

# Evidence for sialidase hydrolyzing gangliosides GM<sub>2</sub> and GM<sub>1</sub> in rat liver plasma membrane

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Rat liver plasma membrane removed sialic acid from mixed bovine brain gangliosides more efficiently than from sialyllactose and orosomucoid with an optimal pH of 4.5. When individual gangliosides, each labeled with [<sup>14</sup>C]sialic acid or [<sup>3</sup>H]sphingosine, were tested, not only GD<sub>1a</sub> and GM<sub>3</sub> but also GM<sub>2</sub> and GM<sub>1</sub>, both of which had been considered to resist mammalian sialidases, were desialylated. The products of GM<sub>2</sub> and GM<sub>1</sub> hydrolysis were identified as asialo-GM<sub>2</sub> and asialo-GM<sub>1</sub>, respectively, by thin-layer chromatography.

*Sialidase      Ganglioside      (Rat liver)      Plasma membrane*

## 1. INTRODUCTION

We previously demonstrated that in rat liver cytosolic sialidase differs from lysosomal (intralysosomal) sialidase in optimal pH and substrate specificity: the former hydrolyzes oligosaccharides, glycopeptides, glycoproteins and gangliosides at near neutral pH [1] while the latter hydrolyzes only oligosaccharides and glycopeptides at acidic pH [2]. According to [3], however, still another sialidase may be present in rat liver plasma membrane and desialylate gangliosides rather efficiently, although no study has since been made to identify the enzyme. The purpose of the present paper is to show that rat liver plasma membrane in fact possesses sialidase attacking not only GM<sub>3</sub> but also GM<sub>2</sub> and GM<sub>1</sub>, which have been shown to resist numbers of mammalian sialidases [4–6] including the one from rat liver cytosol [1].

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**Abbreviations:** NeuAc, *N*-acetylneuraminic acid; UDP-Gal, UDP-galactose; UDP-GalNAc, UDP-*N*-acetylgalactosamine

## 2. MATERIALS AND METHODS

### 2.1. Materials

Plasma membrane was isolated from the liver of male Wistar rats (200–250 g) according to [7]. Golgi membrane was isolated from rat liver and transplantable rat hepatoma (AH-109A, solid form) [8] as described in [9]. Standard gangliosides GM<sub>1</sub> (porcine brain), GM<sub>2</sub> (Tay-Sachs brain) and GD<sub>1a</sub> (bovine brain) were kindly provided by Dr A. Makita, Hokkaido University, Sapporo, Japan. GM<sub>1</sub> and GD<sub>1a</sub> were isolated from bovine brain mixed gangliosides (Sigma, St. Louis, MO) by chromatography on Iatrobeads (Iatron, Tokyo) [10]. GM<sub>3</sub> (human liver) and GM<sub>2</sub> (Tay-Sachs brain) were generous gifts from Drs Y. Suzuki and M. Matsumoto, Shizuoka College of Pharmacy, Shizuoka, and from Dr S. Handa, Tokyo Medical and Dental University, Tokyo, respectively. Lactosylceramide was from Calbiochem (La Jolla, CA) and  $\beta$ -*N*-acetylhexosaminidase (jackbean) from Seikagaku Kogyo (Tokyo). Asialo-GM<sub>1</sub> and asialo-GM<sub>2</sub> were prepared by hydrolyzing GM<sub>1</sub> and GM<sub>2</sub>, respectively, with *Arthrobacter ureafaciens* sialidase (Nakarai, Kyoto) in the presence of bile salts [11]. Silica gel thin-layer plates (Kieselgel

60) were purchased from Merck (Darmstadt). CMP-[ $^{14}\text{C}$ ]NeuAc was obtained from New England Nuclear (Boston, MA) and CMP-NeuAc prepared as described in [12]. UDP-Gal and UDP-GalNAc were the products of Sigma. For the sources of other materials, see [1].

