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MODIFIED ASSAY-PROCEDURE FOR GUANOSINE DIPHOSPHATE-L-FUCOSE:2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSIDE-(1→4)-α-L-FUCOSYLTRANSFERASE WITH THE AID OF SYNTHETIC PHENYL 2-ACETAMIDO-2-DEOXY-4-*O*-α-L-FUCOPYRANOSYL-3-*O*-β-D-GALACTOPYRANOSYL-β-D-GLUCOPYRANOSIDE AS A REFERENCE COMPOUND*

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ABSTRACT

Starting from phenyl 2-acetamido-2-deoxy-4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside (**1**), chemical syntheses were developed for phenyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (**4**) and phenyl 2-acetamido-2-deoxy-4-*O*-α-L-fucopyranosyl-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (**8**). Thin-layer chromatography in the solvent system 6:4:1:5 (v/v) 2-propanol–ethyl acetate–ammonium hydroxide–water clearly separated the synthetic trisaccharide **8** (R_F 0.69) from synthetic disaccharide **4** (R_F 0.78), fucose (R_F 0.56), and GDP-fucose (which remained at the origin). Based upon this observation, a modified method for the determination of GDP-L-fucose:*N*-acetylglucosaminide-(1→4)-α-L-fucosyltransferase was developed that employed the synthetic disaccharide **4** as an acceptor, and compound **8** as an authentic reference-compound. This modified assay-procedure can simultaneously monitor possible competing reactions which may interfere with determination of α-(1→4)-L-fucosyltransferase activity; these include phosphorylase and α-L-fucosidase activities, and incorporation of α-L-[^{14}C]-fucose into endogenous acceptors of enzyme preparations. Thus, the modified assay-procedure should facilitate determination of α-(1→4)-L-fucosyltransferase.

INTRODUCTION

Glycosyltransferases, which are membrane-bound enzymes in the cell, are detected as soluble activities in milk, serum, and other body-fluids[2,3]. Hill and co-workers[3] successfully developed an affinity-chromatography technique for the purification of some of these glycosyltransferases. The importance of the purified enzymes, which exhibit strict specificities, has become evident in structural analyses of various

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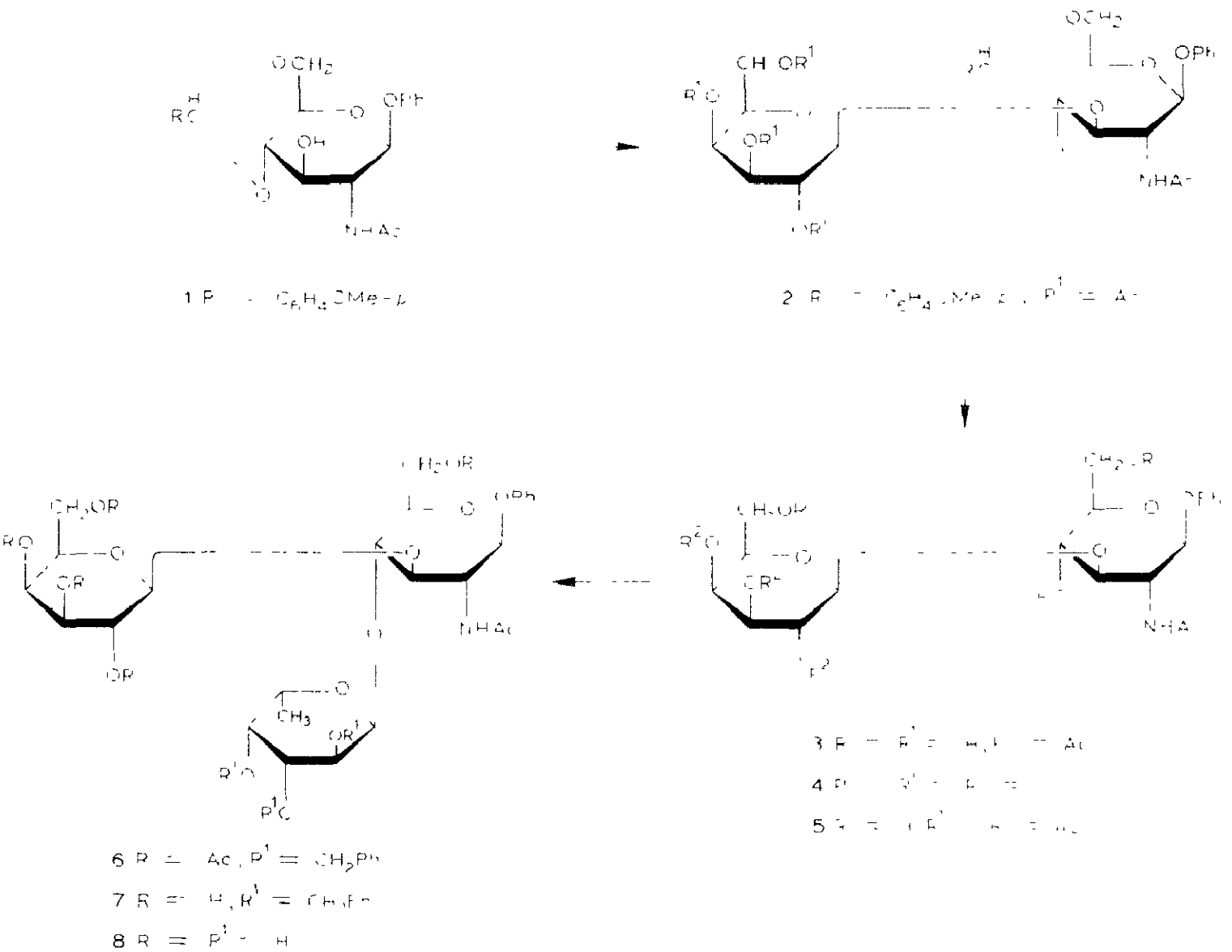
complex saccharides and related compounds. As a result, much attention is being focused on the purification of such enzymes.

