Enzymatic Synthesis of a Blood Group H-Related Glycosphingolipid by an \( \alpha \)-Fucosyltransferase from Bovine Spleen*

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**SUMMARY**

An \( \alpha \)-fucosyltransferase activity has been detected in a purified membrane preparation isolated from bovine spleen which catalyzes the transfer of \( L \)-fucose from GDP-L-[\( ^{14}C \)]-fucose to a tetraglycosylceramide (Lac-nTet-cer, Gal\( \beta \)-1-4GlcNac\( \beta \)-1-3Gal\( \beta \)-1-4Glc-cer) to form the blood group H-related glycosphingolipid. The membrane preparation contained a highly active endogenous nonlipid acceptor, which could be precipitated by 5% trichloroacetic acid or chloroform-methanol-water (v/v/v), whereas there was little endogenous glycosphingolipid acceptor. The optimum pH value for the incorporation of \( L \)-fucose was 6.4 in cacodylate-HCl buffer. The \( K_m \) values were 0.6 mM and 0.36 mM for Lac-nTet-cer and GDP-L-fucose, respectively. The \( ^{14}C \)-labeled product of the reaction was isolated and purified; it migrated with human erythrocyte blood group H-active pentaglycosylceramide. The terminal \( ^{14}C \)-fucose was hydrolyzed 85\% and 55\% by 0.1 N trichloroacetic acid at 100° for 2 hours and Charonia lambs \( \alpha \)-fucosidase (19 hours at 37°), respectively. The \( ^{14}C \)-labeled product inhibited the hemaggulination reaction of O-type cells against eel anti H(O) globulin and formed a precipitin line with Ulex europaeus lectin.

Blood group-active glycosphingolipids are found in the erythrocyte membranes and plasma membranes of most animal cells, but present knowledge of the chemistry, biosynthesis, and functions of these glycosphingolipids is meager. The chemical structures of the A and B blood group-related glycosphingolipids from human erythrocytes have been elucidated by Yamakawa and coworkers. They are characterized by a common carbohydrate content of \( \beta \)-glucose, \( \beta \)-galactose, \( \alpha \)-N-acetylgalactosamine, and \( \alpha \)-fucose. The specificity of a blood group-active glycosphingolipid is determined by its specific sequence of oligosaccharide chains attached to the ceramide moiety and by variation in the anomeric linkages between glycosamine units. It has been reported by different laboratories that a family of enzymes called fucosyltransferases transfers \( L \)-fucose from GDP-L-fucose to oligosaccharides (9-11) and glycoproteins (12-16) related to blood group substances, but biosynthesis in vitro of a blood group H-active glycosphingolipid has not been achieved until now (1).

Recently, Stellner et al. (17) and Kocielak et al. (18) have reported the structure of human erythrocyte H-active glycosphingolipid. The structure of an Le*-related novel glycolipid isolated from human adenocarcinoma has been reported by Yang and Hakomori (19). An \( N \)-acetylgalactosamine-containing tetraglycosylceramide, Lac-nTet-cer, is the common core structure of the following glycosphingolipids: blood group H-active (Fucal-2Gal\( \beta \)-1-4GlcNac\( \beta \)-1-3Gal\( \beta \)-1-4Glc-cer), a novel Le* type (Gal\( \beta \)-1-4(Fucal-3)GlcNac\( \beta \)-1-3Gal\( \beta \)-1-4Glc-cer), B-active isolated from human pancreas (Galal-3(Fucal-2)Gal\( \beta \)-1-4GlcNac\( \beta \)-1-3Gal\( \beta \)-1-4Glc-cer (20)), and a B-type isolated from rabbit erythrocytes (Galal-3Gal\( \beta \)-1-4GlcNac\( \beta \)-1-3Gal\( \beta \)-1-4Glc-cer (21, 22)).

