formaldehyde/mole of compound on oxidation with periodate (Figure 4) shows that the hexenetetrol is substituted on either C-2 or C-6; analogy with the related disaccharide β-D-GNAc-3-hexenetetrols, in which the linkage has been determined (Lloyd and Kabat, 1967), suggests that the position of substitution is C-6.

Lewis R_L 0.71a. Hydrolysis of this oligosaccharide and paper chromatography in solvent 2 showed the presence of fucose, galactose, and glucosamine; analysis (Table III) showed the three sugars to be present in almost equimolar amounts. The oligosaccharide also contains the unsaturated residue, 3-hexenetetrol. β-D-Gal-(1→4)-D-GNAc and β-D-GNAc-(1→6?)-3-hexenetetrols were identified after partial acid hydrolysis. This, together with the identification of 2,3,4,6-tetra-*O*-methyl-D-galactose on methylation analysis (Table IV), showed

TABLE IV: Gas Chromatography of Neutral Methyl Sugars from Methylated Oligosaccharides.

Sample	Times of Elution Relative to Methyl 2,3,4,6-Tetramethyl-α-D-glucoside										Identity of Methyl Sugars from Oligosaccharides
	BDS[a] Column (200°)				PPE[b] Column (200°)			NPGS[c] Column (215°)			
Lewis R_L 0.96		1.20				1.18			1.09		2,3,4,6-Me_4-Gal
Lewis R_L 0.71a	0.54	1.16			0.53	1.14		0.61	1.06		2,3,4-Me_3-Fuc, 2,3,4,6-Me_4-Gal
Lewis R_L 0.71b		1.18	1.56	1.70	1.00(sh)[d]	1.15			1.09	1.33 1.43	2,3,4,6-Me_4-Gal, 1,2,4,5-tetramethyl-3-deoxy alcohols (?)
Lewis R_L 0.41		1.18				1.15			1.08		2,3,4,6-Me_4-Gal
Lewis R_L 0.28	0.54	1.16	1.67		0.54	1.16	1.37(sh)	0.60	1.06		2,3,4-Me_3-Fuc, 2,3,4,6-Me_4-Gal, 1,2,4,5,6-Me_5-galactitol (?)
Lewis R_L 0.17		1.17				1.13			1.07		2,3,4,6-Me_4-Gal
Reference Compounds											
2,3,4-Me_3-Fuc	0.55				0.55			0.60	0.65(w)[e]		
2,3,4,6-Me_4-Gal		1.19				1.16			1.08		
2,3,6-Me_3-Gal	1.94	2.61(m)			1.22	1.76(m)		1.61	1.82(w)	1.98(m)	
2,4,6-Me_3-Gal	2.42(m)	2.64			1.51(sh)	1.70		1.76(m)	1.97		
2,3,4-Me_3-Gal	3.76				1.98			2.40			
3,4,6-Me_3-Gal	2.45	3.65(m)			1.60	2.05(m)		2.45	2.65(sh)		

[a] BDS, butanediolsuccinate (10%) on acid-washed, silanized Chromosorb W. [b] PPE, polyphenyl ether (10%) on acid-washed, silanized Chromosorb W. [c] NPGS, neopentylglycol sebacate (10%) on acid-washed, Chromosorb W. [d] sh = shoulder. [e] Minor peaks are indicated as follows: m = medium, w = weak.

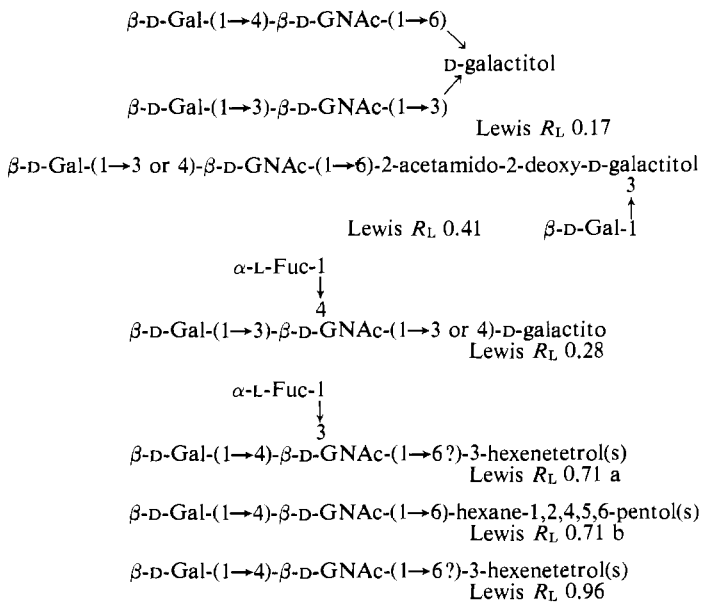

FIGURE 3: Proposed strucures of oligosaccharides from Lewis[a] substance.

the main chain of the oligosaccharide to be β-D-Gal-(1→4)-β-D-GNAc-(1→6?)-3-hexenetetrols. The presence of 2,3,4-tri-*O*-methylfucose and the tetramethylgalactose in the methylation products indicated that the fucose was present as a branch on the GNAc residue. Periodate oxidation data (Figure 4; calcd 5 moles of periodate consumed with the liberation of 2 moles of HCOOH and 1 mole of HCHO, and then overoxidation), support this structure. The *O*-methylglucosamine in the methylation mixture was shown to be 6-*O*-methyl-D-glucosamine by degradation with ninhydrin and the identification of 5-*O*-methylarabinose by gas–liquid partition chromatography (Table V; *cf.* Lloyd *et al.*, 1967). This shows that the GNAc residue is substituted on C-3 and C-4 and since C-4 is substituted by galactose it follows that fucose is linked to C-3 of the GNAc residue. The structure of this oligosaccharide is shown in Figure 3.

Lewis R_L 0.71b. This trisaccharide was a major oligo-

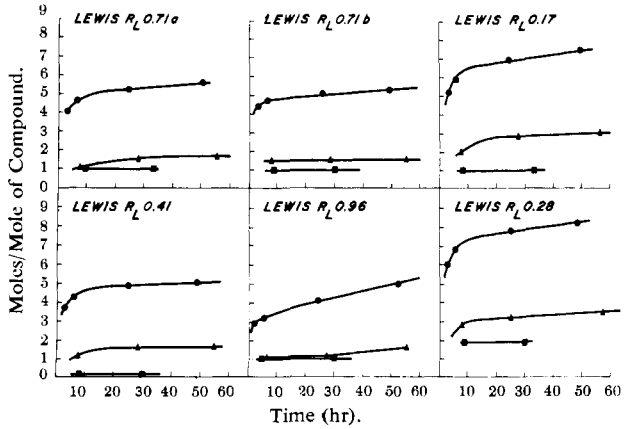

FIGURE 4: Oxidation of oligosaccharides from Le[a] substance by 40 mM sodium periodate. (●—●) IO_4^- uptake; (▲—▲) HCOOH formed; (■—■) HCHO formed.

