Fucosyltransferase Found in Human Milk. Product of the Lewis Blood Group Gene*

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ABSTRACT: A guanosine diphosphate L-fucose, β-D-*N*-acetylglucosaminylsaccharide α-4-L-fucosyltransferase, has been partially purified from human milk. The enzyme catalyzes the transfer of fucose from guanosine diphosphate L-fucose to specific oligosaccharides found in human milk and to certain glycoproteins which are devoid of blood group activity. The product of the biosynthetic reaction was shown by immunochemical techniques to possess Le^a activity.

The enzyme is absent in the milk of Lewis-negative individuals and appears to be the primary product of the Lewis blood group gene.

The blood group specificity of glycoproteins with ABH and Lewis activity is determined by the sequence and linkage of sugars at the terminal nonreducing end of the oligosaccharide side chains (*cf.* Figure 1). Two different oligosaccharide chains have been identified: galactose is linked β(1–3) to *N*-acetylglucosamine in type 1 chains and β(1–4) to *N*-acetylglucosamine in type 2 chains. Fucose-containing oligosaccharides composed of type 1 or type 2 chains have A, B, or H activity but only type 1 oligosaccharides possess Le^a or Le^b activity (Watkins, 1966; Lloyd and Kabat, 1968; Marcus, 1969).

A hypothetical scheme for the biosynthesis of the human ABH and Lewis blood group substances has been proposed on the basis of chemical and genetic studies (Watkins and Morgan, 1959; Watkins, 1966; Ceppelini, 1959). In this scheme, the ABH and Lewis genes control the synthesis of specific glycosyltransferases. These enzymes, in turn, catalyze the final biosynthetic reactions that result in the formation of glycoproteins with blood group activity (*cf.* Grollman, 1967).

The Lewis gene is believed to determine the synthesis of a GDP-L-fucose, β-D-*N*-acetylglucosaminylsaccharide α-4-L-fucosyltransferase.[1] The presence of such an enzyme in the milk of Lewis-positive women and its absence in Lewis-negative individuals suggests that it participates in the biosynthesis of Le^a substance (Shen *et al.*, 1968; Grollman *et al.*, 1968, 1969). The present paper provides evidence that this enzyme catalyzes the transfer of fucose from GDP-fucose to specific glycoproteins.

Experimental Procedure and Results

Materials. [^{14}C]GDP-D-mannose (91 $\mu C_i/\mu$mole) was prepared enzymatically from [^{14}C]mannose (Rosen and Zeleznick, 1966). [^{14}C]GDP-L-fucose (41 $\mu C_i/\mu$mole), L-[^{14}C]fucose and L-[^{14}C]fucose 1-phosphate were prepared from [^{14}C]GDP-L-fucose as previously described (Grollman *et al.*, 1965). Carboxymethylcellulose was purchased from Carl Schleicher & Schuell; Sephadex G-100 from Pharmacia; bovine serum albumin and *N*-ethylmaleimide from Mann Research; sodium borohydride and EDTA from Fisher Chemical; ion-exchange resins from Bio-Rad; Tris buffer from Sigma; NCS™ solubilizer reagent from Nuclear-Chicago; and D-[^{14}C]mannose and [^{14}C]acetic anhydride from New England Nuclear. The α(1–2)-fucosidase was donated by Dr. O. M. Bahl and bovine α-lactalbumin by Dr. R. Hill. The purified oligosaccharides shown in Figure 2 were isolated from human milk and kindly provided by Drs. Adeline Gauhe and Victor Ginsburg. A naturally occurring glycoprotein (OG) without A, B, H, or Lewis blood group activity (Vicari and Kabat, 1969), isolated from a human ovarian cyst, was a gift from Dr. Elvin Kabat. Blood group substances with A, B, Le^a, and H activity were isolated from human pseudomucinous cysts as described previously (Marcus and Grollman, 1966; Marcus and Cass, 1968), and glycoprotein W-ll was a gift from Dr. W. Watkins. ^{14}C-Labeled blood group A substance was prepared by chemical acetylation of a blood group A substance which had been treated with an α-*N*-acetylgalactosamine deacetylase (Marcus *et al.*, 1964). The characterization of the caprine anti-Le^a serum used to identify the biosynthetic products in these experiments has been reported previously (Marcus and Grollman, 1966).