In our laboratory, we have initiated a program of study of substrate specificity of this class of enzymes, and their purification. In our approach, an attempt is also being made to improve the assay procedure for certain glycosyltransferases by employing synthetic compounds as reference compounds. For example, the availability of *o*-nitrophenyl 2-*O*-α-L-fucopyranosyl-β-D-galactopyranoside, synthesized in our laboratory[4], led to the development of a simple assay-procedure for α-(1→2)-L-fucosyltransferase[5], the H-gene specific enzyme. In this report, we describe an efficient assay-procedure for another α-(1→4)-L-fucosyltransferase, the Le-gene specific enzyme present in human milk[3,6]. For our enzymic studies, we have synthesized phenyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (**4**), the acceptor for α-(1→4)-L-fucosyltransferase, and also the compound phenyl 2-acetamido-2-deoxy-4-*O*-α-L-fucopyranosyl-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (**8**), the expected product of the reaction catalyzed by α-(1→4)-L-fucosyltransferase.



EXPERIMENTAL

Materials and methods. 1,4-Dithiothreitol and MES buffer were obtained from Calbiochem–Behring Corp., LaJolla, California, U.S.A. Permafluor III was a product

of Packard Instrument Co., Downers Grove, IL, U.S.A. Bio-Solv was obtained from Beckman Instrument, Inc., Fullerton, CA, U.S.A. GDP-L-[^{14}C]fucose (192 mCi/mmol) was obtained from New England Nuclear, Boston, MA, U.S.A. All other materials were of the highest quality available commercially.

Melting points were determined with a Fisher–Johns apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer 241 polarimeter at room temperature. Ascending t.l.c. was conducted on plates coated with a 0.25-mm layer of silica gel 60 PF-254 (E. Merck, Darmstadt, Germany); the components were located by exposure to u.v. light, or by spraying the plate with 5% sulfuric acid in ethanol and heating. Elemental analyses were performed by Robertson Laboratory, Florham Park, New Jersey, U.S.A. I.r. spectra were recorded with a Perkin–Elmer 297 spectrophotometer, and n.m.r. spectra with a Varian XL-100 instrument at 100 MHz, with Me_4Si as the internal standard.

*Phenyl 2-acetamido-2-deoxy-4,6-*O*-(*p*-methoxybenzylidene)-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-β-*D*-glucopyranoside* (**2**). — A solution of phenyl 2-acetamido-2-deoxy-4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside[7] (**1**; 3.735 g, 9 mmol) in 1:1 (v/v) benzene–nitromethane (500 mL) was boiled until 100 mL of the solvent had distilled. The temperature of the solution was adjusted to 60°, and mercuric cyanide (2.0 g, 7.9 mmol) was added; then a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (3.7 g, 9 mmol) in 1:1 (v/v) benzene–nitromethane (40 mL) was added dropwise, with stirring, during 4 h, under nitrogen, and the solution was stirred under nitrogen for a further 24 h at 60°. Mercuric cyanide (1.0 g, 4 mmol) and a solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (1.8 g, 4.4 mmol) in 1:1 (v/v) benzene–nitromethane (20 mL) were added, and the solution was stirred for an additional 24 h. T.l.c. in 5:1 (v/v) chloroform–acetone then showed complete disappearance of the starting material. The mixture was cooled, successively washed with ice-cold, saturated, aqueous sodium hydrogencarbonate (2 × 50 mL), 30% aqueous potassium iodide solution (2 × 50 mL), and water (100 mL), dried (anhydrous sodium sulfate), and evaporated to a thick syrup that was dissolved in the minimum volume of hot ethanol, and the solution kept overnight at room temperature, giving crystalline compound. One recrystallization from hot ethanol gave pure compound **2** in 72% yield (4.83 g); m.p. 216°, $[\alpha]_D$ −3.7° (*c* 1, chloroform); ν_{max}^{KBr} 3290 (NH), 1745 (OAc), 1650 (amide), and 760 and 705 cm^{-1} (Ph); n.m.r. data (acetone-d_6): δ 1.92, 1.94, 1.98, 2.08, and 2.11 (5 s, 5 × 3 H, 5 Ac), 3.80 (s, 3 H, OMe), 5.37 (d, 1 H, *J* 8 Hz, H-1), 5.39 (d, 1 H, *J* 3.5 Hz, H-4′), 5.64 (s, 1 H, benzylic H), and 6.90–7.60 (m, 9 H, aromatic).

Anal. Calc. for $C_{36}H_{43}NO_{16}$: C, 57.98; H, 5.81; N, 1.88. Found: C, 57.73; H, 5.88; N, 1.88.

*Phenyl 2-acetamido-2-deoxy-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-*D*-galactopyranosyl)-β-*D*-glucopyranoside* (**3**). — A suspension of compound **2** (3.0 g) in 80% acetic acid (200 mL) was stirred for 20 min at 60°, and the clear solution obtained was cooled to room temperature; acetic acid was evaporated off under diminished pressure, and the last traces of acetic acid were removed by repeated co-evaporation with

toluene, affording a residue that crystallized from ethyl acetate–hexane to give **3** (1.72 g, 68%); m.p. 209–210°, $[\alpha]_D$ −1.7° (*c* 1, chloroform); t.l.c. (ethyl acetate) R_F 0.52; ν_{max}^{KBr} 3450 (OH), 3395 (NH), 1740 (OAc), 1655 (amide), and 760 and 705 cm^{-1} (Ph); n.m.r. data (acetone-d_6): δ 1.92, 1.95, 2.02, 2.10, and 2.16 (s each, 5 × 3 H, 5 Ac), and 6.96–7.40 (m, 5 H, Ph).

Anal. Calc. for $C_{28}H_{37}NO_{15}$: C, 53.58; H, 5.94; N, 2.23. Found: C, 53.68; H, 5.97; N, 2.26.

*Phenyl 2-acetamido-2-deoxy-3-O-β-*D*-galactopyranosyl-β-*D*-glucopyranoside* (**4**). — A solution of crystalline **3** (1.75 g) in 1:1 (v/v) chloroform–methanol (25 mL) containing 0.1M sodium methoxide solution (1 mL) was kept for 6 h at room temperature, and then overnight at 5°, made neutral with acetic acid, and evaporated, followed by a few additions and evaporations of dry toluene. The residue precipitated from ethanol–ether to give amorphous **4** in 85% yield; $[\alpha]_D$ +2.8° (*c* 0.5, Me_2SO); n.m.r. data (Me_2SO-d_6): δ 1.93 (s, 3 H, NAc), 5.21 (d, 1 H, *J* 8 Hz, H-1), and 6.94–7.54 (m, 5 H, Ph).