We have previously reported the biosynthesis in vitro of the tetraglycosyleramide (23) and its conversion to blood group B-active pentaglycosylceramide (24) in rabbit bone marrow. The present studies are concerned with the biosynthesis of the blood group H-active pentaglycosylceramide from the tetraglycosylceramide (Lac-nTet-cer). The reaction catalyzed by the \( \alpha \)-fucosyltransferase obtained from bovine spleen is

\[
\text{GDP-L-[\( ^{14}C \)]Fuc + Gal-GlcNac-Gal-Glc-cer Mn}^{2+} \text{ or Mg}^{2+} \rightarrow \text{L-fucose.}
\]

The abbreviations used are: Lac-nTet-cer, galactosyl-\( \beta \)-1-4-galactosyl-\( \beta \)-1-3-N-acetylgalactosaminyl-\( \beta \)-1-4-galactosyl-\( \beta \)-1-3-galactosyl-\( \beta \)-1-4-glycosylceramide; Lea, N-acetylgalactosaminyl-\( \beta \)-1-3-galactosyl-\( \beta \)-1-4-glycosylceramide; B-active, N-acetylgalactosaminyl-\( \beta \)-1-3-galactosyl-\( \beta \)-1-4-glycosylceramide; Fenta, galactosyl-\( \beta \)-1-3-galactosyl-\( \beta \)-1-4-N-acetylgalactosaminyl-\( \beta \)-1-3-galactosyl-\( \beta \)-1-4-glycosylceramide; Forssman-active, N-acetylgalactosaminyl-\( \beta \)-1-3-N-acetylgalactosaminyl-\( \beta \)-1-3-galactosyl-\( \beta \)-1-4-glycosylceramide.
Subcellular distribution of GDP-fucose: Lac-N-Tet-cer α-fucosyltransferase activity during bovine spleen fractionation

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Major subcellular organelle</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
<tr>
<td>Total homogenate</td>
<td>Mixture of all</td>
</tr>
<tr>
<td>First residue (BSR1)</td>
<td>Cell debris, nuclei, etc.</td>
</tr>
<tr>
<td>Second supernatant (BSS2)</td>
<td>Light membranes</td>
</tr>
<tr>
<td>Second residue (BSR2)</td>
<td>Mitochondria, Golgi apparatus, heavy endoplasmic reticulum</td>
</tr>
<tr>
<td>1.25 M sucrose top junction (BSWG)</td>
<td>Golgi rich membrane fraction plus endoplasmic reticulum</td>
</tr>
<tr>
<td>1.25 M sucrose buffy coat (BSUP)</td>
<td>Golgi apparatus, endoplasmic reticulum, mitochondria</td>
</tr>
<tr>
<td>1.25 M sucrose pellet (BSP)</td>
<td>Mitochondria, endoplasmic reticulum</td>
</tr>
</tbody>
</table>

**Materials and Methods**

The following materials were obtained from commercial sources: Unisil (Clarkson Chemical Co.); G 3634 A, Tween 80, Tween 20, Myrij 59, Brij 98, and Brij 355P (Atlas Chemical Industries); sodium taurocholate (Calbiochem); Triton X-100 (Packard Instrument Co.); Triton CF-54 (Rohm and Haas, Philadelphia), Cetyl-Cliquot (Fisher Scientific Co.); and human anti-A and anti-B blood group sera (Hyland Division Travenol Laboratories). The following materials were obtained as gift samples: Ulex europeus lectin (Dr. Toshiaki Osawa, University of Tokyo), eel anti-H(O) globulin (Dr. G. F. Springer, Northwestern University, Chicago, Ill.), Charonia lampas α-fucosidase (Dr. T. Okuyama, Seikagaku Kogyo Institute, Tokyo), and lacto-N-tetraose and lacto-N-fucopentaoses I, II, and III (Dr. Akira Kobata, Kobe University).

**Acceptors**—Lactosylceramide was isolated from bovine spleen.

**Donors**—Unlabeled GDP-fucose was prepared according to published methods (31, 33). Fraction BSWG contained very little contamination with mitochondria and consisted mostly of endoplasmic reticulum.