Analyses. Fucose was determined by the cysteine–sulfuric acid method (Dische and Shettles, 1948) and blood group specificity by inhibition of hemagglutination as described elsewhere (Marcus and Grollman, 1966).

Paper Chromatography. Descending paper chromatography was carried out using the following solvent systems: solvent I, ethanol–1 M ammonium acetate (7:3); solvent II, propanol–ethyl acetate–water (7:1:2); solvent III, butanol–pyridine–water (6:4:3). Reducing sugars on paper chromatograms were detected by the silver nitrate reagent (Anet and Reynolds, 1954).

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[1] Abbreviation used is: GDP-fucose, guanosine diphosphate L-6-deoxygalactose.

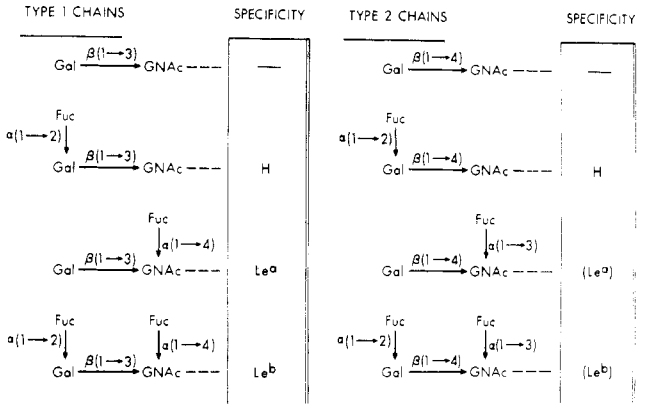

FIGURE 1: Structure of the blood group antigenic determinants. The abbreviations used are as follows: Fuc = L-fucopyranosyl; Gal = D-galactopyranosyl; GNAc = 2-deoxy-2-acetamido-*N*-acetyl-D-glucopyranosyl. The symbols enclosed in parentheses indicate that the immunological activity is very weak.

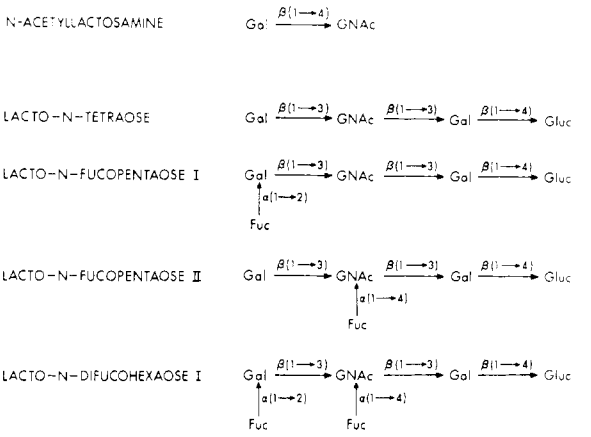

FIGURE 2: Structures of some oligosaccharides found in milk. The abbreviations used are given the legend to Figure 1.

Preparation of an "Inactive Acceptor" Glycoprotein. Fucose was removed from a glycoprotein with blood group A activity (J. S.) by a modified Smith degradation procedure (Abdel-Akher *et al.*, 1952). This glycoprotein (10 mg), containing 23% fucose, was dissolved in 2.0 ml of a 0.04 M solution of sodium periodate and allowed to stand in the dark at 4° for 24 hr. Excess ethylene glycol was added, and the reaction mixture was dialyzed overnight against water. The solution was made 0.06 M in sodium phosphate (pH 6.0), 10 mg of sodium borohydride was added, and the resulting solution was allowed to stand overnight at room temperature. The reaction mixture (pH 8.5) was dialyzed overnight against water, an equal volume of 1 N HCl was added, and the acidic solution was allowed to stand for 24 hr at room temperature. The final product was dialyzed against water and lyophilized. The resulting glycoprotein contained 0.7% fucose by weight and did not inhibit hemagglutination by anti-Le^a or anti-A sera when tested at a concentration of 1 mg/ml.

