AN ENZYMATIC BASIS FOR SECRETOR STATUS AND BLOOD GROUP SUBSTANCE SPECIFICITY IN HUMANS

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About 80 per cent of the population secretes soluble blood group substances with A, B, and O(H) specificities corresponding to each individual's blood type; the remaining 20 per cent, the "nonsecretors," do not.[1] Most nonsecretors, however, do secrete large amounts of Le^a-specific blood group substance, a glycoprotein similar in composition to the A, B, and O(H) substances of secretors.[2] For the purposes of this communication, the most important difference between the substances is that the antigenic determinants of A, B, and O(H) substances contain L-fucose linked α-(1 $\rightarrow$ 2) to D-galactosyl residues, while they are absent in Le^a substance.

In a scheme for the biosynthesis of blood group substances, Watkins and Morgan proposed that differences among individuals resulted from the genetically determined presence or absence of specific transglycosylases.[3, 4] In their hypothesis, the product of the "*H* gene" is an enzyme that catalyzes the addition of L-fucose to D-galactosyl residues to form α-(1 $\rightarrow$ 2) linkages and takes part in the synthesis of all blood group substances containing this particular grouping. The absence of the enzyme in the secretory organs of nonsecretors results in their inability to synthesize glycoproteins with A, B, or O(H) structures. This scheme was recently supported by the finding that the presence of 2′-fucosyllactose in human milk is directly correlated with the secretor status of the donor, and is found in the milk of secretors only.[5] The structure of this trisaccharide as well as the structures of other sugars found in human milk are shown in Table 1. 2′-Fucosyllactose itself is synthesized by transfer of L-fucose from GDP-L-fucose to the galactosyl residue of lactose.[8] The simultaneous absence in nonsecretors of glycoproteins containing *O*-α-L-fucopyranosyl-(1 $\rightarrow$ 2)-*O*-β-D-galactopyranosyl structures and 2′-fucosyllactose, which has the same structure, suggests that the fucosyltransferase responsible for the synthesis of the trisaccharide is also a key factor in the synthesis of soluble blood group substances and that its presence or absence determines secretor status. It was possible to test this hypothesis directly because fucosyltransferases occur in human milk in soluble form, as demonstrated by the experiments reported in this communication.[9]

In the present study, milk samples from secretors and nonsecretors were assayed for their ability to transfer L-fucose from GDP-L-fucose to the 2-position of galactosyl residues. As discussed below, this transfer is catalyzed by milk from secretors but not by milk from nonsecretors. Thus, as originally hypothesized by Watkins and Morgan,[3, 4] secretor status in humans appears to be determined by the presence or absence of a specific enzyme which can be described as a GDP-L-fucose: β-D-galactosylsaccharide α-2-L-fucosyltransferase.

Materials and Methods.—Materials: GDP-L-fucose-C^{14} was enzymatically synthesized from GDP-D-mannose-C^{14}.[10] GDP-D-mannose-C^{14} was enzymat-

TABLE 1. *Some oligosaccharides of human milk.*[6, 7]

Compound	Structure
I. Lactose	Gal-β-(1→4)-Gl
II. 2′-Fucosyllactose	Fu-α-(1→2)-Gal-β-(1→4)-Gl
III. 3-Fucosyllactose	Gal-β-(1→4) \ Gl / Fu-α-(1→3)
IV. Lacto-difucotetraose	Fu-α-(1→2)-Gal-β-(1→4) \ Gl / Fu-α-(1→3)
V. Lacto-*N*-tetraose	Gal-β-(1→3)-NAG-β-(1→3)-Gal-β-(1→4)-Gl
VI. Lacto-*N*-fucopentaose I	Fu-α-(1→2)-Gal-β-(1→3)-NAG-β-(1→3)-Gal-β-(1→4)-Gl
VII. Lacto-*N*-fucopentaose II	Gal-β-(1→3) \ NAG-β-(1→3)-Gal-β-(1→4)-Gl / Fu-α-(1→4)
VIII. Lacto-*N*-difucohexaose I	Fu-α-(1→2)-Gal-β-(1→3) \ NAG-β-(1→3)-Gal-β-(1→4)-Gl / Fu-α-(1→4)
IX. Lacto-*N*-difucohexaose II	Gal-β-(1→3) \ NAG-β-(1→3)-Gal-β-(1→4) / \ Fu-α-(1→4) Gl / Fu-α-(1→3)

