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## Structures and Serological Activities of Three Oligosaccharides Isolated from Urines of Nonstarved Secretors and from Secretors on Lactose Diet†

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**ABSTRACT:** Normal food intake or lactose diet was found to induce an excretion of fucose-containing oligosaccharides in urines of ABO(H)-secretors. The urines of A- and B-secretors were found to contain the trisaccharides 3-O-(2-acetamido-2-deoxy- $\alpha$ -D-galactopyranosyl)-2-O-( $\alpha$ -L-fucopyranosyl)-D-galactose and 3-O-( $\alpha$ -D-galactopyranosyl)-2-O-( $\alpha$ -L-fucopyranosyl)-D-galactose, respectively. The urines of the O(H)-secretors

contained the disaccharide 2-O- $\alpha$ -L-fucopyranosyl-D-galactose. The three oligosaccharides represent nonreducing terminals of the A, B, and H(O) antigenic determinants which was confirmed by inhibition studies in the appropriate precipitin systems. The structures of the isolated oligosaccharides were established by sugar analysis, methylation analysis, optical rotation, and alkaline degradation studies.

**F**ractionation by gel chromatography of urinary ultrafiltrates from starved secretors revealed 6-deoxyhexose (fucose) patterns characteristic of each ABO blood group (Lundblad, 1966). Lactodifucotetraose was found to be the characteristic compound in O(H)-secretor urine (Lundblad, 1968; Björndal and Lundblad, 1970) and two blood-group-active pentasaccharides accounted for the main difference between the 6-deoxyhexose patterns from A- and B-secretors (Lundblad, 1967; Björndal and Lundblad, 1970; Lundblad and Svensson, 1973b). After normal food intake the excretion of 6-deoxyhexose was significantly increased and gel chromatographic fractionation revealed the presence of additional low molecular weight 6-deoxyhexose-containing compounds (Lundblad, 1966). We now report a closer examination of

these 6-deoxyhexose-containing components and demonstrate that food intake as well as lactose ingestion induces an endogenous formation of oligosaccharides characteristic of the blood group of the individual.

### Materials and Methods

**Urine.** Nine healthy males, divided equally between blood groups A, B, and O, gave urine specimens at 2-hr intervals in the afternoon, following a normal meal. Similar samples were collected after a 24-hr starvation. Two individuals, an A- and an O(H)-secretor, were starved for 24 hr and then given 100 g of lactose *per os*. Three consecutive samples of urine were then collected as before. This experiment was repeated with the A-secretor, but 100 g of sucrose was substituted for lactose.

**Preservation.** Bacterial growth was prevented by the addition of phenylmercuric nitrate (30 ml of saturated solution/l.).

**Antisera.** Human anti-A blood-grouping serum (lot A 8356-2) and human anti-B blood-grouping serum (lot B 93481) were purchased from Ortho Diagnostics.