Anal. Calc. for $C_{20}H_{29}NO_{11}$: C, 52.28; H, 6.36; N, 3.05. Found: C, 52.08; H, 6.44; N, 3.04.

*Phenyl 2-acetamido-6-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-*D*-galactopyranosyl)-β-*D*-glucopyranoside* (**5**). — Acetyl chloride (173 mg, 2.2 mmol) in dry dichloromethane (5 mL) was added dropwise to a stirred solution of imidazole (300 mg, 4.4 mmol) in dry dichloromethane (10 mL) maintained at 0°. After 5 minutes, the imidazole hydrochloride was removed by filtration, the filtrate added to compound **3** (1.126 g, 2 mmol), and the solution refluxed for 3 days. Dichloromethane (100 mL) was added, and the solution was washed with water (2 × 25 mL), dried (magnesium sulfate), and evaporated to a syrup that was purified by chromatography on a column of silica gel, eluting with pure ethyl acetate, to give **5** (805 mg, 67%) which crystallized from ethyl acetate–hexane: m.p. 121–122°, $[\alpha]_D$ −6.1° (*c* 2, chloroform), t.l.c. (ethyl acetate) R_F 0.74; n.m.r. data (acetone-d_6): δ 1.94–2.18 (cluster of singlets, 18 H, 5 AcO + NHAc) and 6.96–7.50 (m, 5 H, Ph).

Anal. Calc. for $C_{30}H_{39}NO_{16}$: C, 53.81; H, 5.87; N, 2.09. Found: C, 53.65; H, 5.88; N, 2.10.

*Phenyl 2-acetamido-6-O-acetyl-2-deoxy-3-O-(2,3,4,6-tetra-O-acetyl-β-*D*-galactopyranosyl)-4-O-(2,3,4-tri-O-benzyl-α-*L*-fucopyranosyl)-β-*D*-glucopyranoside* (**6**). — Compound **5** (890 mg, 1.33 mmol) was dissolved in a mixture of dichloromethane (15 mL), *N*,*N*-dimethylformamide (3 mL), tetraethylammonium bromide (0.555 g, 2.65 mmol), and diisopropylethylamine (0.5 mL, 2.89 mmol). A solution of freshly prepared 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide (1.32 g, 2.66 mmol) in dichloromethane (5 mL) and dry $HCONMe_2$ (1 mL) was added, and the mixture was stirred under a nitrogen atmosphere for 5 days at room temperature. Dichloromethane (200 mL) was added, and the solution was successively washed with sodium hydrogencarbonate solution and water, dried (anhydrous Na_2SO_4), and evaporated. The residue was purified by chromatography on a column of silica gel, eluting with 3:1 (v/v) ethyl acetate–hexane, to provide **6** in 73% yield (1.05 g): m.p. 183–184°,

$[\alpha]_D$ −66.2° (*c* 0.5, chloroform); t.l.c. (3:1 ethyl acetate–hexane) R_F 0.71; ν_{max}^{KBr} no OH absorption; n.m.r. data (acetone-d_6): δ 1.39 (d, 3 H, *J* 6.5 Hz, C-Me), 1.92–2.11 (cluster of singlets, 18 H, 5 AcO + NAc), 5.28 (d, 1 H, *J* 3 Hz, H-4′), 5.42 (d, 1 H, *J* 3.5 Hz, H-1″), and 6.9–7.6 (m, 20 H, aromatic).

Anal. Calc. for $C_{57}H_{67}NO_{20}$: C, 63.03; H, 6.22; N, 1.29. Found: C, 62.82; H, 6.22; N, 1.31.

*Phenyl 2-acetamido-2-deoxy-3-*O*-β-*D*-galactopyranosyl-4-*O*-(2,3,4-tri-*O*-benzyl-α-*L*-fucopyranosyl)-β-*D*-glucopyranoside* (**7**). — A solution of compound **6** (200 mg) in absolute methanol (4 mL) and chloroform (4 mL) containing 0.1M sodium methoxide solution (0.2 mL) was kept overnight at room temperature, and then made neutral with acetic acid, and evaporated. The residue was compound **7** in 86% yield, but t.l.c. showed the presence of two spots that could not be separated by column chromatography. The ^{1}H-n.m.r. spectrum indicated that it still possessed one *O*-acetyl group. Increase in the concentration of sodium methoxide, or increase in the time, gave *O*-acetyl-free products, but the phenyl group was also removed. Consequently it was used as such for the next operation.

*Phenyl 2-acetamido-2-deoxy-4-*O*-α-*L*-fucopyranosyl-3-*O*-β-*D*-galactopyranosyl-β-*D*-glucopyranoside* (**8**). — A solution of **7** (200 mg) in acetic acid (30 mL) was hydrogenolyzed in the presence of Pd/C (10%) for 2 days. The suspension was filtered, and the filtrate evaporated to dryness, to afford a solid residue which was purified by chromatography on a column of silica gel, eluting with 65:35:8 (v/v) chloroform–methanol–water, to provide amorphous trisaccharide **8** in 74% yield; $[\alpha]_D$ −74.4° (*c* 0.5, MeOH); t.l.c. (65:35:8 chloroform–methanol–water) R_F 0.57; ν_{max}^{KBr} 3380 (OH), 1640 (amide), and 750 and 700 cm^{-1} (phenyl); n.m.r. data (MeOH-d_4): δ 1.21 (d, 3 H, *J* 6.5 Hz, CMe), 1.99 (s, 3 H, NHAc), 4.46 (d, 1 H, *J* 7.0 Hz, H-1′), 5.10 (2 H, H-1,1″), and 6.96–7.4 (m, 5 H, Ph).

