

## Mucin Biosynthesis Revisited

THE ENZYMATIC TRANSFER OF Gal IN  $\beta$ 1,3 LINKAGE TO THE GalNAc MOIETY OF THE CORE STRUCTURE  $R_1$ -GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O- $R_2$ \*

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Synthetic glycosides containing the core, -GlcNAc $\beta$ 1,6GalNAc $\alpha$ -, acted as acceptors for  $\beta$ -galactosyltransferase of human ovarian tumor. A significant amount of Gal was transferred from UDP-Gal (100 nmol) to the  $\alpha$ -benzylglycoside of LacNAc $\beta$ 1,6GalNAc (LGBn) (25.1 nmol of Gal) and the  $\alpha$ -ortho-nitrophenylglycosides of LacNAc $\beta$ 1,6GalNAc (22.0 nmol of Gal), GlcNAc $\beta$ 1,6GalNAc (15.5 nmol of Gal), and Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc (25.9 nmol of Gal); LacNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn (where Bn is benzyl) was almost inactive (only 1.2 nmol of Gal), indicating the Gal transfer to the  $\alpha$ -GalNAc moiety. The product from LGBn was isolated in microgram quantities and identified by fast atom bombardment mass spectrometry as LacNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn. The  $\alpha$ GalNAc: $\beta$ 1,3Gal transferase was present in high concentration in ovarian tumor tissue (ovarian cancer serum  $\rightarrow$  1.4; ascitic fluid  $\rightarrow$  0.9; tumor  $\rightarrow$  17.4). Asialo Cowper's gland mucin (ACGM) at 5 mg/ml reaction mixture inhibited the transfer of Gal to LGBn (25.2 and 53.4% respectively for 2 and 18 h incubation at 37 °C); inhibition by LGBn was 13.4 and 24.5%, respectively. In contrast to the inhibition by ACGM (25.2–31.6%), there was substantial increase (13.4–35.7%) in the inhibition by LGBn, when the incubation for 2 h at 37 °C was continued for 40 h at 4 °C, indicating the high affinity of LGBn for the enzyme at lower temp.  $K_m$  for LGBn in presence of ACGM was 7.6 mM and in absence, 2.7 mM;  $K_m$  for ACGM ( $M_r$  200,000) in presence of LGBn was 16.1  $\mu$ M and  $K_i$  for ACGM (as the inhibitor) was 41.7  $\mu$ M. In comparison with two normal ovarian tissues, the enzyme was found to be low (55–67%) in three ovarian tumors and high (146–260%) in two ovarian and one uterus tumors, as measured with ACGM; the synthetic acceptors showed similar activities. The enzyme had nearly the same extent of activity in the pH range 6–8. Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP had the highest affinity for the enzyme. The present study demonstrates the feasibility of  $\beta$ 1,3Gal attachment on  $\alpha$ GalNAc, which has already been substituted by  $\beta$ 1,6GlcNAc, then elongated by  $\beta$ 1,4Gal and also terminated by  $\alpha$ 1,3Fuc.

GlcNAc $\beta$ 1,3(GlcNAc $\beta$ 1,6)GalNAc requires substitution on the C-3 hydroxyl of GalNAc prior to substitution on the C-6 hydroxyl (1). Although the linear sugar chain, GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Ser (Thr), has been detected in the mucous glycoproteins of human seminal plasma (2), human meconium glycoproteins (3), and human K-casein (4), there is no information available concerning the activity of  $\beta$ 1,3 Gal-transferase (Gal to  $\alpha$ -GalNAc) toward the above linear structure (1). The formation of [ $^{14}$ C]GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn<sup>1</sup> was noticed by Williams *et al.* (1) when canine submaxillary gland microsomes were incubated with Gal $\beta$ 1,3GalNAc $\alpha$ -O-Bn and UDP-[ $^{14}$ C]GlcNAc; this disaccharide was assumed to arise from the action of  $\beta$ -galactosidase on the core trisaccharide. When we examined the ovarian tumor microsomal preparation for GlcNAc transferase activity with the acceptor GalNAc $\alpha$ -O-Bn, one major and one minor radioactive product corresponding, respectively, to authentic GlcNAc $\beta$ 1,3GalNAc $\alpha$ -O-Bn and GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn were identified after paper chromatography (5). In view of the fact that the biosynthetic pathways, whereby the carbohydrate moieties of glycoproteins are formed, are determined by the substrate specificities of glycosyltransferases, the present investigation was undertaken to look at the activity of  $\beta$ -galactosyltransferase on the disaccharide core, -GlcNAc $\beta$ 1,6GalNAc $\alpha$ -. We report that human ovarian tumor extract contains  $\beta$ -galactosyltransferase which galactosylates the above structure, and the linkage of Gal has been established as  $\beta$ 1,3 to GalNAc by FAB mass spectrometry on the product isolated from the acceptor, LGBn.

### EXPERIMENTAL PROCEDURES

Sera and ascitic fluid were collected from ovarian cancer patients admitted to Roswell Park Cancer Institute. Ovarian tumor tissues were obtained during surgical procedures from patients with ovarian cancer. Sera, ascitic fluid, and tissues were stored frozen at -70 °C until use.

**Assay of the Enzymatic Transfer of [ $^{14}$ C]Gal from UDP [ $^{14}$ C]Gal to the Synthetic Acceptors**—The incubation mixture in duplicate contained 100 mM MOPS, pH 6.3, 0.5% Triton X-100, 25 mM MnCl<sub>2</sub>, 1 mM UDP-Gal, 3 mM acceptor, 0.125  $\mu$ Ci of UDP-[U- $^{14}$ C]Gal (specific activity 301 mCi/mmol) and the enzyme in a total volume of 0.10 ml; the control incubation mixture contained everything except the acceptor. At the end of incubation at 37 °C for 20 h, the mixture was diluted with 1 ml of water and passed through Dowex-1-Cl column (1 ml in a Pasteur pipette). The column was washed twice with 1 ml of water; the breakthrough and wash, which contained the  $^{14}$ C-galactosylated product, were collected together in a scintillation vial and counted for radioactivity using the scintillation mixture 3a70 (Research Products International, Mount Prospect, IL) and Beckman

Studies on mucin biosynthesis have indicated that the synthesis of the cores Gal $\beta$ 1,3(GlcNAc $\beta$ 1,6)GalNAc and

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<sup>1</sup> The abbreviations used are: Bn, benzyl; ONP, o-nitrophenyl; MOPS, 4-morpholinepropanesulfonic acid; ACGM, asialo Cowper's gland mucin; Lac, lactose; Fuc, fucose; LGBn, LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn.

LS 9000. Corrections were made by subtracting the radioactivity of control incubation mixture.

GlcNAc: $\beta$ 1,3Gal transferase was assayed using the protocol as above, using GlcNAc (200 mM) as acceptor as described by Sheares and Carlson (6).

**Separation of  $^{14}$ C-Galactosylated Products Formed from the Acceptor Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn**—The incubation mixture (1.0 ml) contained 0.1 M MOPS, pH 6.3, 0.5% Triton X-100, 25 mM MnCl<sub>2</sub>, 1 mM UDP-Gal, 6 mM Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, 1.25  $\mu$ Ci of UDP-[U- $^{14}$ C]Gal (301 mCi/mmol), and 400  $\mu$ l of ovarian tumor 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction. After incubation at 37 °C for 20 h, the mixture was chromatographed on a Bio-Gel P2 column (1.0  $\times$  116.0 cm), equilibrated with 0.1 M pyridine acetate, pH 5.4. In order to obtain more information on the nature and size of the [ $^{14}$ C]Gal-containing products, a portion of the concentrated material from each of the two separated products was mixed with synthetic standards (1.5  $\mu$ m each), Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, Gal $\beta$ 1,4GlcNAc $\beta$ 1,6Gal $\beta$ 1,3GalNAc $\alpha$ -O-Bn, and Gal $\beta$ 1,4GlcNAc $\beta$ -O-Bn and then fractionated on the same Bio-Gel P2 column. Both the radioactivity and carbohydrate (anthrone reaction) in the fractions were monitored.