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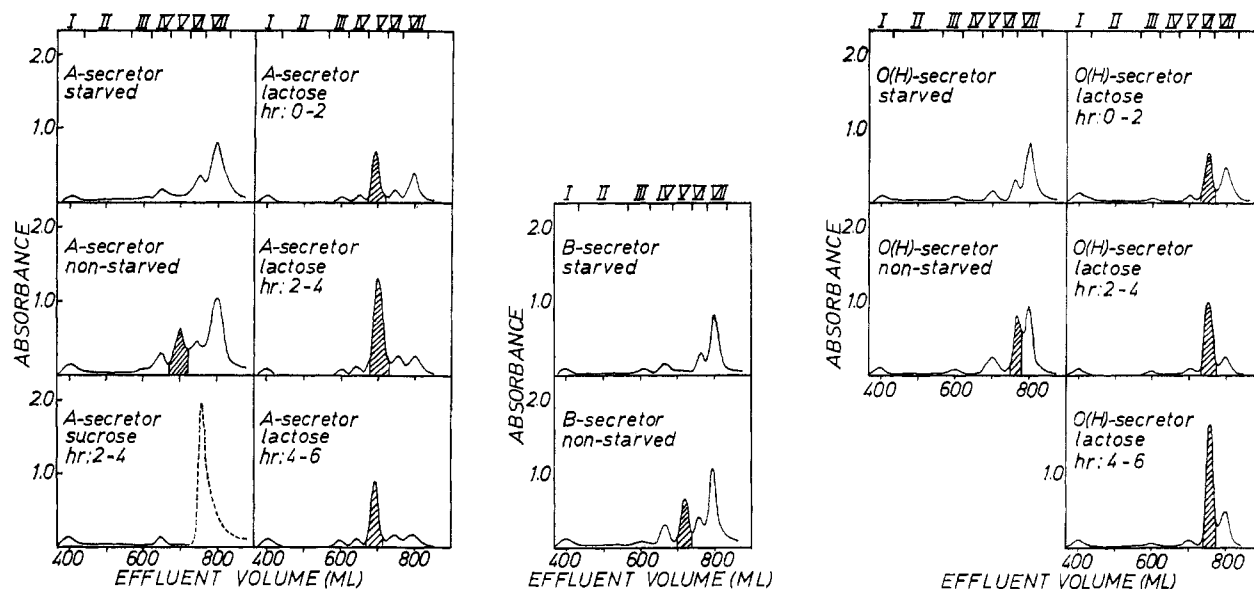


FIGURE 1: Elution profiles of 6-deoxyhexose containing oligosaccharides by gel chromatography. Urine samples were collected from different ABH-secretors at 2-hr intervals following a meal (nonstarved), fasting for 24 hr (starved) or on disaccharide diets (100 g of lactose or sucrose *per os*), following a 24-hr starvation. The samples were ultrafiltered, concentrated tenfold, and applied separately to a column (2.8  $\times$  145) of Sephadex G-25 (fine). Fractions were eluted with distilled water (void volume = 400 ml) and the 6-deoxyhexose content was analyzed by the cystine-sulfuric acid method. The material in regions indicated with vertical lines was pooled for further analysis. The curve (---) represents the material from regions VI and VII that did not react as 6-deoxyhexose with the method used: (a, left) urines from A-secretor group; (b, middle) urines from B-secretor group; (c, right) urines from O(H)-secretor group.

**Antigens.** Blood-group A substance was obtained from an ovarian cyst and was a gift from Dr. T. Kristiansen, Institute of Biochemistry, Uppsala University. Human blood-group B substance (Beach phenol insoluble), which has been described earlier (Schiffman *et al.*, 1964), was a gift from Dr. E. A. Kabat, Columbia University, New York.

**Oligosaccharides.** Urinary A and B difucosyl pentasaccharides from A- and B-secretors have been previously prepared and characterized (Lundblad, 1967; Björndal and Lundblad, 1970; Lundblad and Svensson, 1973b). The reduced oligosaccharides MSS AR<sub>L</sub> 0.52 and Beach PI R<sub>L</sub> 0.44<sup>1</sup> from soluble blood-group A and B substances have also been described earlier (Lloyd *et al.*, 1966a,b; Lundblad *et al.*, 1972) and were gifts from Dr. E. A. Kabat.

**Analytical Methods.** The ultrafiltration of filtered urine samples was performed at 4° using Visking <sup>2</sup>/<sub>32</sub>-in. dialysis tubing (Berggård, 1962). Gel chromatography was carried out using a Sephadex G-25 (fine) column (2.8  $\times$  145 cm; void volume: 400 ml). Preparative zone electrophoresis in 2 M acetic acid was carried out using the block method described by Kunkel (1954) on Pevikon C 870 (Müller-Eberhard, 1960). Whatman No. 1 papers were used for descending paper chromatography in the following solvent systems: 1-butanol-pyridine-water (6:4:3, v/v) (a), ethyl acetate-acetic acid-water (3:1:1, v/v) (b), 1-propanol-ethyl acetate-water (6:1:3, v/v) (c), ethyl acetate-pyridine-acetic acid-water (5:5:1:3, v/v) (d), and 1-butanol-acetic acid-water (4:1:5, v/v) (e).