Anal. Calc. for $C_{26}H_{39}NO_{15} \cdot 2\ H_2O$: C, 48.67; H, 6.75; N, 2.18. Found: C, 48.58; H, 6.64; N, 2.07.

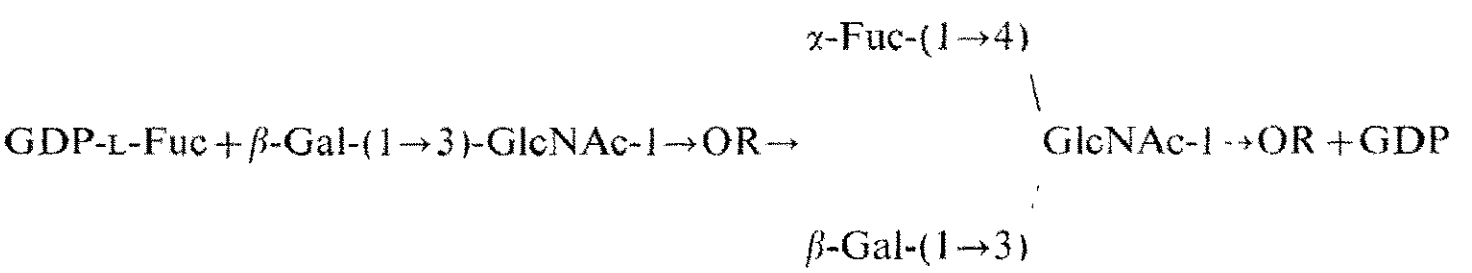
Preparation of the crude enzyme solution. — Human milk was obtained from a single donor. Partial purification of the enzyme preparation was achieved by a modification of the procedure used by Jarkovsky *et al.*[6]. Following the removal of the milk fat, and precipitation of the protein by means of ammonium sulfate, the protein was dialyzed against 5mM Tris buffer (pH 6.8) containing 0.5mM 1,4-dithiothreitol. This preparation was clarified by centrifugation at 10,000*g* for 30 min at 4°. A 20-mL sample of human milk was used as the starting material, and, after partial purification, the volume was 14.5 mL. This preparation was stored, until used in the enzyme assays.

Enzyme assay. — The enzyme-assay mixture (0.1 mL, final vol.) contained 5 μmol of MES buffer (pH 6.8), 0.5 μmol of $MnCl_2$, 0.2 nmol of GDP-L-[^{14}C]fucose, 0.5 μmol of phenyl 2-acetamido-2-deoxy-3-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (**4**) and 10–40 μg (estimated by ultraviolet absorption) of the milk-enzyme preparation. The assay mixtures were incubated for 30 min at 37°, and reactions were terminated by placing samples on ice, followed by addition of 0.2 mL of absolute ethanol. In some cases, the ethanol–terminated assay-mixture was evaporated to

dryness with a stream of nitrogen, and further treated with purified α-L-fucosidase from human serum[8]; for this treatment, 0.1 mL of 150mM acetate buffer, pH 5, containing serum fucosidase was added to the assay tube, and this mixture was incubated for up to 6 h at 37°, and the reaction terminated by adding 0.2 mL of ethanol. The ethanol-treated reaction mixtures were centrifuged at 2000*g* for 15 min at 4° to precipitate the protein as a pellet. The supernatant liquors from ethanol-treated mixtures were spotted on thin-layer plates (75 mm thickness) of silica gel, along with authentic, reference trisaccharide **8**, and the chromatograms were developed with 6:4:1:5 (v/v/v/v) isopropyl alcohol–ethyl acetate–ammonium hydroxide–water. After being developed for 2.5 to 3 h, the chromatograms were dried, and viewed under ultraviolet light. Areas containing the authentic reference compound and fucose were separately removed by scraping, and quantified by liquid scintillation spectrometry using 17:2:1 (v/v/v) toluene–Permafluor III–Bio-Solv (10 mL). Routinely, all enzyme assays were performed in duplicate, with four different control-assays. These consisted of a zero-time control, in which the assay mixture was assembled at 4° and immediately terminated, and control assays without enzyme, without substrate (GDP-L-[^{14}C]fucose), or without acceptor [β-Gal-(1→3)-β-GlcNAc-1→O-Ph]. A unit of α-(1→4)-L-fucosyltransferase activity is defined as 1 pmol of α-L-[^{14}C]fucose incorporated per hour.

RESULTS AND DISCUSSION

Recently, we reported[5] for the determination of GDP-L-fucose:galactoside 2′-fucosyltransferase in human serum a modified method which employed *o*-nitrophenyl β-D-galactopyranoside and phenyl 2-acetamido-2-deoxy-3-*O*-α-D-galactopyranosyl-β-D-galactopyranoside as acceptors. The principal advantages of this method over the previous procedure[9] (which used aryl glycosides as acceptors) are elimination of the need for a radiochromatogram scanner to locate reaction-products and a shorter development time for chromatography. Based on similar strategy, we have now developed a modified assay-procedure for α-(1→4)-L-fucosyltransferase, which is known to catalyze the following reaction.



Compounds containing the β-Gal-(1→3)-GlcNAc sequence have frequently been used as acceptors for this enzyme[3]. For our purposes, we aimed at the preparation of β-Gal-(1→3)-β-GlcNAc-1→OPh (**4**), a disaccharide having a chromophore, as an acceptor for the enzyme. It is also apparent that, when compound **4** is used as the

acceptor for the enzyme, the expected product, catalyzed by the enzymic reaction will have the structure β-Gal-(1→3)-β-GlcNAc-1→OPh.

4
↑
1
α-Fuc

As a result, we also developed the chemical synthesis of the expected product **8**. For the chemical synthesis of the acceptor **4**, as well as of reference compound **8**, phenyl 2-acetamido-2-deoxy-4,6-*O*-(*p*-methoxybenzylidene)-β-D-glucopyranoside[7] (**1**) was chosen as a suitable starting-material. Reaction of **1** with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide in the presence of mercuric cyanide in 1:1 benzene–nitromethane provided **2**, which was isolated crystalline in 72% yield. The infrared and n.m.r. spectra supported the structure assigned to the compound. Treatment of **2** with aqueous acetic acid for 20 min at 60° gave crystalline **3**, and deacetylation of **3** under the usual conditions provided phenyl 2-acetamido-2-deoxy-3-*O*-β-D-galacto-pyranosyl-β-D-glucopyranoside (**4**).

