Glycobiology vol. 7 no. 6 pp. 753–768, 1997

The enzymatic sulfation of glycoprotein carbohydrate units: blood group T-hapten specific and two other distinct Gal:3-O-sulfotransferases as evident from specificities and kinetics and the influence of sulfate and fucose residues occurring in the carbohydrate chain on C-3 sulfation of terminal Gal

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Enzymatic 3-O-sulfation of terminal β-Gal residues was investigated by screening sulfotransferase activity present in 37 human tissue specimens toward the following synthesized acceptor moieties: Galβ1,3GalNAcα-O-Al, Galβ1,4GlcNAcβ-O-Al, Galβ1,3GlcNAcβ-O-Al, and mucin-type Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn structures containing a C-3 methyl substituent on either Gal. Two distinct types of Gal: 3-O-sulfotransferases were revealed. One (Group A) was specific for the Galβ1, 3GalNAcα- linkage and the other (Group B) was directed toward the Galβ1,4GlcNAc branch β1,6 linked to the blood group T hapten. Enzyme activities found in breast tissues were unique in showing a strict specificity for the T-hapten. Galβ-O-allyl or benzyl did not serve as acceptors for Group A but were very active with Group B. An examination of activity present in six human sera revealed a specificity of the serum enzyme toward β1,3 linked Gal, particularly, the T-hapten without β1,6 branching. Group A was highly active toward T-hapten/acrylamide copolymer, anti-freeze glycoprotein, and fetuin O-glycosidic asialo glycopeptide; less active toward fetuin triantennary asialo glycopeptide; and least active toward bovine IgG diantennary glycopeptide. Group B was moderately and highly active, respectively, with the latter two glycopeptides noted and least active with the first two. Competition experiments performed with Galβ1,3GalNAcα-O-Al and Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn having a C-3 substituent (methyl or sulfate) on either Gal reinforced earlier findings on the specificity characteristics of Group A and Group B. Group A displayed a wider range of optimal activity (pH 6.0–7.4), whereas Group B possessed a peak of activity at pH 7.2. Mg^{2+} stimulated Group A 55% and Group B 150%, whereas Mn^{+2} stimulated Group B 130% but inhibited Group A 75%. Ca^{2+} stimulated Group B 100% but inhibited Group A 35%. Group A and Group B enzymes appeared to be of the same molecular size (<100,000 Da) as observed by Sephacryl S-100 HR column chromatography. The following effects upon Gal: 3-O-sulfotransferase activities by fucose, sulfate, and other substituents on the carbohydrate chains were noted. (1) A methyl or GlcNAc substituent on C-6 of GalNAc diminished the ability of Galβ1,3GalNAcα-O-Al to act as an acceptor for Group A. (2) An α1,3-fucosyl residue on the β1,6 branch in the mucin core structure did not affect the activity of Group A toward Gal linked β1,3 to GalNAcα-. (3) Lewis x and Lewis a terminals did not serve as acceptors for either Group A or B enzymes. (4) Elimination of Group B activity on Gal in the β1,6 branch owing to the presence of a 3-fucosyl or 6-sulfo group on GlcNAc did not hinder any action toward Gal linked β1,3 to GalNAcα. (5) Group A activity on Gal linked β1,3 to GalNAc remained unaffected by 3′-sulfation of the β1,6 branch. The reverse was true for Group B. (6) The acceptor activity of the T-hapten was increased somewhat upon C-6 sulfation of GalNAc, whereas, C-6 sialylation resulted in an 85% loss of activity. (7) A novel finding was that Galβ1,4GlcNAcβ-O-Al and Galβ1,3GlcNAcβ-O-Al, upon C-6 sulfation of the GlcNAc moiety, became 100% inactive and 5- to 7-fold active, respectively, in their ability to serve as acceptors for Group B.

Key words: human tissues/glycoprotein/galactose:sulfotransferase/specificities/kinetic properties

Introduction

Sulfated complex carbohydrates play important roles in many biological processes. The high endothelial venule associated ligands for L-selectin, GLYCAM-1 and CD34, are sulfated, fucosylated and sialylated mucin-like glycoproteins (Imai *et al.*, 1993). The determinant structures of GLYCAM-1 were identified as 3′-sialyl, 6′-sulfo Lewis x and 3′-sialyl, 6-sulfo Lewis x attached as a β1,6 branch from the blood group T-hapten, Galβ1,3GalNAcα-O-Ser/Thr (Hemmerich and Rosen, 1994; Hemmerich *et al.*, 1994, 1995; Crommie and Rosen *et al.*, 1995). 3′-Sialyl, 6-sulfo Lewis x showed an enhanced L-selectin binding activity *in vitro* (Scudder *et al.*, 1994). The selectin binding activity of 3′-sulfo Lewis x is equal to or more than that of 3′-sialyl Lewis x (Brandley *et al.*, 1993; Chen *et al.*, 1994). Recently, Bertozzi *et al.* (1995) found 6,6′-disulfo lactose to be more potent than either 3′-sialyl Lewis x or 3′-sialyl Lewis a in binding to L-selectin. Baenziger and his group discovered an important role for sulfation in biological processes by demonstrating that oligosaccharides bearing a terminal β1,4-linked 4-O-sulfo GalNAc are recognized by a receptor in hepatic endothelial cells, resulting in the rapid removal of glycoproteins bearing these structures from the blood (Fiete *et al.*, 1991; Baenziger *et al.*, 1992; Smith *et al.*, 1993). An evolutionary conservation of these sulfated oligosaccharides on vertebrate glycoprotein hormones controlled their cir-

culatory half-life (Manzella *et al.*, 1995). The N-linked carbohydrate of human urinary-type plasminogen activator was also shown to contain 4-O-sulfo GalNAcβ1,4GlcNAcβ- as the predominant terminal structure (Bergwerff *et al.*, 1995).

Sulfate occurs in various types of glycoproteins. Spiro and Bhoyroo (1988) and DeWaard *et al.* (1991) found 3-O-sulfo Gal as well as 6-O-sulfo GlcNAc residues in the N-linked complex carbohydrate units of human, calf and porcine thyroglobulins. Carcinoembryonic antigen and its counterparts, normal fecal antigen and nonspecific cross-reacting antigen, were shown to possess a significant amount of 6-O-sulfo GlcNAc as an outer chain moiety of N-linked complex-type chains (Yamashita, 1987; Yamashita *et al.*, 1989; Fukushima *et al.*, 1995). Shilatifard *et al.* (1993) found that the viral envelope glycoprotein produced by human Molt-3 cells persistently infected with HTLV-IIIB, contained 6-O-sulfo GlcNAc. Lo-Guidice *et al.* (1994) identified sulfate linked either to C-3 of Gal or C-6 of GlcNAc in branched mucin-type chains containing α1,2 and α1,3 linked Fuc with α2,3 linked NeuAc in the respiratory mucins of cystic fibrosis. Kato and Spiro (1989) were the first in characterizing the Gal: 3-O-sulfotransferase which is present in calf thyroid gland. Recently Lo-Guidice *et al.* (1995) identified, in human respiratory mucosa, a sulfotransferase which catalyzes the 3-O-sulfation of terminal Gal in Galβ1,4GlcNAc containing mucin carbohydrate chains. We have shown earlier that the presence of sulfate on C-3 of Gal in the Galβ1,3/4GlcNAcβ- moieties increased the efficiency of these acceptors by 4- to 5-fold for Lewis and plasma type α1,3-L-fucosyltransferases (Chandrasekaran *et al.*, 1992). The present article communicates our findings on the existence of at least three distinct Gal: 3-O-sulfotransferases in humans as evident from specificity and kinetic analyses. We also report on the nature of some unique controls of these activities by fucose, sulfate, and other residues occurring in mucin type structures.

Results and discussion

Localization of sulfate after enzymatic transfer as the C-3 position of Gal in the acceptor

The enzyme source for this experiment was the Triton X-100 extract from OT (PW). The [^{35}S] products resulting from Galβ1,3GalNAcα–O-Al and Galβ1,3GlcNAcβ-O-Al coincided with authentic synthesized compounds, namely, 3-O-sulfoGalβ1,3GalNAcα–O-Al and 3-O-sulfoGalβ1,3GlcNAcβ-O-Al (see Figure 1A,B). The autoradiography (Figure 2, lanes 1 and 2) indicates that the [^{35}S] products from Galβ1,3GalNAcα–O-Al and Galβ1,3GlcNAcβ-O-Al move as single spots on TLC and have the same mobility. We have also characterized by TLC the product as 3–O-sulfoGalβ1,3GalNAcα–O-Al and not 6-sulfoGalβ1,3GalNAcα–O-Al using the extracts of some specimens from each group (Table I) and the acceptor Galβ1, 3GalNAcα–O-Al (data not shown).

[^{35}S] Sulfated compounds arising from GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn, Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn, and Galβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Me

The Biogel P2 elution profile of the product from GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn showed two peaks

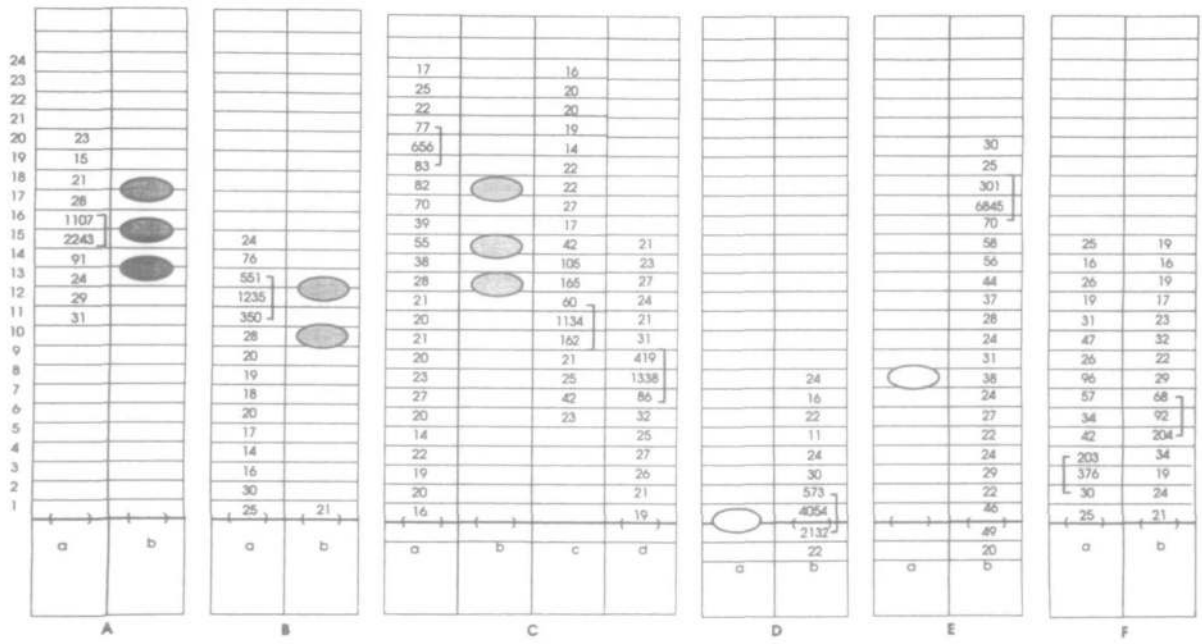

Fig. 1. Thin layer chromatography of the [^{35}S] sulfated compounds resulting from the various acceptors. (for details, see *Materials and methods*). (**A**) a, the [^{35}S] compound resulting from Galβ1,3GlcNAcβ-O-Al. b, The authentic standards in the order of increasing mobility 6-sulfoGalβ1,3GlcNAcβ-O-Al, 3-sulfoGalβ1,3 GlcNAcβ-O-Al, and Galβ1,3GlcNAcβ-O-Al respectively. Solvent: butanol/acetic acid/H_2O (3/2/1). (**B**) a, the [^{35}S] compound resulting from Galβ1,3GalNAcα–O-Al. b, The authentic standards in the order of increasing mobility 6-sulfoGalβ1,3GalNAcα–O-Al and 3-sulfoGalβ1,3GalNAcα–O-Al respectively. Solvent: butanol/acetic acid/H_2O (3/2/1). (**C**) a, The Peak A radioactive product from Galβ1,3(GlcNAcβ1,6)GalNAcα–O-Bn. b, The authentic standards 6-sulfoGalβ1,3GalNAcα–O-Bn, 3-sulfoGalβ1,3GalNAcα–O-Bn, and Galβ1,3GalNAcα–O-Bn. c, The Peak B radioactive product from Galβ1,3GlcNAcβ1,6)GalNAcα–O-Bn. d, The radioactive product from Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn. Solvent: butanol/acetic acid/H_2O (3/2/1). (**D**) a, The standard Galβ1,4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Me. b, The radioactive product from the above acceptor. Solvent: butanol/acetic acid/H_2O (3/2/1). (**E**) a and b as in (**D**). Solvent: $CHCl_3$/CH_3OH/H_2O (5/4/1). (**F**) a, the Peak A radioactive product from fetuin-O-glycosidic asialoglycopeptide. b, The Peak B radioactive product from fetuin-O-glycosidic asialo glycopeptide. Solvent: n-butanol/acetic acid/H_2O (3/2/1)

