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The Use of Hydrophobic Synthetic Glycosides as Acceptors in Glycosyltransferase Assays

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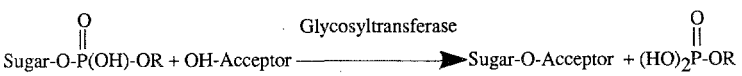
A general method is described for the assay of glycosyltransferase activity, which makes use of synthetic glycoside acceptors attached to hydrophobic aglycones. The products formed by incubation of an enzyme with acceptor and radiolabelled sugar-nucleotide can then be rapidly (one minute) separated from interfering radioactivity by adsorption on to reverse-phase C-18 cartridges. After aqueous washing, products are easily isolated by elution with methanol. The utility of the method for the assay of β(1-4)galactosyltransferase, α(1-2)fucosyltransferase and *N*-acetylglucosaminyltransferase I and V is demonstrated.

Glycosyltransferases are a class of enzymes that catalyze the transfer of a glycosyl-residue, usually from a sugar nucleotide donor, to the hydroxyl group of an acceptor saccharide which generally forms part of a glycoprotein, glycolipid or polysaccharide [1-3].



The activity of glycosyltransferases is most commonly assayed by incubating the sugar nucleotide, radioabelled in the sugar moiety, with a source of the enzyme and an appropriate acceptor. Labelled products are then quantified after separation from the unreacted donor and its degradation products; the sugar phosphate and the free sugar. Separation is carried out in a variety of ways. These include high voltage paper electrophoresis, TLC, gel-filtration or HPLC, which are tedious and time consuming; or by ion-exchange chromatography, which is prone to interference by the detergents (such as Triton X-100) frequently used to solubilize the enzymes [3-6]. The assays are further

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complicated by the fact that the acceptors for many of the enzymes are complex oligosaccharides which are often very difficult to purify, even in milligram amounts, from natural sources.

In this paper, we report a general method to assay glycosyltransferases which makes use of synthetic glycoside acceptors attached to hydrophobic aglycones. By virtue of their hydrophobicities, the labelled products from an enzyme incubation are readily separated from unreacted sugar-nucleotide and by-products by adsorption on a reverse-phase C-18 cartridge, from which they are eluted by methanol after aqueous washing. The assay, which we refer to here as the Sep-Pak assay, is rapid, requiring usually less than one minute processing time per sample, has excellent sensitivity due to the low background achieved and requires no specialized equipment. Triton X-100 does not interfere and the assay works well for both crude cell homogenates and purified enzymes. We demonstrate here the suitability of this method for the assay of *N*-acetylglucosaminide β(1-4)galactosyltransferase, β-galactoside α(1-2)fucosyltransferase and *N*-acetylglucosaminyltransferases I and V. Some of these data have already been presented in a preliminary form [7, 8].

Experimental Procedures

Materials

Bovine milk UDP-Gal:β-D-*N*-acetylglucosaminide β(1-4)galactosyltransferase (EC 2.4.1.90), specific activity 4.3 U/mg protein; UDP-Gal; UDP-GlcNAc; β-NADH; ATP; phosphoenolpyruvate; rabbit muscle L-lactic dehydrogenase (EC 1.1.1.27) Type II, specific activity 920 U/mg; rabbit muscle pyruvate kinase (EC 2.7.1.40) Type III, specific activity 500 U/mg; 4-methylumbelliferyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**2**); phenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**3**); *p*-nitrophenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**4**); phenyl β-D-galactopyranoside (**5**); and *n*-octyl β-D-glucopyranoside were from Sigma (St. Louis, MO, USA). 8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**1**) [9]; 8-methoxycarbonyloctyl β-D-galactopyranoside (**6**) [10]; 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (**7**) [9]; 8-methoxycarbonyloctyl 3,6-di-*O*-(α-D-mannopyranosyl)-β-D-mannopyranoside (**10**) [Alton G, Newton EM, Srivastava V, Palcic MM, Hindsgaul O, unpublished results]; octyl 6-*O*-[2-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-β-D-glucopyranoside (**16**); and octyl 6-*O*-[2-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-α-D-mannopyranosyl]-7-deoxy-D-*glycero*-D-*gluco*-heptopyranoside (**17**) [11] were prepared by chemical synthesis. Uridine diphospho-D-[6-^{3}H]galactose (17.3 Ci/mmol), guanosine diphospho-L-[U-^{14}C]fucose (129 mCi/mmol) and ACS liquid scintillation cocktail were from Amersham (Oakville, Ont. Canada). Uridine diphospho-*N*-acetyl-D-[1-^{14}C]glucosamine (9.5 mCi/mol) was from New England Nuclear (Lachine, Que., Canada) or synthesized as previously described [12]. Sep-Pak C-18 reverse phase cartridges were obtained from Waters (Mississauga, Ont., Canada), and were conditioned before use by washing with 10 ml of methanol and 20 ml of water. GDP-Fuc was synthesized according to the method of Nunez *et al.* [13]. Frozen rabbit livers and porcine submaxillary glands were obtained from Pel-Freeze Biologicals (Rogers, AR, USA). A crude extract of the soluble form of GDP-Fuc:β-D-galactoside α(1-2)fucosyltransferase was prepared from porcine submaxillary glands [14], UDP-GlcNAc:α-D-mannoside (GlcNAc to Manα1-3)β(1-2)-*N*-acetylglucosaminyltransferase (GnT I) was a Triton X-100 ex-

tract of rabbit liver acetone powder [3] and UDP-GlcNAc:α-D-mannoside (GlcNAc to Manα1-6)β(1-6)-*N*-acetylglucosaminyltransferase (GnT V) was a Triton X-100 extract from hamster kidneys [8, 12]. Protein concentrations were estimated using the Bio-Rad protein assay kit which is based on the method described by Bradford [15] using bovine serum albumin as a standard.