Colorimetric methods were used for determination of 6-deoxyhexose (Dische and Shettles, 1948), total hexose (Scott and Melvin, 1953), and hexosamine (Rondle and Morgan, 1955). Sugar analyses were performed by glc (Sawardeker *et al.*, 1965) and ms (Golovkina *et al.*, 1966). The absolute con-

figurations of the sugars were determined by optical rotation. The hexosamine present was also identified as described by Stoffyn and Jeanloz (1954) and *N*-acetylhexosamine as previously described (Lundblad, 1967). Optical rotations were recorded with a Perkin-Elmer Model 141 polarimeter. Methylation analysis has been reported in detail (Björndal *et al.*, 1970). Glc was carried out on glass columns (200  $\times$  0.3 cm) containing (a) 3% w/w of ECNSS-M on Gas Chrom Q (170–200°) and (b) 3% UC-W 98 (200°) on Gas Chrom Q or on S.C.O.T. columns coated with 3% OV-225 (175°). A Perkin-Elmer 900 gas chromatograph with flame ionization detector was used. For ms, a Perkin-Elmer 270 glc-ms instrument fitted with the appropriate column was used. Mass spectra were recorded at an ionization potential of 70 eV, an ionization current of 80  $\mu$ A and an ion source temperature of 80°.

Permethylated disaccharide alditols were investigated by glc-ms as previously described (Lundblad and Svensson, 1973a).

**Alkaline Degradation of Oligosaccharides.** A solution of the oligosaccharide (2.0 mg) and *myo*-inositol (internal standard, 1.0 mg) in 1.0 M aqueous sodium hydroxide (2 ml) was kept at room temperature for 16 hr. The solution was neutralized with acetic acid and the product was reduced with sodium borohydride and acetylated. The resulting alditol acetates were analyzed by glc-ms as described above.

Quantitative precipitin analyses and precipitin inhibition studies (Kabat, 1961) were performed on a microscale (1–5  $\mu$ g of N) analyzing the washed precipitate for nitrogen by the ninhydrin method (Schiffman *et al.*, 1964).

## Results

**Effect of Lactose, Sucrose, and Normal Food on the Urinary Excretion of 6-Deoxyhexose-Containing Oligosaccharides.** Each of the urine samples collected was concentrated tenfold after ultrafiltration and applied to a column of Sephadex G-25 (fine). The eluted fractions were analyzed for 6-deoxyhexose

<sup>1</sup> Abbreviations used are: Fuc, L-fucose; GalNAc, 2-acetamido-2-deoxy-D-galactose; Gal, D-galactose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; Glc, D-glucose; p, pyranose; glc, gas-liquid chromatography; ms, mass spectrometry; R<sub>L</sub>, R<sub>Lactose</sub>.

TABLE I:  $R_{\text{lactose}}$  Values of Three Urinary Oligosaccharides.

Oligosaccharide	$R_{\text{lactose}}$ Value in Solvent				
	a	b	c	d	e
Urine A trisaccharide	1.05	1.15	1.19		
Urine B trisaccharide	0.85	0.84	0.97		
Urine O(H) disaccharide	1.60	1.73		1.22	1.84

(fucose). The results are shown in Figure 1. The 6-deoxyhexose patterns observed for the urine of starved secretors were essentially identical with those reported previously (Lundblad, 1966, 1967). In urines of the nonstarved A- and B-secretors a large additional peak was observed in region V but in the O(H)-secretors the peak in region VI was increased.

Following a lactose diet the 6-deoxyhexose peak in region V was increased for the A-secretor and the peak in region VI for the O(H)-secretor. Furthermore, the small 6-deoxyhexose peak in region III was slightly enhanced for the A-secretor. No significant change was observed in the 6-deoxyhexose content in region IV from the A-secretor and region V from the O(H)-secretor, known to contain the urinary A-pentasaccharide and the O(H)-tetrasaccharide.

For the A-secretor on a sucrose diet, the gel chromatographic profile was different. No peak was observed in region V and the material in regions VI and VII did not react to 6-deoxyhexose with the cysteine-sulfuric acid reagent.

