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Biosynthesis of GM1b and similar neolactoseries gangliosides by a partially purified chicken skeletal muscle sialyltransferase. Effect of sphingomyelin and acetylcholine

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An α2 → 3 glycolipid galactosyl sialyltransferase (SAT3/4) has been partially purified from embryonic chicken skeletal muscle. It is preserved in 50 mM Hepes buffer (pH 6.8) containing 1% Triton CF-54 and 20% glycerol at −70 °C for a period of 6 months without loss of activity. The SAT3/4 preparation transfers sialic acid to nLcOse$_4$Cer, nLcOse$_6$Cer and GgOse$_4$Cer with respective K_m values of 1.4, 0.83 and 0.45 mM. The activity is stimulated 2–3-fold at high substrate concentration and 6–8-fold at low substrate concentration; 0.01 and 0.005 μmol for asialo GM1 and 0.025 and 0.01 μmol for other glycolipids in the presence of phosphatidylcholine (PC) and sphingomyelin (SM) at an optimum concentration 0.75%. A higher concentration is inhibitory. SM from chicken muscle is more effective than that from bovine brain and the stimulation is qualitatively proportional to that of the saturated fatty acyl content of SM. Free fatty acids (palmitic and stearic), their sodium salts, other choline compounds including choline chloride, phosphorylcholine and acetylcholine either do not have any effect or are inhibitory. Acetylcholine, even in the presence of SM and PC, is strongly inhibitory (70%).

Introduction

Sialyltransferases comprise a group of enzymes that catalyze the sequential addition of sialic acid from the nucleotide sugar derivative to the non-reducing terminus of glycoconjugate acceptors. Gangliosides, glycoproteins and oligosaccharides may occur in a variety of structures, including NeuAcα(2 → 3/2 → 6)Gal β1 → 3GalNAc, NeuAcα(2 → 3/2 → 6)Gal β(1 → 3/1 → 4)GlcNAc, NeuAcα2 → 8NeuAcα2 → 3Galβ [1–7]. For each of these structures a separate SAT appears responsible for catalyzing the reaction [8]. β-Galactoside α2 → 3 SAT purified from human colorectal carcinoma [9] and rat liver [10] has specificity for terminal Galβ1 → 3GalNAc and Galβ(1 → 3/1 → 4)GlcNAc respectively [11], but the pure enzyme from porcine submaxillary gland [12] and human placenta [13] transfers sialic acid only to a terminal Galβ1 → 3GalNAc structure [14,12] (SAT4 as designated by Basu et al. [15]). A subsequent attempt to purify CMP-NeuAc : Gal β1 → 4 GlcNAc SAT (SAT3, Basu et al. [15]) from Triton X-100 extract of human placenta together with Galβ1 → 3GalNAcα2 → 3 SAT was unsuccessful. SAT3 activity was lost during the purification procedure; most likely because of detergent inactivation. This indicates that SAT3 differs from SAT4: an interpretation confirmed by differential antibody inhibition [12]. CMP-NeuAc : Galβ1 → 4GlcNAcα2 → 3 SAT has been characterized in embryonic chicken muscle [16] and has been successfully solubilized with preservation of activity for up to 6 months [17,18]. We abbreviate the enzyme as SAT3/4 since it has alternative acceptors

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Abbreviations: Hepes, 4-2 (hydroxyethyl)-1 piperazineethane sulfonic acid; GM1b, IV^3 NeuAc GgOse$_4$Cer; LM1, IV^3 NeuAc nLcOse$_4$Cer; nLcOse$_4$Cer, Gal β(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcCer; GgOse$_4$Cer, Gal β(1 → 3)GalNAcβ(1 → 4)Galβ(1 → 4) GlcCer;nLcOse$_6$Cer, V^4 Gal IV^{3}GlcNAc-nLcOse$_4$Cer; PC, phosphatidylcholine; SM, sphingomyelin; PS, phosphatidylserine; SAT, sialyltransferase. GM1, GD1a, GD1b and GT1b were designated following Svennerholm, L. (1963) J. Neurochem. 10, 613–623. GgOse$_4$Cer, LcOse$_4$Cer are short designations recommended by the IUPAC-IUB Commission on Lipid Nomenclature (1977) Eur. J. Biochem. 79, 11–21.