Methods

Acceptor evaluations

400 nmol of **1** or **2**, 900 nmol of **3** or **4** were incubated with 100 nmol UDP-Gal, 0.37 μCi of UDP[^{3}H]Gal, 7mU of β(1-4)galactosyltransferase, 107 μmol sodium cacodylate, pH 7.5, and 10 μmol $MnCl_2$ in a total volume of 1.07 ml. Following incubation at 37°C for 3 h, each reaction mixture was diluted to 5 ml with water and applied to a conditioned Sep-Pak C-18 cartridge attached to a 5 ml syringe. The unreacted UDP-Gal and its hydrolysis products were eluted from the cartridge with 20-25 ml water or until constant counts near background were reached. Radiolabelled reaction products were eluted in 15 ml methanol, evaporated to dryness, dissolved in 5 ml of water and reapplied to another Sep-Pak cartridge. The cartridge was washed with 5 × 5 ml fractions of water, which were collected directly in scintillation vials, then with 5 ml fractions of methanol. After addition of ACS liquid scintillation cocktail, the samples were counted in a Beckman LS 1801 scintillation counter. The data were obtained as dpm, by using a series of standards to generate quench curves relating the counting efficiency to H number for the isotopes used, as specified by the manufacturer. This compensates for the different counting efficiencies of the aqueous and methanol eluents (25 and 18%, respectively) for tritium.

α(1-2)Fucosyltransferase incubations contained in a volume of 0.69 ml: 5.9 μmol of **5**, 2.2 μmol of **6**, or 0.7 μmol of **7**, 0.03 μCi GDP-[U-^{14}C]fucose, 30 μmol $MnCl_2$, 0.2 μmol ATP, 12.5 μmol sodium cacodylate buffer pH 6.0, and an extract of fucosyltransferase which contained 3.4 mg protein. After incubation, at 37°C for 6 to 20 h, the reaction mixture was quenched with 5 ml of water and applied to a conditioned Sep-Pak cartridge. The cartridge was washed with 5 × 5 ml of water, then 15 ml of methanol to elute radiolabelled reaction products. The methanol fraction was evaporated to dryness, reapplied to a Sep-Pak cartridge and washed with 5 ml fractions of water which were collected in scintillation vials. The cartridges were then washed with 5 ml fractions of methanol, which were also collected in vials and counted as described above. The counting efficiencies for the aqueous and methanol eluents were 89 and 86% respectively.

β(1-4)Galactosyltransferase assays

Bovine milk β(1-4)galactosyltransferase activity was estimated spectrophotometrically, using the method of Fitzgerald *et al.* [16]. Cuvettes containing 107 μmol sodium cacodylate buffer pH 7.5, 10 μmol $MnCl_2$, 100 nmol UDP-Gal, 50 μmol KCl, 1.07 μmol phosphenolpyruvate, 200 nmol NADH, 50 U pyruvate kinase, 50 U lactate dehydrogenase and 20-308 nmol of **1** or 17-250 nmol **2** were equilibrated to 37°C in a Beckman DU-8 spectrophotometer. β(1-4)Galactosyltransferase (0.7-1.7 μg) was added such that the total volume was 1.07 ml, and the decrease in absorbance monitored at 340 nm. Initial rates of product formation were estimated using a millimolar extinction coefficient of 6.2 $mM^{-1}cm^{-1}$ for NADH absorbance. The kinetic paramters V_{max} and K_M were evaluated from the initial rate data using a computer program based on the statistical method of Wilkinson [17].

The radiochemical assay was done in the same manner as the spectrophotometric assay, except the incubations were carried out in plastic liquid scintillation vials and contained 0.12 to 0.21 μg of enzyme and 0.3-0.6 μCi of UDP-[6-^{3}H]Gal in addition to the other components. Following incubation at 37°C, 5 ml of water was added to vials and the samples applied immediately to a Sep-Pak cartridge. The cartridges were washed with water until background levels were achieved and then sample eluted in 2 × 5 ml fractions of methanol and counted in 10 ml of ACS cocktail.

α(1-2)Fucosyltransferase assays

These assays were done in plastic scintillation vials which contained 12.5 μmol sodium cacodylate pH 6.0, 0.01 μCi of GDP-[U-^{14}C]-L-fucose, 9 nmol GDP-Fuc, 30 μmol $MnCl_2$, 0.2 μmol ATP, 0.39-7.4 μmol of **5**, 50-360 nmol of **6**, or 20-360 nmol of **7** as acceptors. An extract of fucosyltransferase containing 14 mg of protein was added such that the total volume was 0.62 ml. The mixtures were incubated at 37°C for 1 h, 5 ml of water was added and the samples applied to a Sep-Pak cartridge. The cartridge was washed until eluent reached 50-70 cpm, then radiolabelled products were eluted with 5 ml of methanol and counted in 10 ml ACS cocktail.