**Preparation of Enzyme**—A preparation of Golgi-rich membranes was obtained according to a modification of the method of Morré (31). The bovine spleen tissues were collected fresh from Gentner Packing Co. (South Bend, Ind.) and were placed immediately in ice. Homogenization was performed within 1 hour after dissection, and all steps were carried out between 0° and 8°. The homogenization medium (SDMT) contained 0.5 M sucrose, 1% dextran (average molecular weight, 225,000); Sigma Chemical Co.), 5 mM mepropoic acid, 1 mM MgCl₂, and 0.04 M Tris-maleate buffer, pH 6.4. A Polytron 20ST (Kinetotrace, Lucerne, Switzerland) was used to homogenize in lots of 20 g of minced tissue in 0 volumes of SDMT solution. Homogenization was performed for 50 to 60 s with dial speed 4.8 at 4°-5°. The homogenate (1.8 liters) was centrifuged for 30 min at 9,000 × g. The supernatant fluid (BSS1, 1.1 liters) was centrifuged for 45 min at 70,000 × g. The second supernatant (BSS2, 1.05 liters) was decanted. The reddish brown phase of the pellet (BSR2) was rehomogenized in 2 volumes of 0.32 M sucrose and layered on 2 volumes of 1.25 M sucrose, and centrifuged for 30 min at 83,000 × g. A pinkish white layer (BSG) was collected from the junction of 0.32 M and 1.2 M sucrose by means of a disposable Pasteur pipette. The pellet that sedimented at the bottom of the tube and the buffy coat on top of that were designated BSP and BSUP, respectively. The BSG fraction was resuspended in 3 volumes of distilled water and centrifuged at 9,000 × g for 20 min to remove plasma membranes (31). The pellet was resuspended in 23 ml of 0.32 M sucrose and used as the enzyme source (BSWG fraction). This fraction contained little mitochondrial contamination as determined by electron microscopic examination and the INT-sucinate dehydrogenase marker enzyme test (31-33). The membrane fraction (BSWG) retained its α-fucosyltransferase activity for at least 6 months when stored at -18°. Only 10 to 15% loss of activity was observed after freezing and thawing.

**Enzyme Assays**—Complete incubation mixtures contained the following components (in micromoles) in final volumes of 0.025 to 0.05 ml: acceptor lipid (Lac-N-Tet-cer), 0.05 to 0.1; detergent G-3634 A, 0.1 to 0.2 mg; MgCl₂, 0.25; cacodylate-Cl buffer, pH 6.4, 10; GDP-L-[¹⁴C]fucose, 0.012 to 0.024 (2.0 × 10⁵ dpm per umol); and enzyme fraction BSWG, 50 to 100 μg of protein (estimated by the method of Lowry et al. (34)). The mixtures were incubated for 30 to 45 min at 37°, and the reaction was stopped by adding 0.1 ml of chloroform-methanol (2:1). The tubes were then centrifuged at 2000 × g for 15 min. Except for the protein disc, which sedimented at the junction of the upper and lower layers, the whole liquid content of the tube was spotted on Whatman No. 3MM paper and then assayed by a double chromatographic method (35) for glycosphingolipid biosynthesis and by the high voltage electrophoresis method (24) for oligosaccharide biosynthesis. The appropriate areas of each chromatogram were quantitatively determined by liquid scintillation techniques with the Packard and Beckman scintillation counters, models 3375 and DFM-100, respectively.

**Results**

The distribution of GDP-L-fucose: Lac-N-Tet-cer α-fucosyltransferase was determined in different subcellular fractions obtained from bovine spleen (Table 1). The electron microscopic studies for characterizing subcellular fractions were performed according to previously published methods (31, 32). The morphometric identification of different fractions was in good agreement with the fraction identification based on the assay of marker enzymes (31, 33). Fraction BSWG contained very little contamination with mitochondria and consisted mostly of endo-
suggests that cu-fucosyltransferase is also endogenous to endo-
action without Lac-nTet-cer decreased markedly (95%). The
rate of reaction was proportional to enzyme concentration be-
tween 0.5 and 2.5 mg of protein (measured by the method of
per ml of incubation volume, and remained constant with time
in cacodylate-HCl buffer. The enzyme preparation (Fraction
BSWG) was treated with 0.025 M EDTA for 5 min and then
dialyzed against 0.32 M sucrose solution for 1 hour. As indicated
in Table II, there was a 95% loss of activity when EDTA (5
mM) inhibited 30% of the original activity, whereas at higher
concentration (10 mM) it inhibited the reaction completely
(98 to 99%).