To synthesize **8** from **3**, it was necessary in the first step, to protect, selectively, O-6 of the 2-acetamido-2-deoxy-D-glucopyranose residue. Compound **5** was obtained in 67% yield by preferential acetylation using 1-acetylimidazole. Halide-ion catalysis[10] for glycopyranoside formation was employed to introduce an α-linked L-fucopyrano-syl group into **5**. Then, the chromatographically pure **5** was treated with 2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl bromide in the presence of tetraethylammonium bromide in dichloromethane containing diisopropylethylamine (to neutralize the hydrogen bromide liberated). The reaction residue was purified by chromatography in a column of silica gel, to give pure **6** in 73% yield. The n.m.r. spectrum of **6** showed a clear doublet at δ 5.42 (d, 1 H, *J* 3.5 Hz, H-1″), establishing that the L-fucopyranosyl group in **6** had the α configuration. *O*-Deacetylation of **6** gave **7** which, on hydrogeno-lysis in glacial acetic acid in the presence of pallidium–charcoal as catalyst, produced the desired trisaccharide **8** after purification by chromatography in a column of silica gel.

In order to develop a facile assay-procedure for the title fucosyltransferase, it was important to find a chromatographic technique that would consistently separate the acceptor **4**, reference compound **8**, L-fucose, and GDP-L-[^{14}C]fucose. Indeed, it is essential that the reference compound **8** should be clearly separated from L-fucose and GDP-L-fucose; as mentioned later, the monosaccharide L-fucose can be expected as a byproduct in the enzyme assay for α-(1→4)-L-fucosyltransferase. These compounds were effectively separated on thin-layer plates of silica gel that were developed with 6:4:1:5 (v/v/v/v) 2-propanol–ethyl acetate–ammonium hydroxide–water. The aryl compounds **4** (R_F 0.78) and **8** (R_F 0.69) were clearly separated, and visibilized under ultraviolet light (see Fig. 1). Fucose (R_F 0.56) could be detected after the chromatogram had been sprayed with 5% sulfuric acid in ethanol and then

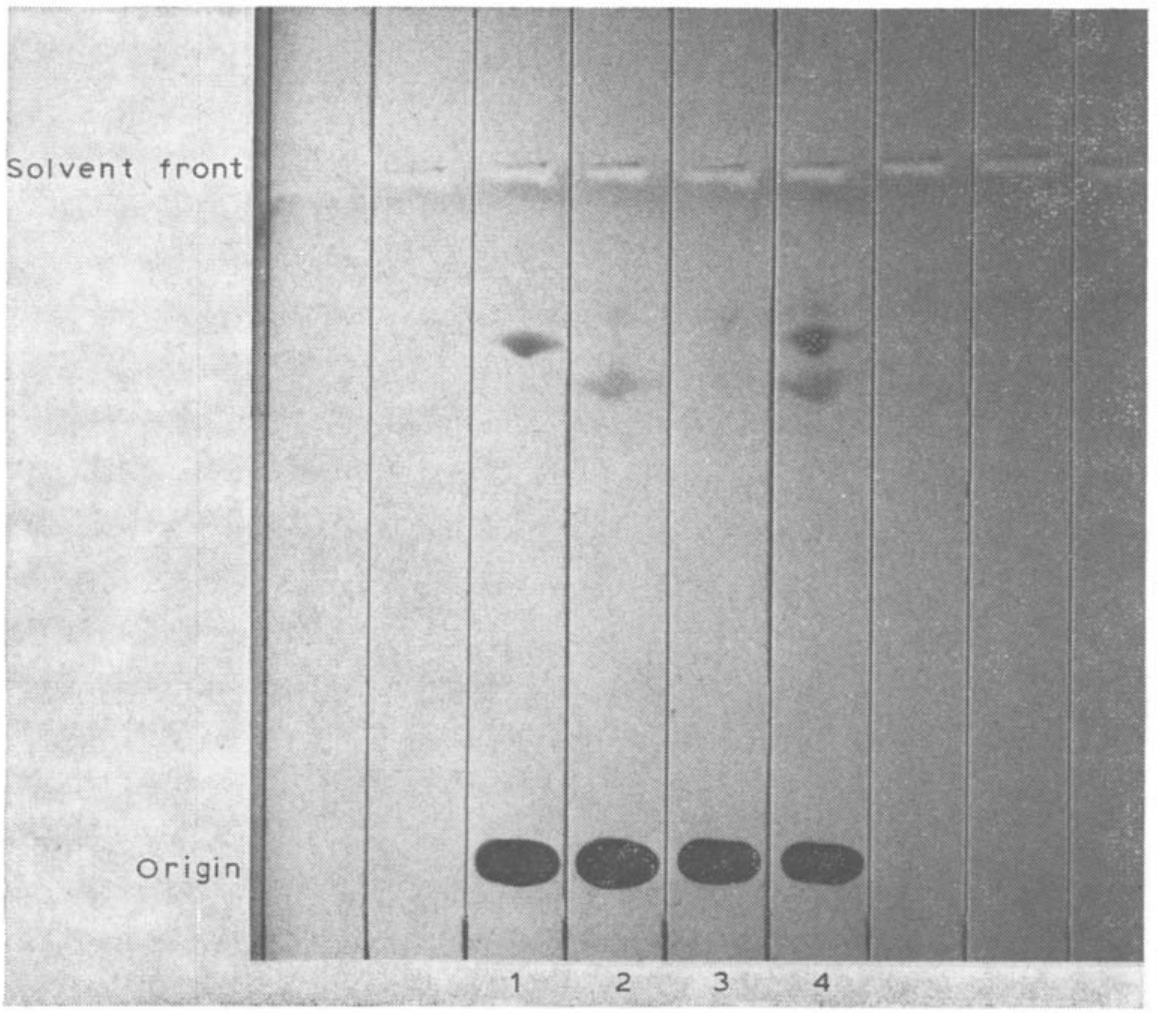


Fig. 1. Thin-layer chromatogram of the components of the reaction mixture of the α-(1→4)-L-fucosyltransferase assay. Chromatographic conditions are given in Materials and methods. [From left to right: lanes 1, 2, and 3 contained the authentic compounds (0.2 μmol) α-L-fucose, β-Gal-(1→3)-β-GlcNAc-1→OPh, and β-Gal-(1→3)-[α-L-Fuc-(1→4)]-β-GlcNAc-1→OPh, respectively. Lane 4 contained all three compounds. All lanes also contained 5 μmol of MES buffer (pH 6.8) and 2 μmol of $MnCl_2$. Spots were visibilized under ultraviolet light. GDP-L-[^{14}C]fucose was located at the origin.]

heated for 5–10 min at 110° (see Fig. 2). GDP-L-[^{14}C]fucose remained at the origin in this chromatographic system.