GnT I Assays

The assays for GnT I were done in scintillation vials and contained in 1.5 ml: 0.4 μCi UDP-[^{14}C]GlcNAc, 21 nmol UDP-GlcNAc, 10 μmol $MnCl_2$, 50 μmol sodium cacodylate pH 6.5, 0.75% Triton X-100, and 0.11-1.4 μmol of **10** as acceptor. An extract of GnT I containing 3.7 mg of protein was added and the mixture was incubated at 37°C for 1 h. Samples were applied to a Sep-Pak cartridge after the addition of 5 ml of water. The cartridges were washed with water until the eluent reached 50-70 cpm, then radiolabelled products were eluted with 5 ml of methanol and counted in 10 ml ACS cocktail.

GnT V Assays

The assays for GnT V activity in crude hamster kidney extracts were carried out as microassays in a total volume of 20 μl. The details have been previously reported [8, 12].

Routine Sep-Pak assay

After incubation of an enzyme extract and an appropriate donor and acceptor, the reaction mixture was diluted to 5 ml with water, then applied to a freshly conditioned Sep-Pak cartridge. The cartridge was washed with 20 ml of water. An additional 5 ml water wash was collected in a scintillation vial and counted to ensure that elution of labelled donor was complete. Radiolabelled products were then eluted with 2 × 5 ml methanol and counted separately. The second 5 ml fraction rarely contained significant radioactivity except when large amounts of enzyme were present. The time required for aqueous washing and methanol elution is under one minute. Control assays which contain all the components except acceptor were used to establish blanks. We have not observed interference by endogenous hydrophobic acceptors in our crude fractions. These would give high counts in methanol eluents for control incubations without added acceptor, and in such instances a boiled enzyme solution would serve as an appropriate control. For routine assays cpm rather than dpm can be used for data analysis. Sep-Paks can be reused after regeneration according to the manufacturers recommendations.

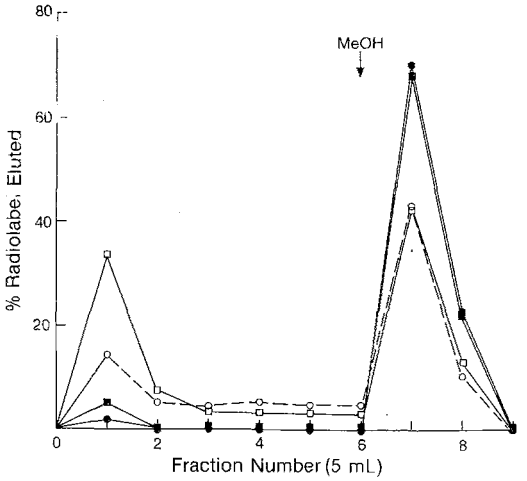
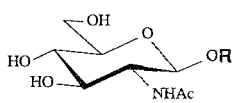


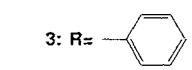
Figure 1. Elution profiles of radiolabelled reaction products of β(1-4)galactosyltransferase on Sep-Pak C-18 cartridges. The methanol eluents from Sep-Paks of preparative incubations of **1** (●), **2** (■), **3** (○) and **4** (□), as described for acceptor evaluations in the experimental procedures were evaporated to dryness, dissolved in 5 ml water and reapplied to Sep-Pak cartridges. The cartridges were washed with water, then methanol; 5 ml fractions were collected and counted. Recovery is expressed as percentage of the amount loaded on the cartridge.

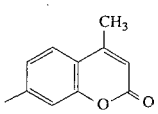
Table 1. Evaluation of the suitability of hydrophobic glycosides as acceptors in the Sep-Pak assay of β(1-4)galactosyltransferase.[a]

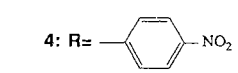
Acceptor	Dpm (product loaded)	Dpm (H_2O wash)	Dpm (methanol eluate)	% Recovery
1	743 240	20 100 (2.7%)	705 550 (94.9%)	97.6
2	531 570	41 050 (7.7%)	490 280 (92.2%)	99.9
3	323 740	121 840 (37.6%)	176 320 (54.5%)	92.1
4	352 020	188 920 (53.7%)	197 750 (56.2%)	109.0



1: R= $-(CH_2)_8COOMe$







[a] The products of incubations carried out for 3 h as described for acceptor evaluations in the experimental procedures were isolated on Sep-Pak cartridges, evaporated, dissolved in 5 ml water and loaded on a Sep-Pak cartridge.

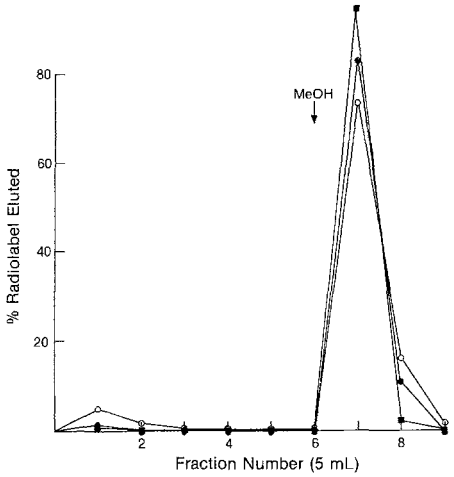
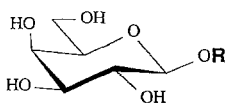
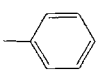


Figure 2. Elution profiles of radiolabelled reaction products of α(1-2)fucosyltransferase on Sep-Pak C-18 cartridges. The methanol eluents from Sep-Paks of preparative incubations of **5** (■), **6** (●) and **7** (○) were evaporated to dryness, dissolved in 5 ml water and reapplied to Sep-Pak cartridges. The cartridges were washed with water, then methanol; 5 ml fractions were collected and counted. Recovery is expressed as a percentage of the amount loaded on the cartridge.