**Optimal Conditions**—As shown in Table II, the rate of re-
action without Lac-nTet-cer decreased markedly (95%). The
rate of reaction was proportional to enzyme concentration be-
tween 0.5 and 2.5 mg of protein (measured by the method of
Lowry et al. (34) using crystalline bovine albumin as standard)
per ml of incubation volume, and remained constant with time
of incubation up to 1 hour. A pH optimum of 6.4 was observed
in cacodylate-HCl buffer. The enzyme preparation (Fraction
BSWG) was treated with 0.025 M EDTA for 5 min and then
dialyzed against 0.32 M sucrose solution for 1 hour. As indicated
in Table II, there was a 95% loss of activity when EDTA (5
mm) was used in the incubation mixture. However, in the presence
of Mn
+ (5.0 mm) or Mg
+ (5.0 mm) only 50 to 70% of the
original activity was obtained, when EDTA-dialyzed enzyme
was used in the incubation mixture. Addition of either Ca
+ or
Zn
+ in the incubation mixture containing dialyzed enzyme
stimulated the reaction 5- to 8-fold, whereas Cu
+ , Fe
+ , Co
+ ,
Ni
+ , or Cd
+ were inactive. GTP at lower concentration (0.5
mm) inhibited 30% of the original activity, whereas at higher
concentration (10 mm) it inhibited the reaction completely
(98 to 99%).

**Detergent Specificity**—Many detergents were tested (Table
III), and the rate of reaction was optimal at a concentration of 5
mg per ml (Fig. 1) with cationic detergent G-3634-A. An anionic
detergent, sodium taurocholate, was about 36% as effective as
G-3634-A, whereas Triton CF-54, Cutsucm, and Triton X-100
were only about 5 to 10% as active.

**Donor Specificity**—UDP-[U-14C]glucose, UDP-[U-14C]galactose,
UDP-N-acetyl-[1-14C]glucosamine, UDP-N-acetyl-[1-14C]galac-
tosamine, and CMP-[14C]sialic acid could not replace GDP-L-
[14C]fucose under the present incubation conditions (Table
IV). The effect of varied concentrations of GDP-L-[14C]fucose
on the rate of reaction is shown in Fig. 2, and the calculated
K
m value is 0.36 mm.

**Acceptor Specificity**—The most active glycosphingolipid acceptors
were (Table V) Lac-nTet-cer (Galβ1-4GlcNAcβ1-3Galβ1-
4Glc-cer) and rabbit erythrocyte B-active pentaglycosylceramide
(Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-cer). The effect of varied
concentrations of Lac-nTet-cer on the rate of reaction is

| Detergent      | [14C]Pentaglycosylceramide
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>No detergent</td>
<td>1.48</td>
</tr>
<tr>
<td>G-3634-A</td>
<td>32.84</td>
</tr>
<tr>
<td>Sodium taurocholate</td>
<td>11.88</td>
</tr>
<tr>
<td>Triton CF-54</td>
<td>2.62</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>3.05</td>
</tr>
<tr>
<td>Cutsucm</td>
<td>2.97</td>
</tr>
<tr>
<td>Myrj 59</td>
<td>1.06</td>
</tr>
<tr>
<td>Brij 98</td>
<td>0.92</td>
</tr>
<tr>
<td>Brij 35 SP</td>
<td>1.26</td>
</tr>
<tr>
<td>Tween 20</td>
<td>1.2</td>
</tr>
<tr>
<td>Tween 80</td>
<td>0.75</td>
</tr>
<tr>
<td>Triton CF-54-Tween 80 (2:1)</td>
<td>2.61</td>
</tr>
</tbody>
</table>

**TABLE II**

**Requirements for GDP-fucose:Lac-nTet-cer α-fucosyltransferase**

In Experiment A, the complete incubation mixture contained
the following components (in micromoles unless otherwise stated)
in final volumes of 0.025 ml: Lac-nTet-cer, 0.05; detergent G-3634-
A, 0.125 mg; cacodylate HCl buffer, pH 6.4, 10; MgCl₂, 0.125;
GDP-L-[14C]fucose, 0.012 (2.6 X 10⁶ dpm per μmol); and enzyme
fraction BSWG as indicated, 54 μg of protein. After incubation
at 37° for 45 min, the mixtures were assayed by the double chromato-
graphic method as described in the text. In Experiment B,
conditions were the same as described in A, except that EDTA-
diaiyzed BSWG, 46 μg, was used as enzyme source.