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transferring sialic acid to the 3-hydroxyl of terminal galactose of both the ganglio (β1-3Gal link to GalNAc) and the neolactotetraose (β1-4Gal link to GlcNAc) glycosphingolipids, and thus, has activities of both the SAT-3 and SAT-4 enzymes, as classified by Basu et al. [15]. The designation SAT3/4 allows for the possibilities that different SAT enzyme proteins or differential regulation determine substrate specificity. Studies employing membrane preparations and solubilized enzyme from chicken skeletal muscle [16–18] and brain [19] suggest that the same enzyme catalyzes the transfer reactions (i) lacto-*n*-tetraosylceramide: CMP-NeuAc → LM1, (ii) lacto-*n*-hexaosylceramide : CMP-NeuAc → NeuAc-nLcOse$_6$Cer and (iii) GgOse$_4$Cer (asialo GM1) : CMP-NeuAc → GM1b. The last reaction is particularly relevant since GM1b has recently been found in normal human brain [20].

We have partially purified and stabilized the β-galactoside ($\alpha 2 \rightarrow 3$) SAT(s) or SAT3/4 as a major preliminary step in achieving its purification to homogeneity as we explore its role in muscle development. Here we describe: (a) the partial purification and characterization of SAT3/4 from chicken muscle; (b) the stimulation of SAT3/4 activity by PC and SM and; (c) a possible physiological regulation by acetylcholine.

Materials and Methods

Eggs were obtained from the Animal Genetics Laboratory, University of Connecticut, Storrs, CT, and incubated in our laboratory. Radioactive CMP-sialic acid was purchased from Amersham, Arlington Heights, IL. Biosil-A was obtained from Bio-Rad, Richmond, CA. PC, PS, phosphatidylethanolamine, SM (containing different fatty acyl group), sphingosine, phosphatidylinositol, phosphorylcholine, palmitic and stearic acid and their sodium salts, choline chloride, acetylcholine, Triton CF-54, CMP-NeuAc and glycerol were obtained from Sigma, St. Louis, MO. Precoated TLC plates from E. Merck were obtained through A.H. Thomas and DEAE-Sephadex A 25 (40–120 mesh) was purchased from Pharmacia, Piscataway, NJ. Neuraminidase was obtained from Cooper Biomedical, Malvern, PA; 3% OV-275 and 3% OV-17 from Supelco, Bellefonte, PA; Sep-pak C18 cartridges from Waters Associates, Milford, MA, and BCA-reagent from Pierce, Rockford, IL. All neutral and acidic glycolipids as well as SM from bovine brain and chicken skeletal muscle were isolated and purified in our laboratory and found homogeneous by thin-layer chromatography using at least three different solvent systems. CDP-hexanolamine was a generous gift from Dr. J.C. Paulson, UCLA, Los Angeles, CA.

Preparation of asialo-GM1 and lacto-N-neohexaosylceramide. GM1 was purified from bovine brain [21]; sialosyl(*N*-acetyl and *N*-glycolyl)lacto-*N*-neohexaosyl ceramide and lacto-*N*-neotetraosylceramide were isolated and purified from bovine erythrocytes [22]. All gangliosides were desialylated with 0.5 N formic acid and the asialo compounds were purified as described earlier [16].