**Formation of  $^{14}$ C-Galactosylated Product from the Acceptor Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn in the Presence of Phenylthiogalactose, an Inhibitor of  $\beta$ -Galactosidase**—The incubation mixture was the same as described above except for the inclusion of phenylthiogalactose (10 mM).

**Large Scale Isolation of  $^{14}$ C-Galactosylated Products from LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, for Complete Characterization by FAB Mass Spectrometry**—The incubation mixture was scaled up proportionately to a total volume of 10 ml except for UDP-[ $^{14}$ C]Gal (7.5  $\mu$ Ci). After incubation for 20 h at 37 °C and then removing the insoluble material by centrifugation, the supernatant was passed through a column of Dowex-1-Cl (42 ml bed volume); the column was washed with water until the effluent showed no radioactivity. The pool of breakthrough solution and water wash was concentrated by flash-evaporation to dryness, dissolved in 2 ml of water, and then fractionated on the Bio-Gel P2 column (1.0  $\times$  116.0 cm). The separated [ $^{14}$ C]Gal-containing products were further purified by rechromatography on the same column.

The  $^{14}$ C-galactosylated products, when GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP, 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP were used as acceptors, were isolated using the same format as described above.

**Thin Layer Chromatographic (TLC) Identification of the  $^{14}$ C-Galactosylated Products Formed from the Acceptors Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP, and 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn**—The TLC was run in analytical silica gel glass plates with *n*-butanol:acetic acid:H<sub>2</sub>O (3:2:1) as the solvent system along with authentic standards. The standards were located on the plates by spraying with sulfuric acid in ethanol and then heating at 100 °C. The radioactive compound was located by scraping the silica gel from 1-cm-width segments into scintillation vials, soaking in 2 ml of water, and then liquid scintillation counting.

**Preparation of Asialo-CGM**—The translucent gel was collected from boar semen (transported on ice from Cornell University, Ithaca, NY), dissolved in 6 M guanidine HCl, and reduced and alkylated as described by Mendicino *et al.* (7). This preparation (in batches of 10 ml) was fractionated on Sepharose CL-6B (2.6  $\times$  95.0 cm) with 2 M

guanidine HCl in 20 mM Tris-HCl, pH 8.0, as the eluent. The fractions of the first eluting peak (from the sialic acid assay by the resorcinol method (8)) containing the CGM were pooled and dialyzed exhaustively against water at 4 °C. This dialyzed preparation was adjusted to 0.05 N HCl and heated for 1 h at 80 °C. After further dialysis against water for removing sialic acid, this sample was lyophilized. This asialo-CGM preparation contained in 1 mg, 0.24  $\mu$ mol galactose, 0.80  $\mu$ mol *N*-acetylgalactosamine, and 0.06  $\mu$ mol sialic acid. Galactose was estimated in asialo-CGM by galactose oxidase method (9), after 1 N HCl hydrolysis and the removal of the acid and galactosamine with Dowex-1-HCO<sub>3</sub> and Dowex-50H<sup>+</sup>, respectively. Galactosamine was estimated by the method of Ludowig and Benmaman (10) after 6 N HCl hydrolysis and removal of acid in a vacuum dessicator over NaOH pellets.

**Competition between LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn and Asialo-CGM as the Acceptors for  $\alpha$ -GalNAc: $\beta$ 1,3Gal Transferase**—A microsomal preparation from human ovarian tumor was used as the enzyme source. The tissue was homogenized in Brinkmann Polytron homogenizer with 5 volumes (w/v) of 50 mM Tris-HCl, pH 7.5, 0.25 M sucrose and centrifuged at 27,000  $\times$  g for 15 min. The microsomal pellet was obtained by centrifugation of the 27,000  $\times$  g supernatant at 100,000  $\times$  g for 2 h at 4 °C and then solubilized with 1% Triton X-100 in 50 mM Tris-HCl, pH 7.5, containing 10 mM MgCl<sub>2</sub>, 1 mM UMP, and 0.1 M NaCl (7).

The first incubation mixture (0.5 ml) contained MOPS, pH 6.3 (50  $\mu$ mol), MnCl<sub>2</sub> (2.5  $\mu$ mol), UDP-Gal (0.5  $\mu$ mol), NAD (2.5  $\mu$ mol), Galactono  $\gamma$ -lactone (5.0  $\mu$ mol), UDP-[ $^{14}$ C]Gal (0.625  $\mu$ Ci), asialo-CGM (2.5 mg), and 0.2 ml of the solubilized microsomal extract. The second incubation mixture contained LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (1.5  $\mu$ mol) instead of asialo-CGM. The third one contained both LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (1.5  $\mu$ mol) and asialo-CGM (2.5 mg). They were incubated at 37 °C for 22 h. Then each was subjected to fractionation on a Bio-Gel P2 column (1.0  $\times$  116.0 cm), equilibrated with 0.1 M pyridine acetate, pH 5.4. The fractions were monitored for radioactivity; fractions under each peak were pooled, and the radioactivity present in each peak was determined.

**Influence of ACGM on the  $K_m$  of LGBn**—Incorporation of [ $^{14}$ C]Gal into LGBn (varying concentration) was measured simultaneously both in the presence and absence of asialo-CGM (500  $\mu$ g) with ovarian tumor microsomal extract (200  $\mu$ g of protein) as the enzyme source under the standard incubation conditions using Sep-Pak C-18 cartridge (Waters Chromatography Division) procedure (11) as follows: The reaction mixture (100  $\mu$ l) after incubation at 37 °C for 18 h was diluted with 1.0 ml of water and applied to the Sep-Pak C-18 cartridge, which had been solvated by washing with 5 ml of methanol followed by 25 ml of water. After the sample entry, the cartridge bed was washed with 25 ml of water. The bound radioactive product was eluted with 5 ml of methanol and counted directly after mixing with 14 ml of 3a70 scintillation mixture.

**Determination of  $K_i$  for ACGM (Competitive Inhibitor) and Also  $K_m$  for ACGM (in Presence of LGBn)**—Enzyme incubation was done as above under standard conditions. Incorporation of [ $^{14}$ C]Gal into both ACGM (varying concentration) and LGBn (1.5 mM) was measured by first isolating a mixture of the  $^{14}$ C products from both acceptors free of UDP-[ $^{14}$ C]Gal by Dowex-1-Cl method. Prior to this experiment, we had established beyond any doubt that ACGM does not bind to Dowex-1-Cl and a complete recovery of ACGM in the water wash of Dowex-1-Cl (X-8 200–400 mesh) is obtained.

TABLE I  
Ovarian cancer  $\beta$ 1,3 galactosyltransferase activity

Source	Incorporation of [ $^{14}$ C]galactose into the acceptor		
	GlcNAc	LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn	Gal ↓ $\beta$ 1,3 LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn
	cpm $\times 10^{-4}$		
Ovarian cancer serum 1	1.71	0.21	ND <sup>a</sup>
Ovarian cancer serum 2	2.12	0.27	ND
Ovarian cancer patient ascitic fluid	3.01	0.13	ND
Ovarian tumor extract 80% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction:			
(a) No addition to reaction mix (RM)	2.11	2.61	0.38
(b) RM plus NAD (5 mM)		2.77	
(c) RM plus ATP (7 mM)		0	
(d) RM plus UDP-GlcNAc (1 mM)		2.43	

<sup>a</sup> ND, not determined.