TABLE V: Gas Chromatography of the Arabinose Methyl Ethers Formed by the Ninhydrin Treatment of the *O*-Methylglucosamines from Methylated Oligosaccharides.[a]

Sample	Relative Retention Time[b]
O-Methylarabinose from Lewis R_L 0.28	0.42 (s), 0.51 (m), and 1.15 (trace)
O-Methylarabinose from Lewis R_L 0.71a	0.42 (s), 0.50 (m), and 1.15 (trace)
5-*O*-Methylarabinose	0.42 (s), 0.50 (m)
2-*O*-Methylarabinose	0.37 (s), 0.52 (m)

[a] The method is described by Lloyd *et al.* (1967). [b] Times of methyl *O*-trimethylsilylglycosides relative to methyl *O*-trimethylsilyl-α-D-glucopyranoside on BDS column at 150°.

saccharide isolated from the alkaline degradation and has an unusual terminal reduced residue. Acid hydrolysis and paper chromatography showed the presence of galactose, glucosamine, and a third component (R_{Gal} 1.24 in solvent 2) which was not galactitol or 3-hexenetetrol but had the same R_F as hexane-D-*ribo*-1,2,4,5,6-pentol (3-deoxy-D-glucitol). Confirmation that the compound was a deoxyalditol was obtained after oxidation with periodate by the colorimetric identification of malonaldehyde (1.15 moles/mole of oligosaccharide), using 3-deoxy-D-glucose as standard (Cynkin and Ashwell, 1960). Since this was produced by oxidation of the intact oligosaccharide and since 1 mole of formaldehyde was also formed (Figure 4), it follows that the unknown is a hexane-1,2,4,5,6-pentol which is substituted on C-6 by the other sugar residues (Figure 5). Analytical data (Table III) showed galactose and glucosamine to be present in equimolar amounts. Partial acid hydrolysis showed the presence of a β-D-Gal-(1→4)-D-GNAc sequence in the oligosaccharide. 2,3,4,6-Tetra-*O*-methylgalactose was identified after methylation and hydrolysis (Table IV). Another methylated sugar, presumably hexane-1,2,-4,5-tetra-*O*-methylpentol, was also present; this component was resolved into two peaks on the BDS and NPGS columns. The identification of two hexanetetrols, isomeric on C-2, in oligosaccharides containing this residue (Lloyd and Kabat, 1967) suggests that the two peaks were the methylated D-*lyxo*- and D-*xylo*-pentols.

Lewis R_L 0.41. Analysis of this compound showed that it contained 2 moles of galactose, 1 mole of *N*-acetyl-D-glucosamine, and 2 moles of nitrogen. The extra mole of nitrogen was accounted for by the identification of 2-acetamido-2-deoxy-D-galactitol by hydrolysis and gas–liquid partition chromatography of the products as their trimethylsilyl derivatives. After hydrolysis of the methylated oligosaccharides the only neutral methyl sugar identified was tetra-*O*-methylgalactose (Table IV) demonstrating that both of the D-galactose residues are terminal. Periodate oxidation data (Figure 4; calcd

5 moles of periodate reduced and 2 moles of HCOOH and no HCHO formed) indicated that the 2-acetamido-2-deoxy-D-galactitol was substituted on both C-6 and C-3. All the galactose but no GNAc was destroyed in the oxidation. The results are compatible with a structure in which the 2-acetamido-2-deoxy-D-galactitol is substituted by D-galactose or β-D-Gal-(1→3 or 4)-D-GNAc on C-3 (and *vice versa* on C-6). To distinguish between these two possibilities an attempt was made to remove the D-galactosyl residues from the oligosaccharide with β-galactosidase from *Escherichia coli* (Wallenfels *et al.*, 1959) to give a product in which substitution on the 2-acetamido-2-deoxy-D-galactitol could be studied. In a trial experiment, however, only a portion of the D-galactose was removed. In the structure shown in Figure 3, the galactose is placed on C-3 of the 2-acetamido-2-deoxy-D-galactitol in agreement with the finding that β-D-Gal-(1→3)-D-GalNAc has been isolated from partial acid hydrolysates of A, B, H, and Le[a] substances (Rege *et al.*, 1963).

Lewis R_L 0.28. Hydrolysis of this oligosaccharide and paper chromatography in solvents 2 and 3 showed the presence of fucose, glucosamine, galactose, and galactitol. Colorimetric analysis (Table III) for the first three sugars and their determination, together with galactitol, by gas–liquid partition chromatography of their trimethylsilyl derivatives showed that the constituents were present in almost equimolar amounts. Identification of 2,3,4,6-tetra-*O*-methylgalactose and 2,3,4-tri-*O*-methylfucose (Table IV) after hydrolysis of the methylated oligosaccharide demonstrated that, except for the presence of galactitol, the structure of this oligosaccharide closely resembled that of Lewis R_L 0.71a. The identification by partial acid hydrolysis of β-D-Gal-(1→3)-D-GNAc, however, showed that Lewis R_L 0.28 has a type 1 chain whereas Lewis R_L 0.71a has a type 2 structure. The *O*-methylglucosamine in the methylation mixture was shown to be 6-*O*-methylglucosamine by degradation with ninhydrin to 5-*O*-methylarabinose (Table V), showing that L-fucose is linked to C-4 of the *N*-acetylglucosamine residue. Periodate oxidation data (Figure 4; calcd 7 moles of periodate reduced with the formation of 3 moles of HCOOH and 2 moles of HCHO and then overoxidation) confirm this structure and show that the chain is linked to either C-3 or C-4 of the galactitol residue. Figure 3 shows the structure of this oligosaccharide.

Lewis R_L 0.17. Analytical data (Table III) showed that this oligosaccharide contained 2 moles of GNAc and 2 moles of galactose for every mole of galactitol. The identification of these sugars was confirmed by hydrolysis and chromatography in solvents 2 and 3. Methylation and hydrolysis gave only 2,3,4,6-tetra-*O*-methylgalactose (Table IV) and *no* tri-*O*-methylgalactose, thus showing that both galactose residues are unsubstituted and terminal. This structure is supported since periodate oxidation destroyed all of the galactose but none of the *N*-acetylglucosamine residues. The action of β-galactosidase from *E. coli* (Wallenfels *et al.*, 1959) on the oligosaccharide was then studied. A sample (3.5 mg) was treated with β-galactosidase (1.4×10^7 units as standardized with *o*-nitrophenyl β-D-galactopyranoside;

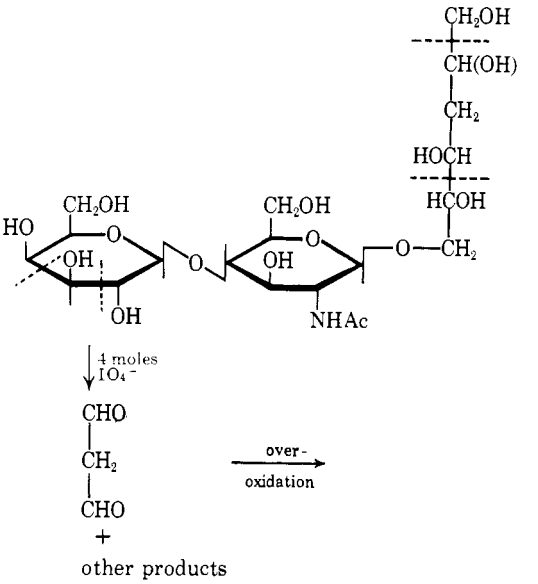

FIGURE 5: Scheme for the oxidation of Lewis R_L 0.71 b by periodate.