Assay for Fucosyltransferase Activity. The standard reaction mixture contained 15 $\mu\mu$moles of [^{14}C]GDP-L-fucose (41 μCi/μmole), 4 μmoles of $MnCl_2$, 10 μmoles of Tris-acetate (pH 6.8), 10 μg of "inactive acceptor" glycoprotein, and 30–40 μg of enzyme protein in a final volume of 0.2 ml. Reactions were incubated at 37° for 3 hr, applied directly to Whatman No. 3MM filter paper, and subjected to paper chromatography in solvent I for 8–15 hr. The radioactivity remaining at the origin following chromatography was considered to represent newly fucosylated glycoprotein.

When monosaccharides or oligosaccharides were used as acceptors, the "inactive acceptor" glycoprotein was replaced in this assay by 30–50 μg of the appropriate sugar. Such reactions were incubated at 37° for 15 hr, deionized by sequential treatment with Dowex AG 1-X8 resin in the chloride form followed by a mixed-bed MB3 resin (AG 501-X8), and finally subjected to paper chromatography using solvent II.

Immunoprecipitation of Biosynthetic Product. The complete reaction mixture containing the biosynthetic product was heated in a boiling-water bath for 1 min then chilled. NaCl (0.02 ml, 9%) and K-PO_4 buffer (0.02 ml, 0.2 M, pH 7.4) were added. Either a goat anti-Le^a serum or an unrelated goat antiserum (100 μl) was added to duplicate reactions and incubated at 37° for 1 hr. A quantity of rabbit anti-goat γG-globulin, sufficient to precipitate all of the goat γG-globulin, was then added and the reaction was allowed to stand overnight at 4°. Precipitates were collected by centrifugation at 12,000*g* for 15 min at 4°, washed twice with cold saline, and dissolved in 1 ml of the NCS reagent. For the inhibition studies, lacto-*N*-fucopentaose I or lacto-*N*-fucopentaose II (Figure 2) was incubated with the anti-Le^a serum before adding it to the reaction mixture.

Determination of Radioactivity. Radioactivity was detected on paper chromatograms by means of a Vanguard strip scanner. ^{14}C-Labeled compounds were counted in a Packard Tri-Carb liquid scintillation spectrometer using either 10 ml of a toluene solution composed of 400 mg of 2,5-diphenyloxazole and 10 mg of *p*-bis[2′-(5′-phenyloxazolyl)]benzene per 100 ml or 10 ml of the scintillation mixture described by Bray (1960).

Preparation of Enzyme. Human milk was obtained from fourteen different donors at various stages of lactation and stored at −20°. Each enzyme purification was performed on milk obtained from single individuals. Whole milk (50–100 ml) was centrifuged for 30 min at 10,000*g*, the coagulated lipid was removed manually, and 51.6 g of solid ammonium sulfate was added per 100 ml of defatted milk. After standing for 1 hr, the precipitate was collected by centrifugation, resuspended in 10 ml of 5 mM Tris buffer (pH 6.8) containing 2 mM glutathione, and dialyzed overnight against a buffer of the same composition.

The enzyme was separated from endogenous acceptor glycoproteins and lactalbumin by column chromatography on carboxymethylcellulose as described in the legend to Figure 3. The fraction containing fucosyltransferase activity (IV) was used for the studies to be described (Figure 3A). Protein corresponding to peak IV in fucosyltransferase activity or in its elution pattern from carboxymethylcellulose was not observed in milk obtained from Lewis-negative individuals (Figure 3B). A summary of a representative purification procedure is given in Table I. Similar results were obtained in samples from ten different Lewis-positive donors, representing individuals of the O, A, or Le^a phenotypes.