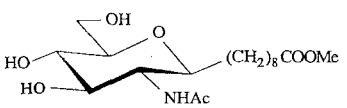
Table 2. Evaluation of the suitability of hydrophobic glycosides as acceptors in the Sep-Pak assay of α(1-2)fucosyltransferase.[a]

Acceptor	Dpm (product loaded)	Dpm (H_2O wash)	Dpm (methanol eluate)	% Recovery
5	21 030	570 (2.7%)	20 430 (96.4%)	99.7
6	13 460	500 (3.7%)	12 980 (96.4%)	100.1
7	36 320	3 250 (8.96%)	33 010 (90.9%)	99.9



5: R= 

6: R= $-(CH_2)_8COOMe$

7: R=


[a] The products of incubations carried out for 6 to 20 h as described for acceptor evaluations in the experimental procedures were isolated on Sep-Pak cartridges, evaporated, dissolved in 5 ml water and loaded on a Sep-Pak cartridge.

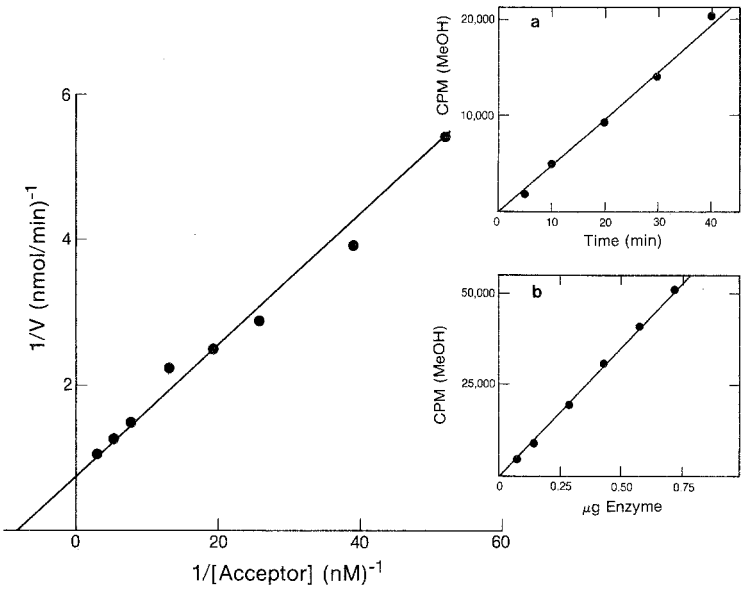


Figure 3. Double reciprocal plot for **1** as a substrate for β(1-4)galactosyltransferase. Incubations contained 20-308 nmol **1**, 0.18 μg of enzyme, 100 nmol UDP-Gal, 0.4 μCi UDP-[^{3}H]Gal and coupling components in a total volume of 1.07 ml, as described in the methods section. Reaction rates were determined by measuring radiolabelled product formation for 14 min at 37°C. The kinetic parameters V_{max} and K_M obtained using the Wilkinson method [17] were 1.38 ± 0.089 nmol/min and 134 ± 17 μM, respectively.

A. Effect of incubation time on β(1-4)galactosyltransferase activity. Incubations contained 206 nmol **1**, 0.12 μg enzyme, 100 nmol UDP-Gal, 0.37 μCi UDP-[^{3}H]Gal and coupling components in a total volume of 1.07 ml. The products from reactions for the indicated period of time were isolated on Sep-Pak cartridges, eluted with methanol and counted.

B. Effect of enzyme protein concentration on β(1-4)galactosyltransferase activity. Incubations contained 206 nmol **1**, 100 nmol UDP-Gal, 0.5 μCi UDP-[^{3}H]Gal, coupling components and enzyme in a total volume of 1.07 ml. The products from incubations at 37°C for 10 min for the indicated concentrations of enzyme were isolated on Sep-Pak cartridges, eluted with methanol and counted.

Results and Discussion

In principle, any structure that is an acceptor for a glycosyltransferase is useful in the Sep-Pak assay, provided that the glycosylated product is partially adsorbed on to the reverse-phase matrix under conditions where the labelled donor and degradation products are eluted. In order to obtain meaningful and reproducible kinetic parameters, however, we operationally define a "suitable acceptor" as one where over 90% of the product remains adsorbed to the Sep-Pak (and is later eluted with methanol) during a water wash sufficient to achieve an eluate radioactivity indistinguishable from background, usually 15 to 25 ml.