**Isolation of Oligosaccharides.** The fractions corresponding to regions V from the A- and B-secretors and region VI from the O(H)-secretors were pooled as indicated in Figure 1 and purified further by preparative zone electrophoresis at pH 1.9. The stationary material from the A- and B-secretors was fractionated by preparative paper chromatography using the solvent systems a, b, and c in turn. The corresponding material from the O(H)-secretors was purified by using the solvent systems a and b only.

Treated in this way, urines from each group of secretors gave one main 6-deoxyhexose-containing fraction, which had a different mobility in the solvent system a. The  $R_L$  values of the components are given in Table I and the nomenclature proposed is  $AR_L$  1.05,  $BR_L$  0.85, and  $O(H)R_L$  1.60, after their mobility in system a. These compounds were passed through a column of Bio-Gel P2 (minus 400 mesh). Analysis for 6-deoxyhexose in the eluted fractions revealed a symmetric peak for each compound. The fractions were pooled and lyophilized. The yields were 10–20 mg/l. of urine for all three compounds.  $AR_L$  1.05 and  $O(H)R_L$  1.60 were also isolated from urines obtained after lactose ingestion in approximately the same yields.

**Characterization of the Oligosaccharides.**  $AR_L$  1.05  $[\alpha]_D +79^\circ$  ( $c$  1.8, water) from A-secretors yielded on acid hydrolysis (Lundblad and Svensson, 1973b) 2-acetamido-2-deoxy-D-galactose, D-galactose, and L-fucose in the relative molar proportions 1.0:1.0:1.0. These sugars accounted for 95% of the dry weight of the oligosaccharide. Methylation analysis of the oligosaccharide, after reduction with sodium borodeuteride, gave 2,3,4-tri-O-methyl-L-fucose, 1,4,5,6-tetra-O-methyl-D-galactitol-1-d, 2-acetamido-(N-methyl)-2-deoxy-3,4,6-tri-O-methyl-D-galactose, and 2-acetamido-2-deoxy-3,4,6-tri-O-methyl-D-galactose (Table II, column A).

Treatment of the oligosaccharide with aqueous sodium hydroxide released 1 mol of 2-acetamido-2-deoxy-D-galactose, demonstrating that this sugar was linked to the 3 posi-

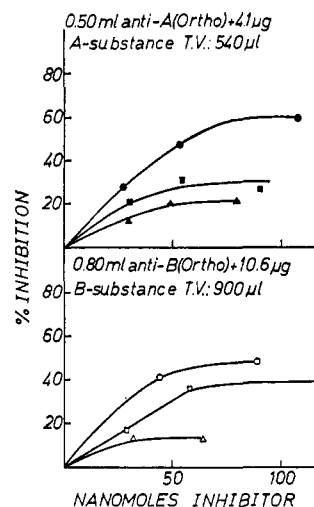


FIGURE 2: Inhibition studies (see text for method) of the oligosaccharides  $AR_L$  1.05 and  $BR_L$  0.85. Urine A pentasaccharide ( $\blacktriangle$ ), urine B pentasaccharide ( $\triangle$ ), urine A trisaccharide ( $\blacksquare$ ), urine B trisaccharide ( $\square$ ), MSS  $AR_L$  0.52 ( $\bullet$ ), and Beach P1  $R_L$  0.44 ( $\circ$ ).

tion of the reducing D-galactose residue (Whistler and Be-Miller, 1958).

$BR_L$   $[\alpha]_D +49^\circ$  ( $c$  1.5, water) from B-secretors yielded D-galactose and L-fucose in the relative molar proportions 2.0:1.0 on acid hydrolysis. The two sugars accounted for 100% of the dry weight. Methylation analysis of the reduced (sodium borodeuteride) oligosaccharide yielded equimolar proportions of 2,3,4-tri-O-methyl-L-fucose, 2,3,4,6-tetra-O-methyl-D-galactose, and 1,4,5,6-tetra-O-methyl-D-galactitol-1-d (Table II, column B). The oligosaccharide released 1 mol of D-galactose on treatment with aqueous sodium hydroxide which showed that this sugar was linked to the 3 position of the branched D-galactose residue.