Wallenfels (1962)) in 3.6 ml of Tris buffer (pH 7.2) and 200 μl of 1 M NaCl. After 3 days the mixture was chromatographed on a charcoal–Celite (1:1) column (2 g). Washing with water gave 280 μg of galactose (20%) and by further elution with an ethanol gradient two more, partially overlapping, peaks were obtained. The last peak (1.5 mg) contained twice as much glucosamine as galactose and was probably a partially hydrolyzed product. The other peak (1.4 mg; eluted with 7–8% ethanol) contained 2.15 moles of GNAc/mole of galactitol and only 0.31 mole of galactose. Since the original oligosaccharide had 2.04 moles of galactose and 1.72 moles of GNAc per mole of galactitol, it is probable that this fragment is the D-GNAc-[D-GNAc]-D-galactitol which would be expected from the branched structure shown in Figure 3. In agreement with this, the product had the same R_F as authentic β-D-GNAc-(1→3)-[β-D-GNAc-(1→6)]-D-galactitol in solvent 2 (R_L 0.70). Chromatography also showed the presence of a small amount of a slow-moving component which would account for the galactose in the sample. Periodate oxidation studies under the usual conditions (40 mM IO_4^-) did not distinguish between the branched and straight-chain structures; the formation of 1 mole of HCHO/mole of oligosaccharide (Figure 4) showed, however, that the galactitol residue is substituted on C-6 or C-2. Oxidation in very dilute periodate (0.4 mM) gave more information. Clancy and Whelan (1959) showed that under these conditions the sugar alcohol moieties of glucobiitol sugars were preferentially oxidized. Lewis R_L 0.17 reduced only 2 moles of 0.4 mM sodium periodate/mole of oligosaccharide (Figure 6) in contrast to the 6–7 moles reduced in 40 mM periodate (Figure 4). This result is compatible only with a structure in which the galactitol residue has more than one substituent. An oligosaccharide with galactitol substituted on C-2 would consume 3 moles of periodate under these conditions and one with the substituent on C-6 would reduce 4 moles as did a reference

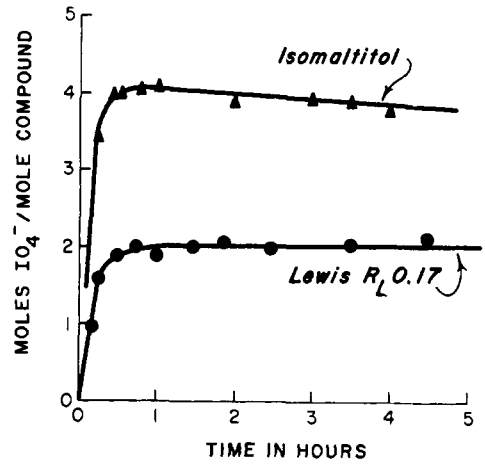

FIGURE 6: Oxidation of reduced oligosaccharides in 0.4 mM sodium periodate. Reduction of periodate was determined spectrophotometrically at 225 mμ.

compound, α-D-glucopyranosyl-(1→6)-D-glucitol (isomaltitol) (Figure 6). The product from the treatment of Lewis R_L 0.17 with 0.4 mM periodate was used to determine the presence of type 1 or type 2 chains by partial acid hydrolysis. This eliminated β-D-GNAc-galactitols from the hydrolysate, which would interfere with recognition of the latter, since they have about the same R_F values as the β-D-Gal-D-GNAc disaccharides. Paper chromatography of the partial hydrolysate in solvent 1 showed the presence of both β-D-Gal-(1→3)-D-GNAc and β-D-Gal-(1→4)-D-GNAc. The structure of the oligosaccharide is shown in Figure 3.

Immunochemical Studies

The precipitation of Le^a substance (N-1-1) by a goat anti-Le^a serum (Marcus and Grollman, 1966) is shown in Figure 7. Although N-1-1 has a relatively low fucose content (8.5%), it precipitates almost as well as the immunizing antigen (MT) which has a much higher fucose content (17%). An Le^a substance containing sialic acid

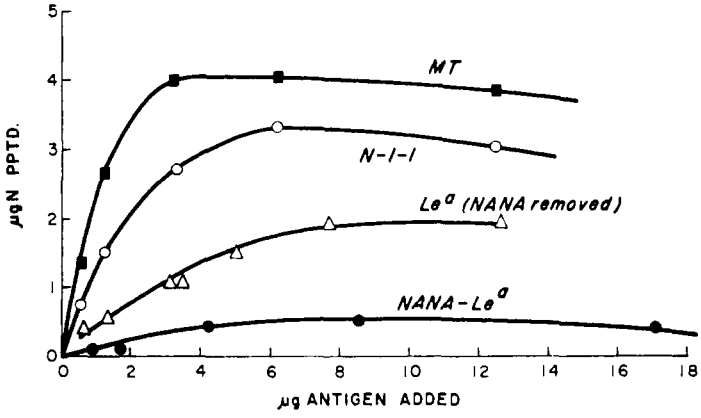

FIGURE 7: Precipitation of goat anti-Le^a serum by blood-group substances: MT, immunizing antigen; N-1-1, see Table I; NANA-Le^a, sialic acid containing Le^a substance (Pusztai and Morgan, 1961); Le^a (NANA removed), Le^a substance from Pusztai and Morgan after hydrolysis at pH 2. The total volume was 175 μl; 25 μl of goat anti-Le^a serum (Marcus and Grollman, 1966).

TABLE VI: Inhibition of Le^a-Anti-Le^a Hemagglutination by Oligosaccharides.