| Enzyme and incubation mixture | [14C]Pentaglycosylceramide
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Experiment A: Undialyzed BSWG</td>
<td></td>
</tr>
<tr>
<td>Complete</td>
<td>40.4</td>
</tr>
<tr>
<td>Minus Lac-nTet-cer</td>
<td>1.3</td>
</tr>
<tr>
<td>Minus G-3634-A</td>
<td>7.7</td>
</tr>
<tr>
<td>Minus metal</td>
<td>47.4</td>
</tr>
<tr>
<td>Plus EDTA (5 mm)</td>
<td>1.8</td>
</tr>
<tr>
<td>Plus EDTA (5 mm) + MgCl₂ (10 mm)</td>
<td>40.7</td>
</tr>
<tr>
<td>Plus GTP (0.5 mm)</td>
<td>40.3</td>
</tr>
<tr>
<td>Plus GTP (10 mm)</td>
<td>0.6</td>
</tr>
<tr>
<td>Experiment B: EDTA-dialyzed BSWG</td>
<td></td>
</tr>
<tr>
<td>Minus metal</td>
<td>3.9</td>
</tr>
<tr>
<td>Plus MgCl₂ (5.0 mm)</td>
<td>31.4</td>
</tr>
<tr>
<td>Plus MnCl₂ (0.0 mm)</td>
<td>21.4</td>
</tr>
</tbody>
</table>

**Fig. 1.** Effect of detergent concentration on the rate of the reaction. Conditions were the same as described in Table II, except that different concentrations of G-3634-A (cationic detergent) were used as indicated. The values on the figure were corrected for endogenous values, 0.2 to 2.2 nmol/ml/hour.

**TABLE III**

**Detergent specificity of bovine spleen GDP-fucose:Lac-nTet-cer α-fucosyltransferase**

The conditions were the same as those described in Table II, except that different detergents (100 μg) and a different batch of enzyme Fraction BSWG were used in a total incubation volume of 0.045 ml. The mixtures were assayed by the method described in the text.
ceramide or triglycosylceramides containing terminal galactose, 5% as active as Lac-nTet-cer. Incorporation of [i4C]fucose into N-acetylgalactosamine, or N-acetylglucosamine were only 2 to 3.

The acceptor specificity of bovine spleen a-fucosyltransferase was determined first separately and then with mixtures of two substrates. The quantity of n-[14C]fucose (63.1 nmol) incorporated into the mixture was close to the theoretical value (42.5 nmol) calculated (36) if a single enzyme were involved. The K_m value for pentaglycosylceramide was 1.5 mM.

The acceptor specificity of bovine spleen a-fucosyltransferase was tested with a number of different oligosaccharides (Table VII). N-Acetyllactosamine and lacto-N-tetraose were the most active acceptors. Lactose and lacto-N-fucopentaoses I, II, and III were almost equally active at 8.0 mM concentration. The significance of these results is considered under "Discussion."

Isolation and Characterization of 14C-Labeled Product—The labeled product was isolated from a large scale (40-fold) incubation mixture. The radioactive product (53 nmol) was eluted from the double chromatographic method as described in the text. The inset shows Woolf plot of the same data.

Table IV

<table>
<thead>
<tr>
<th>Donor</th>
<th>Specific activity</th>
<th>Amount added</th>
<th>[14C] Labeled sugar incorporated</th>
<th>dpm/μmol</th>
<th>ν/μmol</th>
<th>nmol/mg protein/45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDP-[U-14C]glucose</td>
<td>2.0</td>
<td>27</td>
<td>0</td>
<td>0</td>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td>UDP-[U-14C]galactose</td>
<td>1.9</td>
<td>25</td>
<td>3.58</td>
<td>0.06</td>
<td>0.44</td>
<td>0.08</td>
</tr>
<tr>
<td>UDP-N-acetyl-[14C]glucosamine</td>
<td>1.13</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0.005</td>
<td>0</td>
</tr>
<tr>
<td>UDP N-acetyl-[14C]galactosamine</td>
<td>2.1</td>
<td>18</td>
<td>0.34</td>
<td>0.06</td>
<td>0.44</td>
<td>0.08</td>
</tr>
<tr>
<td>GDP-L-[U-14C]fucose</td>
<td>1.56</td>
<td>22</td>
<td>35.99</td>
<td>0.06</td>
<td>0.44</td>
<td>0.08</td>
</tr>
<tr>
<td>CMP-[14C]sialic</td>
<td>2.4</td>
<td>17</td>
<td>0</td>
<td>0</td>
<td>0.005</td>
<td>0</td>
</tr>
</tbody>
</table>