By using appropriate controls for the transferase assay, competing reactions that may interfere with the quantification of transferase activity can be detected; these include phosphorylase activity (which liberates L-fucose from GDP-L-[^{14}C]-fucose) and fucosidase activity (which cleaves L-fucose from the product formed). The presence of these interfering enzymic activities, particularly the GDP-L-fucose hydrolyase activity in human milk, has been clearly demonstrated by Clamagirand-Mulet *et al.*[11]. The incorporation of labeled L-fucose into endogenous glycoprotein can be approximated by measuring the radioactivity associated with the protein precipitated by absolute ethanol. The enzyme source for the aforementioned experiments was a 10-fold dilution of the frozen milk-preparation. When quantities of the enzyme of 0–40 μg were assayed, the amount of ethanol-precipitated protein was insignificant. When 80 μg, or more, of this enzyme source was assayed, the ethanol-precipitated protein contained $<1\%$ of the incorporated α-L-[^{14}C]fucose. Thus, under typical, α-(1→4)-L-fucosyltransferase-assay conditions, endogenous acceptor proteins do not interfere. The rate of reaction of α-(1→4)-L-fucosyltransferase was

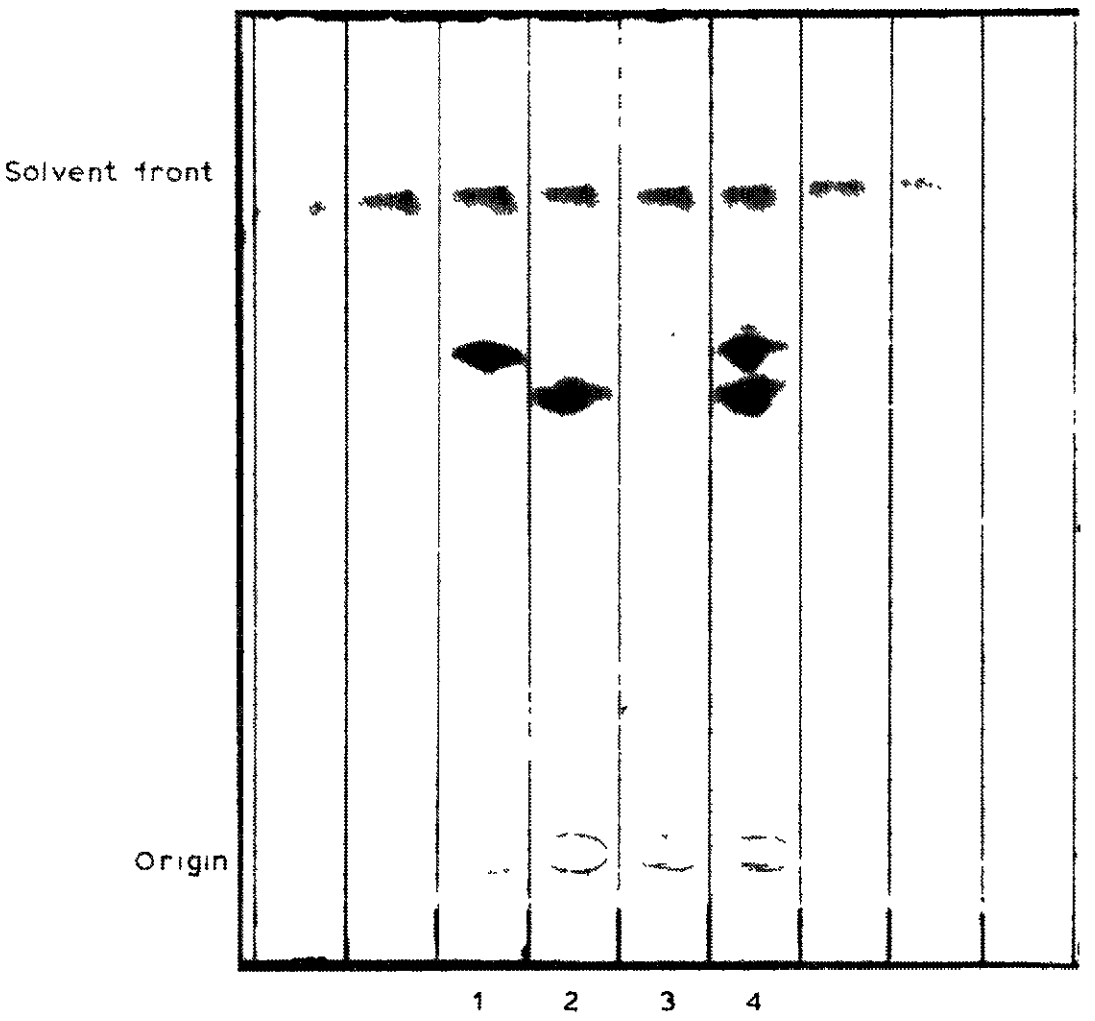


Fig. 2. Thin-layer chromatogram of the components of the reaction mixture of an α-(1→4)-L-fucosyltransferase assay. [This is the same chromatographic plate shown in Fig. 1. The spots on this plate were visibilized by spraying with 5% H_2SO_4 in ethanol, and heating the plate to produce charring of the organic compounds.]