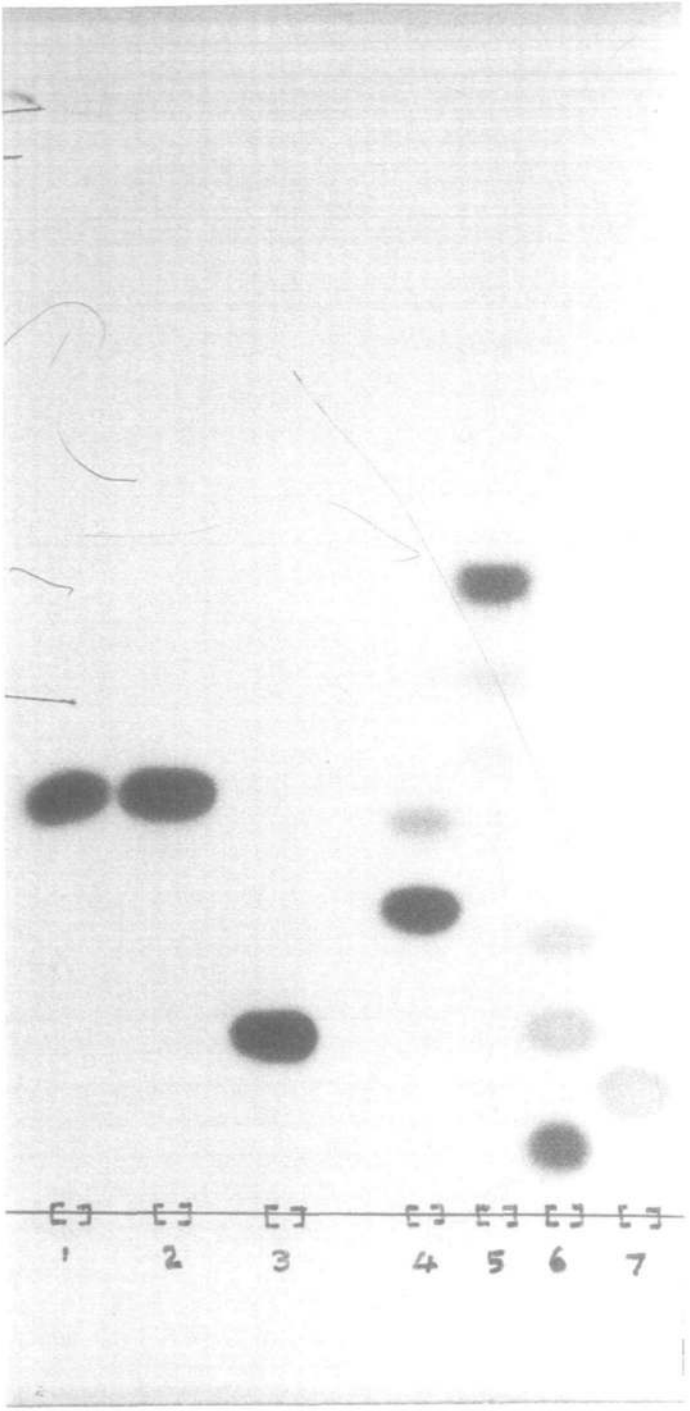

Fig. 2. Autoradiographic localization on TLC of the [^{35}S] sulfo compounds isolated from the various acceptors by Biogel P2 chromatography. [^{35}S] sulfo compounds arising from the following: lanes 1, Galβ1,3GalNAcα–O-Al. 2, Galβ1,3GlcNAcβ-O-Al. 3, Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn. 4, Galβ1,3(GlcNAcβ1,6)GalNAcα–O-Bn Biogel P2-B. 5, Galβ1,3(GlcNAcβ1,6)GalNAcα–O-Bn Biogel P2-A. 6, Fetuin O-glycosidic asialo glycopeptide Biogel P2-A. 7, Fetuin O-glycosidic asialo glycopeptide Biogel P2-B.

(data not shown). TLC results (Figure 1C, lanes a and c; Figure 2, lanes 4 and 5) indicated that the Peak A compound apparently larger than Peak B compound in size had surprisingly faster mobility in comparison to Peak B compound, suggesting that the former may arise from an impurity in the GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn preparation (probably due to some incomplete removal of the hydroxyl protecting groups from GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn during chemical synthesis). The Peak B material for all practical purposes moved as a single spot with mobility less than the [^{35}S] products from the disaccharide allyl glycosides (compare lanes a and c in Figure 1C; compare lanes 1 and 2 with lane 4 in Figure 2).

The [^{35}S] product from the Galβ1,4GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn acceptor emerged from the Biogel P2 column as a single peak in front of the Peak B product from GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn, conforming to the expectation (data not shown). TLC results (Figure 1C, lane d; Figure 2, lane 3) indicate its mobility as being slower than the Peak B product from GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn and its single entity as evident from its movement as a single spot (Figure 2 lane 3).

The [^{35}S] sulfated product from Galβ1,4(Fucα1,3) GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Me emerged from the Biogel P2 column as a single peak in front of the product from Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn, as anticipated (data not shown). It stayed almost at the origin on TLC in n-butanol/acetic acid/water (3:2:1) but moved faster than the parent compound in chloroform/methanol/water (5:4:1) (Figure 1D,E). We also observed that the chemically synthesized 3-sulfo derivatives of Galβ1,4GlcNAcβ1, 6(Galβ1,3)GalNAcα–O-Bn migrated faster than the parent compound in this solvent system.

The [^{35}S] sulfated glycopeptide arising from fetuin O-glycosidic asialoglycopeptide

Two peaks of radioactivity were seen upon chromatography on a Biogel P2 column (Figure 1A). Peak A had a lower mobility than Peak B on TLC (Figure 1F). Autoradiography of the TLC indicates that Peak A material gives an intense spot at 1.0 cm from the origin along with two weak spots (Figure 2, lane 6) and Peak B showed one weak spot (Figure 2, lane 7).

Two distinct Gal: 3-O-sulfotransferases in human tissues

We have examined 37 tissue specimens for Gal: 3-O-sulfotransferase activity employing five different synthetic acceptors (see Table I). From the results reported in Table I the activities present in these tissues can be divided into two distinct groups. The activities in both normal and tumor breast tissues (without any exception), almost all of the primary ovarian tumor tissues, three metastatic tissues from ovary, and one endometrial tumor fell into one category. Further, the activities present in both normal and tumor colon tissues (without any exception), both omentum tumors, four metastatic ovaries, and two endometrial tumors formed a second category (see Table I).

The first group (Group A) Gal: 3-sulfotransferase activity was strictly specific for the T-hapten, Galβ1,3GalNAcα–, since the 3-O-MeGalβ1,4GlcNAcβ1,6 (Galβ1,3)GalNAcα–O-Bn acceptor was nearly as active as Galβ1,3GalNAcα–O-Al, whereas the other acceptors, Galβ1,4GlcNAcβ-O-Al, Galβ1,3GlcNAcβ-O-Al and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn yielded either negligible or very low activity. It is important to emphasize the observation that this Gal: 3-O-sulfotransferase specific for T-hapten is either almost absent or extremely low in normal breast tissues. A

Table I. Two distinct Gal:3-O-sulfotransferases in human tissues as evident from the reactivity with synthetic acceptors

Tumor tissues	Gal: 3-sulfotransferase activity, incorporation of [^{35}S] sulfate (cpm × 10^{-3}) into the acceptors catalyzed by 1 mg protein				
	Galβ1,3 GalNAc α-O-Al	Galβ1,4 GlcNAc β-O-Al	Galβ1,3 GlcNAc β-O-Al	3-O-MeGalβ1,4 GlcNAcβ1,6 (Galβ1,3) GalNAcα-O-Bn	Galβ1,4 GlcNAcβ1,6 (3-OMeGalβ1,3) GalNAcα-O-Bn
Group A:					
BN 7869	0.2	0	0		
BT 7549	36.1	0	0	28.7 (79.4)	0.3 (0.9)
BT 7764	301.7	2.4 (0.8)	0.8 (0.3)	274.3 (90.9)	
BN 7764	18.9	0	0		
BT 7803	58.8	0.7 (1.2)	0.5 (0.9)	55.5 (94.5)	
BN 7803	11.7	0	0		
BT 8565	36.8	0.4 (1.0)	0.4 (1.0)	38.5 (104.5)	
BT 8640	201.5	1.5 (0.7)	0.6 (0.3)	239.2 (118.7)	
BT 8600	79.3	6.3 (7.9)	3.5 (4.4)	83.7 (105.6)	
OT 7770	122.9	5.8 (4.7)	4.0 (3.2)	92.0 (74.9)	
OT 7753	218.1	1.9 (0.9)	1.6 (0.7)	231.4 (106.1)	
OT 7915	80.2	4.9 (6.2)	10.0 (12.5)		
MO 7885	170.1	7.5 (4.4)	3.7 (2.2)	164.3 (96.6)	
MO 7855	32.7	1.7 (5.2)	1.2 (3.7)	29.8 (91.2)	3.2 (9.7)
MO 8207	33.6	4.3 (12.7)	2.3 (6.9)	33.2 (99.0)	8.1 (24.1)
EMT 7588	6.9	0.1 (1.4)	0.1 (1.4)	7.5 (109.6)	0.7 (10.4)
Group B:					
MOM 8057	23.5	15.0 (63.8)	7.2 (30.6)		
MO 8157	11.3	2.8 (24.8)	1.4 (12.4)		
MO 8066	6.3	3.5 (55.6)	1.9 (30.2)		
OT 7827	5.9	1.3 (22.0)	0.8 (13.6)		
MO 8237	335.3	152.5 (45.5)	76.6 (22.8)	160.0 (47.7)	523.6 (156.2) [343.3]
EMT 8653	222.3	138.7 (64.6)	72.0 (32.4)	66.8 (30.1)	313.5 (141.0) [218.3]
OMT 7770	65.9	11.0 (16.7)	9.6 (14.6)	46.8 (71.7)	60.5 (91.8) [549.7]
EMT 7979	92.4	21.8 (23.6)	12.1 (13.0)	53.7 (58.1)	94.8 (102.5) [434.3]
MO 8022	118.6	24.9 (21.0)	11.8 (9.9)	104.1 (87.8)	132.7 (111.9) [532.9]
CT 8458	129.0	54.9 (42.6)	29.0 (22.5)	57.1 (44.3)	127.6 (98.9) [232.2]
CT 8661	184.7	96.0 (52.0)	64.9 (35.1)	46.2 (25.0)	281.8 (152.6) [293.5]
CT 8059	764.7	449.1 (58.7)	186.1 (24.3)	285.6 (37.3)	1196.5 (156.5) [266.6]
CT 8467	328.9	210.8 (64.1)	103.8 (31.6)	165.2 (50.2)	558.4 (169.8) [264.9]
CT 8365	346.9	173.7 (50.1)	74.3 (21.4)	153.1 (44.1)	527.9 (152.2) [303.8]
CN 2845	15.5	11.2 (72.3)	5.8 (37.7)	3.6 (23.5)	31.4 (203.2) [281.1]
CN 23273	6.9	4.5 (65.1)	3.2 (46.1)	3.5 (50.5)	24.3 (354.0) [543.8]
CN 23269	35.4	23.4 (66.1)	19.6 (55.4)	8.9 (25.1)	61.7 (174.1) [263.4]
CN 25385	35.8	22.6 (63.0)	11.4 (31.8)		
CN 28375	2.6	1.4 (53.8)	0.9 (34.6)		
CN 2914	5.9	4.4 (74.6)	2.8 (47.5)		
CN 3165	3.2	2.1 (65.6)	1.4 (43.8)		

The values in parentheses are the activities expressed as percentage of the activity toward Galβ1,3GalNAcα-O-Al for each tissue sample. The values in brackets (see the last column) are activities expressed as percentage of the activity towards Galβ1,4GlcNAcβ-O-Al.
BN, Breast normal; BT, breast tumor; OT, ovarian tumor; MO, metastatic ovary; MOM, metastatic omentum; EMT, endometrial tumor; OMT, omentum tumor; CN, colon normal; CT, colon tumor.

comparison of breast tumor tissues with normal breast tissue from the same patient (BN and BT 7764; BN and BT 7803) indicates that there is a 5- to 15-fold increase in this T-hapten specific Gal: 3-O-sulfotransferase activity associated with this malignancy.