The abbreviations used above are as follows: Gl for D-glucose, Gal for D-galactose, NAG for N-acetyl-D-glucosamine, and Fu for L-fucose.

ically prepared[11] from D-mannose-1-C^{14} (27 μc per μmole) purchased from the Nuclear-Chicago Corporation. Standard milk oligosaccharides were generously provided by Dr. Adeline Gauhe of the Max Planck Institute. 2-α-L-Fucopyranosyl-D-galactose (2-fucosylgalactose) and 2-α-L-fucopyranosyl-D-talose (2-fucosyltalose) were obtained by alkaline hydrolysis of 2′-fucosyllactose.[6]

Chromatography: Descending paper chromatography was carried out with Whatman 1 or 3 MM paper using the following solvents: (*A*) ethyl acetate–pyridine–water (2:1:2), (*B*) 1-propanol–ethyl acetate–water (7:1:2), (*C*) 1-butanol–pyridine–water (6:4:3), and (*D*) ethyl acetate–pyridine–acetic acid–water (5:5:1:4). Sugars were located on paper with $AgNO_3$ reagent;[12] C^{14}-activity was detected with a Vanguard chromatogram scanner. Radioactivity in the peaks was determined by placing appropriate sections of the chromatograms in vials containing 10 ml of Bray's solution[13] which were then counted with a scintillation counter. Background activity was determined with sections of similar size cut from both sides of the peak.

Milk samples: Milk (or colostrum) was obtained from donors and frozen until used. The secretor status of the donors was determined by saliva tests.[1] The milk samples were centrifuged at 5000 × *g* for 10 minutes at 4° and congealed

fat was removed with a spatula. The defatted samples were then tested directly for enzymatic activity, relying on the endogenous sugars to act as acceptors. In order to eliminate endogenous acceptors, other samples were further treated as follows: defatted milk, 10 ml, was centrifuged at 105,000 $\times$ *g* for one hour. The supernatant fluid was put on a 2 $\times$ 25-cm column of Sephadex G25 previously equilibrated with 0.05 *M* Tris buffer, pH 6.8, and 0.005 *M* glutathione, and the column was eluted with the same buffer. The fractions containing protein (as determined by absorption at 280 mμ) were pooled and ammonium sulfate was added to 80 per cent saturation. The precipitate was collected by centrifugation, dissolved in 5 ml of Tris-glutathione buffer, and the resulting solution dialyzed overnight against 1 liter of the same buffer.

Results.—Formation of radioactive oligosaccharides: The incorporation of labeled L-fucose from GDP-L-fucose-C^{14} into neutral oligosaccharides with milk from both secretors and nonsecretors is shown in Figure 1. The incorporation is linear with time for at least two hours, and 20–50 per cent of the added C^{14}-activity can be recovered in the neutral oligosaccharide fraction after four hours. Hydrolysis of the oligosaccharides in 0.5 *N* H_2SO_4 for ten minutes at 100° resulted in the formation of free fucose as the only radioactive product. The experiment shown in Figure 1 was repeated with milk from ten secretors and seven nonsecretors. Although the relative sizes of the radioactive peaks that were formed, and the total activity incorporated, varied somewhat from sample to sample, the same general pattern was always observed. Both types of milk