Purification of muscle sialyltransferase. SATs from skeletal muscle were solubilized as described earlier [18] and passed through a CDP-hexanolamine agarose column preequilibrated with 50 mM Hepes/1% Triton CF-54 (w/v). The column was washed thoroughly with the 50 mM Hepes buffer (pH 6.8) containing 1% Triton CF-54 and the enzyme was eluted using the same buffer containing 1 M NaCl and 20% glycerol (v/v). The enzyme was precipitated by addition of 60% ammonium sulfate, centrifuged at $15\,000 \times g$ for 30 min in a Sorvall RC5B centrifuge and the supernatant was carefully aspirated. The pellet was resuspended in Hepes/20% glycerol/1% Triton CF-54 and dialyzed overnight against the same buffer. The solution was centrifuged at $15\,000 \times g$ for 30 min and the clear supernatant was used as the source of the enzyme. The enzyme preparation was purified to 388-fold as compared to whole homogenate. Protein was assayed according to Smith et al. [23] with the following modification. Turbidity appeared after incubation at 37 °C and this was clarified by addition of 5–10 μl of 10% SDS in each tube prior to reading in a spectrophotometer.

The partially purified enzyme stored at −70 °C was assayed periodically at different intervals with asialo GM1 as substrate. The enzyme solution was maintained at 0–4 °C during the assay period (0–96 h) because a single freezing and thawing caused a 30% reduction in enzyme activity.

Assay system. Except where indicated differently, the incubation system contained the following components (in μmol): glycolipid substrate, 0.005–0.01 (for asialo GM1) and 0.01–0.025 (others); CMP-^{14}C NeuAc, 0.016 ($2 \cdot 10^6$ cpm/μmol); $MgCl_2$, 0.25; EDTA, 0.5 mM; Triton CF-54, 80 μg; and the enzyme preparation, 20–30 μg of protein in a final volume of 40 μl. Unless otherwise mentioned, all the phospholipids were added at a concentration of 0.25% (g/100 ml). After incubation at 37 °C for 2–3 h the reaction was terminated with the addition of 10 μl of chloroform/methanol, 1 : 2 (v/v) and the mixture was spotted on a Whatman 3MM paper and assayed by double chromatography [24] with 1% borax (descending) and chloroform/methanol/water, 60 : 40 : 9 (v/v, ascending). A separate assay method using a Sep-pak C18 cartridge is described below. An incubation system lacking glycolipid substrate served as control for activity with endogenous substrate. 50 mM HEPES buffer was added to the control and other samples whenever necessary to attain a final total volume of 55 μl.

Sep-pak assay method. At the end of the incubation, the tubes were transferred in ice, and the reaction mixture was brought to a final volume of 100 μl with

distilled water and mixed. Solvent (diethyl) ether (300 μl) was added, mixed thoroughly and centrifuged at 3000 rpm for 10 min. The upper layer was aspirated carefully and the lower layer washed again with an equal volume of ether. Excess ether was removed under a stream of nitrogen. To each tube, 250 μl of 0.1 M KCl solution was added, mixed by vortex and passed through a Sep-pak C18 cartridge previously equilibrated with 0.1 M KCl. The column was washed with 15 ml of distilled water and the radioactive glycolipid was eluted with 6.5 ml of chloroform/methanol, 2 : 1 (v/v). The eluate was dried under nitrogen, dissolved in 100 μl of chloroform/methanol, 2 : 1 (v/v), 60 μl spotted on 1 in. × 1 in. Whatman No. 3 chromatographic paper and counted in a Beckman LS-3133P counter.

Isolation and purification of radioactive products. After overnight incubation, the mixture was resolved through a Sep-pak cartridge and passed through a DEAE-Sephadex A25 (acetate form, 0.5 × 15 cm) column. The column was eluted successively with (i) 5 vol. of chloroform/methanol/water, 30 : 60 : 8 (v/v) followed by sodium acetate in the order of; (ii) 10 vol. of 0.0055 M (iii) 5 vol. of 0.025 M and (iv) 5 vol. of 0.055 M in chloroform/methanol/water 30 : 60 : 8 (v/v). After complete removal of solvent and dialysis, the radioactivity of the fractions was determined and their glycolipid content assayed by TLC. The radioactive glycolipid, in elution fraction (ii) was further purified via a Biosil-A column.