The second group (Group B) exhibited Gal: 3-O-sulfotransferase activity whose specificity appears to be directed toward Galβ1,4GlcNAc occurring as the β1,6 branch from blood group T-hapten. In addition, this enzyme category is not stringent in its requirement for the type of N-acetylhexosamine to which Gal is β linked. The above interesting findings became evident as follows. The Galβ1, 4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn acceptor was 1.5- to 3.0-fold more active than Galβ1,3GalNAcα–O-Al and 2.5- to 5.5-fold more active than Galβ1,4GlcNAcβ-O-Al. Both Galβ1,4GlcNAcβ-O-Al and Galβ1,3GlcNAcβ-O-Al were less active (16.7–74.6% and 9.9–55.4%, respectively) as compared to Galβ1,3GalNAcα–O-Al.

It is apparent from the data reported in Table I that among the two types of Gal: 3-O-sulfotransferases identified as above, only one type appears to be associated with a particular tissue. The 3-O-sulfotransferase activities present in both normal (BN7869, BN7764, and BN7803) and cancerous breast tissues (BT7549, BT7764, BT7803, BT8600, and BT8640) exhibited specificity toward the acceptor structure Galβ1,3GalNAcα–O-Ser/Thr. The colon tissues both normal (CN2845, CN23273, CN23269, CN25385, CN28375, CN2914, and CN3165) and cancer (CT8458, CT8661, CT8059, CT8467, and CT8365) contained 3-O-sulfotransferase activity whose specificity directed most favorably toward N-acetyllactosamine linked β1,6 to α-GalNAc. As compared to the normal breast tissue, the tumor breast tissue from the same patient (compare BN7764 and BT77642; BN7803 and BT7803) showed 5–15 fold 3-O-sulfotransferase activity. The elevation of Gal: 3-O-sulfotransferase activity in breast tumor tissue is much more pronounced if one considers the fact that fat is the most pre-

dominant constituent of normal breast. Furthermore, the level of sulfotransferase activity in colon tumor specimens was much higher than in normal colon specimens (the range of activity 129.0→764.7 (tumor) and 2.6→35.8 (normal)). The ovarian tumor specimens OT7770, OT7753, and OT7915 exhibiting high level of sulfotransferase activity fell into the Group A category whereas the endometrial tumor specimens EMT8653 and EMT7979 having high sulfotransferase activity belonged to Group B; the exceptions were the very low active specimens OT7827 and EMT7588. The three highly active metastatic ovary specimens MO7885, MO7855, and MO8207 belonged to Group A. On the other hand, one highly active specimen MO8237 and the two very low active specimens MO8157 and MO8066 appear to belong to Group B. The above discrepancy may be attributed to the possible difference in the metastatic status of these tissue specimens.

Human serum Gal: 3-O-sulfotransferase as a third category

We have screened the activity of six sera (two normal, two colon cancer, and two breast cancer) using the five acceptors employed above and the results are presented in Table II. All of the sera were found to be most active toward Galβ1,3GalNAcα–O-Al. Contrary to the first category of tissues, the activities in sera were far less active (14.0–40.4% only) with 3-O-MeGalβ1,4GlcNAcβ1,6 (Galβ1,3)GalNAcα–O-Bn in comparison to Galβ1,3GalNAcα–O-Al. Surprisingly, the enzyme in sera was considerably more active toward Galβ1,3GlcNAcβ-O-Al (15.7%–24.6%) than either Galβ1,4GlcNAcβ-O-Al (3.2–6.1%) or Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn (1.9–6.0%). These data would suggest that Gal:3-O-sulfotransferase occurring in human serum does not fall into either Group A or Group B reported in Table I. It appears that the specificity of the human serum enzyme is directed mostly toward β1,3 linked Gal which is not a part of a branched mucin-type chain.

Differentiation of Gal: 3-O-sulfotransferases using mono- and disaccharide glycosides as acceptors

We found that the two distinct groups of Gal: 3-O-sulfotransferases identified in human tissues, using disaccharide and branched mucin-type tetrasaccharide glycosides as acceptors, can also be distinguished using the allyl and benzyl glycosides of galactose and the T-hapten. In order to illustrate this point, we arbitrarily chose two tissue sources each from Group A and Group B, as reported in Table I. The data are reported in Table III. The breast tumor enzyme (BT 7764) was almost nonreactive with allyl and benzyl galactosides, whereas other enzyme (MO 8207) belonging to the same group (Group A; see Table I) displayed some activity with both Galβ-O-Al (13.0%) and Galβ-O-Bn (22.9%), as compared to that with Galβ1,3GalNAcα–O-Al. On the contrary, Group B (see Table I) enzymes MOM 8057 and CN 23269 were 173.7 and 244.5% active, respectively, with Galβ-O-Bn in comparison to their activity toward Galβ1,3GalNAcα–O-Al. They were either equally (MOM 8057: 100.5%) or more active (CN 23269: 138.0%) with Galβ-O-Al. It is interesting to note that Galβ1,3GalNAcα–O-Al is a better acceptor than Galβ1,3GalNAcα–O-Bn for both groups of enzymes (Groups A and B), whereas Galβ-O-Bn is a better acceptor than Galβ-O-Al for Group B enzymes.

Activity of human tissue Gal: 3-O-sulfotransferases with high molecular weight glycoconjugates: (see Table IV)

Three enzyme sources from Group A (BT 7549, MO 7855, and MO 8207) and four enzyme sources from Group B (MOM 8057, CN 25385, CN 23273, and CN 23269) were arbitrarily chosen for examining their activity toward the T-hapten-acrylamide copolymer (Galβ1,3GalNAcα–O-Al/AA–CP), anti-freeze glycoprotein (consisting of Galβ1,3 GalNAcα–O-Ser/Thr carbohydrate units), an O-glycosidic asialo glycopeptide as well as a N-glycosidic triantennary asialo glycopeptide isolated from fetuin and an N-glycosidic diantennary glycopeptide from bovine IgG. As anticipated, the three Group A enzyme sources were more active than the Group B when T-hapten-acrylamide copolymer (4- to 8-fold active) and anti-freeze glycoprotein (5- to 15-fold active) were used as the acceptors. In contrast to this, Group B showed 3- to 25-fold and 9- to 34-fold greater activity, respectively, toward fetuin triantennary asialoglycopeptide and bovine IgG diantennary glycopeptide as compared to the activity of Group A enzymes toward the respective glycopeptide.

A difference between Group A and Group B enzymes is best illustrated when their activity with high molecular weight acceptors is expressed as percent of the activity toward T-hapten

Table II. Human serum Gal: 3-O-sulfotransferase activity towards synthetic acceptors

Acceptors	Gal: 3-O-sulfotransferase activity, incorporation of [^{35}S] sulfate (cpm × 10^{-3}) into the acceptors by 15 μl serum					
	HS1	HS2	HS3	HS4	HS5	HS6
Galβ1,3GalNAcα-O-Al	21.3 (100.0)	10.9 (100.0)	16.5 (100.0)	11.5 (100.0)	11.2 (100.0)	6.0 (100.0)
Galβ1,4GlcNAcβ-O-Al	0.7 (3.2)	0.7 (6.1)	1.0 (5.8)	0.6 (5.3)	0.4 (3.9)	0.3 (5.5)
Galβ1,3GlcNAcβ-O-Al	5.2 (24.6)	2.3 (21.2)	3.1 (18.9)	1.8 (15.7)	2.1 (18.3)	1.2 (20.1)
3-O-MeGalβ1,4GlcNAc β1,6(Galβ1,3)GalNAcα O-Bn	5.5 (25.9)	1.9 (17.0)	2.3 (14.0)	3.3 (28.9)	3.7 (32.7)	2.4 (40.4)
Galβ1,4GlcNAcβ1,6 (3-O-MeGalβ1,3) GalNAcα-O-Bn	0.5 (2.5)	0.7 (6.2)	0.3 (1.9)	0.6 (5.0)	0.4 (3.5)	0.4 (6.0)

HS1 and HS2, Fresh sera from normal individuals; HS3 and HS4, fresh sera from colon cancer patients; HS5 and HS6, fresh sera from breast cancer patients. The values in parentheses are activities expressed as percentage of the activity toward Galβ1,3GalNAcα-O-Al for each serum.

Table III. Mono- and disaccharide glycosides as acceptors in differentiating Gal:3-O-sulfotransferases present in human tissues

Tissue	Gal: 3-O-sulfotransferase activity, incorporation of [^{35}S] sulfate (cpm) into the acceptors			
	Galβ1,3GalNAcα-O-Al	Galβ1,3GalNAcα-O-Bn	Galβ-O-Al	Galβ-O-Bn
BT 7764	63242 (100.0)	38389 (60.7)	333 (0.5)	853 (1.3)
MO 8207	27092 (100.0)	11832 (43.7)	3516 (13.0)	6195 (22.9)
MOM 8057	21958 (100.0)	8946 (40.7)	22058 (100.5)	38144 (173.7)
CN 23269[a]	15855 (100.0)	4626 (29.2)	21885 (138.0)	38773 (244.5)

In this experiment, the radioactive product (the [^{35}S] sulfated compound) from each acceptor including Galβ1,3GalNAcα-O-Al was quantitated by TLC (see Experimental procedures) since the products from Galβ1,3GalNAcα-O-Bn, Galβ-O-Al, and Galβ-O-Bn could not be eluted from Dowex-1-Cl under our experimental conditions. The values in parentheses are activities expressed as percentage of the activity toward Galβ1,3GalNacα-O-Al for each tissue sample.
[a]As compared to Galβ-O-Bn, the acceptors Galβ-O-pNP and Galβ-O-Me were 35.9% and 9.9% active, respectively, when tested with CN 23269.

acrylamide copolymer (compare the values in parentheses for each enzyme source in Table IV). The Group A enzymes (BT 7549, MO 7855, and MO 8207) appear to be highly specific for the Galβ1,3GalNAcα– structure as evident from their facile interaction with anti-freeze glycoprotein and fetuin O-glycosidic asialo glycopeptide. In this context, it is of importance to note the following. The above enzymes also showed considerable activity toward fetuin triantennary asialo glycopeptide comprising three terminal Gal units and were least active with bovine IgG diantennary glycopeptide, comprised mostly of one terminal Gal. The Group B enzymes were 2- to 3-fold and 4- to 6-fold more active with fetuin-O-glycosidic asialo glycopeptide (MW < 1000 Da) when compared respectively to the T-hapten-acrylamide copolymer (MW ~ 40,000) containing ~40 T-haptens (Chandrasekaran *et al.*, 1995a) and anti-freeze glycoprotein (average MW 16,000) containing ~20 T-haptens (DeVries, 1970). These results suggest that the specificity of Group B enzymes is not directed toward mucin type glycoproteins containing the T-hapten structures. This observation is further substantiated by our earlier finding (see Table I) that Galβ1,3GalNAcα–O-Al is 2- to 4-fold more active than the mucin type 3-O-MeGalβ1,4GlcNAcβ1, 6(Galβ1,3)GalNAcα–O-Bn acceptor when tested with CN 25385, CN 23273, and CN 23269.

Activity of human tissue Gal: 3-O-sulfotransferases toward Le^x, Le^a terminal and related structures (see Table V)

The Group A sources BT 7549 and OT (PW) and the Group B sources CT 8059 and CT 8467 were used arbitrarily in these experiments.

When C-6 of GalNAc in T-hapten is substituted with a methyl group or GlcNAc moiety acceptor activities toward group A enzymes were less (BT 7549: 42.5% and 16.8%; OT (PW): 73.4% and 26.4%, respectively) in comparison to Galβ1,3GalNAcα–O-Al. Elongation of the β1,6 branch in Galβ1,3(GlcNAcβ1,6) GalNAcα–O-Bn either by β1,4 linked Gal or by both β1,4 linked Gal and α1,3 linked Fuc increased the acceptor ability (BT 7549: 16.8%→73.0% and 112.1% respectively; OT (PW): 26.4%→83.6%). The Le^x terminal structures, namely, Galβ1,4 (Fucα1→3)GlcNAcβ1,6Man$_3$, Galβ1,4(Fucα1,3)GlcNAcβ1,4Galβ-O-Me, and Galβ1,4(Fucα1,3) GlcNAcβ-O-Bn, and the Le^a terminal structures, namely, Galβ1,3(Fucα1,4)GlcNAcβ-O-Al, Galβ1,3(Fucα1,4) GlcNAcβ-O-Bn, and Galβ1,3(Fucα1,4) GlcNAcβ-O-pNP, were inactive as acceptors for BT 7549. This enzyme was also inactive with Galβ1,4GlcNAcβ1,6 Man$_3$. OT (PW) enzyme was not active toward Galβ1, 3(Fucα1,4)GlcNAcβ-O-Bn, Galβ1,4GlcNAcβ1,6GalNAcα–O-pNP, Galβ1,3GlcNAcβ1,3Galβ1,4Glcβ-O-pNP and Galβ1,4 GlcNAcβ1,3Galβ1,4 Glcβ-O-pNP. These results would imply that the biological role of Group A enzymes is normally restricted to 3-O-sulfation of the T-hapten structures.