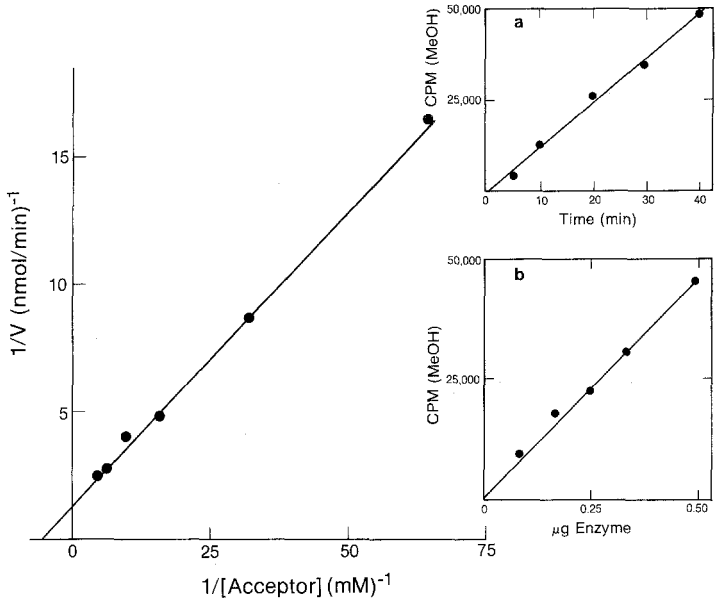


Figure 4. Double reciprocal plot for **2** as a substrate for β(1-4)galactosyltransferase. Incubations contained 17-250 nmol **2**, 0.21 μg of enzyme, 100 nmol UDP-Gal, 0.56 μCi UDP-[^{3}H]Gal and coupling components in a total volume of 1.07 ml, as described in the Methods section. Reaction rates were determined by measuring radiolabelled product formation for 10 min at 37°C. The kinetic parameters V_{max} and K_M obtained using the Wilkinson method [17] were 0.83 ± 0.12 nmol/min and 168 ± 24 μM, respectively.

A. Effect of incubation time on β(1-4)galactosyltransferase activity. Incubations contained 239 nmol **2**, 0.21 μg of enzyme, 100 nmol UDP-Gal, 0.57 μCi UDP-[^{3}H]Gal and coupling components in a total volume of 1.07 ml. The products from the reaction for the indicated period of time were isolated on Sep-Pak cartridges, eluted with methanol and counted.

B. Effect of enzyme protein concentration on β(1-4)galactosyltransferase activity. Incubations contained 239 nmol **2**, 100 nmol UDP-Gal, 0.57 μCi UDP-[^{3}H]Gal, coupling components and enzyme in a total volume of 1.07 ml. The products from incubations at 37°C for 12 min for the indicated concentrations of enzyme were isolated on Sep-Pak cartridges, eluted with methanol and counted.

To assess the suitability of a potential acceptor, a "preparative-scale" synthesis of the product is first carried out, using an available source of enzyme where the product is isolated using the routine Sep-Pak procedure. After evaporation of the methanol from the eluate, the labelled product is redissolved in water and re-isolated on a Sep-Pak cartridge to determine whether it satisfies the suitability criteria noted above. The results of such evaluation for the four hydrohobic 2-acetamido-2-deoxy-β-D-glucopyranosides **1-4** as acceptors for β(1-4)galactosyltransferase are summarized in Fig. 1 and Table 1. The conclusion from these data is that only the 8-methoxycarbonyloctyl and 4-methylumbelliferyl acceptors **1** and **2** are satisfactory, while the phenyl and *p*-nitrophenyl glycosides **3** and **4** are not suitable as acceptors for quantifying enzyme activity. These results contrast with the similar evaluation of the phenyl and 8-methoxylcarbonyloctyl galactopyranosides (**5** and **6**), as acceptors for a crude α(1-2)fucosyltransferase extract from porcine submaxillary glands, where both products are sufficiently hydrophobic

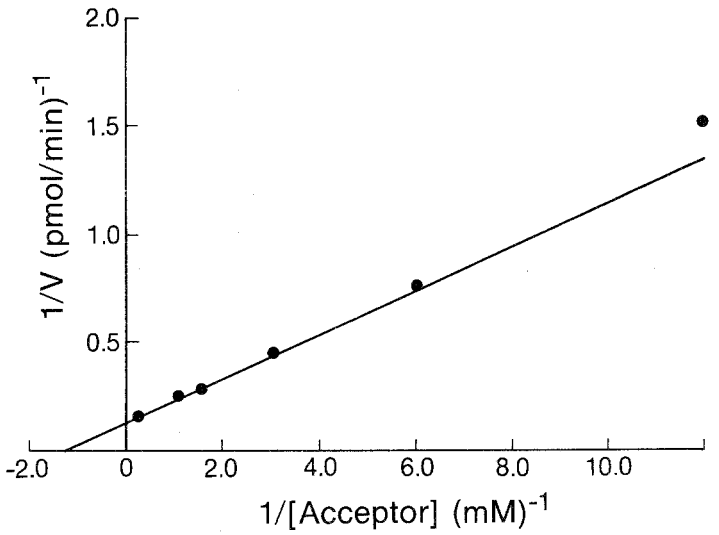


Figure 5. Double reciprocal plot for **5** as an acceptor for α(1-2)fucosyltransferase. Incubations contained in 0.62 ml: 0.39-7.4 μmol of **5**, 9 nmol GDP-Fuc, 0.01 μCi GDP-[^{14}C]Fuc, an extract of fucosyltransferase containing 14 mg protein and buffer components as described in the Methods section. Reaction rates were determined by measuring radiolabelled product formation for 1 h at 37°C. The kinetic parameters V_{max} and K_M obtained by using the Wilkinson method [17] were 2.6 ± 0.1 pmol/min and 4.4 ± 0.4 mM respectively.