Identification of product by TLC and autoradiogram. An aliquot of the radioactive product containing 5000 cpm was mixed with authentic bovine brain GM1 (5 μg) and spotted on a TLC plate along with a standard of GM1 and a standard mixture of human brain gangliosides. After developing the plate with chloroform/methanol/0.25% $CaCl_2$, 60 : 40 : 9 (v/v) the plate was surveyed by autoradiography. The ganglioside standards were detected after spraying with resorcinol-HCl [25].

Neuraminidase treatment of the radioactive product. A portion (3000 cpm) of the enzymic product was dissolved in 50 mM acetate buffer (pH 5.0) containing 200 munit of neuraminidase from *Clostridium perfringens* (1 unit is the amount of enzyme which will liberate 1 μmol of neuraminic acid from sialosyllactose at 37 °C/min). The [^{14}C]NeuAc was characterized by descending paper chromatography with 1% borax as described previously [16].

Permethylation analysis. Approx. 50 μg of radioactive product was methylated and separated on an LH20 column [26,27]. The combined methylated glycolipid fractions were hydrolyzed with 0.7 N H_2SO_4 in 80% aqueous acetic acid [28] and acetylated according to Bjorndal et al. [29]. The partially-methylated alditol acetates were analyzed in a Perkin-Elmer 3920 gas chromatogram as described earlier [16].

TABLE I

Effect of glycerol concentration

TO = 0 h; T24 = 24 h; T48 = 48 h; T96 = 96 h. Note: Expressed as the means ± S.E. from a set of three experiments.

[a] Glycerol concentration (%)	Enzyme activity (nmol/mg protein per h)			
	T0	T24	T48	T96
–	6.0 ± 0.5	4.5 ± 0.3	1.5 ± 0.2	0.8 ± 0.1
12.5	5.5 ± 0.5	6.7 ± 0.6	6.3 ± 0.6	3.9 ± 0.4
25	5.9 ± 0.5	9.0 ± 0.8	6.3 ± 0.5	4.2 ± 0.5
37.5	5.7 ± 0.4	5.9 ± 0.5	3.3 ± 0.3	2.9 ± 0.3
50	5.3 ± 0.4	5.5 ± 0.4	3.3 ± 0.3	1.9 ± 0.2

[a] Enzyme contained 1% Triton CF-54.

Results

Stabilization of α 2,3-SAT. As was the case with the solubilized enzyme(s) [18] the enzyme preparation required 20% glycerol and 1% Triton CF-54 (Table I and II) for stability while an increased concentration of either was inhibitory. There were two apparent pH optima; one at pH 6.0 or more (cacodylate) and another near pH 7.0 (Hepes) (Table III). Hepes (pH 6.8) was used throughout the study since it enabled lengthy preservation. With our assay conditions, the enzyme was active for 2 weeks with a maximum activity at 1–2 days and decreasing thereafter. The reaction velocity was linear for up to 5 h. Both sodium chloride and ammonium sulfate, which are used during purification, inhibited the enzyme activity in proportion to concentration (results not shown).

PC and SM produced a similar activation, PS and sphingosine a lesser stimulation, and the other phospholipids were either ineffective or inhibitory (Table IV). The optimum concentration of both PC and SM (Figs. 1 and 2) was 0.75%, with any further increase in concentration inhibiting activity. A comparison of the effect of sphingomyelins of differing fatty acyl composi-

TABLE II

Effect of detergent concentration

TO = 0 h; T24 = 24 h; T48 = 48 h; T96 = 96 h. Note: Expressed as means ± S.E. from a set of three experiments.