The Group B enzymes CT 8059 and CT 8467 were more active (140.9% and 152.8%, respectively) toward Galβ1, 4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn than they were toward Galβ1,3GalNAcα–O-Al. On α1,3-fucosylation the ability of the resulting pentasaccharide, Galβ1,4(Fucα1,3) GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Me, to act as an acceptor was reduced by ~50% (CT 8059: 140.9%→61.2%; CT 8467: 152.8%→75.7%).

Since Galβ1,4(Fucα1,3)GlcNAcβ-O-Bn showed extremely low activity (~5%) with CT 8059 and CT 8467, it is suggestive that the Gal linked β1,3 to GalNAcα– in the Galβ1, 4(Fucα1,3)GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Me acceptor must be the moiety being 3-O-sulfated. The group B (CT 8059 and CT 8467) enzymes also failed to act on acceptors terminating in Le^a structures.

Sulfated compounds as acceptors for Gal: 3-O-sulfotransferases

The results are reported in Table VI. The group A enzyme BT 7764 displayed 95.5% and 123.9% activity with 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn and Galβ1,3(6-sulfo)GalNAcα–O-Al, respectively, compared to its activity with Galβ1,3GalNAcα–O-Al. When the C-6 hydroxyl of GalNAc in Galβ1,3GalNAcα- is substituted by sialic acid instead of sulfate the activity dropped to 15.2%. The Group B enzyme MOM 8057 was 140.8% active with Galβ1, 4GlcNAcβ1,6(3-sulfo Galβ1,3)GalNAcα–O-Bn as compared to its activity with Galβ1,3GalNAcα–O-Al.

The most noteworthy finding, which would be of utmost biological significance, emerged when the Group B sources CT 8059, CT 8467, and CT 8365 were tested with the Galβ1,3(6-sulfo)GlcNAcβ-O-Al and Galβ1,4(6-sulfo)GlcNAcβ-O-Al acceptor; C-6 sulfation of N-acetylglucosamine completely abolished the acceptor ability of the β1,4 linked Gal but increased the acceptor ability of a β1,3 linked Gal. The Galβ1,3(6-sulfo)GlcNAcβ-O-Al acceptor was ~1.5-fold and ~5- to 7-fold more active, respectively, than Galβ1,3GalNAcα–O-Al and Galβ1,3GlcNAcβ-O-Al (see the values in brackets in Table VI). But, as noted with Galβ1,4(Fucα1,3)GlcNAcβ1, 6(Galβ1,3)GalNAcα–O-Me, Galβ1,4(6-sulfo)GlcNAcβ1,6

Table IV. Action of Gal: 3-O-sulfotransferase(s) present in human tissues on high molecular weight glycoconjugates

Glycoconjugates	Gal: 3-O-sulfotransferase activity*, incorporation of [^{35}S] sulfate (cpm × 10^{-3}) into glycoconjugates by 1 mg protein						
	BT 7549	MO 7855	MO 8207	MOM 8057	CN 25385	CN 23273	CN 23269
Galβ1,3GalNAcα-O-A1/AA-CP (100 μg)	7.11 (100.0)	5.72 (100.0)	6.32 (100.0)	1.47 (100.0)	1.19 (100.0)	0.83 (100.0)	1.17 (100.0)
Anti-freeze glycoprotein (100 μg)	6.25 (87.9)	4.72 (82.5)	6.32 (100.0)	1.03 (70.1)	0.55 (46.2)	0.41 (49.4)	0.48 (41.0)
Fetuin triantennary asialoglycopeptide (200 μg)	1.17 (16.5)	1.98 (34.6)	4.33 (68.5)	13.93 (947.6)	24.65 (2071.4)	13.14 (1583.1)	25.76 (2201.7)
Bovine IgG diantennary glycopeptide (200 μg)	0.15 (2.1)	0.15 (2.6)	0.32 (5.1)	3.24 (220.4)	4.79 (402.5)	2.89 (348.2)	5.15 (440.2)
Fetuin O-glycosidic asialoglycopeptide (200 μg)	8.75 (123.1)	7.76 (135.7)	6.30 (99.7)	3.37 (229.3)	3.53 (296.6)	1.89 (227.7)	3.35 (286.3)

The values in parentheses are activities expressed as percentage of the activity toward Galβ1,3GalNAcα-O-A1/AA-CP.
*The radioactive products were measured by Dowex-1-Cl method (see *Materials and methods*)

(Galβ1,3)GalNAcα–O-Bn also served as an acceptor for the Group B enzyme CT 8467, indicating that Gal linked directly to GalNAc must be the site of 3-O-sulfation in this case.

Differentiation of Group A and Group B Gal: 3-O-sulfotransferases based on the difference in their activities over a pH range

Three enzyme sources from Group A (BT 7549, BT 7764, and MO 8207) were screened for their activity over the pH range 5.2→8.4 (Tris-maleate buffer) using Galβ1,3GalNAcα–O-Al as the acceptor. All three sources showed optimal activity over a wide pH range 6.0–7.4 (Figure 3A). In contrast, Group B enzyme exhibited a peak of activity at pH 7.2 as evident from testing CN 23269 with three different acceptors, Galβ1,3GalNAcα–O-Al, Galβ1,4GlcNAcβ-O-Al, and Galβ1,3GlcNAcβ-O-Al, and MOM 8057 with Galβ1,3GalNAcα–O-Al (Figure 3B). These enzymes showed <40% activity at pH 6.0 and ~55% activity at pH 6.4. Since the activity profiles of CN 23269 with the three different acceptors were almost iden-

Table V. Specificities of Gal: 3-O-sulfotransferases present in tumor tissues towards fucosylated and related synthetic compounds

Compounds	Gal: 3-O-sulfotransferase activity*, incorporation of [^{35}S] sulfate (cpm × 10^{-3}) into the test compounds catalyzed by 1 mg protein			
	BT 7549	OT (PW)	CT 8059	CT 8467
Galβ1,3(6-O-Me)GalNAcα-O-A1	15.3 (42.5)	12.4 (73.4)		
GalB1,3(GlcNAcβ1,6) GalNAcα-O-Bn	6.1 (16.8)	4.5 (26.4)		
Galβ1,4GlcNAcβ1,6 (Galβ1,3)GalNAcα-O-Bn	26.5 (73.0)	14.2 (83.6)	199.5 (140.9)	124.3 (152.8)
Galβ1,4(Fucα1,3)GlcNAc β1,6(Galβ1,3)GalNAcα-O-Me	40.5 (112.1)		86.7 (61.2)	61.6 (75.7)
Galβ1,4(Fucα1,3)GlcNAc β1,6Manβ1,6Manβ1,6Man	0			
Galβ1,4(Fucα1,3) GlcNAcβ1,4Galβ-O-Me	0			
Galβ1,4(Fucα1,3) GlcNAcβ-O-Bn	0		9.1 (6.4)	4.5 (5.5)
Galβ1,3(Fucα1,4) GlcNAcβ-O-Al	0		1.7 (1.2)	1.1 (1.3)
Galβ1,3(Fucα1,4) GlcNAcβ-O-Bn	0	0	0.2 (0.1)	0.1 (0.1)
Galβ1,3(Fucα1,4) GlcNAcβ-O-pNP	0		0.2 (0.1)	0.1 (0.1)
Galβ1,4GlcNAcβ1,6Man β1,6Manβ1,6Man	0			
Galβ1,4GlcNAcβ1,6 GalNAcα-O-pNP		0.6 (3.6)		
Galβ1,3GlcNAcβ1,3 Galβ1,4Glcβ-O-pNP		0		
Galβ1,4GlcNAcβ1,3 Galβ1,4Glcβ-O-pNP		0		

*The radioactive products were measured by Dowex-1-Cl method (see *Materials and methods*).
The values in parentheses are the activities expressed as percentage of the activity toward Galβ1,3GalNAcα-O-A1 for each tissue sample.

tical (Figure 3B), it is apparent that these activities are most likely catalyzed by a single enzyme. The above results thus demonstrate very clearly that Group A enzyme is quite different from Group B and we are dealing with two distinct 3-O-sulfotransferases in human tissues.

Influence of divalent metal ions on Group A and Group B as well as serum Gal: 3-O-sulfotransferases

The effect of Mg^{2+}, Mn^{2+}, and Ca^{2+} on the activities of Group A (BT 7764) (see Figure 4A), Group B (23269) (see Figure 4B) and Group C (HS1) enzymes (see Figure 4C) were examined. A maximum stimulation of BT 7764 activity (~55% increase) was attained at 10 mM Mg^{2+} and then dropped slowly reaching its original level of activity at 40 mM. On the contrary, Mn^{2+} stimulated BT 7764 activity only by ~20% at 10 mM then a steady decline in activity was seen with increasing Mn^{2+} concentration, reaching a level of 25% at 50 mM. Ca^{2+} stimulated BT 7764 activity (maximum: ~30% increase) at 5 mM. The activity then dropped to 65% at 10 mM and maintained this level at higher concentrations of Ca^{2+}.

CN 23269 enzyme activity was stimulated by Mg^{2+} reaching 2.5-fold at 30 mM and was still maintained at a 2.0-fold level at 50 mM. In contrast to the Group A enzyme, Mn^{2+} stimulated CN 23269 activity (2.3-fold at 10 mM). Activity was maintained at this level up to 20 mM, then declined to its original level at 50 mM. Stimulation of CN 23269 activity by Ca^{2+} was similar to that by Mg^{2+}, reaching a maximum of 2.2-fold at 30 mM. This level was nearly maintained up to 50 mM Ca^{2+}.

Serum enzyme (HS1) activity was stimulated by Mg^{2+} (maximum increase: ~50%) at 5 mM then steadily declined reaching the original level at 30 mM, whereas Mn^{2+} at 5 mM stimulated this activity by ~50%, and then declined sharply reaching a 30% level at 50 mM. Ca^{2+} caused a 33% increase in activity at 10 mM; the activity declined slowly reaching the original level at 30 mM, and 75% activity was seen at 40–50 mM. The results indicate that serum enzyme mostly resembles Group A enzyme, especially when considering the effect of the Mn^{2+} and Mg^{2+} divalent cations.

A distinct difference thus exists between Group A and Group B enzymes when considering the effects of the divalent cations as follows. (1) With Group A enzyme Mn^{2+} showed some stimulation (~20%) at 10 mM and then adversely affected the activity (75% inhibition at 50 mM). With Group B enzyme a high stimulation resulted at 10 mM (2.3-fold activity). This stimulation declined slowly and disappeared at 40 mM, but there was no inhibition of activity. (2) Mg^{2+} stimulated Group A enzyme by ~55% at 10 mM. This stimulation declined to zero at 40 mM. The Group B enzyme was stimulated by both Mg^{2+} and Ca^{2+} even up to 50 mM concentration of these cations. On the other hand, Ca^{2+} showed a slight (30%) stimulation of Group A enzyme at 5 mM, but after a sharp decline in the level of activity at 10 mM no effect was seen up to 50 mM.