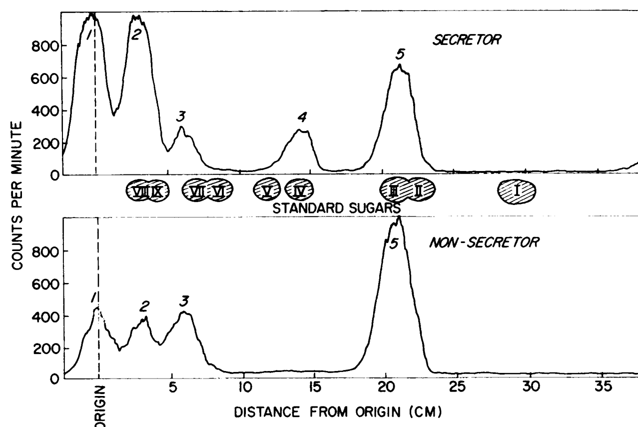

FIG. 1.—Labeled oligosaccharides formed by incubation of GDP-L-fucose-C^{14} with milk from a secretor and a nonsecretor.

The Roman numerals in the standard sugars refer to the oligosaccharides of Table 1.

The reaction mixtures contained 1 mμmole GDP-L-fucose-C^{14} (40,000 cpm), 0.25 μmole $MgCl_2$, 0.25 μmole Tris buffer pH 6.8, and 30 μl of defatted milk in a final volume of 0.05 ml.

After incubation at 37° for 4 hr, the reactions were stopped by heating at 100° for 1 min. The reaction mixtures were deionized by passage through an ionexchange column(1 ml) containing equal parts of Bio-Rad AG 3 $\times$ 4 (OH-form) and Bio-Rad AG 50w x 8 (H^+ form).

The neutral oligosaccharides in the effluents were chromatographed as $1^1/_2$-in. bands with solvent *A* for 40 hr and the resulting chromatograms scanned for C^{14}-activity as shown above.

produced peaks 1, 2, 3, and 5. Peaks 2 and 3 did not correspond to any of the standard sugars and were separated from them by longer chromatography or by the use of different solvent systems. Peak 5 appeared to coincide with standard 3-fucosyllactose. Peak 4 coincided with lacto-difucotetraose and was formed only by milk from secretors. The formation of labeled products in these experiments depends on endogenous acceptors—the oligosaccharides present in the milk samples—as well as the appropriate transglycosylase. Peak 4 was formed by the milk of nonsecretors if 2′-fucosyllactose, which is absent from their milk,[5] was added to the incubation mixtures (cf. Table 3).

Detection of L-fucosyl residues in α-(1 → 2) linkages: As shown in Table 1, L-fucose occurs in three different linkages: α-(1 → 3) to D-glucose, α-(1 → 4) to N-acetyl-D-glucosamine, and α-(1 → 2) to D-galactose. To determine if the labeled oligosaccharides contained L-fucose-C^{14} in α-(1 → 2) linkages, advantage was taken of the alkali stability of this bond. Kuhn, Baer, and Gauhe[6] reported that 2-fucosylgalactose and 2-fucosyltalose are formed in good yield by the action of alkali on the oligosaccharides of milk that contain 2-α-L-fucopyranosyl-D-galactopyranosyl groupings. These sugars which include 2′-fucosyllactose, lacto-difucotetraose, lacto-N-fucopentaose I, and lacto-N-difucohexaose I are degraded to 2-fucosylgalactose which is alkali-stable because of its 1-2 glycosidic bond but which can undergo a base-catalyzed Lobry de Bruyn-Alberda van Ekenstein transformation resulting in a partial conversion to 2-fucosyltalose. A 60 per cent yield of 2-fucosylgalactose and a 14 per cent yield of 2-fucosyltalose was obtained from 2′-fucosyllactose.[6]

Peaks 5 in Figure 1 were eluted from chromatograms and hydrolyzed with alkali. The hydrolysis products are shown in Figure 2. Radioactive peaks corresponding to 2-fucosylgalactose[14] and 2-fucosyltalose were formed from