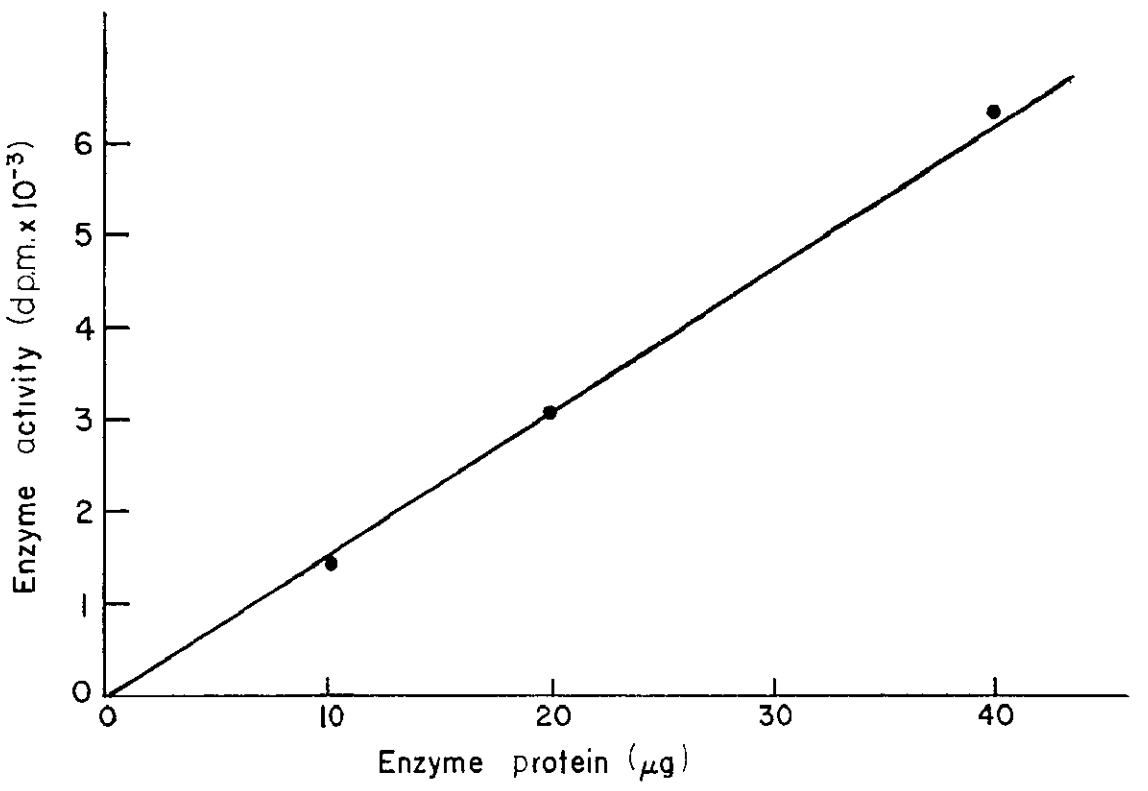


Fig. 3. α-(1→4)-L-Fucosyltransferase activity as a function of enzyme protein. [Standard assays were assembled with various quantities of milk protein.]

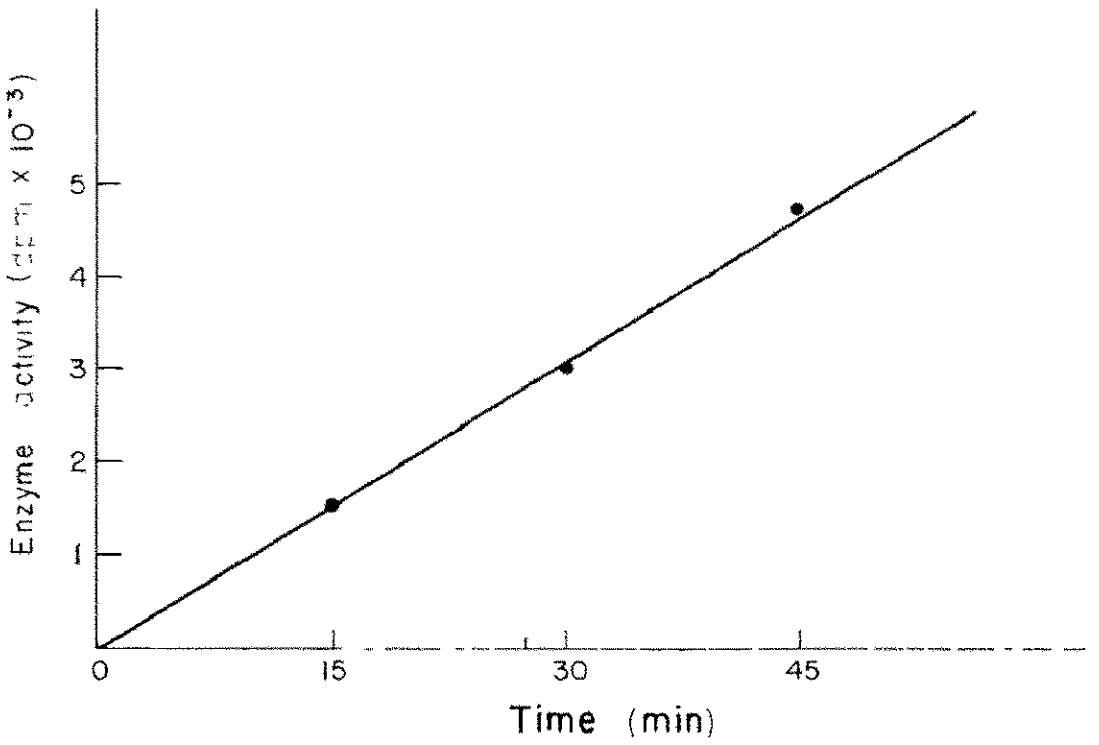


Fig. 4. α-(1→4)-L-Fucosyltransferase activity as a function of incubation time. [Standard assays were assembled, and incubated for the time period indicated.]