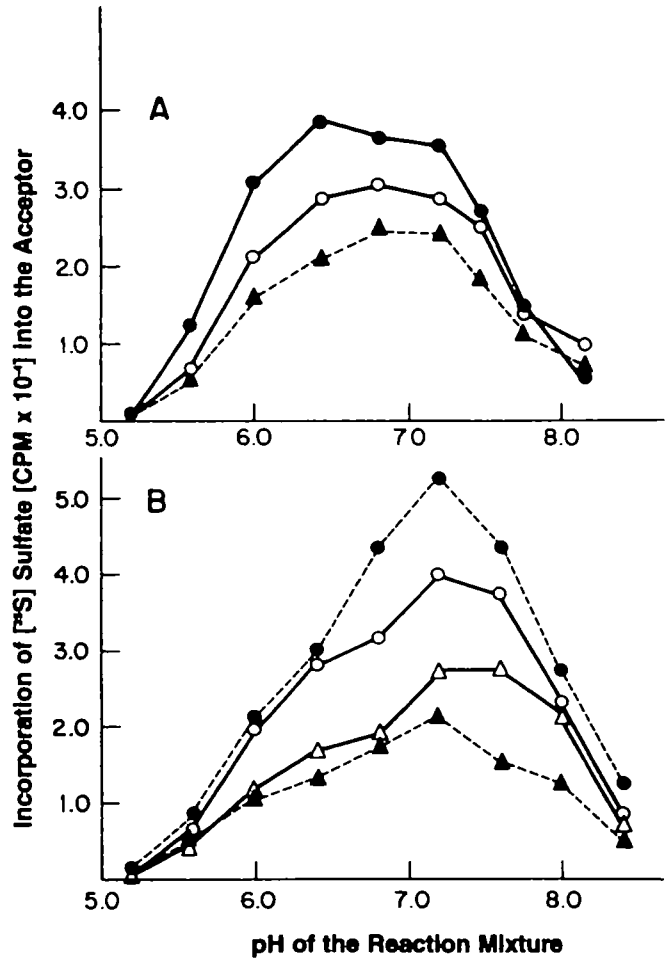

Fig. 3. Gal: 3-O-sulfotransferase activity over a pH range. (A) •, BT 7549 (Galβ1,3GalNAcα–O-Al as acceptor); ○, BT 7764 (Galβ1,3GalNAcα–O-Al as acceptor); ▲, MO 8207 (Galβ1,3GalNAcα–O-Al as acceptor). (B) •, MOM 8057 (Galβ1,3GalNAcα–O-Al as acceptor); ○, CN 23269 (Galβ1,3GalNAcα–O-Al as acceptor); △, CN 23269 (Galβ1,4GlcNAcβ-O-Al as acceptor); ▲, CN 23269 (Galβ1,3GlcNAcβ-O-Al as acceptor).

Table VI. Synthetic sulfated compounds as acceptors for Gal: 3-O-sulfotransferases present in tumor tissues

Compounds	Gal: 3-O-sulfotransferase activity[a], incorporation of [^{35}S] sulfate (cpm × 10^{-3}) into the test compounds catalyzed by 1 mg protein				
	MOM 8057	BT 7764	CT 8059	CT 8467	CT 8365
Galβ1,4GlcNAcβ1,6 (3-sulfo-Galβ1,3) GalNAcα-O-Bn	55.8 (140.8)				
3-SulfoGalβ1,4 GlcNAcβ1,6(Galβ1,3) GalNAcα-O-Bn		70.4 (95.5)			
Galβ1,3(6-sulfo) GalNAcα-O-Al		91.3 (123.9)			
Galβ1,3(NeuAcα2,6) GalNAcα-O-Bn		11.2 (15.2)			
Galβ1,4(6-sulfo) GlcNAcβ1,6(Galβ1,3) GalNAcα-O-Bn				66.8 (82.1)	
Galβ1,4(6-sulfo) GlcNAcβ-O-Al			0	0	0
Galβ1,3(6-sulfo) GlcNAcβ-O-Al			215.2 (152.0) [625.5]	117.5 (144.4) [457.0]	106.8 (148.0) [691.6]

The values in parentheses are the activities expressed as percentage of the activity toward Galβ1,3GalNAcα-O-Al for each tissue sample.
The values in brackets are the activities expressed as percent of the activity towards Galβ1,3GlcNAcβ-O-Al for each tissue sample.
[a]The ratioactive products were quantitated by the TLC method (see *Materials and methods*).

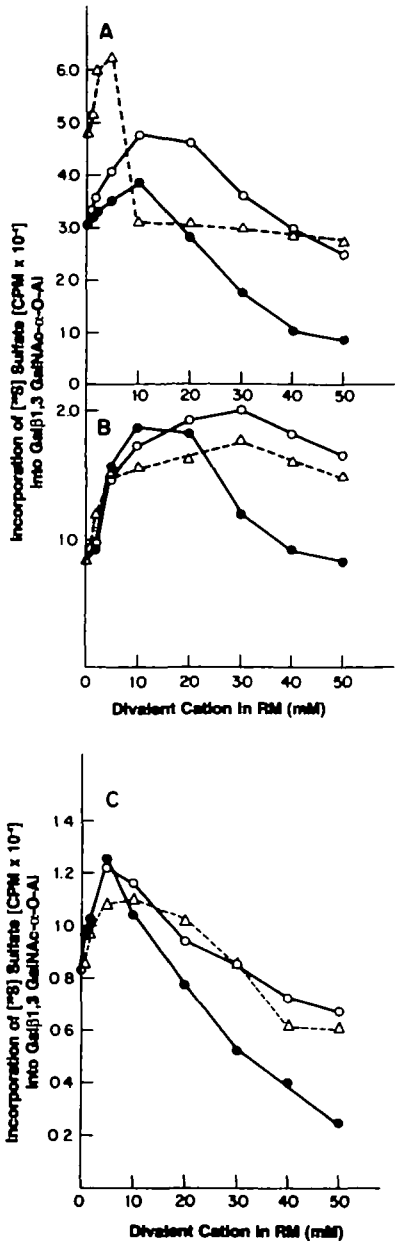

Fig. 4. The effect of the divalent cations Mg^{2+}, Mn^{2+}, and Ca^{2+} on Gal: 3-O-sulfotransferase activities. (A) BT 7764 as the enzyme source. ○, Mg^{2+}; •, Mn^{2+}; △, Ca^{2+}. (B) CN 23269 as the enzyme source (symbols as in A). (C) HS1 as the enzyme source (symbols as in A).

Clear distinction of two Gal: 3-O-sulfotransferases in human tissues utilizing the differential effect of Mn^{2+}

Activity of the Group A enzyme (BT 7764) was monitored with 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn acceptor while activity of the Group B enzyme (MOM 8057) was measured separately using three different acceptors, namely, Galβ1,3GalNAcα–O-Al, 3-O-MeGalβ1,4GlcNAcβ1, 6(Galβ1,3)GalNAcα–O-Bn and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn. As noted earlier, the activity of the Group A enzyme (BT 7764) declined with increasing concentrations of Mn^{2+}, reaching a 30% level at 50 mM.

Irrespective of the acceptor used, the Group B enzyme (MOM 8057) displayed similar activity profiles, reaching maximum stimulation (>2.0-fold activity) at 10 mM Mn^{2+} then dropping to the original level. These results further substantiate our earlier determination made from the measurement of the activity over a pH range that 3-O-sulfation of Gal linked β1,4 to GlcNAc or β1,3 to GalNAc (GlcNAc) is catalyzed by the same enzyme present in Group B sources and that this enzyme is quite different from the Group A enzyme.

Molecular size of Group A and Group B Gal: 3-O-sulfotransferases

We know thus far that Group A and Group B Gal: 3-O-sulfotransferases are two distinct enzymes. So it was interesting to know whether they are also different in molecular size. We have chosen arbitrarily BT 7764 to represent Group A (Figure 5A) and MOM 8057 to represent Group B (Figure 5B) for this experiment. A measurement of their activities in the effluent fractions from Sephacryl S-100 HR column indicated that there was no apparent difference in either their elution positions or elution profiles from the column. Both were slightly included in the column as evident from the void volume measured with Blue Dextran 2000. Both appeared to be much larger than BSA (MW 66,000 Da) used as a reference in this column chromatography. These results would suggest that these two distinct enzymes may be related at a molecular level.

Further distinction of Group A and Group B Gal: 3-O-sulfotransferases through acceptor competition (for details, see Materials and methods*)*

When Group A enzyme (BT 7764) activity was measured with Galβ1,3GalNAcα–O-Al in the presence of varying concentrations (1–10 mM) of 3-sulfoGalβ1,4 GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn, Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3) GalNAcα–O-Bn, or Galβ-O-Bn (Figure 6A), it was found that only 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn, which has the T-hapten structure (Galβ1,3GalNAc) available for enzyme action, served as a competitive inhibitor (Ki: 4.76 mM) (see Figure 6A,B), whereas the other acceptors did not show any inhibition of this enzyme activity. These results would imply that this Group A enzyme is entirely specific for the T-hapten structure.

Group B enzyme (MOM 8057) activity was followed with the Galβ1,3GalNAcα–O-Al acceptor in the presence of varying concentrations (1–10 mM) of 3-sulfo Galβ1, 4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn, Galβ1,4GlcNAcβ1, 6(3-sulfoGalβ1,3)GalNAcα–O-Bn, Galβ1,4GlcNAcβ-O-Bn, or Galβ-O-Bn (see Figure 6C). All four compounds served as competitive inhibitors for this enzyme (see Figure 6C,D). The most effective competitive inhibitors were the last three compounds (Ki: 2.27 mM, 2.99 mM, and 1.83 mM, respectively), whereas the first compound, which served as the only competitive inhibitor for the Group A enzyme, was the least effective competitive inhibitor for the Group B enzyme (Ki: 6.90 mM) (Figure 6D).

The influence of sulfated acceptors on the Km of neutral acceptors for Gal: 3-O-sulfotransferases (see Materials and methods *for details)*

BT 7764 and BT 8640 were used as the Group A enzyme sources. The Km for the neutral acceptor 3-O-MeGalβ1, 4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn, which is specific for the Group A enzyme, was measured in the presence of the

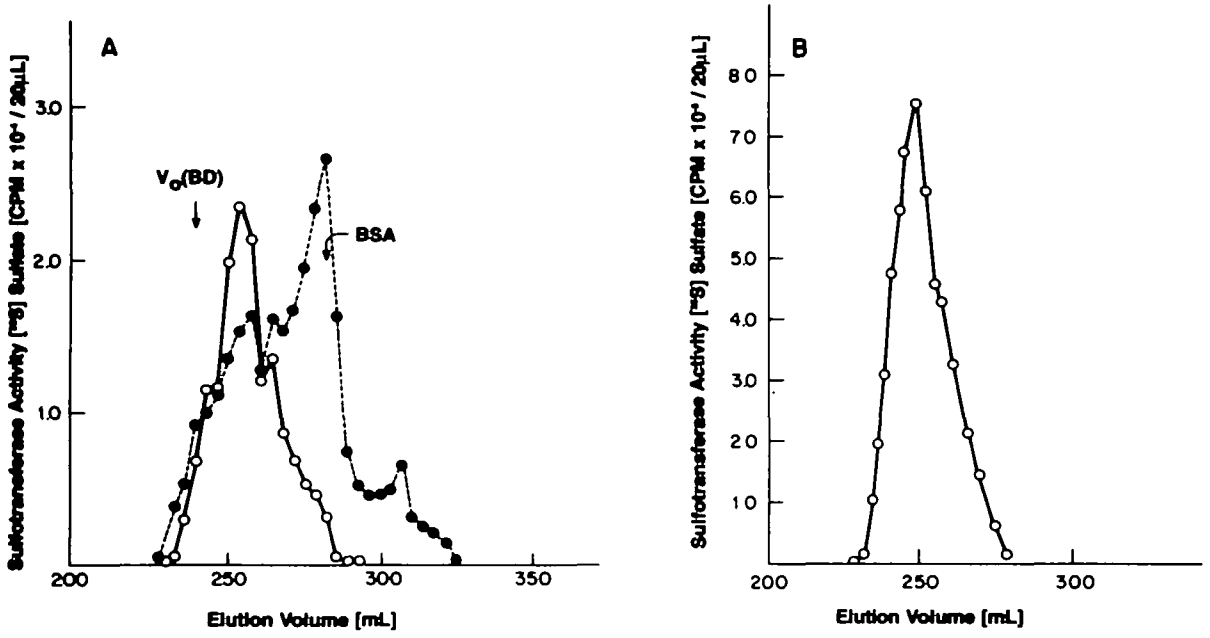

Fig. 5. Chromatography of Gal: 3-O-sulfotransferases on Sephacryl S-100 HR column. (A) Group A: BT 7764 enzyme activity (O); protein by BCA method (•). (B) Group B: MOM 8057 enzyme activity (O).

sulfated acceptor, 3-sulfoGalβ1,4 GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn (4.0 mM), which is also specific for this enzyme. When BT 7764 was the enzyme source (see Figure 7A,B) the Km for the neutral acceptor increased from 1.33 mM to 8.00 mM in presence of the sulfated acceptor and with BT 8640 (see Figure 7C,D), Km increased from 1.48 mM to 8.00 mM. The results indicate that this sulfated acceptor competes well with the neutral acceptor for the Group A enzyme.

For the study of Group B, CT 8059 was used as the enzyme source. Both 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3) GalNAcα–O-Bn were separately used as neutral acceptors to study this enzyme.