TABLE II

[ $^{14}$ C]Galactosylation of synthetic glycosides containing the core structure, -GlcNAc $\beta$ 1,6GalNAc $\alpha$ -, by human ovarian tumor crude enzyme fraction

Synthetic glycoside	Incorporation of [ $^{14}$ C]Gal
	nmol/100 nm UDP-Gal
LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (3.0 mM)	25.1
LacNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn (3.0 mM)	1.2
GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP (3.0 mM)	15.5
LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP (3.0 mM)	22.0
4-Fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (1.5 mM)	2.9
Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP (3.0 mM)	25.9

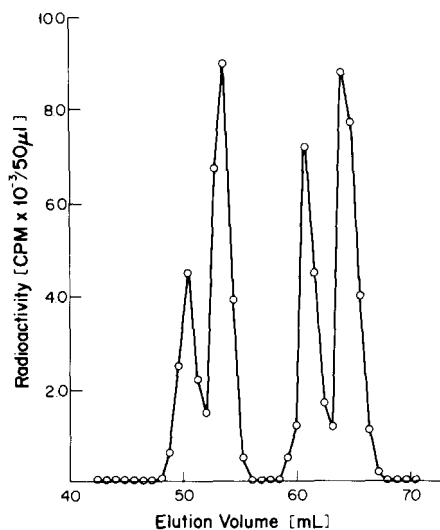


FIG. 1. Fractionation of [ $^{14}$ C]-galactosylated products formed from the acceptor LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn. The incubation mixture (1.0 ml) was chromatographed on a Bio-Gel P2 column (1.0  $\times$  116.0 cm) 200–400 mesh and eluted with 0.1 M pyridine acetate, pH 5.4; 50  $\mu$ l from fractions (0.9 ml) were subjected to radioactive scintillation counting.

After careful lyophilization to dryness, the samples were dissolved in 100  $\mu$ l of water; an aliquot (10  $\mu$ l) was applied as a 1-cm streak to analytical silica gel plates and the two [ $^{14}$ C] products were separated using the solvent system *n*-butanol:acetic acid:water (3:2:1) and quantitated by scraping off the gel from 0.5-cm-width segments into scintillation vials, soaking in 1 ml of water, and then liquid scintillation counting. The [ $^{14}$ C] product from ACGM remained at the origin, whereas that from LGBN moved 1.5 cm from the origin under the present experimental conditions.

**Affinity of ACGM and Synthetic Acceptors for Human Ovarian Tumor Microsomal  $\alpha$ -GalNAc: $\beta$ 1,3Gal Transferase**—The rate of [ $^{14}$ C]Gal incorporation into various acceptors was measured by quantitating the product formed at different incubation periods under the standard incubation conditions as above. The [ $^{14}$ C] product was quantitated by Dowex-1-Cl method.

**Influence of pH on the Activity of Ovarian Tumor  $\alpha$ -GalNAc: $\beta$ 1,3Gal Transferase**—Enzyme incubation was done in 50 mM Tris maleate buffer of the pH range 5.2–8.4 under the standard incubation conditions, and the incorporation of [ $^{14}$ C]Gal into the acceptors was determined by Dowex-1-Cl method.

**Analysis of the Relative Affinities of LGBN and ACGM for Human Ovarian Tumor Microsomal  $\alpha$ -GalNAc: $\beta$ 1,3Gal Transferase**—Enzyme incubation was done with LGBN (3 mM) and ACGM (500  $\mu$ g) alone and in presence of each other at four different incubation conditions: (i) 37  $^{\circ}$ C for 2 h, (ii) 37  $^{\circ}$ C for 2 h followed by 4  $^{\circ}$ C for 40 h, (iii) 37  $^{\circ}$ C for 18 h, (iv) 37  $^{\circ}$ C for 18 h followed by 4  $^{\circ}$ C for 40 h. Incorporation of [ $^{14}$ C]Gal into both ACGM and LGBN was measured by first isolating the [ $^{14}$ C] products by Dowex-1-Cl method followed by separation and quantitation of the two [ $^{14}$ C] products by thin layer chromatography as described above.

**The Level of  $\alpha$ -GalNAc: $\beta$ 1,3Gal Transferase in Normal Ovarian Tissues and Ovarian Tumors, as Measured with Various Acceptors**—

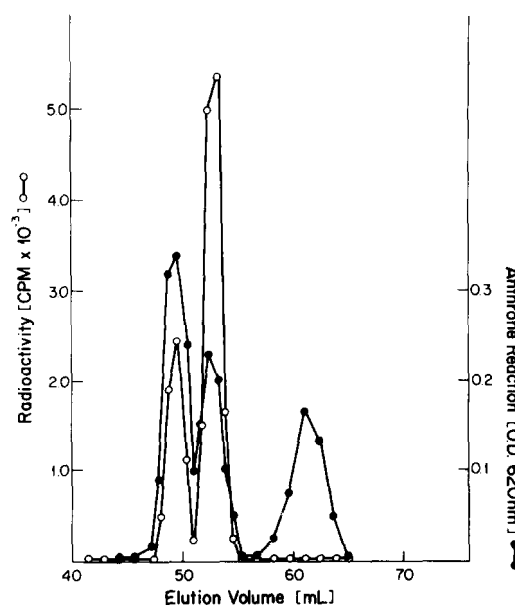


FIG. 2. Identification of [ $^{14}$ C]-galactosylated products by cochromatography with authentic synthetic compounds. A mixture of peaks A and B materials of Fig. 1 and authentic (1.5  $\mu$ m each) LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, LacNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn, and LacNAc $\beta$ -O-Bn (sample volume, 1.0 ml) was fractionated on Bio-Gel P2 column as described in the legend to Fig. 1. The elution of the standards was monitored by the anthrone reaction.

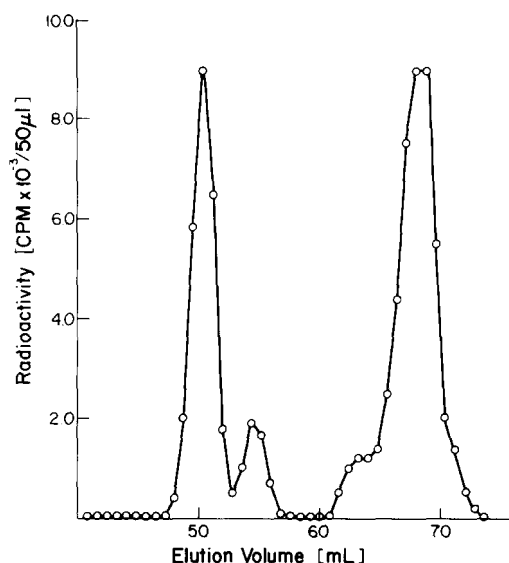


FIG. 3. Formation of [ $^{14}$ C]-galactosylated products from LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn in presence of an inhibitor of  $\beta$ -galactosidase, phenylthiogalactose. Fractionation as described in the legend to Fig. 1.

Six tumor specimens and two normal specimens were examined simultaneously for a strict comparison. Three specimens (from National Disease Research Interchange, Philadelphia) were transported on dry ice and then stored at  $-70^{\circ}$ C.

No. 2162—Normal ovarian tissue from 35-year-old patient, snap-frozen within 1 h after surgical removal.