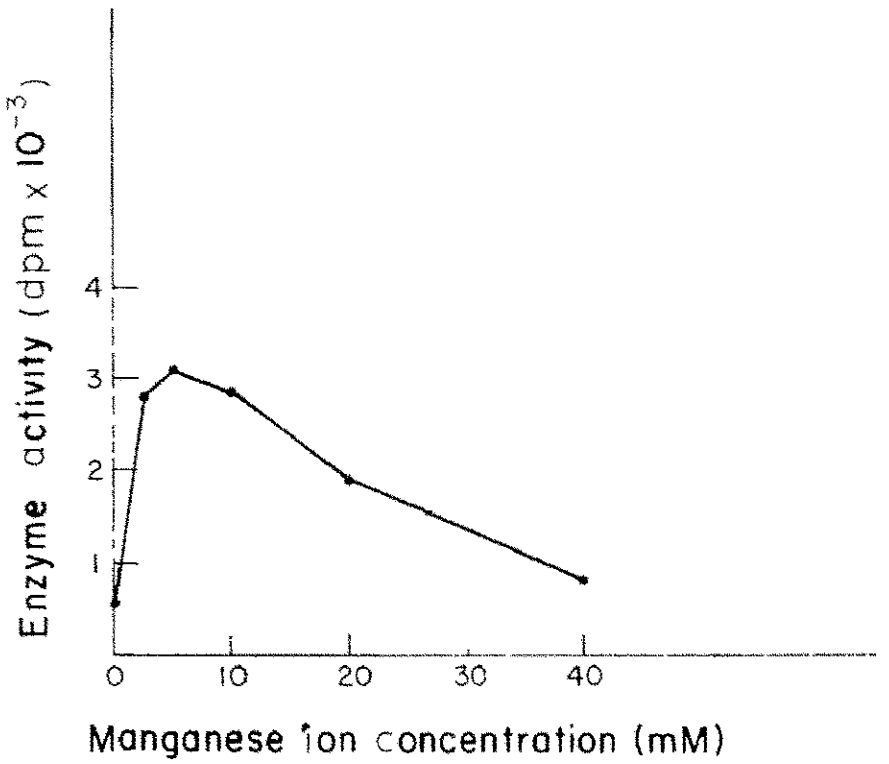


Fig. 5. α-(1→4)-L-Fucosyltransferase activity as a function of the concentration of manganese. [Standard assays were assembled that contained various amounts of manganese chloride.]

linear with respect to protein, from 0–40 μg (see Fig. 3), and with respect to time from 0 to 45 min (see Fig. 4). For these experiments, radioactively labelled end-products were quantitatively recovered from scraped spots having labelled L-fucose in the trisaccharide **8**.

Because it has been reported that manganese enhances the activity of α-(1→4)-L-fucosyltransferase, the assay system described herein was used to measure the activity of this enzyme in the presence of various concentrations of manganese.

Fig. 5 shows that α-(1→4)-L-fucosyltransferase activity is dependent on the concentration of manganese. Enzyme activity is enhanced, reaches a maximal level at 5mM, and falls off rapidly at higher concentrations of manganese. A similar effect of $MnCl_2$ on the activity of this fucosyltransferase has recently been reported by Prieels *et al.*[12].

In addition to α-(1→4)-L-fucosyltransferase, human milk has been shown to contain α-(1→2)- and α-(1→3)-L-fucosyltransferase activity[11-14]. Thus, formation of α-Fuc-(1→2)-β-Gal-(1→3)-β-GlcNAc-1→OPh can be expected when the synthetic compound **4** is used as the acceptor for the crude, milk-enzyme preparation. In order to preclude the presence of α-(1→2)-L-fucose linkages in the end product of this assay, two experiments were performed. First, the milk-enzyme preparation was assayed for α-(1→2)-L-fucosyltransferase activity[5]. Under enzyme conditions that would cause the production of 200 times the detectable level of α-(1→4)-L-fucosyltransferase activity, no α-(1→2)-L-fucosyltransferase was detected in this milk preparation. Clamagirand-Mulet *et al.*[11] and Shen *et al.*[14], also reported that it is difficult to detect α-(1→2)-L-fucosyltransferase activity in milk, regardless of the secretor status of the donor. The apparent absence of α-(1→2)-L-fucosyltransferase from this enzyme preparation suggests that L-fucose could only be incorporated into the disaccharide acceptor in α-(1→4) linkage position, as O-3 of the acceptor **4** is not available. The lack of α-(1→2)-L-fucosyltransferase activity in this milk preparation, and the presence of an α-(1→4)-linked L-fucose in the end product, are further supported by the results of an experiment that utilized purified α-L-fucosidase from human serum; this enzyme effectively cleaves[8] compounds that contain L-fucose-linked α-(1→2)-Gal. When the assay mixture of α-(1→4)-L-fucosyltransferase, reported herein, was treated with serum α-L-fucosidase, neither an increase in L-fucose labeling nor a decrease in end-product labeling was observed. This same L-fucosidase preparation (diluted 30-fold) released detectable levels of L-fucose from the end product, α-L-Fuc-(1→2)-β-Gal-1→$OC_6H_4NO_2$-*p*, of the α-(1→2)-L-fucosyltransferase assay[5]; this suggests that L-fucose is not linked to α-(1→2)-Gal in the end product of the assay described herein. These results, along with the fact that the end product comigrated with the authentic trisaccharide **8**, indicate that compound **4** can serve as an acceptor for the production of an end product that contains L-fucose in α-(1→4) linkage.

The Lewis gene is considered to determine the synthesis of GDP-L-fucose:*N*-acetylglucosaminide (1→4)-α-L-fucosyltransferase. Degraded glycoproteins derived from individuals with blood-type A serve as the acceptor for α-(1→4)-L-fucosyltransferase. Alternatively, appropriate oligosaccharides derived from human milk have been used as acceptors for this enzyme. For these saccharide acceptors of high molecular weight, paper-chromatographic techniques, followed by strip-scanning methods, are usually used for the detection of radioactivity. Prieels *et al.*[12] described for the enzyme an assay procedure that uses a small column of Dowex-1 X8 resin

for the isolation of radioactively labeled products. However, the method described herein is a quick and effective alternative for the detection of α-(1→4)-L-fucosyltransferase.

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