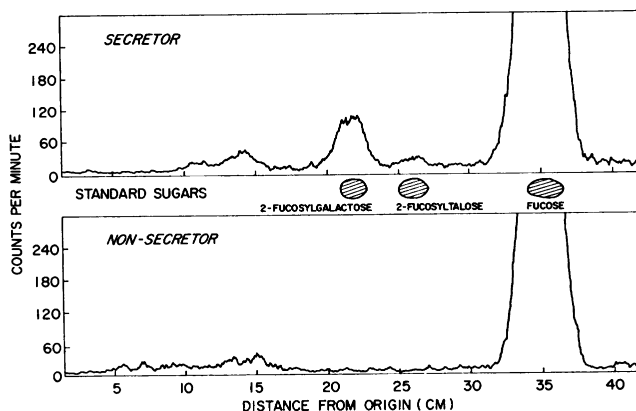

FIG. 2.—Labeled products derived from peak 5, Fig. 1, by alkaline hydrolysis.
The labeled material of peak 5, Fig. 1, was eluted from the chromatograms with water, evaporated to dryness, taken up in 0.1 ml of 0.05 M Na_2CO_3, and heated for 20 min at 100° under nitrogen.[6]
The hydrolysate was deionized as described in the legend of Fig. 1, and the neutral sugars separated by chromatography with solvent A for 17 hr.
The resulting chromatograms were scanned for C^{14}-activity as shown above.

TABLE 2. *GDP-L-fucose: β-D-galactosylsaccharide α-2-L-fucosyltransferase activity of human milk.*

Milk	Addition	2-Fucosylgalactose formed (cpm)
Secretor	—	1610
Secretor	Boiled nonsecretor milk	1520
Nonsecretor	—	0
Nonsecretor	Boiled secretor milk	0

The reaction mixtures contained 4 mμmole GDP-L-fucose (160,000 cpm), 1.0 μmole $MgCl_2$, 1.0 μmole Tris buffer, pH 6.8, and 0.06 ml of defatted milk in a final volume of 0.2 ml. In addition, two mixtures contained 0.06 ml of boiled (5 min at 100°) milk as indicated above. After incubation for 4 hr at 37°, 0.4 ml of 0.1 *M* Na_2CO_3 was added and the incubation mixtures heated for 20 min at 100° under nitrogen. After deionization, as described in the legend of Fig. 1, the neutral sugars were chromatographed with solvent *A* for 17 hr and the C^{14}-activity in the 2-fucosylgalactose area of the chromatogram was determined.

the hydrolysis of peak 5 derived from the secretor but not from the nonsecretor incubation mixtures. In other experiments, 2-fucosylgalactose was obtained in yields of 2–10 per cent by alkaline hydrolysis of peak 5 formed in incubation mixtures containing milk from secretors, but none was obtained from incubation mixtures containing milk from nonsecretors. The formation of peak 5 by sephadex-treated enzyme depends on lactose as an acceptor (cf. Table 3), and it therefore appears likely in the case of nonsecretors that peak 5 is 3-fucosyllactose, while in the case of secretors it is mainly 3-fucosyllactose with a small amount (3–15%) of 2′-fucosyllactose based on the yield of 2-fucosylgalactose after alkaline hydrolysis.

Similar results were obtained after alkaline hydrolysis of the combined radioactive oligosaccharides of Figure 1: labeled 2-fucosylgalactose was obtained by alkaline hydrolysis of the oligosaccharides formed by ten samples of secretor milk in yields of 0.5–5 per cent of the total C^{14}-activity in the oligosaccharides. No labeled 2-fucosylgalactose was obtained from the oligosaccharides formed by seven samples of nonsecretor milk. From these results it may be concluded that milk from secretors, in contrast to milk from nonsecretors, incorporates some L-fucose into oligosaccharides in α-(1 → 2) links to D-galactosyl residues. The inability of milk from nonsecretors to form this bond is not due to lack of endogenous acceptors, as demonstrated by the mixing experiment shown in Table 2. The addition of oligosaccharides from the milk of a secretor to nonsecretor milk did not result in the linkage of the fucose to galactose. Furthermore, there is no enzyme inhibitor in milk from nonsecretors because, in separate experiments, the expected amounts of radioactive 2-fucosylgalactose were obtained when the two types of milk were mixed in varying proportions.