[a] Detergent (Triton CF-54) concentration (%)	Enzyme activity (nmol/mg protein per h)			
	T0	T24	T48	T96
–	2.6 ± 0.3	1.5 ± 0.2	0	0
0.25	3.6 ± 0.3	4.8 ± 0.3	1.7 ± 0.2	1.1 ± 0.2
0.5	4.6 ± 0.4	7.0 ± 0.7	3.8 ± 0.3	2.8 ± 0.3
0.75	6.0 ± 0.6	8.3 ± 0.7	7.0 ± 0.5	5.1 ± 0.5
1	6.0 ± 0.5	8.7 ± 0.9	7.4 ± 0.5	5.2 ± 0.6
1.25	4.9 ± 0.4	7.7 ± 0.8	6.0 ± 0.6	3.8 ± 0.5
1.5	4.5 ± 0.4	6.5 ± 0.7	5.0 ± 0.4	3.0 ± 0.2

[a] Enzyme contained 20% glycerol.

TABLE III

Effect of buffer

T0 = 0 h; T24 = 24 h; T48 = 48 h; T96 = 96 h. Note: Expressed as the means ± S.E. from a set of three experiments.

pH	Buffer	Enzyme activity (nmol/mg protein per h)			
		T0	T24	T48	T96
5	cacodylate	3.7±0.4	3.7±0.3	2.5±0.2	1.3±0.2
5.5		4.9±0.5	6.2±0.6	4.0±0.4	1.6±0.2
6		5.1±0.4	7.1±0.7	5.4±0.6	4.8±0.5
6	Hepes	5.0±0.5	5.9±0.5	4.9±0.6	1.1±0.2
6.5		5.7±0.4	6.9±0.6	5.5±0.4	4.5±0.4
7		6.1±0.5	7.8±0.8	5.8±0.6	5.0±0.5
7	phosphate	4.6±0.4	5.6±0.6	4.7±0.5	3.4±0.4
7.5		3.3±0.4	5.0±0.5	4.2±0.4	2.5±0.3
8		3.3±0.3	3.8±0.4	2.9±0.2	0.9±0.2

TABLE IV

Effect of different choline compounds, sphingosine and fatty acids on SAT

Water soluble compounds were dissolved in Hepes buffer (pH 6.8) and added to the incubation mixture. Water-insoluble compounds were directly added to the enzyme solution prior to 3 h incubation. Note: Expressed as the means ± S.E. from a set of three experiments.

Compounds	Enzyme activity (nmol/mg protein per h)
Control	4.7±0.5
Phosphatidylcholine	27.8±2.8
Phosphatidylserine	6.4±0.8
Phosphatidylethanolamine	3.2±0.4
Phosphatidylinositol	2.8±0.3
Sphingomyelin	27.7±2.6
Sphingosine	10.2±1.5
Acetylcholine	1.2±0.2
Phosphorylcholine (Ca salt)	2.0±0.2
Choline chloride	2.7±0.2
Palmitic acid	4.7±0.4
Palmitic acid (Na salt)	4.2±0.4
Stearic acid	4.7±0.6
Stearic acid (Na salt)	4.4±0.4

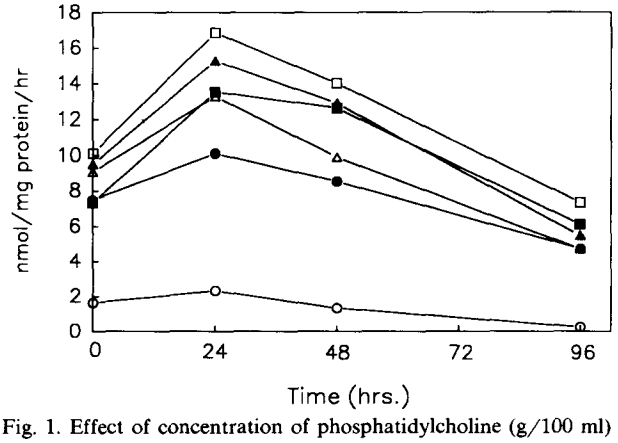

Fig. 1. Effect of concentration of phosphatidylcholine (g/100 ml) on sialyltransferase at different incubation duration. Control, (○); 0.1% (●); 0.25% (△); 0.5% (▲); 0.75% (□); 1.0% (■).