$O(H)R_L$  1.60  $[\alpha]_D -51^\circ$  ( $c$  1.4, water) from O(H)-secre-

TABLE II: Partially Methylated Sugars Obtained from Hydrolysates of Methylated, Reduced (Borodeuteride) Oligosaccharides.

Sugars	$T^a$ OV 225	Rel Molar Proportions <sup>d</sup>		
		$AR_L$ 1.05 A	$BR_L$ 0.85 B	$O(H)R_L$ 1.60 C
		1.05	0.85	1.60
2,3,4-Tri-OMe-L-Fuc	0.58	1.0	1.0	1.0
2,3,4,6-Tetra-OMe-D-Gal	1.19	—	1.0	—
1,3,4,5,6-Penta-OMe-D-Gal-ol-1-d <sup>b</sup>	0.44	—	—	0.9
1,4,5,6-Tetra-OMe-Gal-ol-1-d	0.85	0.9	0.9	—
3,4,6-Tri-OMe-GalNAc	1.02 <sup>c</sup>	+	—	—
3,4,6-Tri-OMe-GalN(Me)Ac	1.10 <sup>c</sup>	+	—	—

<sup>a</sup> Retention times of the corresponding alditol acetates on the OV 225 column relative to that of 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol. <sup>b</sup> 1,3,4,5,6-Penta-OMe-D-Gal-ol-1-d = 1,3,4,5,6-penta-O-methyl-D-galactitol-1-d. <sup>c</sup> Retention times of the corresponding alditol acetates on the UC W-98 column relative to 1,5-di-O-acetyl-2-(N-methylacetamido)-3,4,6-tri-O-methyl-D-glucitol. <sup>d</sup> The relative molar proportions for the partially methylated aminosugar derivatives are not given due to lack of response factors.