Identification of the Biosynthetic Product. The radioactive

TABLE I: Purification of Fucosyltransferase Activity from Human Milk.[a]

Enzyme Fraction	Total Protein (mg)	Total Act. ($\mu\mu$mole/ 3 hr)	Sp Act. ($\mu\mu$mole/ 3 hr per mg of Protein)	Recovery (%)
Whole milk	94	1.4[b]	0.015	100
Ammonium sulfate	84	1.3[b]	0.015	93
Fraction IV eluted from carboxymethylcellulose column	4.6	1.1	0.245	79

[a] Details of the purification procedure and the standard assay procedure for determining fucosyltransferase activity are described under Experimental Procedure. [b] Includes some [^{14}C]fucose incorporated into endogenous acceptor.

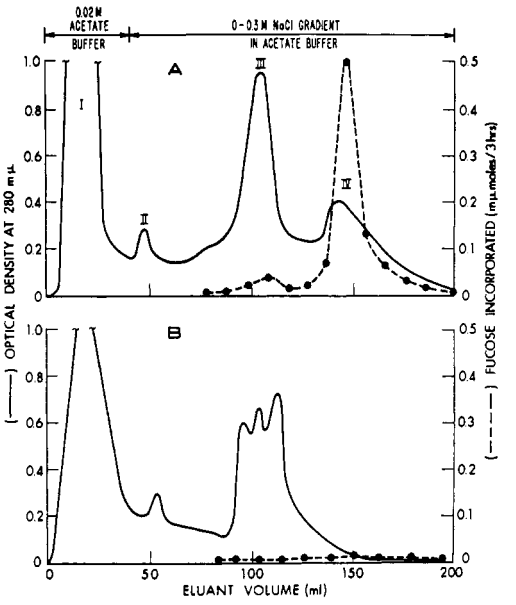

FIGURE 3: Fractionation of L-fucosyltransferase activity on carboxymethylcellulose. (A) Milk obtained from a Lewis-positive individual. (B) Milk obtained from a Lewis-negative individual. The dialyzed ammonium sulfate fraction (5 ml, 14 mg/ml) (*cf.* Experimental Procedure) was adjusted to pH 5.0 with acetic acid and applied to a 1 × 15 cm column of carboxymethylcellulose previously equilibrated with 0.02 M sodium acetate buffer (pH 5.0). The column was washed with 50 ml of the equilibrating buffer and eluted with 300 ml of a linear gradient of NaCl, 0.0–0.3 M in sodium acetate buffer (pH 5.0) at a flow rate of 19 ml/hr. The optical density of the eluted protein was recorded by continuous monitoring at 280 mμ. Individual fractions were dialyzed and samples containing 30 μg of enzyme protein were assayed for fucosyltransferase activity using the standard reaction conditions described under Experimental Procedure. Under similar experimental conditions, a standard sample of bovine lactalbumin was eluted from this column with 90–125 ml of buffer as a symmetrical peak.

biosynthetic product formed under conditions of the standard assay was nondialyzable and could be separated from residual GDP-L-fucose by paper chromatography in solvent I or by column chromatography on Sephadex G-100. These properties were similar to those observed with an authentic sample of ^{14}C-labeled A substance.

Although the product could not be directly precipitated by caprine anti-Le^a serum, at least 40% of the radioactive glycoprotein formed a nonprecipitating complex with anti-Le^a serum which, in turn, could be precipitated by addition of a rabbit antiserum to goat γG globulin (Table II). Only a small amount of radioactivity was precipitated when caprine antiserum of unrelated specificity was used in place of anti-Le^a serum or when the acceptor glycoprotein was omitted from the reaction mixture. The precipitation of radioactivity by the anti-Le^a serum was completely inhibited by the addition of the Le^a-active oligosaccharide, lacto-*N*-fucopentaose II, but not by the addition of lacto-*N*-fucopentaose I (Table II).