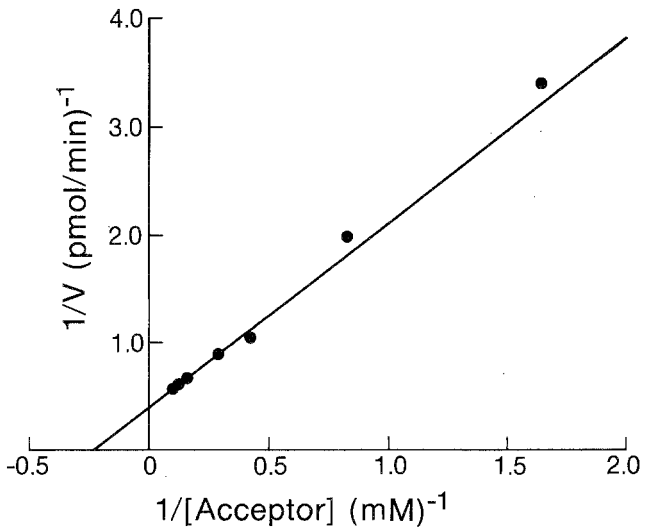


Figure 6. Double reciprocal plot for **6** as an acceptor for α(1-2)fucosyltransferase. Incubations contained in 0.62 ml: 50-360 nmol of **6**, 9 nmol GDP-Fuc, 0.01 μCi GDP-[^{14}C]Fuc, an extract of fucosyltransferase containing 14 mg of protein and buffer components as described in the Methods section. Reaction rates were determined by measuring radiolabelled product formation for 1 h at 37°C. The kinetic parameters V_{max} and K_M obtained by using the Wilkinson method [17] were 8.0 ± 0.1 pmol/min and 0.82 ± 0.03 mM, respectively.

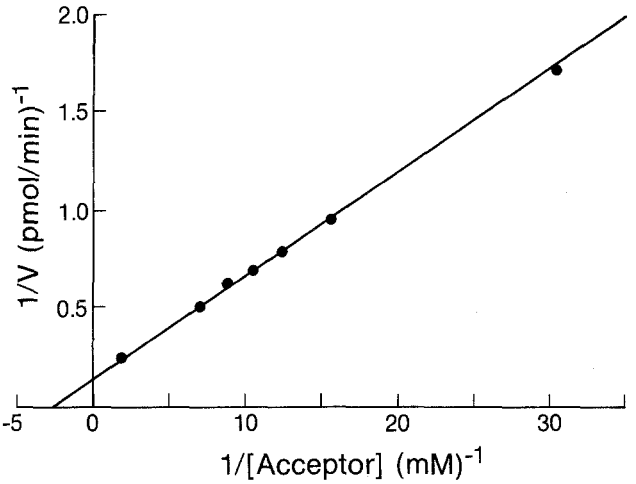


Figure 7. Double reciprocal plot for **7** as an acceptor for α(1-2)fucosyltransferase. Incubations contained in 0.62 ml: 20-360 nmol of **7**, 9 nmol GDP-Fuc, 0.01 μCi GDP-[^{14}C]Fuc, an extract of fucosyltransferase containing 14 mg of protein and buffer components as described in the Methods section. Reaction rates were determined by measuring radiolabelled product formation for 1 h at 37°C. The kinetic parameters V_{max} and K_M obtained by using the Wilkinson method [17] were 7.2 ± 0.1 pmol/min and 0.509 ± 0.008 mM respectively.

(Fig. 2 and Table 2). In this instance, the addition of the fucose residue appears to increase substantially the hydrophobicity of phenyl-β-D-galactopyranoside which was itself only poorly adsorbed during the Sep-Pak procedure, when the water wash was monitored spectrophotometrically or by the phenol-sulfuric acid assay [18]. The disaccharide Galβ1-3GlcNAc-O$(CH_2)_8$COOMe (**7**) was also evaluated and, as expected, found to be satisfactory as an acceptor (Fig. 2 and Table 2).

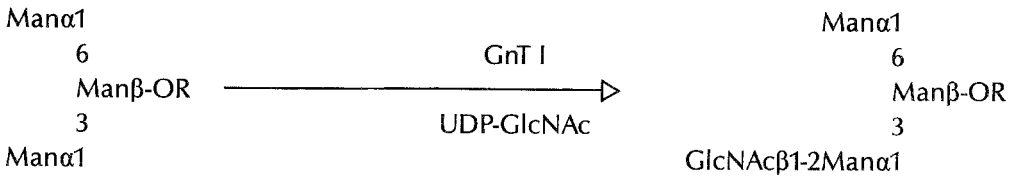
The suitability of acceptors **1** and **2** for the estimation of standard kinetic parameters for purified bovine milk β(1-4)galactosyltransferase, using the Sep-Pak assay is demonstrated in Figs. 3 and 4. The formation of product was linear with both enzyme concentration and time (Figs. 3 and 4, insets A and B). The kinetic parameters V_{max} and K_M obtained with the Wilkinson method [17] for these substrates were 7.7 ± 0.5 μmol/min/mg and 134 ± 17μM for **1**, and 4.0 ± 0.6 μmol/min/mg and 168 ± 24 μM for **2**. Substrates **1** and **2** were evaluated independently using the spectrophotometric method of Fitzgerald *et al.* [16]. The V_{max} and K_M values obtained for these substrates were 6.8 ± 0.5 μmol/min/mg and 118 ± 20 μM for **1** and 6.3 ± 0.8 μmol/min/mg and 163 ± 32 μM for **2**. The kinetic parameters obtained by these two independent assays agree reasonably well, and the K_M for **2** agrees with 220 μM reported previously [19].

The suitability of the Sep-Pak assay for detecting fucosyltransferase activity present in crude extracts from pig submaxillary glands is demonstrated in Figs. 5-7, where V_{max} and K_M are given for compounds **5-7**. The enzyme activities detected with these substrates were again linear with increasing protein concentration and product formation increased linearly with time (data not shown). The products were not structurally characterized but since porcine submaxillary glands are a well known source of α(1-2)fucosyltransferase, this is undoubtedly the activity being detected with these synthetic substrates.