No. 2509—Ovarian tumor from 59-year-old patient, snap-frozen within 1 h after surgical removal.

No. 2645—(a) Ovary (designated normal) and (b) uterus tumor (Leiomyoma) from 41-year-old patient; both were snap-frozen within 3 h after surgical removal.

The remaining four ovarian tumors (A.O., K.K., J.M., and L.M.) were respectively from 57-, 48-, 59- and 64-year-old patients of the Roswell Park Cancer Institute. After surgical removal, these tissues were stored frozen within 1 h at  $-70^{\circ}$ C.

Microsomes were prepared from 2645 a and b and A.O. as described

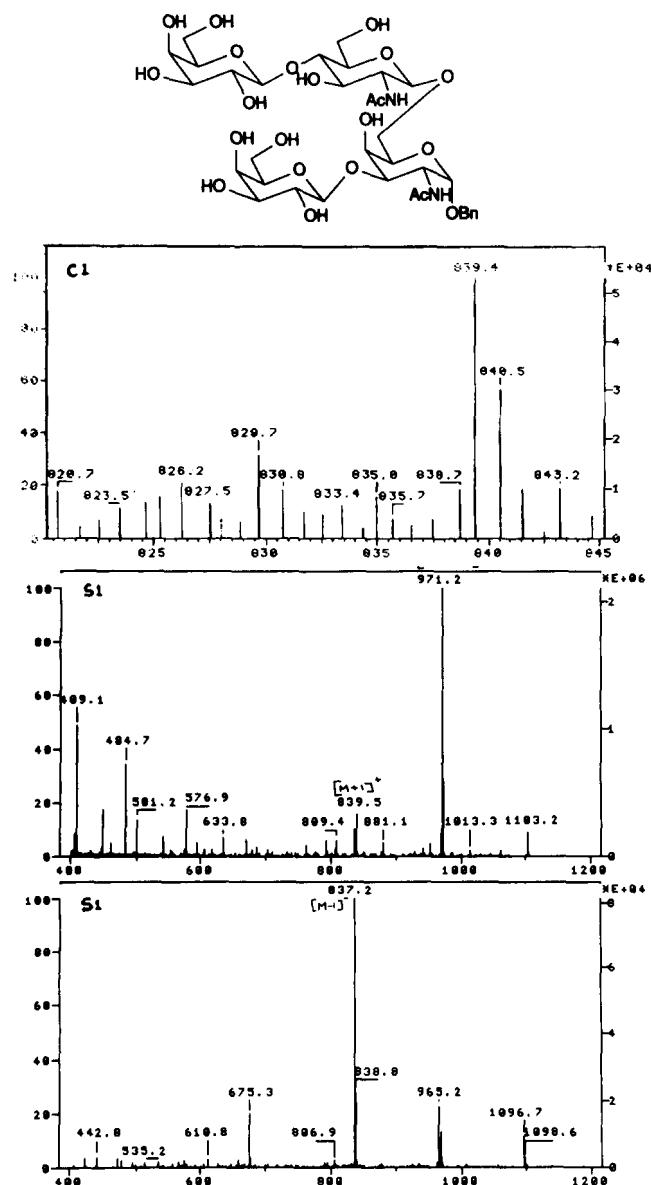


FIG. 4. FAB mass spectral analysis of the  $[^{14}\text{C}]$ Gal-containing tetrasaccharide product and the authentic synthetic compound LacNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn. C<sub>1</sub>,  $[^{14}\text{C}]$ galactose containing tetrasaccharide products from the acceptor LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn  $[M + 1]^+$ ; S<sub>1</sub> and S<sub>2</sub>, synthetic LacNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn  $[M + 1]^+$  and  $[M - 1]^-$ .

above. From other tissues, around 2 g (exact weight obtained) were homogenized with 10 volumes of 50 mM Tris-HCl, pH 7.0, 0.15 M NaCl, adjusted to 1% Triton X-100, and stirred for 4 h at 4 °C. These extracts were centrifuged at  $20,000 \times g$  for 1 h at 4 °C. The supernatants were collected, stored on ice, and the protein was measured by the BCA method (Pierce Chemical Co.). The protein concentration in the extracts was adjusted to exactly 4 mg/ml with the extraction buffer. The enzyme incubation was done under the standard incubation conditions using 20  $\mu$ l of each extract (80  $\mu$ g of protein). Incorporation of  $[^{14}\text{C}]$ Gal into various acceptors was measured by the Dowex-1-Cl method.

## RESULTS

**$\beta$ 1,3-Galactosyltransferase in Ovarian Cancer Sera, Ascitic Fluid, and Ovarian Tumor**—Human ovarian cancer sera and ascitic fluid as well as the soluble fraction of ovarian tumor were assayed for  $\beta$ 1,3-galactosyltransferase activity with GlcNAc and LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn as acceptors, and the results are presented in Table I. As compared with the  $\alpha$ -N-acetylgalactosaminide: $\beta$ 1,3-galactosyltransferase activity,

21		36		92		39	
82		3225		3023		3998	
4150		58		42		1286	
561		15		36		126	
80		6		20		31	
CPM		CPM		CPM		CPM	
A	STD	B	STD	C	STD	D	

FIG. 5. Thin layer chromatographic separation and identification of  $^{14}\text{C}$ -galactosylated products from various acceptors. For details see text. Lanes A and B, the products (Bio-Gel P2 Peaks A and B, respectively) from the acceptor LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn. STD, a mixture of LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, LacNAc $\beta$ 1,6(Gal $\beta$ 1,4)GalNAc $\alpha$ -O-Bn, and Gal $\beta$ 1,4GlcNAc $\beta$ -O-Bn. Lane C, the product from GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP. STD, GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP. Lane D, the product from 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn. STD, 4-fluoro GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn.

303		54
1404		57
114		1800
CPM		CPM
1	STD	2

FIG. 6. Thin layer chromatography of the product (Bio-Gel P2 Peak A) from LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn after treatment with galactosidases. Lane 1, product treated with  $\beta$ -galactosidase (*Aspergillus*). Lane 2, product treated with  $\alpha$ -galactosidase (coffee bean). STD, a mixture of LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, LacNAc $\beta$ 1,6(Gal $\beta$ 1,4)GalNAc $\alpha$ -O-Bn, and Gal $\beta$ 1,4GlcNAc $\beta$ -O-Bn.

the GlcNAc: $\beta$ 1,3-galactosyltransferase activity was 8-fold higher in ovarian cancer sera and >20-fold higher in ascitic fluid. On the other hand, the former activity was found to be slightly higher than the latter in ovarian tumor.

**Reactivity of Ovarian Tumor Galactosyltransferase with Various Acceptors**—Table II presents the data on the transfer of Gal by the ovarian tumor crude enzyme to the various  $\alpha$ -N-acetylgalactosaminides. Both LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn and Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn were very good acceptors followed by LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP and GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP; 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, when used at half-concentration due to its poor solubility, showed significant acceptor ability. As compared with LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, LacNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn did not serve as an acceptor; this also indicated that Gal is transferred to the  $\alpha$ -GalNAc moiety.