Sephadex-treated milk: Treatment of the milk to remove low-molecular-weight material resulted in a preparation which was completely dependent on added acceptors for activity. Secretor and nonsecretor milk yielded similar results as shown in Table 3. The addition of active acceptors resulted in each case in the formation of single radioactive peaks; the chromatographic mobility of these peaks corresponds to products which would result from the transfer of one L-fucosyl residue to the acceptors in an α-(1 → 3) linkage to D-glucose or in

TABLE 3. *Acceptor specificity of the fucosyltransferases of sephadex-treated milk.*

Acceptor added	Product*	Yield (cpm) Secretor enzyme	Yield (cpm) Nonsecretor enzyme
D-Galactose	None	—	—
β-Methyl-D-galactoside	None	—	—
Lactose	3-Fucosyllactose	1,400	1,600
2′-Fucosyllactose	Lacto-difucotetraose	3,100	1,300
Lacto-*N*-tetraose	Lacto-*N*-fucopentaose II	4,100	8,100
Lacto-*N*-fucopentaose I	Lacto-*N*-difucohexaose I	9,400	4,600
Lacto-*N*-fucopentaose II	Lacto-*N*-difucohexaose II	1,400	5,500

The reaction mixtures contained 1 mμmole of GDP-L-fucose-C^{14} (40,000 cpm), 0.25 μmole $MgCl_2$, 0.25 μmole Tris buffer, pH 6.8, and 0.03 ml of sephadex-treated, dialyzed enzyme, and 0.05–0.1 μmoles of the various acceptors in a final volume of 0.05 ml. After incubation for 4 hr at 37° the incubation mixtures were deionized as described in the legend of Fig. 1, and the neutral sugars chromatographed with solvent *A* for varying times depending on the particular acceptor added: 17 hr for D-galactose and β-methyl-D-galactoside, 40 hr for lactose and 2′-fucosyllactose, and 144 hr for lacto-*N*-tetraose, lacto-*N*-fucopentaose I, and lacto-*N*-fucopentaose II.

* Identification of the product is based on the coincidence of the radioactive peaks with standard sugars in solvents *A*, *B*, and *C* (see Fig. 1 for the mobility of the standards in solvent *A*), and the assumption that in each case one fucosyl residue is added to the acceptor. Further characterization was not attempted.

an α-(1 → 4) linkage to *N*-acetyl-D-glucosamine. On the basis of the results reported in the previous section, it may be inferred that the secretor preparation also adds a small fraction of the L-fucosyl residues in α-(1 → 2) linkage to D-galactose in the appropriate acceptors. This inference is supported by the fact that 2-fucosylgalactose is formed in low yield by alkaline hydrolysis of the "3-fucosyllactose" formed by secretors but not from the "3-fucosyllactose" formed by nonsecretors. Further, the chromatographic mobilities of 2′-fucosyllactose, lacto-*N*-fucopentaose I, or lacto-*N*-difucohexaose I are similar to the mobilities of products formed by the other types of additions (see Fig. 1). If these products are present in only small amounts in the secretors, they would be hidden in the main radioactive peak as part of the shoulder and thus undetectable. D-Galactose and β-methyl-D-galactoside did not act as acceptors.