Both phenyl-β-D-galactopyranoside **5** and the disaccharide sequence Galβ1-3GlcNAc **7** are known to be substrates for this enzyme [3].

The *N*-acetylglucosaminyltransferases that control the branching of asparagine-linked oligosaccharides all transfer *N*-acetyl-β-D-glucosamine from UDP-GlcNAc to D-mannose residues of natural glycopeptide acceptors that are at least the size of a hexasaccharide [2]. The finding [20] that much smaller synthetic oligosaccharides can sometimes also act as acceptors makes these enzymes particularly good targets for which to prepare hydrophobic acceptor glycosides. While the heptasaccharide structure **8** is the natural acceptor structure for GnT I, the trisaccharide **9** was shown to be a specific though poor acceptor for the bovine colostrum enzyme [2, 20].

8



9 R = $CH(CH_3)_2$

10 R = $(CH_2)_8COOMe$ **11** R = $(CH_2)_8COOMe$

We have found that the simple synthetic 8-methoxycarbonyloctyl-trimannoside **10**, readily prepared in multigram-quantity [Alton G, Newton EM, Srivastava V, Palcic MM, Hindsgaul O; unpublished results] is an excellent acceptor of GnT I whose activity was detected in Triton X-100 extracts of rabbit-liver acetone powder (Fig. 8). The product of the enzymic reaction has been isolated and shown, by ^{1}H-NMR, to have the expected structure **11**, demonstrating the specificity of the acceptor in detecting GnT I in the presence of other GnT's known to be present in rabbit liver.

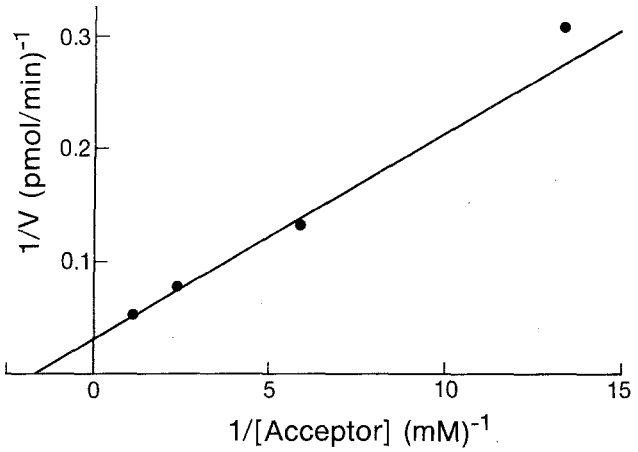


Figure 8. Double reciprocal plot for **10** as an acceptor for GnT I. Incubations contained in 1.5 ml: 0.11-1.4 μmol **10**, 21 nmol UDP-GlcNAc, 0.04 μCi UDP-[^{14}C]GlcNAc, an extract of GnT I contaiing 3.7 mg of protein and detergent and buffer components as described in the methods section. Reaction rates were determined by measuring radiolabelled product formation for 1 h at 37°C. The kinetic parameters V_{max} and K_M obtained by using the Wilkinson method [17] were 32 ± 2 pmol/min and 0.59 ± 0.07 mM, respectively.

The heptasaccharide structure **12** is the smallest natural acceptor for GnT V [2, 5, 21] which allows its elongation to **13**.

GlcNAcβ1-2Manα1
6
Manβ1-4GlcNAcβ1-4GlcNAc **12**
3
GlcNAcβ1-2Manα1

GnT V ↓ UDP-GlcNAc

GlcNAcβ1
6
GlcNAcβ1-2Manα1
6
Manβ1-4GlcNAcβ1-4GlcNAc **13**
3
GlcNAcβ1-2Manα1

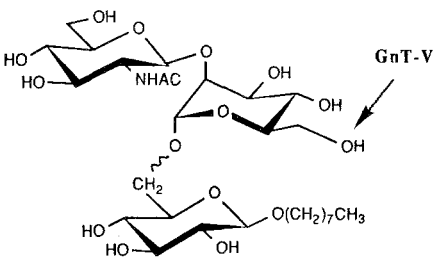
We have previously reported that the simple trisaccharide **14** [22] is also a good substrate for GnT V which catalyzes it conversion to **15** [12].

GlcNAcβ1-2Manα1-6Manβ-O$(CH_2)_8$COOMe **14**

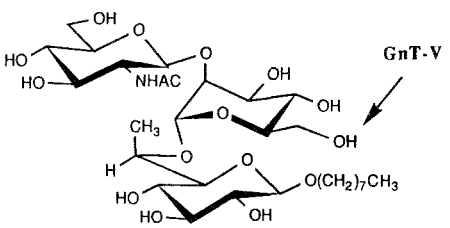
GnT V ↓ UDP-GlcNAc

GlcNAcβ1
6
GlcNAcβ1-2Manα1-6Manβ-O$(CH_2)_8$COOMe **15**

In an ongoing project aimed at delineating the molecular specificity of the combining site of GnT V we have chemically synthesized a large number of analogs of **14** [11]. These analogs were, for synthetic convenience, prepared as *n*-octyl glycosides after verifying that octyl glucoside (*n*-octyl β-D-glucopyranoside) was quantitatively adsorbed on C-18 Sep-Paks from aqueous solution. Both the *n*-octyl-linked trisaccharide **16** and its (*R*)-6-methyl derivative **17** were found to be acceptors for GnT V whose activity was detected in Triton X-100 extracts of hamster kidney. Compound **17** whose conformation about the hydroxymethyl group of the β-D-glucose residue is biased towards the gt rotamer, was found to be more than twice as active as an acceptor for GnT V as the conformationally more flexible **16** (Figs. 9, 10).