**Fractionation of  $^{14}\text{C}$ -Galactosylated Products**—The separation of the  $^{14}\text{C}$ -galactosylated products arising from the acceptor LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn on the Bio-Gel P2 column is

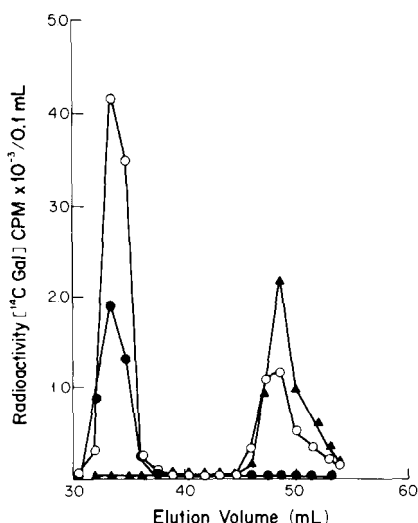


FIG. 7. Separation of the  $^{14}\text{C}$ -galactosylated products formed from the acceptors LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, asialo Cowper's gland mucin, and both acceptors together in the incubation mixture. A composite picture of the elution profiles is presented.  $\Delta$ , LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn as acceptor;  $\bullet$ , asialo-CGM as acceptor;  $\circ$ , both together as acceptors.

TABLE III

Competition between LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn and asialo-CGM as the acceptors for  $\alpha$ -GalNAc: $\beta$ 1,3Gal transferase

Acceptor in the incubation mixture	Incorporation of $^{14}\text{C}$ Gal into the acceptor <sup>a</sup>	
	cpm	nmol Gal
(a) LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (1.5 $\mu\text{mol}$ )	70,290	234
(b) Asialo-CGM (2.5 mg)	51,030	170
(c) A mixture of a and b		
LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (1.5 $\mu\text{mol}$ )	38,056	127
plus asialo-CGM (2.5 mg)	110,240 <sup>b</sup>	368
Inhibition of $^{14}\text{C}$ Gal incorporation into LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn	32,234	107 (45.7%)

<sup>a</sup> For details on separation and quantitation of  $^{14}\text{C}$  galactosylated compounds see Fig. 7.

<sup>b</sup> The increase in  $^{14}\text{C}$ Gal incorporation into ACGM was due to additional product formation, when reaction mixture c was left at 4 °C for 48 h before fractionation as compared with the fractionation of reaction mixture b immediately after incubation at 37 °C. For a detailed explanation, please refer to the experimental results reported in Table IV.

shown in Fig. 1. The first two radioactive peaks emerging from the column were the  $^{14}\text{C}$ -galactosylated products and the rest were UDP- $^{14}\text{C}$ Gal and its degradation products such as  $^{14}\text{C}$ Gal. The second peak was nearly twice as large as the first one, indicating the possible conversion of the product under peak I to the peak II compound by an endogenous  $\beta$ -galactosidase.

**Cochromatography of  $^{14}\text{C}$ -Galactosylated Products and Authentic Compounds**—Fig. 2 shows the rechromatography of peak I and peak II materials along with authentic synthetic compounds Gal $\beta$ 1,4GlcNAc $\beta$ -O-Bn, Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, and Gal $\beta$ 1,4GlcNAc $\beta$ 1,6(Gal $\beta$ 1,3) GalNAc $\alpha$ -O-Bn on the same Bio-Gel P2 column. The radioactive peaks I and II coincided, respectively, with the elution position of cold tetrasaccharide and trisaccharide benzylglycosides, as determined by the anthrone reaction.

**Formation of  $^{14}\text{C}$ -Galactosylated Product in Presence of  $\beta$ -Galactosidase Inhibitor**—When the enzymatic  $^{14}\text{C}$  galactosylation was done in the presence of phenylthiogalactose (an inhibitor of  $\beta$ -galactosidase), the amount of radioactive tetrasaccharide product (see Fig. 3, the first peak) far exceeded

the radioactive trisaccharide product, thus indicating the occurrence of inhibition in the  $\beta$ 1,4Gal hydrolysis, possibly from either the substrate or the radioactive tetrasaccharide product. Furthermore, a considerable qualitative difference in the degradation products from UDP- $^{14}\text{C}$ Gal can also be noticed, in the presence and absence of phenylthiogalactose in the incubation mixture.

**Characterization of  $^{14}\text{C}$ -Galactosylated Product by FAB Mass Spectrometry**—The  $^{14}\text{C}$ -galactosylated products from a large scale (100-fold) incubation mixture were recovered in the breakthrough fraction from Dowex-1-Cl column and followed by separation on Bio-Gel P2 column; the products were further purified by rechromatography of each peak material on the same column. From the specific radioactivity of the donor UDP- $^{14}\text{C}$ Gal in the incubation mixture, the amount of the  $^{14}\text{C}$ -galactosylated tetrasaccharide product was calculated as 134  $\mu\text{g}$ . The  $^{13}\text{C}$  NMR spectrum of the synthetic compound (12) used as reference showed four anomeric resonances at  $\delta$  105.73 (C-1 (Gal $\beta$ 1 $\rightarrow$ 4)), 104.32 (C-1 (GlcNAc $\beta$ 1 $\rightarrow$ 6)), 107.46 (C-1 (Gal $\beta$ 1 $\rightarrow$ 3)) and 99.18 (C-1 (GalNAc $\alpha$ -O-Bn)). The FAB mass spectrum of the synthetic compound (Fig. 4,  $S_1$  and  $S_2$ ) showed  $m/z$ : 839.5  $[M + 1]^+$  and 837.2  $[M - 1]^-$ , which was consistent with the structure assigned. It also gave an ion representing  $[M + \text{cesium}]^+$  at  $m/z$  971.2 and  $[M + \text{iodine}]^+$  at  $m/z$  965.2, as cesium iodide was used for mass calibration. A loss of hexose sugar (mass of 162) from the negatively charged molecular ion  $[M - 1]$  at  $m/z$  837.2 resulted in an ion at  $m/z$  675.3. The purified tetrasaccharide product gave  $m/z$ : 839.4  $[M + 1]^+$  (Fig. 4,  $C_1$ ), proving its identity with the synthetic reference compound.

**Identification of  $^{14}\text{C}$ -Galactosylated Products from Other Acceptors**—Fig. 5 illustrates the TLC identification of  $^{14}\text{C}$ -galactosylated products from Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP, and 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn. As expected, the  $^{14}\text{C}$ -galactosylated large size product (Bio-Gel P2 Peak A) from Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn moved to the region of the tetrasaccharide Gal $\beta$ 1,4GlcNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$ -O-Bn and the other found in the trisaccharide region (Fig. 5, lanes A and B, respectively). The  $^{14}\text{C}$ -galactosylated product from GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP and 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn showed lower mobility (Fig. 5, lanes C and D) as compared with the parent compounds and they were located in the trisaccharide glycoside region.

**Enzymatic Confirmation of the Linkage  $\beta$ 1,3Gal**—The  $^{14}\text{C}$ -galactosylated tetrasaccharide product from Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn was treated separately with  $\beta$ -galactosidase (*Aspergillus*, Calbiochem) and with  $\alpha$ -galactosidase (coffee bean) and then subjected to TLC. When digested with  $\beta$ -galactosidase, most of the radioactivity (85%) (see Fig. 6, lane 1) moved to the region of trisaccharide benzylglycoside. The ability of *Aspergillus*  $\beta$ -galactosidase to cleave  $\beta$ 1,4-linked Gal and its inability to hydrolyze  $\beta$ 1,3Gal were established by incubating for 24 h at 37 °C this enzyme plus  $\beta$ -N-acetylglucosaminidase (bovine kidney, Sigma) with Gal $\beta$ 1,4GlcNAc $\beta$ -O-ONP and Gal $\beta$ 1,3GlcNAc $\beta$ -O-ONP and then estimating the liberated nitrophenol.

When treated with  $\alpha$ -galactosidase, there was no degradation of the radioactive tetrasaccharide product (Fig. 6, lane 2). The TLC data establish the linkage of  $^{14}\text{C}$ Gal in the tetrasaccharide product as  $\beta$ 1,3.