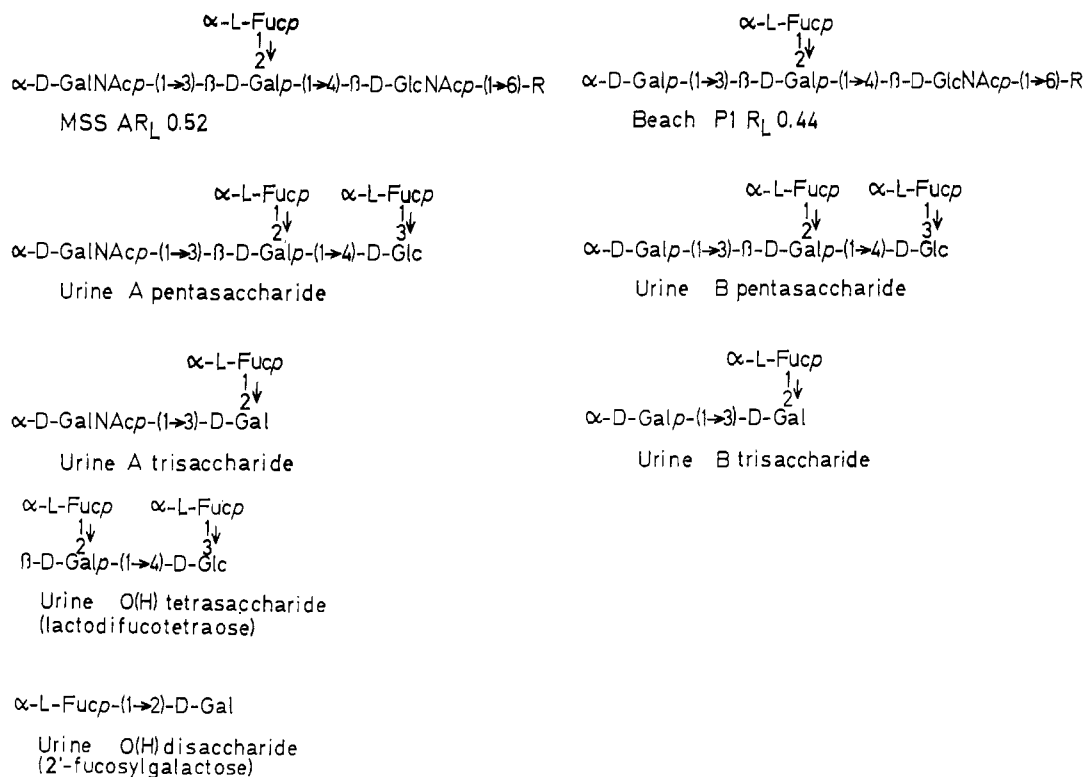


FIGURE 3: Proposed structures of the urinary oligosaccharides. The structure of the two reduced oligosaccharides from soluble blood-group substances and three other urinary oligosaccharides are also included ( $R = 3\text{-hexenetetrol (CH}_2\text{OHCHOHCH=CHCHOHCH}_2\text{OH)}$ ).

tors yielded D-galactose and L-fucose in the relative molar proportions 1.0:1.0 on acid hydrolysis. These sugars accounted for 98% of the dry weight of the material. Methylation analysis of the reduced (borodeuteride) disaccharide yielded comparable amounts of 2,3,4-tri-*O*-methyl-L-fucose and 1,3,4,5,6-penta-*O*-methyl-D-galactitol-1-*d* (Table II, column C). The permethylated disaccharide alditol gave a single peak on glc ( $T_{\text{XE-60}} = 0.39$ , relative to permethylated melibiose). The mass spectrum of the permethylated disaccharide alditol was almost identical with that obtained for the permethylated alditol from 2-*O*- $\alpha$ -L-fucopyranosyl-D-glucose (Lundblad and Svensson, 1973a).

AR<sub>L</sub> 1.05 and BR<sub>L</sub> 0.85 were tested for inhibiting activity in an A anti-A and a B anti-B system, respectively, and compared with the inhibiting activities of the reduced oligosaccharides (MSS AR<sub>L</sub> 0.52 and Beach PI R<sub>L</sub> 0.44 from soluble blood-group substances) and the urinary A and B pentasaccharides. The results are seen in Figure 2. The trisaccharides from both the A- and B-secretors were found to be more active inhibitors than the urinary pentasaccharides of the same blood groups, but less active than the reduced oligosaccharides from the soluble blood-group substances.

## Discussion

The oligosaccharide, AR<sub>L</sub> 1.05, chromatographed as a single peak on Sephadex G-25, was shown to be homogeneous by paper chromatography in three different solvent systems. Sugar analysis and methylation analysis of the reduced compound showed that it was a branched trisaccharide with an L-fucopyranosyl and a 2-acetamido-2-deoxy-D-galactose residue linked to the 2 and 3 positions of a D-galactose unit. Alkaline degradation (Whistler and BeMiller, 1958) of the trisaccharide was carried out to establish the residue attached to the 3 position of the D-galactose unit. The residue eliminated quantitatively

by this procedure was found to be 2-acetamido-2-deoxy-D-galactose, revealing that it was this sugar that was linked to the 3 position.

The optical rotation of  $+79^\circ$  indicated that L-fucopyranoside and 2-acetamido-2-deoxy-D-galactose were  $\alpha$  linked. This was supported by the immunological properties of the trisaccharide. From the above evidence, the A-active trisaccharide has the structure 3-*O*-( $\alpha$ -2-acetamido-2-deoxy-D-galactopyranosyl)-2-*O*-( $\alpha$ -L-fucopyranosyl)-D-galactose. The reduced analog of this trisaccharide has previously been isolated from blood-group A cyst fluid (Lloyd and Kabat, 1964).

The compound BR<sub>L</sub> 0.85 was also found to be homogeneous using the above criteria. Detailed analysis showed that it was a trisaccharide with an L-fucopyranosyl and a D-galactopyranosyl residue linked to the 2 and 3 positions, respectively, of a second D-galactose unit. Immunochemical studies and the low optical rotation of  $+49^\circ$  showed that both the terminal residues were  $\alpha$  linked. Therefore the B-active trisaccharide is 3-*O*-( $\alpha$ -D-galactopyranosyl)-2-*O*-( $\alpha$ -L-fucopyranosyl)-D-galactose.