Oligosaccharide	Min Amt of Oligosaccharide Giving Complete Inhibn	
	μg/ml	μmoles/ml
Lacto-*N*-fucopentaose I[a]	>500	>600
Lacto-*N*-fucopentaose II	30	35
Lacto-*N*-difucohexaose I	110	111
Lewis R_L 0.96	>710	>1380
Lewis R_L 0.71a	174	268
Lewis R_L 0.71b	>650	>1220
Lewis R_L 0.41	>510	>680
Lewis R_L 0.28	49	71
Lewis R_L 0.17	220	242

[a] Lacto-*N*-fucopentaose I: α-L-Fuc-(1→2)-β-D-Gal-(1→3)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-D-Glu

α-L-Fuc 1
↓
4

Lacto-*N*-fucopentaose II: β-D-Gal-(1→3)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-D-Glu

Lacto-*N*-difucohexaose I: α-L-Fuc-(1→2)-β-D-Gal-
α-L-Fuc 1
↓
4
(1→3)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-D-Glu

(17.9%) and 8.4% fucose (Pusztai and Morgan, 1961) precipitates very poorly with this antiserum; the amount of precipitate formed is increased (Figure 7) when the sialic acid is removed at pH 2.0 (Pusztai and Morgan, 1961) to give a product with 8% fucose and 3% sialic acid.

Table VI shows the ability of the oligosaccharides from Lewis substance and some related oligosaccharides from milk to inhibit the hemagglutination of Le^a cells by a human anti-Le^a serum. Lewis R_L 0.28 is the best inhibitor and is almost as active as lacto-*N*-fucopentaose II which is known to be a good inhibitor in this system (Watkins and Morgan, 1962). Two other oligosaccharides, Lewis R_L 0.71a and Lewis R_L 0.17, were partially active and the remainder were inactive at the concentrations tested. Figure 8 shows the inhibition of precipitation of Le^a substance with a goat anti-Le^a serum by the same oligosaccharides. The specificity of this antiserum has been shown to be very similar to human anti-Le^a (Marcus and Grollman, 1966). Again Lewis R_L 0.28 is the best inhibitor, with Lewis R_L 0.17 being poorer but somewhat better than Lewis R_L 0.71a. Thus in the inhibition of both precipitation and hemagglutination, Lewis R_L 0.28, the monofucosyl tetrasaccharide with a type 1 chain, is the best inhibitor, whereas the

corresponding oligosaccharide with a type 2 chain, Lewis R_L 0.71a, is a poor inhibitor.

Figure 9 illustrates the ability of the oligosaccharides to inhibit the cross-reaction between horse antipneumococcal type XIV serum and Lewis[a] substance. The best inhibitors are Lewis R_L 0.41 and Lewis R_L 0.17 which have two terminal D-galactose residues; neither, however, is as good as lacto-*N*-neotetraose (β-D-Gal-(1→4)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-D-Gluc), as would be expected from the data of Kabat (1962b) which showed the importance of the three terminal nonreducing residues of this oligosaccharide as a determinant in the type XIV cross-reaction. Lewis R_L 0.96 and Lewis R_L 0.71b have intermediate activity, and Lewis R_L 0.71a and Lewis R_L 0.28 in which the addition of an L-fucosyl residue introduces a steric block to the reaction with the first two units (*cf.* Kabat, 1962b) are much less active. The greater inhibitory power of oligosaccharides containing β-D-Gal-(1→4)-D-GNAc over those containing β-D-Gal-(1→3)-D-GNAc was confirmed (Watkins and Morgan, 1956; Allen and Kabat, 1959).

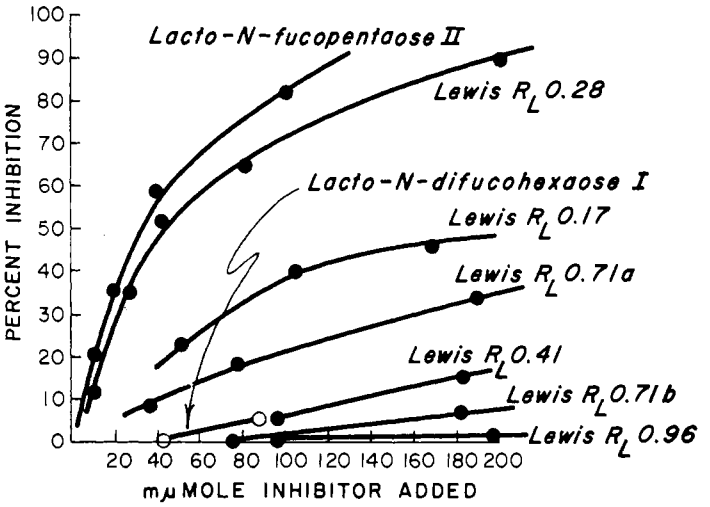

FIGURE 8: Inhibition by oligosaccharides of precipitation of Le[a] substance with goat anti-Le[a] serum. See Table VI for structures of milk oligosaccharides. Goat anti-Le[a] serum (50 μl) plus Le[a] substance (MT) (6.2 μg); total volume, 300 μl.

Discussion

Structures of Oligosaccharides. Two oligosaccharides containing fucose and which might, therefore, be expected to have Le[a] activity were isolated (Figure 3). One of these (Lewis R_L 0.28) has a type 1 chain with L-fucose linked to C-4 of an *N*-acetylglucosamine residue and is terminated by galactitol. Except for the galactitol residue this oligosaccharide is identical in structure with the active oligosaccharide isolated by Rege *et al.* (1964b) by the treatment of Le[a] substances with triethylamine. The other (Lewis R_L 0.71a) has a type 2 structure with L-fucose on C-3 of the *N*-acetylglucosamine residue and is terminated by 3-hexenetetrol; a corresponding oligosaccharide was not isolated by Rege *et al.* The large difference in the inhibitory activities of these two oligosaccharides is discussed below in terms of this inverse arrangement of D-galactosyl and L-fucosyl residues on the *N*-acetylglucosamine. The isolation of only two fucosyloligosaccharides, in low yields, is in contrast to the relatively large amounts isolated from A, B, and H substances by the same technique (Lloyd *et al.*, 1966a). Indeed, the degradation of Le[a] substance gave a much simpler pattern of oligosaccharides, This is probably because (a) the blood-group preparation had a relatively low content of fucose, (b) Le[a] substance is thought to be inherently simpler than A, B, and H substances and to represent an earlier stage in the biosynthesis of blood-group substance (Watkins and Morgan, 1959; Ceppellini, 1959).

The oligosaccharides which were isolated can be accounted for by a mechanism which assumes that many of the alkali-labile bonds linking the carbohydrate chains to the peptide portion of the glycoprotein are split first, releasing oligosaccharide chains (with free reducing ends) into the medium (Lloyd *et al.*, 1966a). Subsequent events, on which the size and nature of the isolated products depend, are governed by competition between: (a) reduction by borohydride to give stable oligosaccharides and (b) elimination from the sugar at the reducing end of the remainder of the chain, thus exposing a new reducing sugar. This new reducing residue then undergoes reactions a and b, and the competitive process continues as the peeling proceeds along the chains. Chains terminated by any of the sugar alcohols are possible, but in practice the peeling reaction predominantly terminates at certain relatively alkali-resistant linkages (*e.g.*, to C-6 and C-2 of galactose). Thus the majority of the oligosaccharides have, at what were their reducing ends, products of the alkaline degradation of galactose (chiefly hexenetetrols and hexanepentols) or galactitol. It naturally follows from this mechanism that all oligosaccharides are the remains of the terminal nonreducing ends of the carbohydrate chains.