Discussion.—The milk of secretors apparently contains several L-fucosyltransferases, one of which is responsible for the formation of all the *O*-α-L-fucopyranosyl-(1 → 2)-*O*-β-D-galactopyranosyl structures found in milk oligosaccharides.[15] The enzyme is weak relative to the other L-fucosyltransferases as shown by the low yields of 2-fucosylgalactose obtained by alkaline hydrolysis of the radioactive oligosaccharides and the products formed from added acceptors by sephadex-treated milk (Table 3). The same enzyme is apparently involved in the synthesis of soluble blood group substances because its presence is correlated with the secretor status of the donor. In view of the reaction that it catalyzes, the enzyme would be the hypothetical product of the "*H* gene" in the scheme of Watkins and Morgan.[3, 4] It would attach L-fucose in an α-(1 → 2) linkage to D-galactosyl residues in the synthesis of blood group substances, converting "precursor" substance to H substance and Le^a substance to Le^b substance. Nonsecretors, who lack this enzyme, would be unable to synthesize H substance (the hypothetical precursor of A and B substance) or Le^b substance.

By analogy, other enzymes involved in the synthesis of milk oligosaccharides may also be involved in the synthesis of blood group substances. For example,

the enzyme that converts lacto-*N*-tetraose to lacto-*N*-fucopentaose II, and also converts lacto-*N*-fucopentaose I to lacto-*N*-difucohexaose I (see Table 3), adds L-fucose in α-(1 → 4) linkages to *N*-acetyl-D-glucosaminyl residues, and would correspond to the product of the "*Le* gene" in the Watkins and Morgan scheme. A study of the presence or absence of oligosaccharides and transglycosylases in individual samples of human milk[16] may shed light on the enzymatic and structural basis of other blood group specificities. Such a study is feasible because of the solubility of the transglycosylases, their absolute dependence on added acceptors, and the relatively simple nature of the products.[17]

Summary.—Human milk contains soluble fucosyltransferases which transfer L-fucosyl residues from GDP-L-fucose to oligosaccharide acceptors. Milk from secretors attaches L-fucose in at least three different linkages: α-(1 → 3) to D-glucose, α-(1 → 4) to *N*-acetyl-D-glucosamine, and α-(1 → 2) to galactose. The last activity is weak relative to the first two and is absent in the milk of nonsecretors. Secretor status in humans can be explained by the presence or absence of the enzyme responsible for this activity, a GDP-L-fucose: β-D-galactosylsaccharide α-2-L-fucosyltransferase.

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[14] For further identification, the peak corresponding to 2-fucosylgalactose was eluted from the paper and rechromatographed in solvents *B*, *C*, and *D*. In each case, the C^{14}-activity cochromatographed with authentic 2-fucosylgalactose. All the radioactivity was recovered as free fucose after hydrolysis of the isolated material in 0.5 *N* H_2SO_4 for 10 min at 100°.

[15] Consistent with the results reported in this paper is the fact that milk from nonsecretors, in addition to lacking 2′-fucosyllactose, does not contain any of the other oligosaccharides of Table 1 that contain this structure—these include lacto-difucotetraose, lacto-*N*-fucopentaose I, and lacto-*N*-difucohexaose I (Kobata, A., unpublished results).

[16] The oligosaccharides of urine also vary with the blood type of the donor. A correlation of blood type with the occurrence of specific oligosaccharides in urine of human A_1 and B secretors was recently reported (Lundblad, A., *Biochim. Biophys. Acta*, **148**, 151 (1967)). It had previously been shown that the urine of secretors has more fucose-containing oligosaccharides than the urine of nonsecretors (Evans, D. A. P., R. B. McConnel, and W. T. A. Donohoe, *J. Lab. Clin. Med.*, **64**, 581 (1964)), and the pattern of these oligosaccharides in secretors varied with the ABO status of the subject (Lundblad, A., *Biochim. Biophys. Acta*, **130**, 130 (1966)).

[17] Incorporation of radioactive sugars into blood group substances has been demonstrated using particulate preparations from hog gastric mucosa (Tuppy, H., and W. L. Staudenbauer, *Nature*, **210**, 316 (1966); Grollman, A. P., and D. M. Marcus, *Biochem. Biophys. Res. Commun.*, **25**, 542 (1966)). However, interpretations of the results are obscured by the particulate nature of the enzymes and acceptors as well as the complexity of the products.