The Km for Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3) GalNAcα–O-Bn was measured in presence of the sulfated acceptors (4.0 mM) Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3) GalNAcα–O-Bn and 3-sulfoGalβ1,4 GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn, separately (Figure 8A,B). Only the former sulfated acceptor exhibited competitive inhibition (Km increased from 5.0 mM to 13.3 mM) (Figure 8B), whereas the other sulfated acceptor did not inhibit at all.

The Km for 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn which is not a specific acceptor for this enzyme was also measured as above (Figure 8C). As seen with the other neutral acceptor, only Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3)GalNAcα–O-Bn, which is specific for this enzyme, served as a competitive inhibitor. Km for this neutral acceptor in the absence of this specific sulfated acceptor was found to be 6.7 mM (Figure 8D). But, in the presence of the specific sulfated acceptor (4.0 mM), Km for the nonspecific neutral acceptor increased from 6.7 mM to 20.0 mM as determined by using concentrations >1.5 mM, and it decreased from 6.7 mM to 0.8 mM when determined by using concentrations <1.5 mM (Figure 8D). These results indicate the phenomenon of competition at high concentration and noncompetition at low concentration.

Competition of branched chain mucin type neutral acceptors for Group B Gal: 3-O-sulfotransferase

CN 23269 was used as the enzyme source and the activity was measured with the Galβ1,3GalNAcα–O-Al acceptor in presence of Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3) GalNAcα–O-Bn and 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn, separately (Figure 9). It was found that the former branched structure was far better than the latter in its competing ability for the enzyme. The results indicate that Group B enzyme specificity is directed toward LacNAc based structures, rather than T-hapten.

In consistence with the results of the present study, it was reported earlier (Kuhns *et al.*, 1995) that the sulfotransferase of rat colonic mucosa transfers sulfate to the 3-position of Gal in Galβ1,3GalNAcα–OBn. The present study provided several lines of evidence for the existence of at least two distinct Gal: 3-O-sulfotransferases in human tissues and, probably, a third distinct enzyme in human serum. The utilization of disaccharide allyl glycosides as well as branched chain mucin-type tetrasaccharides having a blocking group on C-3 of either Gal as the acceptors for screening several human tissue specimens convinced us unequivocally of the existence of two distinct types of Gal: 3-O-sulfotransferases in human. One (Group A) was specific for Galβ1,3GalNAcα– (the blood group T-hapten), and the other (Group B) was specific for Galβ1, 4GlcNAc, which occurs as the β1,6 branch from blood group T-hapten. The specificity of Group A enzyme, which is directed toward the T-hapten, became further evident from the finding that allyl or benzyl β-galactosides (monosaccharide acceptors) did not serve as acceptors for the Group A enzyme, whereas these acceptors were splendidly active with the Group B enzyme. The serum enzyme was far more active with Galβ1,3GalNAcα–O-Al than with either Galβ1,4GlcNAcβ-O-Al or Galβ1,3GlcNAcβ-O-Al. In contrast to the Group A enzyme, which showed the same level of activity even when the T-hapten is part of branched chain mucin-type structure, the

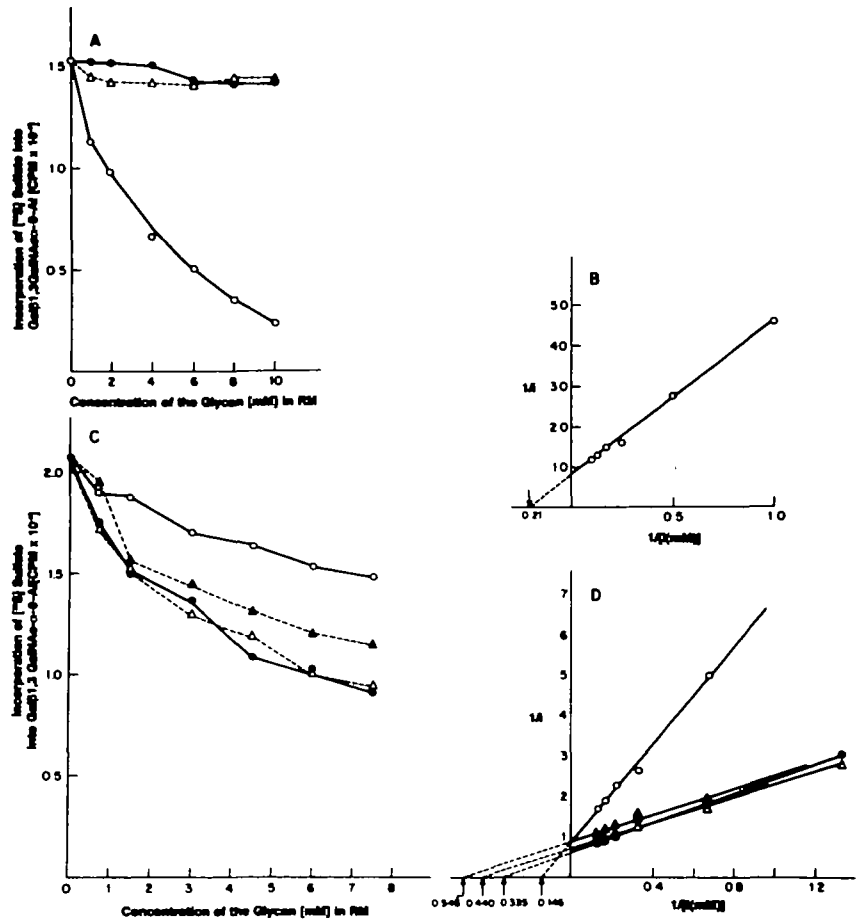

Fig. 6. Acceptor competition for Group A and Group B Gal: 3-O-sulfotransferases. (A) BT 7764 enzyme activity using Galβ1,3GalNAcα–O-Al as the acceptor in presence of increasing concentration of 3-sulfoGalβ1,4GlcNAcβ1,6 (Galβ1,3)GalNAcα-O-Bn (○), Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3) GalNAcα–O-Bn (●), and Galβ-O-Bn (△). (B) Lineweaver-Burke plot to determine Ki for the inhibition of BT 7764 activity by 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn. (C) MOM 8057 activity using Galβ1,3GalNAcα–O-Al as the acceptor in presence of increasing concentrations of 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα– O-Bn (○), Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3)GalNAcα–O-Bn (●), Galβ1,4GlcNAcβ-O-Bn (▲), and Galβ-O-Bn (△). (D) Determination of Ki (Lineweaver-Burke plots) for the inhibition of MOM 8057 activity by the above acceptors (symbols as in C)

serum enzyme was only 14.0–40.4% active with the T-hapten occurring in a branched mucin chain. Further, the serum enzyme was 3- to 8-fold and 3- to 10-fold more active, respectively, with Galβ1,3GlcNAcβ-O-Al as compared to its activity toward Galβ1,4GlcNAcβ-O-Al and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn. The serum enzyme appears to be different from Group A and Group B since it prefers a β1,3 linked Gal in an unbranched chain for its activity. Thus it becomes a third category (Group C) of Gal: 3-O-sulfotransferase. It will be necessary to examine further the specificities of the serum enzyme with various acceptors having Galβ1,3GalNAcα/β- as terminals.

The distinct specificities of Group A and Group B enzymes were further illustrated by examining their interaction with high molecular weight glycoconjugates as the acceptors. Further distinction between Group A and Group B enzymes was demonstrated as follows. (1) Group A enzymes exhibited an optimal activity over a wide range of pH (pH 6.0–7.4), whereas, Group B displayed peak activity at pH 7.2. (2) Group A and Group B were stimulated by Mg^{2+} to different extents (55% and 150%, respectively). (3) Group B activity was stimulated to a level of 230% by Mn^{2+} (10–20 mM) and this stimulation dropped slowly reaching the original level of activity at 50 mM Mn^{2+}. On the contrary, Group A enzyme was stimulated only 20% by Mn^{2+} (10 mM). Activity was then inhibited, reaching a low level of 25% at 50 mM Mn^{2+}. (4) Ca^{2+} stimulated Group B activity by 100%, whereas, Group A was only stimulated by 30% at (5 mM) Ca^{2+}, declining to a level of 65% at higher concentrations. The serum enzyme behaved mostly like Group A toward these divalent metal ions. (5) Only 3-sulfo Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn and not Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3) GalNAcα–O-Bn acted as a competitive inhibitor for Group A enzyme. (6) Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3)GalNAcα-O-Bn was far more effective than 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn as a competitive inhibitor for Group B enzyme. (7) Group B enzyme displayed high affinity toward Galβ1,4 GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn, when compared with 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3) GalNAcα–O-Bn. (8) The Km of Group A enzymes toward the 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn acceptor increased ~6.0-fold in the presence of 3-sulfoGalβ1, 4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn. (9) The Km of Group B enzyme toward the Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn acceptor increased ~2.5-fold in the presence of Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3) Gal-

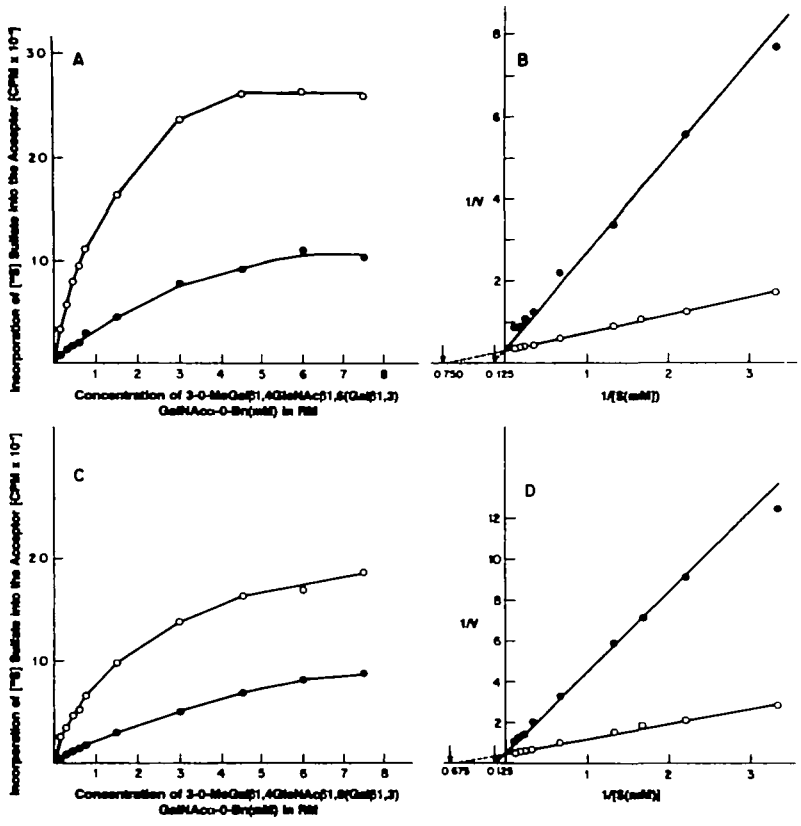

Fig. 7. Influence of sulfated acceptors on the Km of neutral acceptors for Group A Gal: 3-O-sulfotransferase. (**A**) BT 7764 activity as measured with increasing concentrations of 3-O-Me Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn in absence of (O) and in presence of (•) 3-O-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn (4.0 mM). (**B**) Determination of Km by Lineweaver-Burke plot (symbols as in **A**). (**C**) BT 8640 activity was measured under the conditions mentioned in (**A**) (symbols as in A). (**D**) Determination of Km (symbols as in **C**)

NAcα–O-Bn, but was not affected by 3-sulfoGalβ1, 4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn. (10) Galβ1, 4GlcNAcβ1,6(3-sulfoGalβ1,3)GalNAcα–O-Bn inhibited Group B enzyme activity as measured with the 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn acceptor.

The present study allowed a number of observations to be made on the influence of residues such as fucose or sulfate on the activity of Gal: 3-O-sulfotransferases. (1) C-6 substitution of GalNAc by either a methyl or GlcNAc moiety decreased the acceptor ability of Galβ1,3GalNAcα–O-Al toward Group A enzyme. (2) A fucose residue on the β1,6 branch did not affect Group A enzyme activity at all, whose specificity is directed toward the T-hapten. (3) Lewis x and Lewis a terminal structures were inactive as acceptors for Group A and also for Group B enzymes. (4) Elimination of activity toward Gal on the β1,6 branch by an α1,3 linked fucose residue did not prevent Group B activity on Gal linked β1,3 to GalNAcα–. (5) 3′-sulfation of the β1,6 branch did not affect Group A activity toward Gal linked β1,3 to GalNAcα–. (6) C-6 sulfation of GalNAc, in fact, increased to some extent the acceptor activity of the T-hapten, whereas C-6 sialylation dropped acceptor activity to 15%. (7) Group B activity on the β1,6 branch of the mucin-type acceptor was not affected when the β1,3 linked Gal had been 3-O-sulfated. (8) The most important observation, which may have great biological consequence, is the novel finding that Galβ1,4GlcNAcβ-O-Al and Galβ1,3GlcNAcβ-O-Al, upon C-6 sulfation of the GlcNAc moiety, became totally inactive (100%) and 5- to 7-fold active, respectively, in their ability to serve as acceptors for the Group B enzymes. (9) A termination of Group B activity on the β1,6 branch in a mucin-type acceptor due to a 6-sulfo group on the GlcNAc residue did not prevent this enzyme from acting upon the other terminal Gal.