**Competition of Acceptors**—The results obtained when human ovarian tumor microsomal preparation was incubated separately with LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn and asialo-CGM and both substrates together are reported in Fig. 7 and Table III. The elution profiles of the radioactive products from the

TABLE IV

Relative affinities of LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn and asialo-CGM for human ovarian tumor  $\alpha$ -GalNAc: $\beta$ 1,3Gal transferaseThe values in parentheses are the percent of the [ $^{14}$ C]Gal incorporation for 2-h incubation period at 37 °C.

Acceptor in the mixture	Incorporation of [ $^{14}$ C]Gal into the acceptor							
	Incubation at 37 °C for 2 h		Incubation at 37 °C for 2 h followed by incubation at 4 °C for 40 h		Incubation at 37 °C for 18 h		Incubation at 37 °C for 18 h followed by incubation at 4 °C for 40 h	
	LGBn	ACGM	LGBn	ACGM	LGBn	ACGM	LGBn	ACGM
	$\text{cpm} \times 10^{-4}$		$\text{cpm} \times 10^{-4}$		$\text{cpm} \times 10^{-4}$		$\text{cpm} \times 10^{-4}$	
(a) LGBn	1.59		3.32		6.95		7.13	
	(100.0)		(208.8)		(437.1)		(448.4)	
(b) ACGM		1.57		2.52		7.89		8.34
		(100.0)		(160.5)		(502.5)		(531.2)
(c) LGBn plus ACGM	1.19	1.36	2.27	1.62	3.24	5.96	3.06	5.40
	(100.0)	(100.0)	(190.8)	(119.1)	(272.3)	(438.2)	(257.1)	(397.1)
Inhibition (%) <sup>a</sup>	25.2	13.4	31.6	35.7	53.4	24.5	57.1	35.3

<sup>a</sup> The decrease in [ $^{14}$ C]Gal incorporation observed for each acceptor when both acceptors are present in the reaction mixture is expressed as the percent of the incorporation into the acceptor when incubated alone.

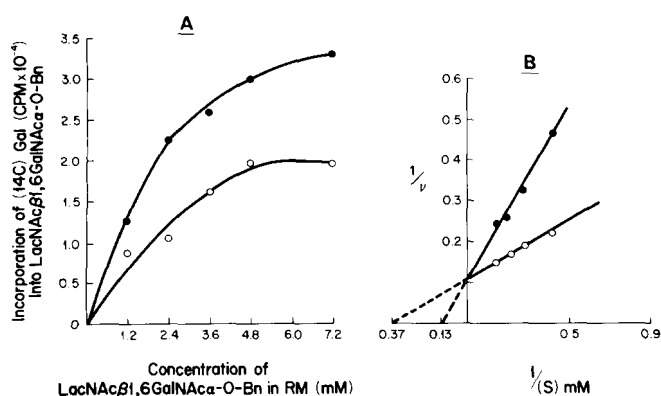


FIG. 8. Influence of asialo-CGM on the  $K_m$  of LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn. Enzyme activity with increasing concentration of LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn was determined both in the presence and absence of asialo-CGM (500  $\mu$ g) under the standard incubation conditions. A, the [ $^{14}$ C] product from LGBn was assayed by Sep-Pak C $_{18}$  cartridge method:  $\bullet$ , in the absence of ACGM;  $\circ$ , in the presence of ACGM. B, the  $K_m$  was determined by Lineweaver-Burk plot:  $\circ$ , in absence of asialo-CGM;  $\bullet$ , in the presence of asialo-CGM.

Bio-Gel P2 column are presented in Fig. 7. A comparison of these profiles illustrates an inhibition of [ $^{14}$ C]Gal incorporation into the synthetic substrate. The data presented in Table III showed that when asialo-CGM (2.5 mg) and LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (1.5  $\mu$ mol) were incubated together, Gal transfer to LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn was inhibited by 45.7%.

Table IV presents the data obtained by analyzing the relative affinities of LGBn and ACGM for human ovarian tumor microsomal  $\alpha$ -GalNAc: $\beta$ 1,3Gal transferase. When both acceptors were incubated together with the enzyme at 37 °C for 2 h, mutual inhibition between the acceptors was observed (LGBn, 25.2%; ACGM, 13.4%). When incubated at 37 °C for 2 h followed by incubation at 4 °C for 40 h, considerable increase in the formation of the [ $^{14}$ C] product was observed with both acceptors (LGBn, 108.8%; ACGM, 60.5%); the inhibition of the acceptor activity increased only slightly with LGBn (25.2–31.6%), whereas in case of ACGM, there was almost 3-fold increase in inhibition (13.4–35.7%). When the incubation was done at 37 °C for 18 h, as anticipated, more product was formed from both acceptors (an increase of 337.1% in case of LGBn and 402.5% with ACGM); a reversal of the mutual inhibition pattern was noticed (LGBn 53.4% and ACGM 24.5% as compared with 31.6 and 35.7%, respectively); additional incubation at 4 °C for 40 h did not bring

any further significant changes.

Fig. 8A shows the incorporation of [ $^{14}$ C]Gal into LGBn both in presence and absence of ACGM. A decrease in [ $^{14}$ C] incorporation is noticed in presence of ACGM. Lineweaver-Burk plot of the above data (Fig. 8B) shows that both curves intercept the y axis at the same point and the intercept on the x axis is considerably decreased in presence of ACGM due to the increase in slope of the curve.  $K_m$  for LGBn in the absence of ACGM was 2.7 mM and in the presence of ACGM, 7.69 mM.

Fig. 9 illustrates the acceptor activity of LGBn in presence of varying concentration of ACGM. A measurement of [ $^{14}$ C]Gal incorporation into both acceptors (Fig. 9A) indicates a gradual increase of [ $^{14}$ C]Gal incorporation with ACGM and a proportional decrease with LGBn. Lineweaver-Burk plot of the inhibitor (ACGM) concentration against the degree of inhibition (Fig. 9B) gave a value of  $K_i = 41.7 \mu$ M by assuming a minimum molecular weight of 200,000 for ACGM. From the Lineweaver-Burk plot of ACGM concentration against [ $^{14}$ C]Gal incorporated into ACGM (Fig. 9C),  $K_m$  for ACGM was found to be 16.1  $\mu$ M.

Fig. 10 illustrates the affinity of various acceptors for the ovarian tumor microsomal enzyme by measuring the [ $^{14}$ C] product formed at different time intervals of incubation. From the curves it is evident that the affinity of Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP as an acceptor for the microsomal enzyme far exceeds that of the other acceptors tested. The acceptor efficiency decreases in the following order: Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP, ACGM, LGBn, GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP, GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn, and 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn.

Fig. 11 shows the influence of pH on the activity of ovarian tumor microsomal  $\alpha$ -GalNAc: $\beta$ 1,3Gal transferase. This was studied with ACGM and LGBn as the acceptors. The enzyme appears to be equally active over a wide pH range 6–8 as observed with both acceptors.