Trisaccharides Lewis R_L 0.96 and Lewis R_L 0.71b differ only in their terminal-reduced residues. Lewis R_L 0.96 has 3-hexenetetrol residues; it has also been isolated from A and H substances. Lewis R_L 0.71b, terminated by 3-deoxypentols, has not previously been detected in the degradation products of blood-group

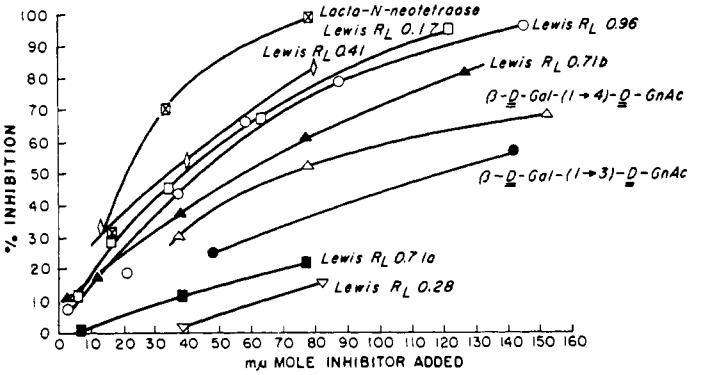

FIGURE 9: Inhibition by oligosaccharides of the cross-reaction between horse antipneumococcal type XIV serum and Le[a] substance. Lacto-*N*-neotetraose = β-D-Gal-(1→4)-β-D-GNAc-(1→3)-β-D-Gal-(1→4)-D-Gluc. Serum H 635 '38 bleeding (20.4 μl), Lewis N-1-1 (12.5 μg); total volume, 150 μl.

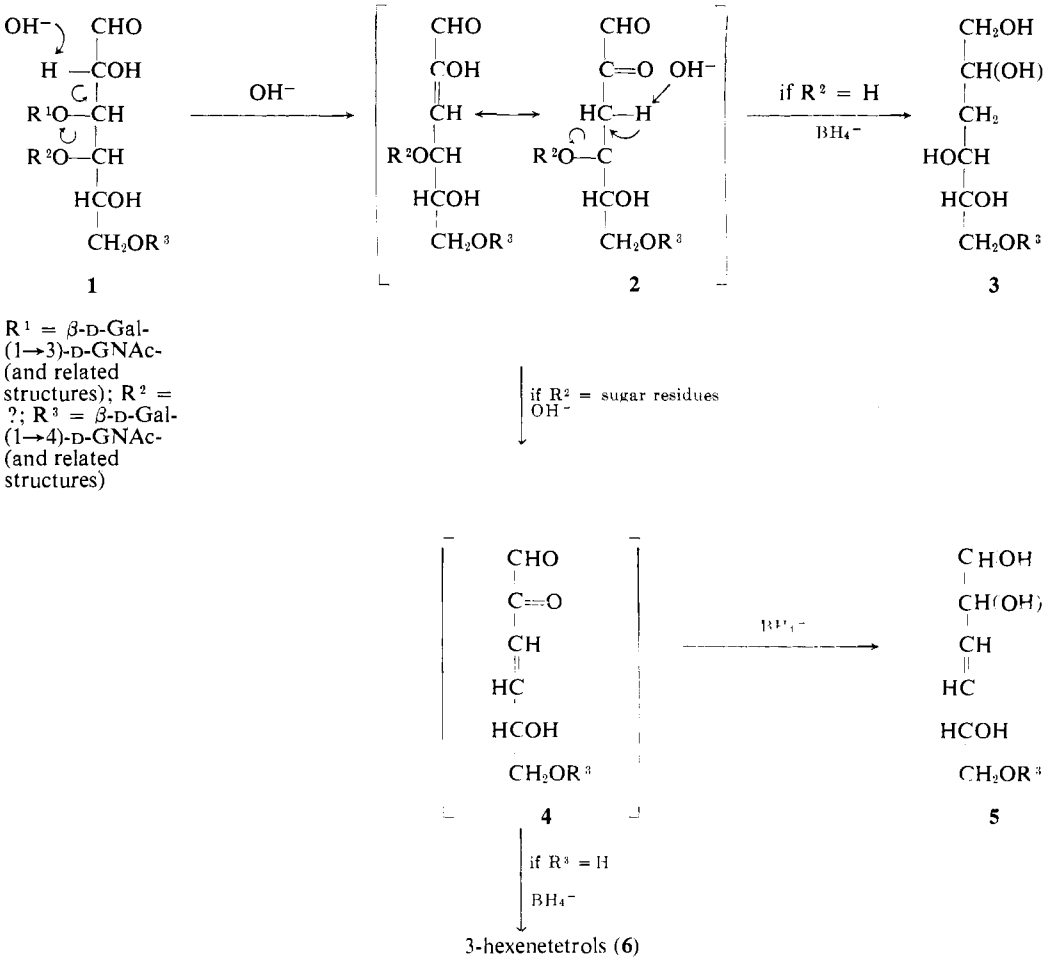

FIGURE 10: Mechanism for the formation of unsaturated and deoxy alcohol residues during the degradation of blood-group substances by NaOH–$NaBH_4$.

substances. These two residues must arise because of structural differences in their respective chains and in particular in the degree of substitution and positions of substituents on the internal galactose residues from which they were formed. It has been suggested previously (Lloyd *et al.*, 1966a) that the unsaturated alcohols are derived from galactose residues involved in branch points in the structure. In Figure 10 is shown a scheme for the possible effect of alkali and sodium borohydride on a multisubstituted, internal galactose residue (**1**) when peeling of the main chain has reached this point. The proposed mechanism postulates: (a) the β elimination of a sugar or sugar chain from C-3 to give the 3-deoxyhexosulose (osone) intermediate (**2**); (b) when C-4 is unsubstituted this intermediate is reduced to give two isomeric hexanepentols (**3**); (c) since OH^- is a poor leaving group many of the branched galactose residues must have a substituent on C-4 also. This residue could be eliminated under the influence of the 2-keto group to give the glycosulos-3-ene (**4**) which would be reduced by borohydride to give the oligosaccharides terminated by 3-hexenetetrols (**5**); these comprise most of the oligosaccharides thus far isolated from A, B, H, and Le^a substances. The presence of free hexenetetrols (**6**) among the reaction products would be accounted for by galactose residues substituted on C-3 and C-4 but not on C-6 (*i.e.*, when R^3 = H). A portion of the original branched structure (**1**) would also be reduced before elimination could occur; the resulting oligosaccharides would have relatively high molecular weights. No examples were isolated from A, B, and H substances although many fractions of very low R_L were present. This scheme assumes the presence of glycosyl-(1→4)-D-galactose linkages in blood group substances although no example of such a sequence has been found either by partial acid hydrolysis or by alkaline degradation. However, apart from explaining the formation of the degradation products, the mechanism is supported by other evidence. First, it is in accord with the available data on alkaline degradation of other carbohydrates. These data have been reviewed by Ballou (1954), Whistler and BeMiller (1958), and Anet (1964). Only the earlier stages of the mechanisms discussed in these reviews are pertinent to this study since most of their examples involve conversions into saccharinic acids which in the presence of borohydride are prevented by reduction to sugar alcohols. There is direct experimental support for the involvement of a 3-deoxyhexosulose (**2**) in the alkaline degradation of 3-*O*-benzyl-D-glucose (Machell and Richards, 1960). Although there is no corresponding