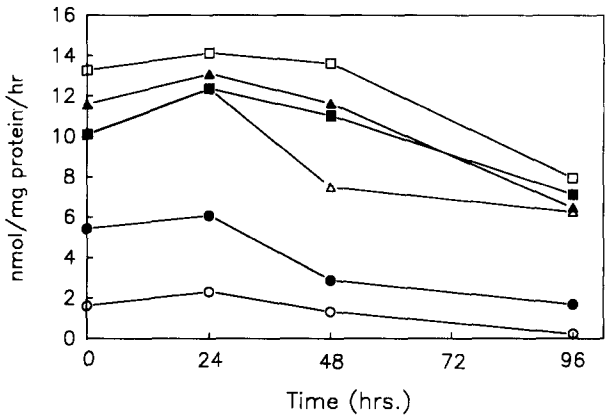

Fig. 2. Effect of sphingomyelin concentration (g/100 ml) on sialyltransferase at different incubation duration. Control, (○); 0.1% (●); 0.25% (△); 0.5% (▲); 0.75% (□); 1.0% (■).

tion upon SAT3/4 activity (Table V) suggested that SM containing saturated fatty acid had the greatest effect. Hence, SM from chicken skeletal muscle, which contains almost entirely saturated fatty acid (18 : 0, 16 : 0), was 1.5-times as effective as bovine brain SM containing 69% nervonic acid (C24 : 1).

K_m. The K_m values for $nLcOse_4Cer$, $nLcOse_6Cer$ and $GgOse_4Cer$ were calculated from the Michaelis-Menten equation using different substrate concentrations: 1.4 mM, 0.83 mM and 0.45 mM for $nLcOse_4Cer$, $nLcOse_6Cer$ and $GgOse_4Cer$, respectively (Fig. 3). Addition of 0.25% SM from bovine brain produced a shift of K_m to 0.9 mM, 0.63 mM and 0.25 mM (data not shown). A substrate competition study [30] suggested that the same enzyme transfers sialic acid to glycolipids containing terminal Galβ1 → 3GalNAc and Galβ1 → 4GlcNAc structure (Table VI).

Effect of sphingomyelin and acetylcholine. The effects of choline-containing compounds including PC, SM, choline chloride, phosphorylcholine, and acetylcholine as well as sphingosine, fatty acids and their sodium salts

TABLE V

Effect of sphingomyelin fatty acyl composition upon sialyltransferase activity

Note: Expressed as the mean ± S.E. from a set of three experiments.

Sphingomyelin	Nature of fatty acid	Enzyme activity (nmol/mg protein per h)
Control	–	4.0±0.5
Palmitoyl	palmitic acid (C16 : 0)	10.5±1.1
Oleyl	oleic acid (C18 : 1)	7.7±0.6
Lignoceryl (bovine erythrocyte)	lignoceric acid (C24 : 0)	10.3±1.2
Bovine brain	stearic and nervonic (C18 : 0, 24%) (C24 : 1, 69%)	7.8±0.8
Chicken muscle	palmitic and stearic (C16 : 0, 60%) (C18 : 0, 30%)	11.6±1.5

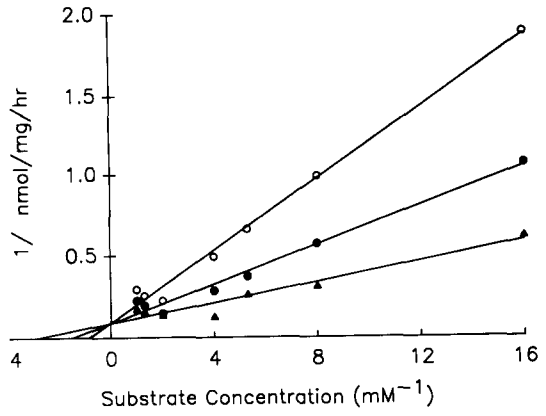

Fig. 3. Determination of K_m of nLcOse$_4$Cer, nLcOse$_6$Cer and GgOse$_4$Cer. nLcOse$_4$Cer, (○); nLcOse$_6$Cer, (●); GgOse$_4$Cer, (▲).

are summarized in Table IV. All of the choline-containing compounds except PC and SM and including acetylcholine were inhibitory. Acetylcholine inhibited even in the presence of SM and PC (Fig. 4). An increase in inhibition was observed with increasing concentration of acetylcholine to a maximum (70%) at a concentration of 1.82 mg/ml (100 μg) without further inhibition at higher concentrations.