16



17

In the foregoing examples we have demonstrated that hydrophobic glycosides of appropriate carbohydrate structure are ideal as acceptors in the assay of glycosyltransferases by virtue of their unique chromatographic behaviour on reverse-phase C-18 supports. The hydrophobic aglycones confer glycolipid-like properties to the acceptors and products. Sep-Pak C-18 cartridges have previously been used in assaying glycosyltransferases which act on isolated glycolipids [23]. The examples presented here demonstrate that eight carbons in the aglycone appear to be sufficient to allow a quantitative enzyme assay. The 4-methylumbelliferyl aglycone is acceptable in the Sep-Pak assay in the case of β(1-4)galactosyltransferase, while a simple phenyl group is

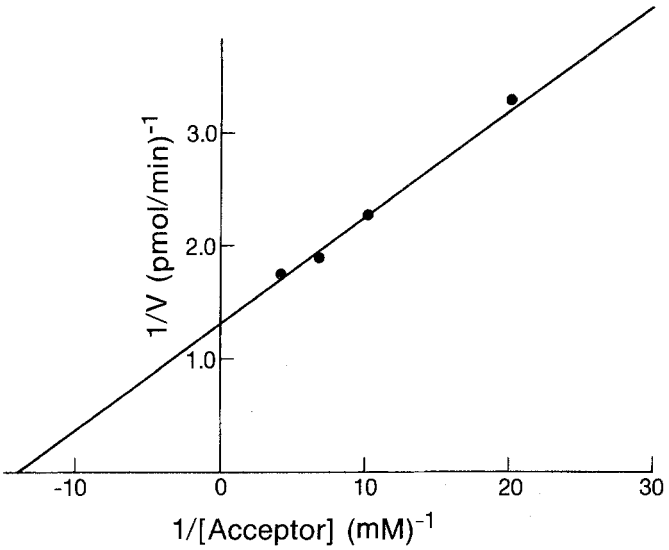


Figure 9. Double reciprocal plot for **16** as a substrate for GnT V. Incubations contained in 0.02 ml: 1-5 nmol **16**, 100 nmol UDP-GlcNAc, 10^6 cpm UDP-[^{3}H]GlcNAc (10 cpm/pmol), 40 nmol ADP, 100 nmol *N*-acetyl-β-D-glucopyranosylamine (a hexosaminidase inhibitor [22]), 0.5% TritonB X-100, 2 μmol MES pH 6.0, 400 nmol EDTA and an extract of GnT V containing 90 μg protein. Reaction rates were determined by measuring radiolabelled product formation for 1 h at 37°C. The kinetic parameters V_{max} and K_M obtained using the Wilkinson method [17] were 0.77 ± 0.04 pmol/min and 72 ± 11 μM, respectively.

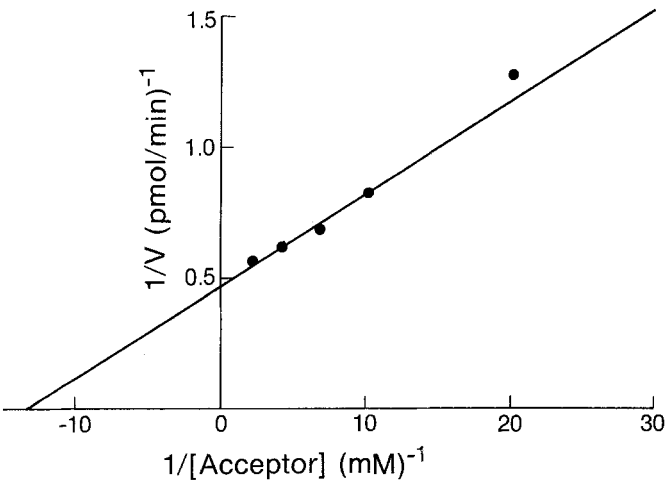


Figure 10. Double reciprocal plot for **17** as a substrate for GnT V. Incubations contained in 0.02 ml: 1-5 nmol **17**, 100 nmol UDP-GlcNAc, 10^6 cpm UDP-[^{3}H]GlcNAc, (10 cpm/pmol), 40 nmol ADP, 100 nmol *N*-acetyl-β-D-glucopyranosylamine (a hexosaminidase inhibitor), 0.5% Triton X-100, 2 μmol MES pH 6.0, 400 nmol EDTA and an extract of GnT V containing 90 μg protein. Reaction rates were determined by measuring radiolabelled product formation for 3 h at 37°C. The kinetic parameters V_{max} and K_M obtained using the Wilkinson method [17] are 2.13 ± 0.08 pmol/min and 75 ± 9 μM, respectively.

borderline, being sufficient in some instances but not in others. The success of the type of Sep-Pak assay presented here requires each new acceptor to be evaluated with respect to the hydrophobicity of the radiolabelled product, as we have described above. The increasing commercial availability of synthetic oligosaccharides, many of which are already attached to hydrophobic aglycones, places the scope of the Sep-Pak assays beyond the synthetic chemistry laboratories.

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