Table V

<table>
<thead>
<tr>
<th>Potential acceptor (4.0 mM)</th>
<th>Name</th>
<th>Structure</th>
<th>[14C]Fucose incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Lactosylceramide</td>
<td>Galβ1-4Glc-cer</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>GloboTri-β-GER</td>
<td>Galα1-4Galβ1-4Glc-cer</td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>GanglioTri-β-GER</td>
<td>GalNAcβ1-4Galβ1-4Glc-cer</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>LacTri-β-GER</td>
<td>GlcNAcβ1-3Galα1-4Glc-cer</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>Lac-nTet-β-GER</td>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glc-cer</td>
<td></td>
<td>40.2</td>
</tr>
<tr>
<td>Penta-β-GER (B-active)</td>
<td>Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc-cer</td>
<td></td>
<td>47.7</td>
</tr>
<tr>
<td>GanglioTet-β-GER</td>
<td>Galβ1-3GalNAcβ1-4Galβ1-4Glc-cer</td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>GM1, monosialoganglioside</td>
<td>Galβ1-3GalNAcβ1-4(NANα2-3)Galβ1-4Glc-cer</td>
<td></td>
<td>0.2</td>
</tr>
</tbody>
</table>

Fig. 2. Effect of GDP-β-[14C]fucose concentration on the rate of formation of pentaglycosylceramide. Incubation conditions were the same as described in Fig. 1, except that the indicated concentrations of GDP-β-[14C]fucose were used. The mixtures were assayed by the double chromatographic method as described in the text. The inset shows Woolf plot of the same data.

Fig. 3. Effect of acceptor concentration on the rate of formation of pentaglycosylceramide. Incubation mixtures were the same as in Table II, except that varied concentrations of Lac-nTet-β-GER and a different batch of enzyme Fraction BSWG (108 mg of protein) in 0.05-ml incubation volumes were used. Incubations were conducted for 30 min at 37°, and the double chromatographic method described in the text was used for assay. The endogenous value was 1.4 nmol/mg/hour. The inset shows Woolf plot of the same data.
TABLE VI
Substrate competition experiments

Conditions were the same as described in Fig. 3, except that the indicated substrates or substrate mixtures were used. The mixtures were incubated at 37°C for 30 min and assayed by the double chromatographic method as described in the text.

<table>
<thead>
<tr>
<th>Substrate (4.0 μmol)</th>
<th>[14C]-Labeled product found</th>
<th>Theoretical for</th>
<th>nmol/mg protein/30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>One enzyme</td>
<td>Two enzymes</td>
<td></td>
</tr>
<tr>
<td>Lac-nTet-cer.........</td>
<td>45.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penta-cer (B-active isolated from rabbit erythrocytes)....</td>
<td>54.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Penta-cer...........</td>
<td>42.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a Calculated using the indicated equation (36).

TABLE VII
Oligosaccharide substrate specificity studies with enzyme Fraction BSWG obtained from bovine spleen

Conditions were the same as described in Table II, except that different potential oligosaccharide acceptors were used as indicated below. The mixtures were incubated for 45 min at 37°C and then assayed by high voltage paper electrophoresis as indicated in the text.

<table>
<thead>
<tr>
<th>Acceptor (8.0 μmol)</th>
<th>[14C]Fucose incorporated</th>
<th>nmol/mg protein/45 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Endogenous</td>
<td>Galβ1-4Glc</td>
<td>0.4</td>
</tr>
<tr>
<td>Lactose</td>
<td>Galβ1-4Glc</td>
<td>17.9</td>
</tr>
<tr>
<td>Lactosamine</td>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glc</td>
<td>131.4</td>
</tr>
<tr>
<td>Lacto-N-tetraose</td>
<td>Galβ1-4GlcNAcβ1-3Galβ1-4Glc</td>
<td>23.6</td>
</tr>
<tr>
<td>Lacto-N-fucopentaose I</td>
<td>Fucα1-2Galβ1-3Galβ1-4Glc</td>
<td>13.5</td>
</tr>
<tr>
<td>Lacto-N-fucopentaose II</td>
<td>Galβ1-3(Fucα1-4)GlcNAcβ1-3Galβ1-4Glc</td>
<td>11.9</td>
</tr>
<tr>
<td>Lacto-N-fucopentaose III</td>
<td>Galβ1-3(Fucα1-3)GlcNAcβ1-3Galβ1-4Glc</td>
<td>12.6</td>
</tr>
</tbody>
</table>

layer chromatography on Silica Gel G (60% recovery). The [14C]-labeled product obtained from Lac-nTet-cer migrated with unlabeled rabbit erythrocyte B-active pentaglycosylceramide (Fig. 4) and moved just behind sheep erythrocyte Forssman-active glycosphingolipid. In a separate experiment [14C]-labeled product was mixed with human H-active pentaglycosylceramide (isolated from human O-type erythrocytes) and subjected to Silica Gel G thin layer chromatography. The [14C]-labeled product co-chromatographed with the diphenylamine-positive (23) band of H-active glycosphingolipid.