Prior to the present investigation there were only a few studies attempting to understand the nature of those enzyme activities responsible for 3-O-sulfation of terminal β-galactose residues. The calf thyroid gland Gal: 3-O-sulfotransferase (Kato and Spiro, 1989) was shown to have an optimal activity at pH 7.0; it was distinctly stimulated by Mn^{2+}; Mg^{2+} could only partially substitute for Mn^{2+} while Ca^{2+} was inhibitory. The highest activity shown by this enzyme was toward Gal β1,4 linked to GlcNAc relative to only 7% toward methyl β-galactoside. The Gal: 3-O-sulfotransferase of human respiratory mucosa (Lo-Guidice *et al.*, 1995) was able to sulfate methyl galactosides exhibiting an optimal activity at pH 6.1 (MES) and at pH 6.4 (MOPS); Mn^{2+} stimulated this activity more than Mg^{2+} but Ca^{2+} was inhibitory. Human colorectal adenoma cells were found to have a sulfotransferase incorporating sulfate on the galactose residue of O-glycan Core 1 (Vavasseur *et al.*, 1994). Subsequently, rat colonic mucosa (Kuhns *et al.*, 1995) was shown to sulfate Galβ1,3GalNAcα– at the 3-position of the Gal residue. This activity was enhanced by divalent cations in the following order: $Sr^{2+} > Mn^{2+} > Ca^{2+} > Mg^{2+}$. This enzyme source also exhibited some sulfotransferase activity with Galβ1,4GlcNAc. The present study was able to clarify the relationship of various Gal: 3-O-sulfotransferase activities to each other by characterizing them as two distinct enzymes present in human tissues with a third

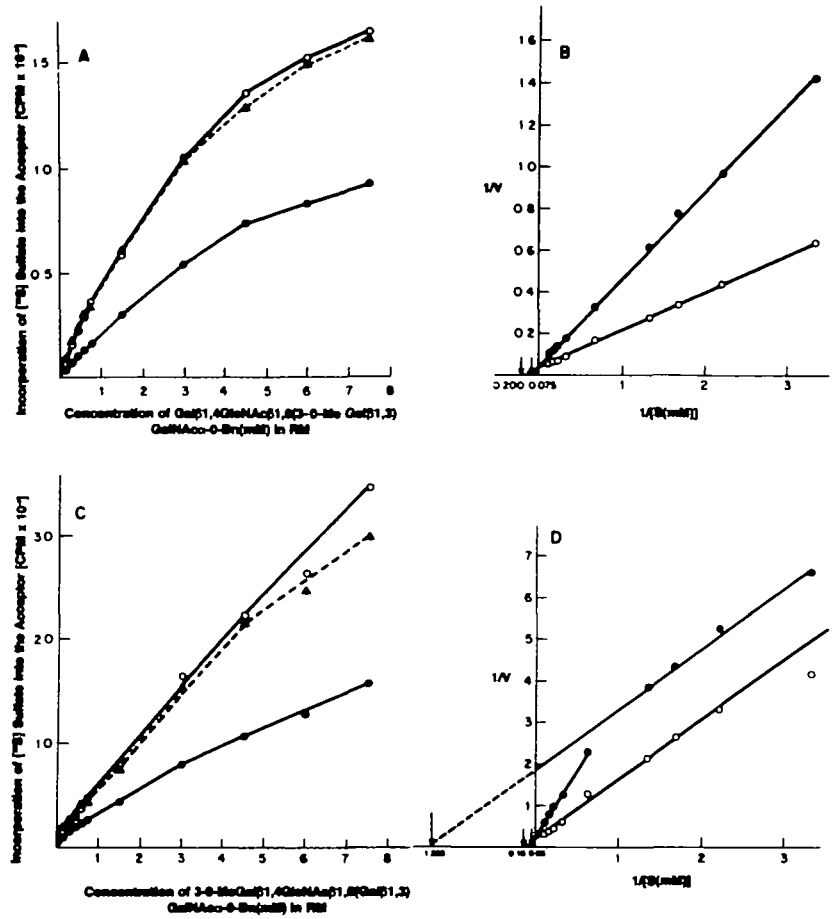

Fig. 8. Influence of sulfated acceptors on the Km of neutral acceptors for Group B Gal: 3-O-sulfotransferase. (**A**) CT 8059 activity as measured with increasing concentrations of Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn in absence (○) and in presence of either Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3)GalNAcα–O-Bn (4.0 mM) (•) or 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn (4.0 mM) (▲). (**B**) Determination of Km by Lineweaver-Burke plot (symbols as in A). (C) CT 8059 activity as measured with increasing concentrations of 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn in absence (○) and in presence of either Galβ1,4GlcNAcβ1,6(3-sulfoGalβ1,3)GalNAc–α-O-Bn (4.0 mM) (•) or 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα–O-Bn (4.0 mM) (▲). (**D**) Determination of Km by Lineweaver-Burke plot (symbols as in C).

enzyme apparent in human serum, differing in acceptor specificities, pH dependent activity profiles, and divalent metal ion activation. Furthermore, another important outcome of the present study is that both Group A and Group B Gal: 3-O-sulfotransferases are increased severalfold in breast and colon cancers, respectively. It has become evident that the two endothelial venule associated ligands for L-selectins, GLYCAM-1 and CD34, are O-linked glycoproteins containing fucose, sialic acid, and sulfate, the essential ingredients for binding with L-selectins (Imai *et al.*, 1991). So a knowledge gained on the specificities of sialyl- (Chandrasekaran *et al.*, 1995a), fucosyl- (Chandrasekaran *et al.*, 1995b, 1996a,b), and sulfotransferases would be helpful in the procurement of ligands for selectins in general.

We found in the present study that among the three types of Gal: 3-O-sulfotransferases identified, only one type is in association with a particular tissue. For example, the 3-O-sulfotransferase acting on Gal linked β1,4 to GlcNAc is present in colon tissue but absent in breast tissue, whereas, the other acting on Gal linked β1,3 to GalNAcα-O-Ser/Thr is present in breast tissue but absent in colon tissue. Furthermore, these enzymes show differences in metal ion activation, pH dependent activity profiles. There are numerous reports on the elevation of T antigen expression in breast cancer (Springer, 1984) and our present observation that 3-O-sulfotransferase exclusively acting on Galβ1,3GalNAcα-O-Ser/Thr is in elevated level in breast tumor tissue is a new insight in glycobiochemistry, indicating the possible involvement of this enzyme in terminating mucin carbohydrate chains. We have shown earlier that among the two lectins PNA and ABA specific for T-epitopes, the binding of ABA to the T-epitope is not affected by 3-O-sulfation of the Gal moiety in T-epitope (Chen *et al.* 1995). This data would indicate that the binding of at least some antibodies and lectins specific for the T-epitopes may not be affected by 3-O-sulfation on the Gal moiety, and so the existence of T-epitopes carrying the 3-O-sulfo group in breast cancer is an interesting possibility and an avenue opened up by our present study. The importance of the present observation in a study of cancer and metastasis lies on the fact, as postulated by Baenziger and his group (Hooper *et al.* 1996), that the addition of a sulfate moiety turns a relatively common structural motif into a unique carbohydrate with the potential to be recognized by a specific receptor or lectin. Myelin-associated glycoprotein recognizes NeuAcα2,3Galβ1,3GalNAc whereas CD33 and sialoadhesion bind both NeuAcα2,3Galβ1,3/4GlcNAc and NeuAcα2,3Galβ1,3GalNAc structures (Sgroi *et al.*

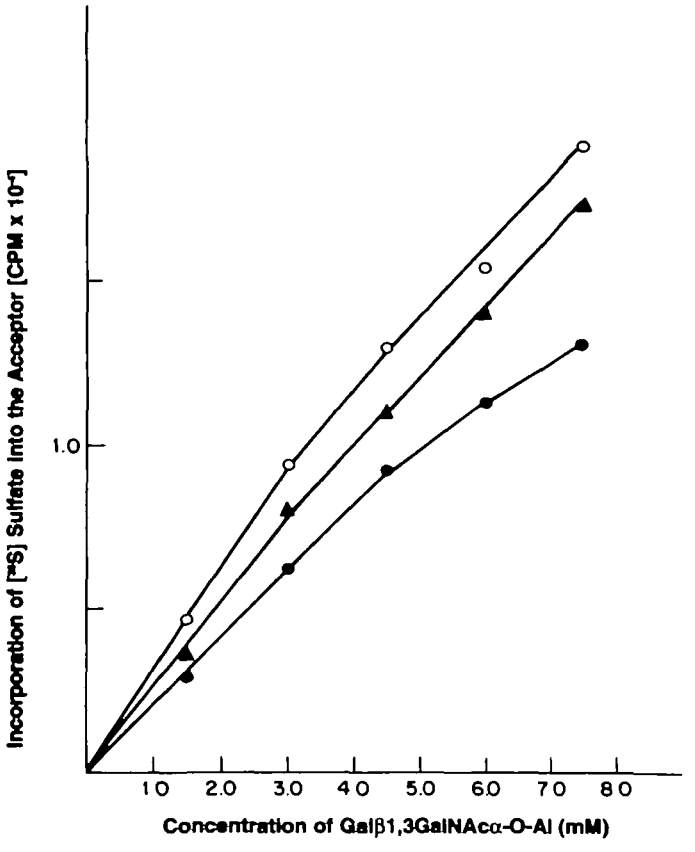

Fig. 9. Competition of branched mucin-type neutral acceptors for Group B Gal: 3-O-sulfotransferase CN 23269 enzyme activity as measured with Galβ1,3GalNAcα–O-Al in absence (O) and in presence of either Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3) GalNAcα–O-Bn (7.5 mM) (•) or 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα– O-Bn (7.5 mM) (▲).

1996). The elevated level of Gal: 3-O-sulfotransferase in tumors, as shown in the present study, would imply the importance of studying the influence of terminal 3-O-sulfoGal-containing structures on the binding of these mammalian lectins.

Recently, it was demonstrated (Sharma *et al.*, 1996) that *in vivo*, in transgenic mice and pigs, the expression of human α1,2-fucosyltransferase results in the production of H-antigen in the endothelial cells of multiple organs and results in a dramatic decrease in the level of the Gal epitope and a reduction in the binding of xeno reactive natural antibodies and of subsequent complement activation. Hence, it is anticipated that the expression of human Gal 3-O-sulfotransferase (Group B enzyme) instead of human α1,2-FT in transgenic mice and pigs would bring out the same kind of results and may serve as a useful enzyme in the area of organ transplantation.

The discovery of GLYCAM-1 and CD_{34}, the two sulfated glycoproteins, which are natural ligands for L-selectin, led to an immense interest in sulfotransferases. Based upon the present study on the specificities of Gal: 3-O-sulfotransferase, we synthesized Galβ1,4(Fucα1,3)GlcNAcβ1,6(3-O-sulfoGalβ1,3) GalNAcα-0-Bn which proved to be far superior to sialyl Lewis x in inhibiting L-selectin binding (Koenig *et al.*, 1996). To the best of our knowledge, this is the first example of a sulfated branched structure serving as a potential inhibitor of L-selectin binding. In this context it is of interest to note that sulfated blood group Lewis[a] was identified as a superior ligand for human E-selectin (Yuen *et al.*, 1994).

Materials and methods

Sera from breast and colon cancer patients and normal individuals were collected at Roswell Park Cancer Institute. Fresh serum (stored on ice for <1 h) was adjusted to contain 2% Triton X-100 and 0.1 M Tris-Maleate pH 6.3 before storing at −20°C until use. This precautionary measure was undertaken to prevent any apparent loss of sulfotransferase activity upon freezing a liquid sample.