example of the formation of glycosulos-3-enes by the alkaline degradation of 3,4-di-*O*-acylglycoses, these compounds are known to be intermediates in the acid rearrangements of various *O*-methyl-3-deoxyglycosuloses (Anet, 1964). Secondly, the isolation of both C-2 isomers of the 3-hexenetetrols and the hexanepentols is good evidence that C-2 is directly involved in the mechanism. Epimerization *via* the Lobry de Bruyn–Alberda van Ekenstein transformation would not account for the two isomers since D-galactose is converted in $NaOH–NaBH_4$ into galactitol without epimerization (K. O. Lloyd and E. A. Kabat, paper in preparation). Thus the creation of a new asymmetric center at C-2 strongly suggests that 2-keto compounds (**2** and **4**) are intermediates in the degradation.

More definitive support for the mechanism would be the actual isolation of an oligosaccharide containing a branched galactosyl residue. This has now been achieved with the isolation of Lewis R_L 0.17 (Figure 3). This oligosaccharide has 2 moles of D-galactose and 2 moles of *N*-acetyl-D-glucosamine per mole of galactitol. The identification of only 2,3,4,6-tetra-*O*-methylgalactose and no tri-*O*-methylgalactose after hydrolysis of the methylated oligosaccharide, the action of β-galactosidase to produce β-D-GNAc-(1→3)[β-D-GNAc-(1→6)]-D-galactitol, and the results of oxidation with dilute periodate all support the branched structure and rule out the alternate straight-chain structure D-Gal-D-GNAc-D-Gal-D-GNAc-D-galactitol. A related oligosaccharide, β-D-GNAc-(1→3)[β-D-GNAc-(1→6)]-D-Gal, has been isolated from hog mucin (Yosizawa, 1962). The identification of both β-D-Gal-(1→3)-D-GNAc and β-D-Gal-(1→4)-D-GNAc linked to a single galactitol residue supports a suggestion made earlier (Lloyd *et al.*, 1966a) to account for the fact that all the oligosaccharides terminated by hexenetetrol residues have only the type 2 chain. This suggestion is that the type 2 chains must be attached to an alkali-stable position on a branched galactose residue with the type 1 chains being substituted on an alkali-labile position such that they would be eliminated and later degraded by alkali. On this basis it is inferred that the β-D-Gal-(1→3)-D-GNAc disaccharide is linked to C-3 of the galactitol residue while the β-D-Gal-(1→4)-D-GNAc is linked to C-6 (Figure 3). This is in agreement with the action of β-galactosidase on Lewis R_L 0.17 and with the isolation of β-D-Gal-(1→4)-D-GNAc-(1→6)-D-Gal and β-D-Gal-(1→4)-D-GNAc-(1→6)-D-Gal-(1→3)-D-GNAc (Aston *et al.*, 1968) and of β-D-Gal-(1→3)-D-GNAc-(1→3)-D-Gal (Rege *et al.*, 1963) by partial acid hydrolysis of blood-group substances.

Oligosaccharide Lewis R_L 0.41 (Figure 3) is believed to be derived from the linkage region involved in joining the carbohydrate chains to the peptide portion. The evidence that the oligosaccharide chains terminate in *N*-acetyl-D-galactosamine which in turn is linked glycosidically to serine and threonine in the backbone is that alkali preferentially destroys these two amino acids in blood-group substances (Anderson *et al.*, 1963; Kabat *et al.*, 1965) and that this destruction is accompanied by loss of *N*-acetyl-D-galactosamine (Kabat *et al.*, 1965). It was shown that $NaOH–NaBH_4$ destroys 84% of GalNAc in B substance, but this is a minimum value since GalNAc is not separated from the 2-acetamido-2-deoxyhexitols by the amino acid analyzer. In the present study, 93% of the GalNAc (determined by a colorimetric method) was destroyed. These results strongly implicate *N*-acetyl-D-galactosamine in the linkage; some D-galactose and *N*-acetyl-D-glucosamine are also destroyed, but this is accounted for by the peeling reaction. The isolation of β-D-GNAc-(1→3)-D-Gal-(1→3)-D-GalNAc by partial acid hydrolysis of blood group substance (Rege *et al.*, 1963) suggests that the D-galactose which is linked to 2-acetamido-2-deoxy-D-galactitol in Lewis R_L 0.41 is not terminal in the majority of the chains but is substituted by the oligosaccharides bearing the antigenic determinants. Lewis R_L 0.41 must, then, have been derived from some unfinished chains. This oligosaccharide is also the first indication that two *N*-acetylhexosamine residues occur in sequence in blood-group substances.

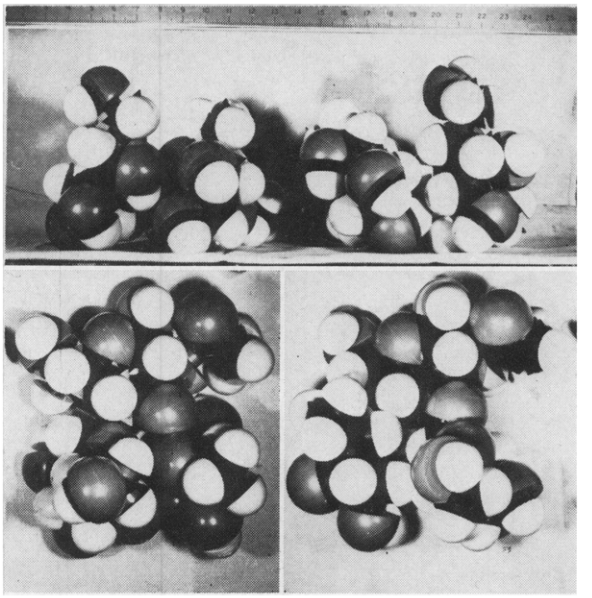

FIGURE 11: Side (upper) and top (lower) views of type 2: β-D-Gal-(1→4)[α-L-Fuc-(1→3)]-D-GNAc (left) and type 1: β-D-Gal-(1→3)[α-L-Fuc-(1→4)]-D-GNAc (right) oligosaccharides arranged with planes of the fucose and galactose perpendicular to the GNAc. Note the hydroxyls between the fucose and galactose rings in the left pair of models as compared with the hydrogens in the right pair of models. Scale indicates Angstrom units.