The biosynthetic product was subjected to column chromatography on Sephadex G-100 to separate it from residual GDP-fucose, dialyzed against water, and lyophilized. This material was subjected to the following chemical and enzymatic degradative procedures designed to determine the nature of the linkage between the incorporated fucose and the glycoprotein: all of the radioactivity could be recovered as free L-fucose after hydrolysis in 2 N HCl for 2 hr at 100° followed by paper chromatography in solvent III. After mild alkaline degradation (Schiffman *et al.*, 1964), 10% of the radioactivity remained in the glycoprotein and 90% of the radioactivity had a chromatographic mobility greater than free fucose in solvent III. No fucosylgalactose could be detected in the radioactive product while, under the conditions used for the degradation, 50% of the fucose present in an ovarian cyst glycoprotein with H activity (W-11) was recovered as fucosylgalactose.

None of the radioactive fucose was released from the glycoprotein by treatment of the biosynthetic product with a purified α(1–2)-fucosidase. Under the conditions employed, enzyme treatment released only fucose from the W-11 glycoprotein and reduced its H activity to less than 15% of its original value as measured by inhibition of hemagglutination.

Properties of the Enzyme. Fucosyltransferase activity was approximately proportional to enzyme concentration up to a concentration of 125 μg/ml, as tested under standard reaction conditions (*cf.* Experimental Procedure). The enzymatic incorporation of L-[^{14}C]fucose into glycoprotein was linear for a period of 3 hr. The maximal incorporation of fucose under the standard incubation conditions was 0.4 $\mu\mu$mole of fucose/3 hr per μg of added glycoprotein acceptor. Incorporation of fucose was dependent on the presence of "inactive acceptor" glycoprotein (Table III) or specific oligosaccharide (Figure 4). Neither L-fucose nor L-fucose 1-phosphate would substitute for GDP-fucose donor in the reaction.

The addition of magnesium or manganese ions is required for maximal activity of the enzyme (Grollman *et al.*, 1969). Other divalent cations did not stimulate the reaction, and manganese was used in all experiments reported in this paper. Although the enzyme showed 60% of the maximal rate in the absence of added divalent cation, the activity was totally inhibited by the presence of 3×10^{-2} M EDTA (Table IV).

TABLE II: Immunoprecipitation of Biosynthetic Reaction Product.[a]

	Additions to Biosynthetic Product				Radioactivity Precipitated	
	Caprine Anti-Le[a] Serum	Serologically Unrelated Caprine Antiserum	Rabbit Anti-Goat γ-Globulin	Oligosaccharide	cpm	% Added
Expt 1						
a	+	0	0	0	21	2
b	+	0	+	0	410	40
c	0	+	+	0	100	9
d	+	0	+	Pentaose II	110	10
e	+	0	+	Pentaose I	405	40
Expt 2						
a	+	0	+	0	367	40
b	0	+	+	0	89	9
c	+	0	+	Pentaose II	100	10

[a] Biosynthetic product was prepared by incubating the standard reaction mixture described under Experimental Procedure at 37° for 24 hr, adding 15 μμmoles of additional [^{14}C]GDP-L-fucose and 30 μg of fresh enzyme at 7 and 14 hr. The procedure for immunoprecipitation, as described in the text, was performed directly in the reaction tube. Less than 10 cpm was precipitated in a control reaction identical with expt 2b, in which the acceptor glycoprotein was omitted from the reaction mixture. Results similar to those shown in this table were obtained by immunoprecipitation of biosynthetic products from four additional enzyme preparations.

The enzyme displayed optimum activity in Tris buffer between pH 7.6 and 8.0, was inhibited 15 and 92%, respectively, by the addition of 3×10^{-5} and 5×10^{-4} M concentrations of *N*-ethylmaleimide, and could be stored for at least 3 months at −40° without substantial loss of activity.

Substrate Specificity. The specificity of the fucosyltransferase was demonstrated by its activity toward oligosaccharide substrates of known composition. The enzyme catalyzed the transfer of fucose from GDP-L-fucose to lacto-*N*-fucopentaose I or to lacto-*N*-tetraose to give radioactive products with the chromatographic mobility of lacto-*N*-difucohexaose I and lacto-*N*-pentaose II, respectively (Figure 4). Lacto-*N*-pentaose II, lactose, fucosyllactose, glucosamine, or glucose did not serve as acceptors when incubated with the enzyme under similar conditions. Some of the enzyme preparations used were capable of transferring small amounts of fucose to *N*-acetyllactosamine, presumably to the 3 position.