^{14}C-product characterization. The enzyme product characterized after purification by DEAE-Sephadex and Biosil-A column chromatography was homogenous with R_F close to that of standard bovine brain GM1 (Fig. 5). In the absence of detergent, it was hydrolyzed by neuraminidase from *Clostridium perfringens* to yield a radioactive product with R_F identical to standard *N*-acetylneuraminic acid indicating that the sialic acid is attached to the terminal galactose of GgOse$_4$Cer. The partially methylated hexitol acetates yielded three peaks identified as 2,4,6-tri-*O*-methylgalactitol-1,3,5-triacetate, 2,3,6-tri-*O*-methylgalactitol-1,4,5-triacetate and 2,3,6-tri-*O*-methylglucitol 1,4,5-triacetate, respectively. The methylated aminosugar was identified as 4,6-di-*O*-methyl-2-deoxy-2-*N*-methylacetamidogalactitol-1,3,5-triacetate. The absence of 2,3,4,6-tetra-*O*-methylgalactitol-1,5-diacetate confirmed the linkage of sialic acid to the 3-position of the nonreducing end of the oligosaccharide.

Fig. 5. (a) Autoradiogram of the radioactive product. (b) Same plate after visualization with resorcinol spray. The plate was developed with $CHCl_3$/MeOH/0.25% $CaCl_2$, 60:40:9 (v/v). Lane 1: Authentic GM1 from bovine brain. Lane 2: Radioactive product with authentic GM1. Lane 3: Ganglioside mixture from human brain.

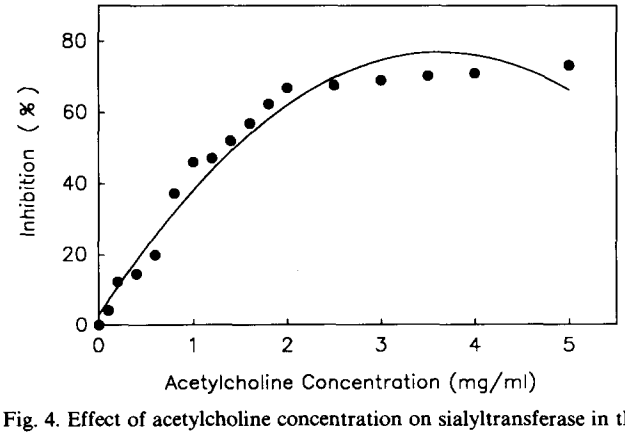

Fig. 4. Effect of acetylcholine concentration on sialyltransferase in the presence of PC and SM (0.25%).

TABLE VI

Glycolipid substrate competition study

x, $v_t = (V_a\ a/k_a + V_b\ b/k_b)/(1 + 1/k_a + b/k_b)$. y, $v_t = v_a + v_b$

Substrate	^{14}C-labeled product formed (nmol/mg protein per h)	Theoreticals for one enzyme ^{x}	Theoreticals for two enzyme ^{y}
nLcOse$_4$Cer	2.1		
nLcOse$_6$Cer	3.4		
GgOse$_4$Cer	7.9		
nLcOse$_4$Cer + nLcOse$_6$Cer	3.1	3.7	5.5
nLcOse$_4$Cer + GgOse$_4$Cer	5.1	4.5	10.0
nLcOse$_6$Cer + GgOse$_4$Cer	6.3	5.5	11.3