O(H)<sub>L</sub> 1.60 was also shown to be homogeneous. Sugar and methylation analysis showed that this compound was 2-*O*-L-fucopyranosyl-D-galactose. This structure was confirmed by mass spectrometry on the permethylated disaccharide alditol (Lundblad and Svensson, 1973a). The optical rotation of  $-51^\circ$  established that the L-fucopyranosyl residue was  $\alpha$  linked to give the structure 2-*O*- $\alpha$ -L-fucopyranosyl-D-galactose. The disaccharide O(H)<sub>L</sub> 1.60 has also been isolated as a hydrolysis product of the human milk oligosaccharide, 2'-fucosyllactose (Kuhn *et al.*, 1958), and can be produced by alkaline degradation of human O(H) blood group substance (Rege *et al.*, 1964). The structures of these compounds are given in Figure 3.

The serologic activity of the reduced oligosaccharides AR<sub>L</sub> 0.52 and BR<sub>L</sub> 0.44 and of the urinary A and B pentasac-

charides was found to be in the same range as previously reported (Lundblad and Kabat, 1971). The presence of the second fucose is believed to prevent a firm attachment of the oligosaccharide to the antibody (Lloyd *et al.*, 1966a,b). The length of the oligosaccharide chain may also contribute to the serological activity. Thus 3-O- $\alpha$ -D-GalNAcp-3-O- $\beta$ -D-Galp-D-GlcNAc is a more potent inhibitor than 3-O- $\alpha$ -D-GalNAcp-D-Gal of A anti-A precipitation (Schiffman *et al.*, 1964). The inhibitory activity of the A and B trisaccharides, on a molar basis, was found to be intermediate to the mono- and difucosyl oligosaccharides as expected from the above discussion.

The three oligosaccharides ( $AR_L$  0.52,  $BR_L$  0.44, and O(H) $R_L$  1.60), shown to be present only in the urines of ABO (H)-secretors, represent the nonreducing termini of the A, B, and O(H) antigenic determinants, respectively (Lloyd and Kabat, 1968; Watkins, 1966). The secretor gene is responsible for the synthesis of an  $\alpha$ -2-L-fucosyltransferase found in human milk, submaxillary glands, and stomach mucosa (Shen *et al.*, 1968; Grollman *et al.*, 1969; Chester and Watkins, 1969). This enzyme transfers L-fucose to the 2 position of D-galactose. Another enzyme with a similar or an identical specificity has been demonstrated in serum of all ABO(H)-secretors, irrespective of secretor-nonsecretor status (Schenkel-Brunner *et al.*, 1972). A possible source of this enzyme is the red blood cell or its precursors where the A, B, and O(H) determinants are synthesized independently of the secretor gene (Watkins and Morgan, 1959; Watkins, 1966). We propose that the urinary oligosaccharides are derived from stomach and intestinal mucosal cells and do not represent degraded red blood corpuscle antigens. An interesting observation is that the synthesis of these oligosaccharides appears to be induced by normal food intake or by feeding on lactose. However, a sucrose diet seems to have no effect. This finding indicates that the D-galactose derived from the hydrolysis of lactose is the immediate precursor which readily accepts the different monosaccharides to form the blood-group-specific oligosaccharides. The presence of lactose in normal food would also account for increased synthesis and urinary excretion of 6-deoxyhexose compounds after normal feeding.

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#### Added in proof

After submitting this paper for publication a report entitled "Etude de la structure et de l'activite de groupe sanguin des oligosaccharides isoles de l'urine de sujets sains et pathologiques. Mise en evidence d'une oligosaccharidourie associee a diverses melituries," by G. Strecker, B. Fournet, S. Bouguellet, T. Riaz-Farzad, and J. Montreuil, was presented at Colloque International sur les Glycoconjugues (CNRS) held in Lille (France), June 20-27, 1973. This report describes the effect of galactose ingestion upon the excretion of blood group specific urinary oligosaccharides. The results obtained were analogous to those described above, but in addition small quantities of larger oligosaccharides were obtained.

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