A variety of glycoproteins with blood group activity were tested for their ability to act as fucosyl acceptors (Table V). The only glycoprotein capable of accepting fucose from GDP-L-fucose, in addition to the chemically prepared

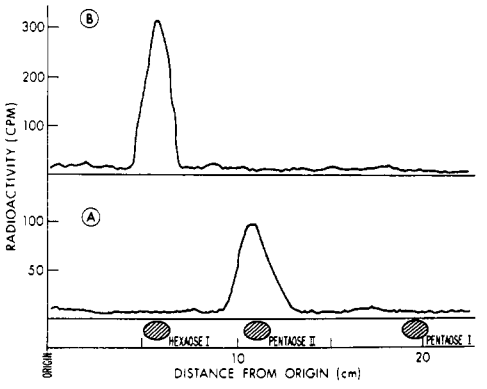

FIGURE 4: The standard reaction mixture, using (A) 40 μg of lacto-*N*-tetraose in place of glycoprotein, was incubated for 15 hr with 30 μg of enzyme protein. The reaction mixture was deionized (*cf.* Experimental Procedure), subjected to paper chromatography in solvent II for 110 hr, and the radioactive products were detected with the chromatogram strip scanner. Standard oligosaccharides were detected with the silver nitrate reagent. (B) Same condition as A except that 40 μg of lacto-*N*-pentaose I were used as acceptor.

TABLE III: Effect of Concentration of Glycoprotein on the Rate of Incorporation of Fucose.[a]

Glycoprotein Concn (μg/ml)	Fucose Incorporated (μμmoles/3 hr)
5	0.47
10	0.76
30	1.28
60	1.70
150	1.61

[a] Components of the standard reaction mixture were incubated for 3 hr with 30 μg of enzyme protein and the indicated amount of "inactive acceptor" glycoprotein. The incorporation of [^{14}C]fucose into glycoprotein was determined as described under Experimental Procedure.

TABLE IV: Effect of Divalent Cations on Fucosyltransferase Activity.[a]

Addition	Concn (mM)	Fucose Incorporated ($\mu\mu$moles/3 hr)
None		1.9
Mn	0.5	2.1
Mn	1.0	3.1
Mn	30	0.5
Mg	0.5	2.0
Mg	1.0	2.6
Mg	3.0	1.2
EDTA	30	0.0

[a] Components of the standard reaction mixture were incubated as described under Experimental Procedure with 30 μg of enzyme protein and the indicated concentration of magnesium or manganese ion.

TABLE V: Incorporation of Fucose into Various Glycoproteins.[a]

Sample	Blood Group Specificity	Source[b]	Maximum Fucose Incorporated ($\mu\mu$moles)
1	*d*	(J. S.)[c]	3.7
2	*d*	(O. G.)	3.6
3	Le^a	(M. T.)	0.9
4	H Le^b	(M. C.)	0.8
5	H	(W-11)	0.7
6	A	(J. S.)	0.7
7	B	(Beach)	0.6
8	Hog H	(23H)	0.9
9	*d*	Bovine serum albumin	0.1

[a] Standard reaction mixtures containing 30 μg of enzyme protein were incubated for 24 hr at 37° to determine the maximum incorporation of fucose. Incorporation of radioactivity was determined as described under Experimental Procedure. [b] Initials represent individuals from whom glycoprotein was originally isolated. [c] Chemically prepared from glycoprotein (J. S.) as described in text. [d] No A, B, H, Le^a, or Le^b activity could be detected by inhibition of hemagglutination when tested at a concentration of 1 mg/ml.