The level of  $\alpha$ -GalNAc: $\beta$ 1,3Gal transferase was measured in two normal ovarian tissues and five ovarian tumors and one uterus tumor, and the results are presented in Table V. When the enzyme assay was done with ACGM as the acceptor, an increase of 160, 148, and 46%, respectively, in activity was seen with uterus tumor and the ovarian tumors J.M. and K.K., but a decrease of 45, 33, and 38% was found with the ovarian tumors A.O., No. 2509, and L.M., respectively. The results obtained with the acceptor LGBn were similar; 25, 106, and 48% increase with uterus tumor, J.M., and K.K. but a decrease of 22, 30, and 22% with A.O., No. 2509, and L.M.,

FIG. 9. The acceptor activity of LGBn in presence of increasing concentration of asialo CGM. Determination of  $K_i$  (asialo-CGM as the inhibitor) and  $K_m$  (asialo-CGM as the acceptor in presence of LGBn) were made. For details, see text. A:  $\bullet$ , incorporation of [ $^{14}$ C]Gal into LacNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn  $\circ$ , incorporation of [ $^{14}$ C]Gal into asialo-CGM. B, determination of  $K_i$  for asialo-CGM by Lineweaver-Burk plot of the inhibitor (asialo-CGM) concentration against the degree of inhibition of [ $^{14}$ C]Gal incorporation into LGBn. C, determination of  $K_m$  for ACGM in presence of LGBn in the incubation mixture.

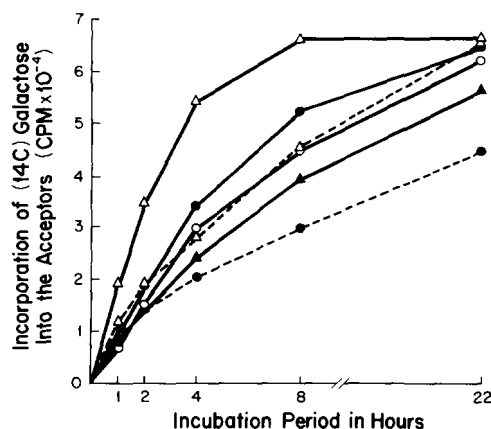
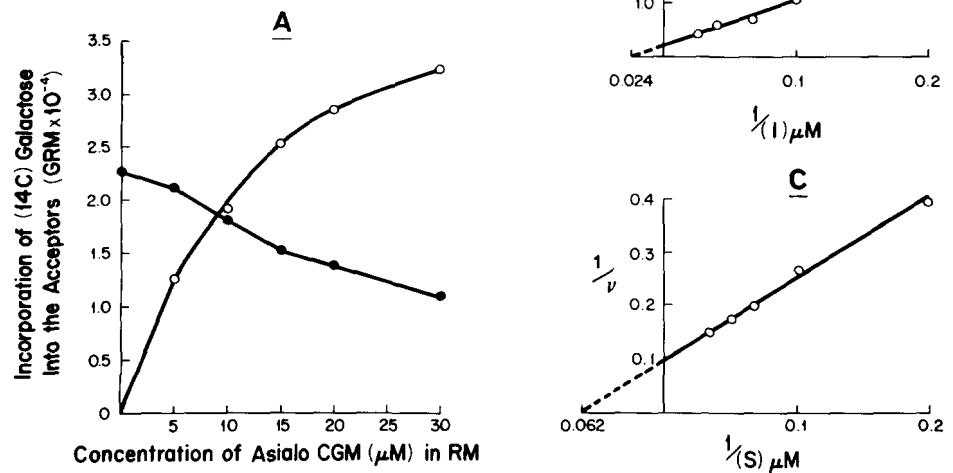


FIG. 10. The affinity of various acceptors for human ovarian tumor microsomal  $\beta$ 1,3Gal transferase. The rate of [ $^{14}$ C]Gal incorporation into various acceptors was measured by quantitating the product formed at different incubation periods under the standard incubation condition. The  $^{14}$ C product was quantitated by Dowex-1-Cl method.  $\circ$ — $\circ$ , Gal $\beta$ 1,4GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (3 mM);  $\bullet$ — $\bullet$ , asialo-CGM (500  $\mu$ g);  $\Delta$ — $\Delta$ , Fuc $\alpha$ 1,3GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (3 mM);  $\triangle$ — $\triangle$ , GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-ONP (3 mM);  $\blacktriangle$ — $\blacktriangle$ , GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (3 mM);  $\bullet$ — $\bullet$ , 4-fluoro-GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn (1.5 mM).

respectively. Other acceptors also showed a similar pattern of activity.

#### DISCUSSION

The present paper shows that  $\alpha$ GalNAc: $\beta$ 1,3Gal transferase of human ovarian tumor is capable of transferring Gal to the  $\alpha$ -GalNAc moiety of synthetic glycosides containing  $\beta$ 1,6GlcNAc-substituted  $\alpha$ -GalNAc. The inhibition of this Gal transfer by asialo-CGM, a macromolecular substrate for GalNAc $\alpha$ -O-Ser (Thr): $\beta$ 1,3Gal transferase, confirms the site of Gal transfer as the  $\alpha$ -GalNAc moiety. The present finding has underscored the previous notion, which is based on the GlcNAc transferase activities of pig and rat colon mucosa (11) that  $\beta$ 1,3 substitution on  $\alpha$ -GalNAc is probably not possible after the  $\beta$ 1,6 substitution. The synthesis and utili-

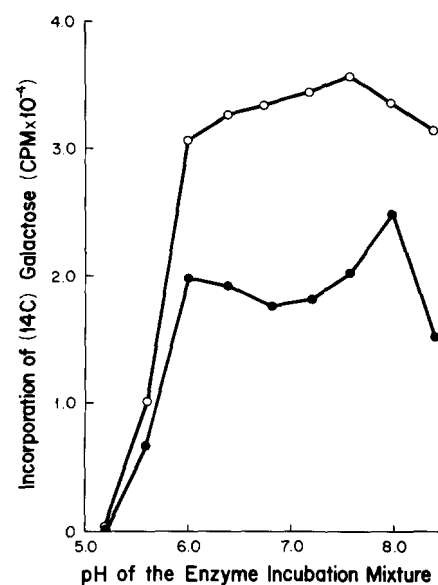


FIG. 11. Influence of pH on the activity of ovarian tumor  $\beta$ 1,3Gal transferase. Enzyme incubation was done in Tris maleate buffer of the pH range 5.2–8.4 under the standard incubation conditions and the incorporation of [ $^{14}$ C]galactose into the acceptors was determined by Dowex-1-Cl method.  $\circ$ , ACGM;  $\bullet$ , LGBn.

zation of glycosides containing  $\beta$ 1,6GlcNAc substituted  $\alpha$ -GalNAc has refined our knowledge in this important area, demonstrating the possibility of  $\beta$ 1,3Gal formation after the introduction of  $\beta$ 1,6GlcNAc on  $\alpha$ -GalNAc. We showed earlier that human ovarian tissue catalyzed the formation of  $\beta$ 1,3GlcNAc link on  $\alpha$ -GalNAc, which had already been substituted by  $\beta$ 1,6GlcNAc (5). The additional information, which is an outcome of the present study, is crucial for understanding the differential expression of glycoconjugate antigens on the cell surface during malignancy; in this context, the ability of this Gal transferase to act on  $\alpha$ -GalNAc moiety flanked by a nongrowing chain Fuc $\alpha$ 1,3GlcNAc is also noteworthy. Earlier, we showed the presence of both  $\beta$ 1,3 and  $\beta$ 1,6 GlcNAc-transferases in human ovarian tissue using the