Discussion

The SAT that catalyzes transfer of NeuAc from CMP-NeuAc to glycosphingolipids with a nonreducing terminus of Galβ1 → 3,4GlcNAc or Galβ1 → 3 GalNAc has been partially purified and stabilized for up to six months: this should enable purification to homogeneity. Purified β-galactosideα2 → 3 SAT [9–14] transfers sialic acid to either a Gal β1 → 3 GalNAc or a Galβ(1 → 3/1 → 4)GlcNAc terminus. Hence, the chicken skeletal muscle SAT3/4 is within this compass and resembles SAT3 of chicken brain [15,19,31]. SM and PC stimulated the partially purified enzyme by 3–6-fold depending on substrate concentration. We found that SM (0.25%) containing saturated fatty acid stimulates more effectively than SM containing unsaturated fatty acids (Table V). This is consistent with the greater stimulation of SAT3/4 by chicken muscle SM (16 : 0, 0, 60% and 18 : 0, 30%) than by bovine brain SM (18 : 0, 24% and 24 : 1, 69%), whereas stimulation of SAT4 from porcine submaxillary gland by PC is reported to depend upon PC fatty acid chain-length [32]. The maximum stimulatory activity was observed between 24–48 h of incubation and subsequently decreased. We think that SM binding affects the enzyme conformation so as to increase its catalytic activity. The time course of SAT3/4 activity suggests that there is a relatively slow formation (over approx. 24 h) of a complex of enzyme with glycolipid and/or nucleotide sugar substrates with a subsequent decline in activity. We attribute this decrease to enzyme denaturation rather than degradation because no proteinase activity was found in our SAT3/4 preparation using ^{14}C-azocasein as substrate and our assay conditions (data not shown). We believe that a complex of SM is formed with the enzyme and/or the nucleotide sugar because of the radioactivity determined (using the double chromatography method) in the region of the product glycolipid in the control lacking acceptor glycolipid (but not in other controls), which indicates formation of a molecular aggregate. Only with the addition of PC or SM was this migration of a radioactive substance of larger size encountered. It is noteworthy that stimulation by PC of the SAT purified from porcine submaxillary gland has been related to enzyme-PC binding [32,33] since this is consistent with our interpretation. The mode of SM action has not yet been studied.

The assay efficacy of the Sep-pak C18 cartridge was very useful. Williams and McCluer [34] applied it to desalting and purifying glycolipids and it has been subsequently applied to assay of glycosidases [35] and of CMP-sialic acid: LacCer SAT (SAT 1) [36]. Though resolution of glycolipid products by double chromatography [24] has been used, the high background levels we found after phospholipid addition (PC and SM) could be easily avoided using Sep-pak C18 presumably because this adsorbent retains only the product GM1b and no other molecular adducts.

Since PC and SM are both cholinophospholipids, we sought to determine which moiety affected stimulation. Choline chloride and phosphorylcholine were inhibitory, indicating that neither choline phosphate nor choline alone would stimulate and the amphipathic phospholipid was necessary. No stimulation occurred with fatty acids (palmitic, stearic) and their salts. Sphingosine was approx. one-third as effective as SM.

The inhibition of activity by acetylcholine is intriguing. Acetylcholine (10 μmol/ml) produced 70% inhibition even in the presence of stimulating phospholipids (0.25%). No further increase in inhibition occurred with increasing concentrations of acetylcholine (to 25 μmol/ml) while there was approx. 73% inhibition in the absence of PC or SM with 15 μg (1.5 μmol/ml) of acetylcholine. Acetylcholine is synthesized and released from nerve terminals [37,38] and O'Brien et al. [39] found that acetylcholine damaged immature chicken muscle. SAT is present in synaptosomal and myelin membranes [40–42]. Ehrlich ascites tumor cells [43] and rat epididymis [44]. Synaptic membranes are particularly rich in sialoglycoconjugates which are predominantly gangliosides [45,46] and have been postulated to participate directly in impulse generation [46] with a role for synaptic sialylation [47–50]. Hence, the inhibition by acetylcholine suggests that sialylation may affect neuromuscular transmission or its maintenance.

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