"inactive" glycoprotein, was the naturally occurring ovarian cyst glycoprotein without blood group activity (OG). In addition, the H-active glycoprotein (W-11), after treatment with α(1–2)-fucosidase, incorporated as much fucose as the "inactive" glycoprotein. The naturally occurring cyst material resembles the chemically prepared glycoprotein in chemical composition and by showing an immunological cross-reaction against pneumococcal type XIV antisera. Other glycoproteins tested incorporated only small quantities of fucose (Table V).

Discussion

The fucosyltransferase described in this paper appears to be identical with the enzyme described by Grollman *et al.* (1968, 1969). It is present in the milk of Lewis-positive donors but absent from all Lewis-negative individuals tested (Grollman *et al.*, 1968; Grollman, 1968). In addition to catalyzing the biosynthesis of oligosaccharides with Le^a and Le^b activity, we find that this enzyme is capable of forming Le^a substance from an inactive glycoprotein. Chester and Watkins (1969) recently described fucosyltransferase activity in particulate preparations from human submaxillary glands using oligosaccharides as acceptors. One of these enzymes was thought to be a 4-fucosyltransferase but the oligosaccharide product was incompletely identified. The antiserum used to identify the biosynthetic product in our experiments is highly specific for the Le^a determinant and is not inhibited by fucose-containing oligosaccharides with A, B, H, or Le^b activity (Grollman and Marcus, 1966; Lloyd *et al.*, 1968).

The precise nature of that portion of fucosylated glycoprotein that was not bound by the anti-Le^a antiserum is obscure. Acid hydrolysis revealed that all of the radioactivity in the biosynthetic product was present as fucose. The absence of fucosylgalactose following mild alkaline hydrolysis indicates that the newly incorporated fucose was not linked to the 2 position of galactose as in H substance. Furthermore, this fucose was not cleaved by an α(1–2)-L-fucosidase which was active against H-active glycoproteins.

Fucose is already known to exist in at least two other linkages in blood group glycoproteins. It may be linked α(1–3) to *N*-acetylglucosamine as depicted in Figure 1, and Aston *et al.* (1968) have isolated fucose-containing oligosaccharides from hydrolysates of blood group substance with fucose linked α(1–6) to galactose. No evidence for fucosyltransferase activity was detected when the partially purified enzyme was tested with oligosaccharides containing *N*-acetylglucosamine with a free 6 position as potential acceptor, but small amounts of 3-fucosyltransferase activity were frequently present. This enzyme could catalyze the incorporation of fucose into the 3 position of type 2 chains and may account for the portion of fucosylated glycoprotein that was not precipitated with the anti-Le^a sera.

Lloyd and Kabat (1968) have recently noted that all of the fucose in blood group glycoproteins cannot be accounted for on the basis of the known linkages. Terminal nonreducing *N*-acetylglucosamine residues are present in the structure proposed by these authors, and it is also possible that the transferase described in this communication might transfer fucose to the 4 position of these residues.

Although the enzyme catalyzed the transfer of fucose to lacto-*N*-fucopentaose I, it was unable to transfer a significant amount of fucose to an H glycoprotein without Le^b activity (W-11) which should possess a similar acceptor site. After the fucose was removed by enzymatic procedures, the glycoprotein became an active acceptor. The failure of W-11 to act as acceptor may be attributable to steric hindrance by the numerous side chains of the glycoprotein and suggests that the fucose residue on the terminal galactose of HLe^b glyco-

proteins is added to the chain subsequent to the addition of fucose to *N*-acetylglucosamine.

The unusual properties of lactose synthetase (Brew *et al.*, 1968; Brodbeck and Ebner, 1966) may be relevant to other glycosyltransferases found in milk. This enzyme has been separated into two components, one of which changes the acceptor specificity of the galactosyltransferase from glucose to *N*-acetylglucosamine (Brodbeck and Ebner, 1966). Further purification of the 4-fucosyltransferase described in this paper may reveal that this enzymatic activity results from the concerted action of two proteins, and it is conceivable that the difference between the various fucosyltransferases found in milk resides in such a "specifier" protein.

Acknowledgments

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