Microimmunodiffusion Reaction—A double microdiffusion plate was set up according to the method of Stellner et al. (17) and our previously published method (23, 35), using Ulex europaeus lectin (37) in the center well of a plastic template (2.5 × 2.5 cm). The [14C]-labeled product was mixed with unlabeled H-active glycosphingolipid (20 μg) in 10 μl of water and placed in Well 2 (Fig. 5a) of the template. Aqueous solutions of the substrate Lac-nTet-cer, B-active pentaglycosylceramide, and GanglioTet-cer were placed in Wells 1, 3, and 4, respectively. The plate was dried and exposed to an x-ray plate for 35 days (negative prints).
The stained plate was exposed to an x-ray plate for 35 days (14C-labeled product) reached 4 to 5 fig/45 ~l on the microscope slide covered with a coverslip. Inhibition of hemagglutination was complete absence of clump formation was not observed until the red blood cells (in 0.85% NaCl) to 35 ~l of a mixture containing electrophoresis and by paper chromatography after reduction of [14C]fucose (85%) was identified by high voltage borate electrolysis according to the method of Stellner et al. (17). The hybridization with 2yo acetic acid, and dried at 37° in a dust-free incubator. The stained plate was exposed to an x-ray plate for 35 days (Fig. 56). The Amido black-stained precipitin line exactly matched the dark band obtained on the x-ray plate.

Microhemagglutination Inhibition Studies—The blood group H specificity of the 14C-labeled product was measured by a modification of previously published hemagglutination inhibition techniques (6, 24). In the absence of any glycosphingolipid a positive hemagglutination reaction (14 to 30 cells per clump) was observed at a magnification of × 150 upon addition of 10 µl of a 2% human O-type cell suspension (obtained from South Bend Medical Foundation) to 5 µl (125 µg of protein) of eel anti-H(O) globulin and 30 µl of 0.85% NaCl on a microscope slide covered with a coverslip. Inhibition of hemagglutination (i.e. no clump formation) of red blood cells was also followed under the microscope after addition of 10 µl of 2% human O-type red blood cells (in 0.85% NaCl) to 35 µl of a mixture containing 125 µg of eel anti-H(O) globulin and 0.6 to 5 µg of the glycosphingolipid to be tested (Table VIII). A gradual decrease in the number of cells per clump was observed under the microscope with increasing concentration of unlabeled H-active glycosphingolipid (isolated from human O-type erythrocytes). Complete absence of clump formation was not observed until the concentration of unlabeled H-active pentaglycosylceramide (or 14C-labeled product) reached 4 to 5 µg/45 µl on the microscope slide.

Hydrolysis of [14C]Fucose with Acid and Enzyme—14C-Labeled product was treated with 0.1 N trichloroacetic acid at 100° for 2 hours according to the method of Stellner et al. (17). The hydrolyzed [14C]fucose (85%) was identified by high voltage borate electrophoresis and by paper chromatography after reduction with NaBH₄.

Hydrolysis with Charonia lampas α-fucosidase (17, 38, 39) was carried out in the presence and absence of sodium deoxycholate (17) and sodium taurocholate. Maximum hydrolysis (55%) of [14C]fucose from 14C-labeled product was obtained without addition of any detergent after 19 hours of incubation.

**Table VIII**

<table>
<thead>
<tr>
<th>Glycolipid added</th>
<th>Amount</th>
<th>Hemagglutination reaction</th>
</tr>
</thead>
<tbody>
<tr>
<td>No glycolipid</td>
<td></td>
<td>++++</td>
</tr>
<tr>
<td>H-active (from O-type cells)</td>
<td>0.6</td>
<td>++</td>
</tr>
<tr>
<td></td>
<td>1.2</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>5.0</td>
<td>-</td>
</tr>
<tr>
<td>14C-Labeled product</td>
<td>2.2 (2444 dpm)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td>4.4 (4888 dpm)</td>
<td>-</td>
</tr>
</tbody>
</table>

allowed to diffuse for 5 days at 4°. The precipitin line was visualized with 2% Amido black, followed by extensive washing with 2% acetic acid, and dried at 37° in a dust-free incubator. The stained plate was exposed to an x-ray plate for 35 days (Fig. 56). The Amido black-stained precipitin line exactly matched the dark band obtained on the x-ray plate.