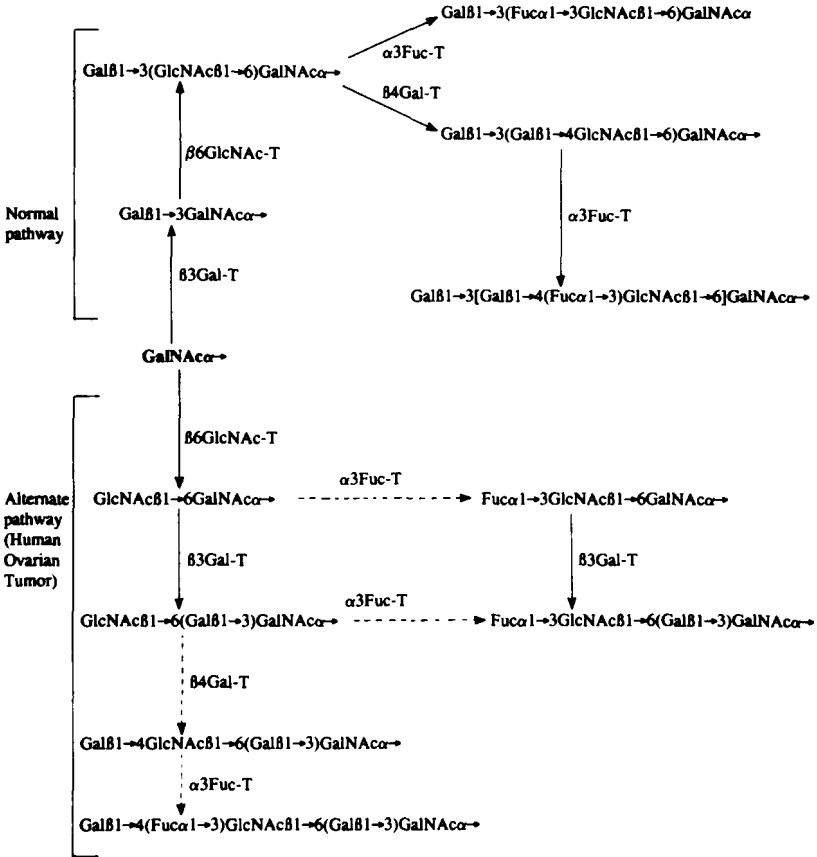
TABLE V

$\alpha$ -GalNAc: $\beta$ 1 $\alpha$ -GalNAc: $\beta$ 1,3Gal transferase level in normal ovary and ovarian tumors as measured with various acceptors

The values in parentheses are the activities expressed as the percentage of the activity present in normal ovarian enzyme source for a particular acceptor.

Acceptor	Incorporation of [ <sup>14</sup> C]Gal into the acceptor							
	Enzyme source: microsomal extract			Enzyme source: extraction of tissue homogenate in Tris saline with 1% Triton X-100				
	Normal ovary 2645	Uterus tumor 2645	Ovarian tumor A.O.	Normal ovary 2162	Ovarian tumors			
					2509	L.M.	J.M.	K.K.
	<i>cpm × 10<sup>-5</sup>/mg protein</i>				<i>cpm × 10<sup>-5</sup>/mg protein</i>			
Asialo-CGM	3.92	10.21 (260.5)	2.17 (55.4)	1.63	1.10 (67.5)	1.02 (62.6)	4.05 (248.5)	2.38 (146.0)
Galβ1,4GlcNAcβ1,6GalNAcα- <i>O</i> -Bn	1.82	2.37 (125.4)	1.48 (78.3)	1.29	0.91 (70.5)	1.01 (78.3)	2.66 (206.2)	1.92 (148.8)
GlcNAcβ1,6GalNAcα- <i>O</i> -Bn	2.71	1.77 (65.3)	1.33 (49.1)	3.02	1.45 (48.0)	1.56 (51.7)	5.90 (195.4)	5.95 (197.0)
Fucα1,3GlcNAcβ1,6GalNAcα- <i>O</i> -ONP	7.15	7.72 (108.0)	0.79 (11.1)	5.89	2.37 (40.2)	4.13 (70.1)	7.10 (120.5)	6.22 (105.6)
GlcNAcβ1,6GalNAcα- <i>O</i> -ONP	5.66	10.10 (178.4)	1.03 (18.2)	4.93	1.59 (32.3)	2.25 (45.6)	8.12 (164.7)	9.48 (192.3)
4-FluoroGlcNAcβ1,6GalNAcα- <i>O</i> -Bn	1.30	1.55 (119.2)	1.26 (96.9)	0.97	0.64 (66.0)	0.73 (75.3)	2.55 (262.9)	1.14 (117.5)

FIG. 12. Proposed scheme for alternate biosynthetic pathway of mucin carbohydrate chains in human ovarian tumor tissue. The broken arrows indicate the hypothetical reactions, of which we have some preliminary evidence for two.



specific acceptors GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn and GlcNAc- $\beta$ 1,3GalNAc $\alpha$ -O-Bn and thus demonstrated the transfer of GlcNAc to  $\alpha$ -GalNAc substituted by  $\beta$ 1,6GlcNAc (5). Under the incubation conditions used in the present study, UDP-GlcNAc did not bring about a decrease in the transfer of Gal to  $\alpha$ -GalNAc substituted by  $\beta$ 1,6GlcNAc. Brockhausen *et al.* (9) reported the formation of GlcNAc $\beta$ 1,6(GlcNAc $\beta$ 1,3)GalNAc $\alpha$ -O-Bn from GlcNAc $\beta$ 1,6GalNAc $\alpha$ -O-Bn at a slow rate by using rat colon mucosal extract. These findings would seem to indicate the possibility that the affinity of Gal trans-

ferase to  $\beta$ 1,6GlcNAc-linked  $\alpha$ -GalNAc supercedes that of  $\beta$ 1,3GlcNAc transferase. Schachter and his associates (13) have shown that the  $\beta$ 1,6GlcNAc transferase activities responsible for the synthesis of mucin Core 2 and Core 4 appear to reside in one enzyme in a given species; they are widely distributed in all tissues in contrast to the limited distribution of  $\beta$ 1,3GlcNAc transferase, thus explaining the predominance of Core 2 in mucins (13). The ability of  $\beta$ 1,3Gal transferase to use  $\beta$ 1,6GlcNAc-substituted  $\alpha$ -GalNAc as an acceptor as shown in the present study illustrates further the dominant nature of  $\beta$ 1,3Gal transferase



in mucin biosynthesis. Brockhausen *et al.* (14) have also reported that rat liver  $\alpha$ -GalNAc: $\beta$ 1,3Gal transferase showed only slightly reduced activity when the 6-hydroxyl of GalNAc was substituted by GlcNAc.

Fig. 12 illustrates our proposed scheme for an alternative biosynthetic pathway of mucin carbohydrate chains in human ovarian tissue. We have also obtained some preliminary evidence<sup>2</sup> that  $\alpha$ 1,3 fucosylation on GlcNAc of mucin-type chains occurs only after the  $\beta$ 1,4-galactosylation of GlcNAc but may also proceed to some extent on GlcNAc in GlcNAc $\beta$ 1,6(Gal $\beta$ 1,3)GalNAc $\alpha$  even in the absence of  $\beta$ 1,4 galactosylation.

An examination of the level of  $\alpha$ -GalNAc: $\beta$ 1,3Gal transferase in human ovarian tumors and normal ovaries using either microsomal extract or Triton X-100-solubilized tissue extract, indicated an enhancement (1.46–2.60-fold activity) of this enzyme in two ovarian tumors (J.M. and K.K.) and uterus tumor (2645) but a reduction (0.55–0.67-fold activity) in three ovarian tumors (A.O., L.M., and 2509), using the glycoprotein acceptor ACGM. An assessment with the synthetic acceptors showed a similar pattern except for some differences in the

affinity of substrates to ovarian enzyme sources. The results thus suggest that the level of expression of this enzyme in ovarian tumor probably varies with the type of ovarian tumor.

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