Blood-Group Activity. Cyst preparation N-1 has high Le^a activity as measured by inhibition of hemagglutination and its ability to precipitate a goat anti-Le^a serum (Marcus and Grollman, 1966) even though its fucose content (8%) is substantially lower than the average value (14%) for Le^a cyst substances. The antiserum precipitates very poorly with a sialic acid containing Le^a substance which also has about 8% fucose. However, when the sialic acid is largely removed, the ability of the antigen to precipitate this serum is increased, suggesting that the sialic acid blocks access of the goat antibodies to the Le^a determinants in the sialylated glycoproteins.

The most active inhibitor isolated from N-1 was the monofucosyl tetrasaccharide, Lewis R_L 0.28. The structure of this oligosaccharide is identical, except in the re-

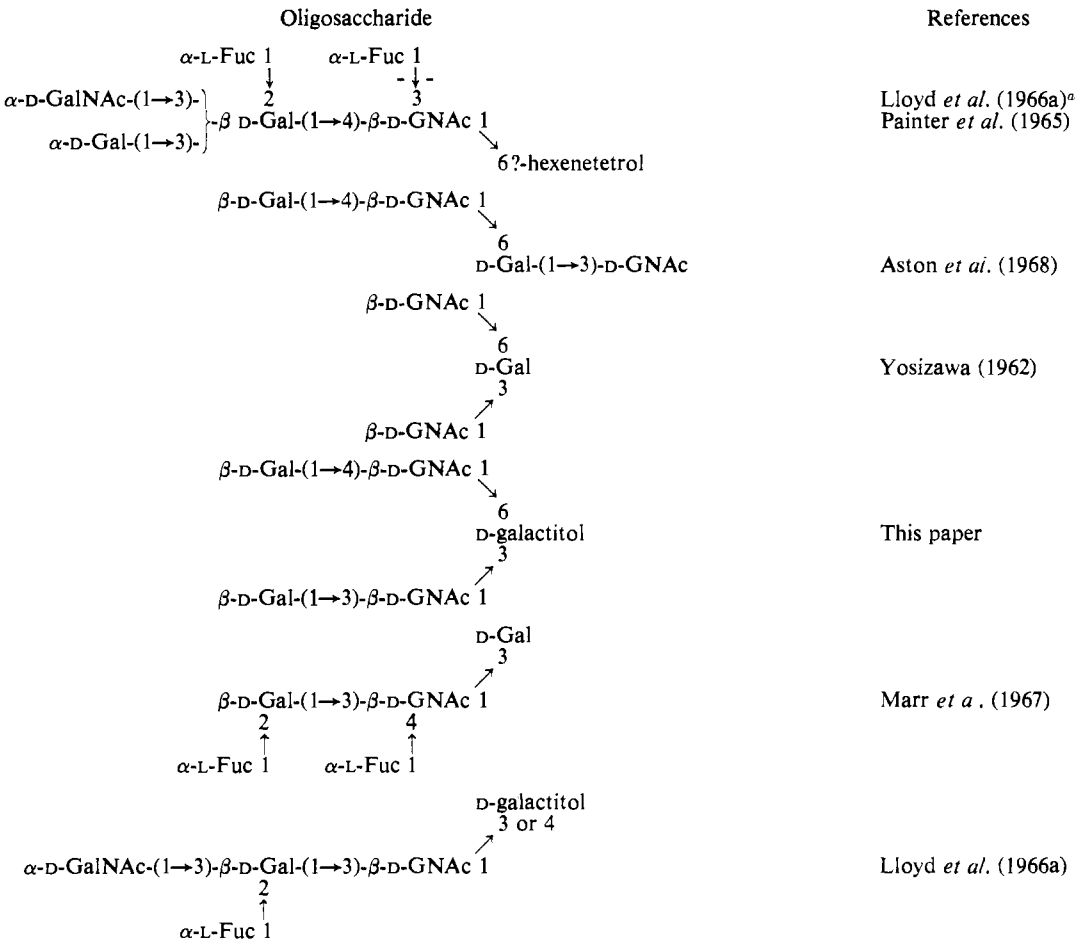

FIGURE 12: Structures of some of the oligosaccharides considered in assembling the composite structures in Figure 13. Lloyd *et al.* (1966a) isolated A, B, and H oligosaccharides of this type with and without L-fucose on the GNAc residue. [a] Painter *et al.* (1965) isolated A, B, and H monofucosyl oligosaccharides without an L-fucose on the D-GNAc residue and having D-GNAc at the reducing end and lacking hexenetetrol.

duced end, with lacto-*N*-fucopentaose II and a trisaccharide isolated from Le[a] substance (Rege *et al.*, 1964b), both of which have high Le[a] activity (Watkins and Morgan, 1962; Rege *et al.*, 1964b). All three oligosaccharides have L-fucose linked to C-4 of D-GNAc in a type 1 chain. Lewis R_L 0.28 is terminated by galactitol and is one unit longer than the trisaccharide isolated by Rege *et al.* (1964b); lacto-*N*-fucopentaose II is two residues longer and has lactose at the reducing end. In this study an oligosaccharide was also isolated in which the substitutions of the L-fucose and D-galactose to the GNAc are reversed. This oligosaccharide (Lewis R_L 0.71a) which has L-fucose linked to C-3 of a D-GNAc residue in a type 2 chain is relatively inactive as an inhibitor (Figure 8 and Table VI). The only other oligosaccharide to show Le[a] activity was Lewis R_L 0.17 which, on the basis of present knowledge of the Le[a] system, would not be expected to inhibit. However, since this sample contains 4% of fucose it is possible that it is contaminated with a highly Le[a]-active impurity.

The very different activities shown by the type 1 and type 2 determinants is an interesting aspect of Le[a] activity. This is in contrast to the A, B, H system where both the chains have approximately equal activities as can be seen by comparing the activities of two A-active oligosaccharides isolated by Lloyd *et al.* (1966a), *i.e.* AR_{IM5} 2.5a and AR_{IM5} 2.5b and by comparing the two H-active monofucosyl trisaccharides isolated by Rege *et al.* (1964a). In the Lewis[b] system, an oligosaccharide with a type 2 structure was inactive (Lloyd *et al.*, 1966b), and activity resides in the type 1 determinant (Marr *et al.*, 1967). This type 2 determinant with an L-fucose residue linked α-(1→3) to GNAc may indicate another genetic marker as yet undetected immunologically.

On a molecular level, the ability of the anti-Le[a] antibody to react preferentially with one of the determinants can be understood in terms of the differences in structure of the two determinants. Examination of molecular models (Figure 11) shows that these determinants can assume conformations which are quite dissimilar, reflecting these structural differences; in addition, however, they can assume other conformations with relatively similar gross contours.