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**Discussion**

The present study, using an isolated membrane preparation from bovine spleen, demonstrates the transfer of α-L-fucose from GDP-L-[14C]fucose to a tetraglycosylceramide acceptor (LacTet-cer) to form an H-active glycosphingolipid. Under different incubation conditions (23) the UDP-Gal: LacTri-cer β-galactosyltransferase (Fig. 6) which catalyzes the synthesis of LacTet-cer (Gal-GlcNAc-Gal-Glc-cer) has also been detected in this membrane fraction (40). Jabbar and Schachter (13) demonstrated the presence of at least two fucoyltransferases in a membrane-bound fraction from porcine liver which incorporated 3H-fucose into various derivatives of human plasma α1-acid glycoprotein. None of these two activities was related to plasma glycoprotein synthesis, whereas the role of the other one is not known. The microsomal fucoyltransferase of rat small intestinal mucosa isolated by Bella and Kim (14) catalyzed the incorporation of fucose into porcine submaxillary mucin and an oligosaccharide which contained β-galactose as the terminal sugar residue. The conclusion that an α(1-2) linkage is formed between fucose and terminal galactose is based primarily on the results obtained after treatment of their 14C-labeled products with Clostridium perfringens α-fucosidase (41). No attempt was made to further characterize the structure of H-related glycoprotein product. The bovine spleen membrane preparation was 6 times more active with N-acetylactosamine than with lacto-N-tetraose, whereas in the rat intestinal mucosal preparation (14) the activities were almost equal. Low but significant activity was observed with all three lacto-N-fucopentaoses (Table VII). The results indicate either that more than one α-fucosyltransferase is present in the bovine spleen membrane preparation or that a single enzyme catalyzes all three reactions. This question can only be solved by solubilization of the membranes and separation of the activities into different fractions. Both LacTri-cer (K_m = 0.6 mm) and Penta-cer (K_m = 1.5 mm) were highly active as substrates. The competition experiment suggested that both reactions were probably catalyzed by the same enzyme. In view of the higher K_m of Penta-cer (1.5 mm), LacTri-cer (K_m = 0.6 mm) is expected to be the physiological substrate. However, the K_m value obtained with a membrane-bound enzyme is an apparent value. A careful survey of the endogenous glycolipids in bovine spleen and erythrocytes might conclusively answer the question of substrate specificity. Further characterization of the enzymatic product, hexaglycosylceramide, obtained from rabbit B-active pentaglycosylceramide is under investigation. As shown in Fig. 6, the product of pentaglycosylceramide is also expected to be a B-active glycosphingolipid if the same α-fucosyltransferase catalyzes both reactions (Table VI). Recently we have isolated a rabbit B-type glycosphingolipid from bovine erythrocytes, and its structural determination is under investiga-
Takashi Okuyama, Georg F. Springer, and Akira Kobata for synthesis of H-active glycosphingolipid occurs in some site other than bone marrow cells, such as spleen, or that cr-fucosyltransferase activities detected in ABO human sera (15, 16) also transfer α-L-fucose to N-acetyllactosamine, lactose, and α-1,3-galactose. The origin of the α-fucosyltransferases in serum is unknown. In recent years it has been postulated (15) that the expression of a H or secretor gene on the erythrocyte surface is probably independent of the secretor gene Se (44). Therefore it is expected that bone marrow cells (11) or some other locus would be the probable site of its synthesis. Independent of secretor status, however, H antigen has been detected in the epithelial cell membrane and also in some endothelial cells (45) of the cardiovascular system. It can be speculated either that synthesis of β-active glycosphingolipid occurs in some site other than bone marrow cells, such as spleen, or that α-fucosyltransferase is synthesized and released from that site in the serum to complete H antigen synthesis at some other site. Using scanning microscopic techniques, Weiss (46) has suggested that the spleen is an encapsulated spongework perfused with blood where the erythrocyte cell surface can be modified. If true, the site of blood group-related glycosphingolipid synthesis may reside in the spleen and is involved with the elongation or termination of carbohydrate chains.

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