Six breast tumors (BT 7549, BT 7764, BT 7803, BT 8565, BT 8640, BT 8600), three normal breast specimens (BN 7869, BN 7764, BN 7803), four ovarian tumors (OT 7770, OT 7753, OT 7915, OT 7827), seven metastatic ovary specimens (MO 7885, MO 7855, MO 8207, MO 8157, MO 8066, MO 8237, MO 8022), three endometrial tumors (EMT 7588, EMT 8653, EMT 7979), one omentum tumor (OMT 7770), one metastatic omentum specimen (MOM 8057), and five colon tumors (CT 8458, CT 8661, CT 8059, CT 8467, CT 8365) were obtained during surgical procedures from patients of Roswell Park Cancer Institute and were stored frozen within 1 h at −70°C until use. A large tissue sample (ovarian tumor from patient PW) was already available in our laboratory (kept frozen at −70°C).

Seven normal colon specimens (CN 2845, CN 23273, CN 23269, CN 25385, CN 28375, CN 2914, CN 3165), which had been snap-frozen within 1 h after surgical removal, were obtained from the National Disease Research Interchange, Philadelphia. These tissues were transported to us on dry ice and then stored at −70°C.

The tissue samples ranging in weight from 1 to 2 g (an exact weight was obtained in each case) were homogenized at 4°C with 4 volumes of 0.1 M Tris-Maleate pH 6.3 using Kinematica. After adjusting the concentration of Triton X-100 to 2% these homogenates were mixed in the cold room for 1 h using Speci-Mix (Thermolyne) and then centrifuged at 20,000 × *g* for 1 h at 4°C. The clear fat-free supernatant was stored frozen at −20°C until use.

Synthetic compounds

The synthesis of many of the compounds used in the present study are already described (Jain *et al.*, 1993; Chandrasekaran *et al.*, 1995a; Jain and Matta, 1996). Details on the synthesis of Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-OBn structures bearing a C-3 block (methyl or sulfate group) on either Gal and other structures employed will be reported elsewhere.

Assay of sulfotransferase

The incubation mixtures run in duplicate contained 100 mM Tris-maleate pH 6.3, 5 mM Mg Acetate, 5 mM ATP, 10 mM NaF, 10 mM BAL, 7.5 mM acceptor (unless otherwise stated) and 0.5 μCi of PAPS (2.4 Ci/mmol) and 10 μl of the tissue extract (50–100 μg protein) in a total volume of 30 μl; the control incubation mixtures contained everything except the acceptor. Incubation was carried out for 2 h at 37°C. Depending on the acceptor used in the incubation, either Dowex-1-Cl fractionation or TLC was employed to measure the radioactive product.

Dowex-1-Cl method (Chandrasekaran et al., *1992)*

The incubation mixture was diluted with 1.0 ml water and passed through a Dowex-1-Cl column (1 ml in a Pasteur pipette). After washing the column twice with 1.0 ml water, the radioactive product ([^{35}S] sulfated compound) was eluted from the column with 3.0 ml of 0.2 M NaCl and its radioactivity measured using 3a70 scintillation mixture (Research Products International, Mount Prospects, IL) and a Beckman LS 9000 instrument. The 0.2 M NaCl eluate from the control reaction mixture (containing no acceptor) always had negligible amount of radioactivity (<100 cpm). The values for the duplicate runs did not vary more than 5%.

TLC method (Chandrasekaran et al., *1995a,b)*

The reaction mixtures (30 ml) were subjected to thin layer chromatography (silica gel GHLF; 250 μm, scored 20 × 20 cm; Analtech, NJ) after a quantitative transfer as 2 cm streaks. The solvent system, chloroform–methanol–water (5:4:1, v/v), was used to separate the [^{35}S] sulfated allyl or benzyl glycoside (migrated >12 cm from the origin) from the much slower migrating [^{35}S] PAPS (<3 cm from the origin). The radioactive content of 1/2 cm width segments of silica scraped into scintillation vials and soaked in 2 ml of water was determined by liquid scintillation. Variation in the values for reaction mixtures run in duplicate were all within 5%.

Isolation and identification of the [^{35}S] sulfate containing products

The synthetic compounds (3.0 μmol each) Galβ1,3GalNAcα-O-Al, Galβ1,3GlcNAcβ-O-Al, GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn,

Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, and Galβ1,4(Fucα1,3) GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Me and also O-glycosidic asialo glycopeptide isolated by us from fetuin (fetuin asialo GP-D-B: 2.0 mg) were incubated separately in 400 μl reaction volume with 100 μl of OT (PW) extract, as described above, for 15 h at 37°C. Each reaction mixture was then diluted with 1.0 ml water and subjected to Dowex-1-Cl fractionation as described above. The 0.2 M NaCl eluates (3 ml in each case) were lyophilized to dryness, each was dissolved in 1.0 ml water and subjected to chromatography on a Biogel P2 column (1.0 × 116.0 cm) using 0.1 M pyridine acetate pH 5.4 as the eluent. The effluent fractions were monitored for radioactivity and the peak containing radioactivity was collected, lyophilized to dryness, dissolved in 200 μl of water, and stored frozen at −20°C for further experimentation.

Aliquots of the above preparations (10–20 μl) were subjected to TLC on silica gel GHLF, as described, in n-butanol:acetic acid:water (3:2:1) solvent using the appropriate synthetic standards (3-O-sulfoGalβ1,3GalNAcα-O-Al; 6-O-sulfoGalβ1,3GalNAcα-O-Al; 3-O-sulfoGalβ1,3GlcNAcβ-O-Al or 6-O-sulfoGalβ1,3 GlcNAcβ-O-Al) along with the acceptor compounds. The authentic standards were located on the TLC plates by spraying with sulfuric acid in ethanol and heating at 100°C. The radioactive compounds were located by scraping 0.5 cm width segments of gel for liquid scintillation counting (see above) as well as by fluorography at −70°C using X-Omat AR film (Eastman Kodak) after spraying the TLC plates with Enhance (Dupont).

Macromolecular and natural acceptors

Acrylamide copolymer of Galβ1,3GalNAcα-O-Al, synthesized by the procedure of Horejsi *et al.* (1978), bovine IgG diantennary glycopeptide and fetuin triantennary asialoglycopeptide were available from earlier studies of this laboratory (Chandrasekaran et al., 1994a,b, 1995a,b). Fetuin O-glycosidic glycopeptide was available in our laboratory as a fraction separated from fetuin N-glycosidic glycopeptides by Biogel P6 column chromatography. This glycopeptide was desialylated by heating at 80°C in 0.1 N HCl for 1 h and then isolated by chromatography of the neutralized solution on a Biogel P2 column (1.0 × 116.0 cm) to remove the free sialic acid. Anti-freeze glycoprotein was a generous gift from Dr. Robert E. Feeny, University of California, Davis

Effect of pH and divalent cations on Gal: 3-O-sulfotransferase activity

(1) Tris-maleate buffer in the range pH 5.2–8.4 (final concentration in reaction mixture:0.10 M) was used under the standard incubation conditions to follow the sulfotransferase activity present in BT 7549, BT 7764, MO 8207, and MOM 8057 using Galβ1,3GalNAcα-O-Al as the acceptor, and in CN 23269 using Galβ1,3GalNAcα–O-Al, Galβ1,4GlcNAcβ-O-Al, and Galβ1, 3GlcNAcβ-O-Al as the acceptors for the reasons stated in *Results and discussion.*

(2) For seeing the effect of divalent cations on Gal: 3-O-sulfotransferase activity the incubation mixture contained varying concentration (1–50 mM) of Mg acetate, Mn acetate or Ca acetate under the standard incubation conditions. BT 7764, CN 23269, and human sera HS1 were used as the enzyme sources. In order to differentiate the effect of Mn^{2+} on the two different enzyme sources BT 7764 and MOM 8057, incubation in presence of Mn acetate (1–50 mM) was carried out with 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn as the acceptor for BT 7764 and with three acceptors, namely, Galβ1,3GalNAcα-O-Al, 3-O-MeGalβ1,4GlcNAcβ1,6 (Galβ1,3)GalNAcα-O-Bn and Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα-O-Bn for MOM 8057 under the standard incubation conditions.

The molecular size of Gal: 3-O-sulfotransferase

The Gal: 3-O-sulfotransferase activities present in BT 7764 and MOM 8057 were subjected to gel filtration in order to understand whether there is any significant difference between the two species. For this purpose 5 ml each of BT 7764 and MOM 8057 extracts were subjected to chromatography separately on a Sephacryl S-100 HR column (2.5 × 114 cm) equilibrated and eluted with 0.1 M Tris Maleate pH 6.3 containing 0.1% Triton X-100. Blue dextran 2000 and BSA (66,000 Da) were used as markers. Protein was assayed by the BCA micromethod (Pierce Chemical Co.). Fractions of 2 ml at a flow rate of 6 ml per h were collected and 20 μl of the alternate fractions were assayed for sulfotransferase activity using Galβ1,3GalNAcα-O-Al as the acceptor.

Testing for competitive inhibition

(1) The effect on BT 7764 Gal: 3-O-sulfotransferase activity by 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, Galβ1,4GlcNAcβ1,6(3-sulfo Galβ1,3)GalNAcα-O-Bn, and Galβ-O-Bn

For studying the effects we took advantage of the fact that only the [^{35}S] sulfated product from the Galβ1,3GalNAcα-O-Al acceptor can be eluted from the Dowex-1-Cl column. Products of the other three acceptors stayed with the column. The concentration of Galβ1,3GalNAcα-O-Al in the reaction mixture was left constant (5 mM) and that of the tested compound was varied from 1 mM to 10 mM and incubated under the standard conditions.

(2) The effect on MOM 8057 Gal: 3-O-sulfotransferase activity by 3-sulfoGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn, Galβ1,4GlcNAcβ1,6(3-sulfo Galβ1,3)GalNAcα-O-Bn, Galβ-O-Bn, and Galβ1,4GlcNAcβ-O-Bn.

As stated in (1), only the [^{35}S] sulfated product from Galβ1,3GalNAcα-O-Al is eluted from Dowex-1-Cl column under the experimental conditions. The reaction mixture contained Galβ1,3GalNAcα-O-Al and the compound under test, as stated above in (1).

The influence of sulfated acceptors on the Km of the neutral acceptors for Gal: 3-O-sulfotransferases

(1) The Km for 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn was determined both in the presence and absence of the competitive inhibitor, 3-sulfo Galβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn (4.0 mM in the reaction mixture), with BT 7764 and BT 8640 as the enzyme sources, separately. The radioactive product from the former was eluted from the Dowex-1-Cl column by 0.2 M NaCl while the latter remained with the column.

(2) The Km values for Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn and 3-O-MeGalβ1,4GlcNAcβ1,6(Galβ1,3)GalNAcα-O-Bn were determined both in the presence and absence of Galβ1,4GlcNAcβ1,6(3-Sulfo Galβ1,3)GalNAcα–O-Bn (4 mM in the reaction mixture) or 3-sulfoGalβ1,4 GlcNAcβ1,6(Galβ1,3)GalNAc-α-O–Bn (4 mM in the reaction mixture) with CT 8059 as the enzyme source.

Competition of branched chain mucin type neutral acceptors for Group B Gal: 3-O-sulfotransferase present in CN 23269

The enzyme activity was followed by varying the concentration of Galβ1,3GalNAcα–O-Al (1.5–7.5 mM) in the absence and presence of Galβ1,4GlcNAcβ1,6(3-O-MeGalβ1,3)GalNAcα–O-Bn (7.5 mM) and 3-O-MeGalβ1,4GlcNAcβ1,6 (Galβ1,3)GalNAcα–O-Bn (7.5 mM) separately under the standard incubation conditions. The radioactive product from Galβ1,3GalNAcα–O-Al was measured after separation by TLC on silica gel GHLF, as described, in chloroform:methanol:water (13:6:1). Unreacted [^{35}S] PAPS remained at the origin. The [^{35}S] product from Galβ1,3GalNAcα–O-Al migrated 4–4.5 cm from the origin and the branched mucin-type acceptors migrated 2–2.5 cm.

Acknowledgments

We thank Dr. Harry Slocum, Dr. Karoly Toth, and Ms. Nancy Reska of Tissue Procurement Facility (RPCI) for their kind cooperation. The editorial assistance of Conrad Piskorz with the manuscript is deeply appreciated. We thank M. Steven Piver, M.D., Chief, Department of Gynecologic Oncology, for his encouragement. This investigation was supported by Grant No. CA63213 awarded by the National Institutes of Health.

Abbreviations

Al, allyl; Bn, benzyl; pNP, para–nitrophenyl; Me, methyl; TLC, thin layer chromatography; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; AA/CP, acrylamide copolymer; BAL, British anti-Lewisite (2,3-dimercaptopropanol).

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Received on November 8, 1996; revised on January 28, 1997; accepted on January 31, 1997