If the models are examined closely a most striking difference is revealed. With both placed in a conformation in which the planes of the galactosyl and fucosyl residues are perpendicular to that of the GNAc ring and with both the fucosyl CH_3 and the galactosyl CH_2OH on the same side the two rings have all of their hydrophobic hydrogens adjacent to one another in the Le[a]

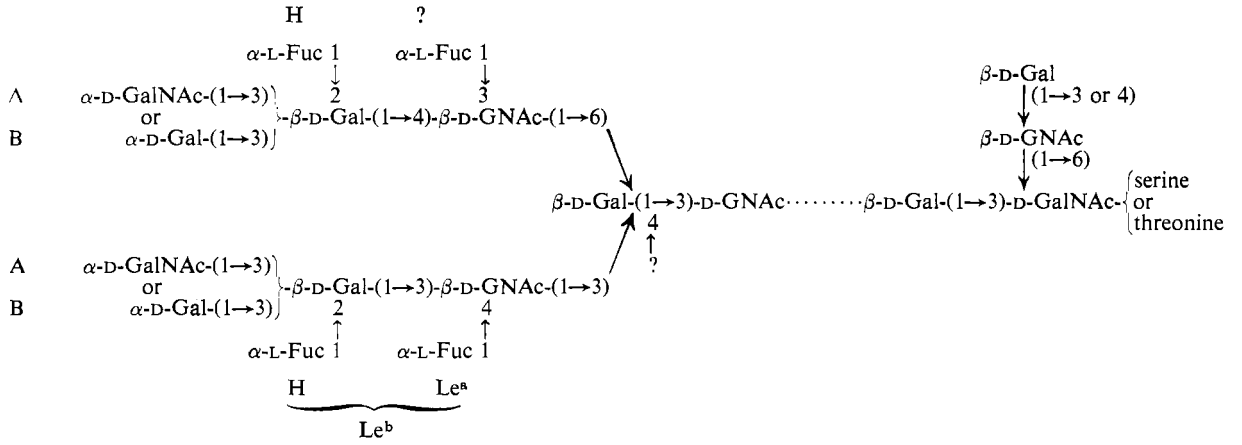

FIGURE 13: Possible composite structure of A-, B-, H-, Le^a-, or Le^b-specific megalosaccharide linked to serine or threonine in blood-group substances. (i) Other end groups present: (a) D-GalNAc- (not associated with A activity) and (b) D-Gal-; (ii) nonreducing ends of chains may be incomplete owing to absence of any gene or to unfinished biosynthesis; (iii) some of the determinants are known not to have two fucosyl residues since monofucosyl oligosaccharides have been isolated in yields comparable, in some instances, to the difucosyl oligosaccharides (Lloyd *et al.*, 1966a).

active oligosaccharide while in the other all of the OH groups are adjacent. This is clearly seen in the side views of Figure 11. Assuming this conformation to be a relatively stable one and with recent observations indicating that hydrophobic residues of sugars are important in their immunochemical specificity (Springer and Williamson, 1962; Kabat, 1962a; Springer *et al.*, 1965; Kabat, 1968), the startling difference in the distribution of the hydrophobic hydrogens in the two models could readily account for the differences in specificity. It has not been possible to produce an arrangement of hydrophobic residues in which the two models were similar.

Structure of the Carbohydrate Portion of Blood-Group A, B, H, Le^a, and Le^b Substances. An important contribution toward understanding the structure of the carbohydrate chains in blood-group substances was the demonstration by Rege *et al.* (1963) that two different chains exist (*i.e.*, type 1 and type 2 chains). It has been proposed (Lloyd *et al.*, 1966a) that these two chains are joined through a branch point to a common chain linking them to the peptide backbone. This suggestion was made to account for the pattern of oligosaccharides produced by alkaline degradation and is inherent in the mechanism discussed above for the formation of hexenetetrols and hexanepentols. The isolation of oligosaccharide Lewis R_L 0.17 offers direct proof that branch points can occur in the carbohydrate chains. Utilizing the branched structure as a basis, the oligosaccharides previously isolated by ourselves and other workers which have either type 1 or type 2 structures can be assembled (Figure 12) into a megalosaccharide containing both types of determinants as shown in Figure 13. The figure also shows the way in which this megalosaccharide may be joined through a linkage region, represented by Lewis R_L 0.41 or a related structure, to serine and threonine residues in the protein moiety of blood-group substances.

The structure shown is a composite of the A, B, H, Le^a, and Le^b determinants, and in individual blood-group substances certain of the residues will be missing, *e.g.*, H determinants lack the terminal α-D-GalNAc or α-D-Gal residues and Le^a determinants have no fucose linked α-(1→2) to D-galactose on the type 1 chain. Although in any one substance this may be the structure of the majority of the chains, other chains will be present: (a) incomplete chains as evidenced by the isolation of β-D-Gal-(1→4)-D-GNAc-(1→6)-hexenetetrol from A, H, and Le^a substances and of α-L-Fuc-(1→2)-D-galactitol from A substance and (b) unbranched determinants as shown by the isolation of

α-L-Fuc 1
↓
2
α-D-GalNAc-(1→3)-β-D-Gal-(1→3)-β-D-GNAc-
(1→3 or 4)-D-galactitol (AR_{IM5} 2.5a)

and

α-L-Fuc 1
↓
2
α-D-GalNAc-(1→3)-β-D-Gal-(1→4)-β-D-GNAc-
(1→2 or 6)-D-galactitol (AR_{IM5} 2.5b)

from A substance and of Lewis R_L 0.28 in this study. Indeed if the mechanism suggested for the formation of hexenetetrol and hexanepentol is correct, then there is considerable heterogeneity in chains with respect to the substitutions on the branched galactose residue. This galactose residue may be (i) monosubstituted to produce oligosaccharides terminated by galactitol, (ii) disubstituted on C-3 and C-4 to form free hexenetetrol, (iii) disubstituted on C-3 and C-6 to produce oligosaccharides with hexanepentol residues, or (iv) trisubstituted on C-3, C-4, and C-6 to form oligosaccharides terminated by hexenetetrol. The predominance of unsaturated oligosaccharides suggests that the majority of the chains are of the last type. The less highly substituted structures would tend to give smaller fragments than the highly branched structures, and the ease of isolation

of low molecular weight oligosaccharides from the former may give an exaggerated estimate of their quantitative contribution to the over-all structure. The isolation of galactitol from A and H substances (Lloyd *et al.*, 1966a) and of *N*-acetyl-D-galactosaminitol from B and Le^a substances indicates that nonreducing end groups of D-galactose and *N*-acetyl-D-galactosamine not accounted for in this structure must be present; the latter may be linked as single sugar side chains on serine and threonine. The lengths of the chains bearing the determinants are not yet established and a gap in the sequence has been inserted in Figure 13 to emphasize this point, although it may be that the chains are no longer than the length shown.

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