## 2.2. Preparation of radioactive gangliosides

[ $^{14}\text{C}$ ]NeuAc-labeled gangliosides, then abbreviated [ $^{14}\text{C}$ ]gangliosides, were prepared according to [13] but with modifications. Golgi membranes were used as the sources of glycosyltransferases [14]. To obtain [ $^{14}\text{C}$ ]GM<sub>3</sub>, the incubation mixture (10 ml) contained CMP-[ $^{14}\text{C}$ ]NeuAc (40  $\mu\text{Ci}$ , 200 mCi/mmol), lactosylceramide (8  $\mu\text{mol}$ ), Triton X-100 (20 mg), sodium cacodylate (1 mmol, pH 6.2) and AH-109A Golgi membrane (28 mg). When GD<sub>1a</sub> labeled in the terminal sialic acid residue was being prepared, the reaction mixture (2.5 ml) contained CMP-[ $^{14}\text{C}$ ]NeuAc (10  $\mu\text{Ci}$ , 10 mCi/mmol), GM<sub>1</sub> (3  $\mu\text{mol}$ ), Tween 80-Triton CF-54 (15 mg, 1/2 by wt), sodium cacodylate (250  $\mu\text{mol}$ , pH 6.2) and liver Golgi (10 mg). After 20 h at 37°C, the mixture was lyophilized, added with 3 ml chloroform-methanol (2:1, v/v) and centrifuged. The extract was then evaporated under N<sub>2</sub> and eluted from a 2.0  $\times$  3.0 cm Sephadex G-25 column with chloroform-methanol-water (60:30:4.5). The radioactive iodine-positive fractions were pooled, evaporated, applied to a 0.25 mm thick silica gel 60 plate and developed with chloroform-methanol-water (60:35:8). The radioactive spot corresponding to GM<sub>3</sub> or GD<sub>1a</sub> was scraped off and extracted with chloroform-methanol-water (10:10:1). The yields of [ $^{14}\text{C}$ ]GM<sub>3</sub> and [ $^{14}\text{C}$ ]GD<sub>1a</sub> were  $2.0 \times 10^6$  and  $2.2 \times 10^5$  cpm, respectively. Half the amount of [ $^{14}\text{C}$ ]GM<sub>3</sub> formed was incubated with UDP-Gal (8  $\mu\text{mol}$ ), UDP-GalNAc (8  $\mu\text{mol}$ ), Triton X-100 (6 mg), Tween 80-Triton CF-54 (12 mg, 1/2 by wt), MnCl<sub>2</sub> (50  $\mu\text{mol}$ ), sodium cacodylate (300  $\mu\text{mol}$ , pH 7.3) and rat liver Golgi (10 mg) in 2.0 ml to obtain [ $^{14}\text{C}$ ]GM<sub>1</sub>. After 20 h at 37°C, the [ $^{14}\text{C}$ ]GM<sub>1</sub> formed was isolated as was [ $^{14}\text{C}$ ]GM<sub>3</sub>. Two-thirds of the radioactivity of [ $^{14}\text{C}$ ]GM<sub>3</sub> used was recovered as [ $^{14}\text{C}$ ]GM<sub>1</sub>. [ $^{14}\text{C}$ ]GM<sub>2</sub> was prepared by incubating half the amount of [ $^{14}\text{C}$ ]GM<sub>1</sub> formed with rat liver  $\beta$ -galactosidase (0.2 U) and sodium acetate (10  $\mu\text{mol}$ , pH 5.0) in 0.1 ml for 36 h at 37°C. The

$\beta$ -galactosidase used was solubilized from the lysosomal fraction [2] and eluted from a Con A-Sepharose column (equilibrated with 10 mM potassium phosphate, pH 6.8, containing 0.5 M NaCl) with 0.5 M methyl- $\alpha$ -mannoside. All these radioactive gangliosides were pure as far as the form of ganglioside was concerned and were diluted to 500 cpm/nmol (GM<sub>3</sub> and GD<sub>1a</sub>) and 1000 cpm/nmol (GM<sub>1</sub> and GM<sub>2</sub>) with the nonradioactive gangliosides before use. [ $^3\text{H}$ ]Sphingosine-labeled GM<sub>1</sub>, GM<sub>2</sub> and GM<sub>3</sub> were obtained according to [15]. Each of the gangliosides was diluted to 500–3000 cpm/nmol with the corresponding ganglioside.

## 2.3. Assay of sialidase activity

When [ $^{14}\text{C}$ ]gangliosides were used as substrate, the standard assay mixture contained 5–20 nmol substrate, 0.1 mg sodium deoxycholate, 5  $\mu\text{mol}$  sodium acetate, pH 4.5, and 50–300  $\mu\text{g}$  plasma membrane in 0.1 ml. The mixture was incubated at 37°C for 30–120 min, and the sialic acid released was determined according to [16]. For the assay with [ $^3\text{H}$ ]gangliosides as substrate, [ $^3\text{H}$ ]ganglioside (500 cpm/nmol) replaced  $^{14}\text{C}$ -substrate and the asialo  $^3\text{H}$ -product was quantitated. After incubation, the reaction mixture was partitioned by adding 0.3 ml water and 2 ml chloroform-methanol (2:1). The lower phase was washed with two-thirds of its volume of chloroform-methanol-water (3:48:47), evaporated and chromatographed on a silica gel plate using chloroform-methanol-water (65:25:4) as developer. The area corresponding to asialo  $^3\text{H}$ -product was scraped off and counted in a liquid scintillation spectrometer. Hydrolysis of nonradioactive substrates was measured as described in [1]. In all these assays, deletion of endogenous substrates by preincubation was not conducted since it resulted in a considerable loss of enzyme activity.

## 2.4. Assays of other enzymes

5'-Nucleotidase [17], Mg<sup>2+</sup>-ATPase [17], alkaline phosphatase [7], cytochrome oxidase [18], glucose 6-phosphatase [18], catalase [18], acid phosphatase [18],  $\beta$ -N-acetylglucosaminidase [18] and sialyltransferase [12] were assayed as described. When  $\beta$ -galactosidase and  $\beta$ -N-acetylglucosaminidase were assayed, 200 nmol of respective 4-MU-glycoside, 10  $\mu\text{mol}$  sodium

acetate, pH 4.5, and plasma membrane sialidase fraction in 0.2 ml were incubated at 37°C. After 30 min, the 4-MU released was measured fluorometrically as described in [1].

### 2.5. Identification of sialidase products

[<sup>3</sup>H]GM<sub>1</sub> or [<sup>3</sup>H]GM<sub>2</sub> (3000 cpm/nmol, 15 nmol) was incubated with plasma membrane (0.5 mg protein) in 0.2 ml in the presence of 0.05% deoxycholate at pH 4.5. After 20 h at 37°C, the reaction mixture was subjected to thin-layer chromatography as described for the assay method. Radioactive asialo products were detected by fluorography [19] and standards were visualized with anthrone reagent. In a 10-fold large-scale experiment with cold GM<sub>1</sub> as substrate, the product recovered from the plate was incubated with rat liver  $\beta$ -galactosidase (0.2 U), deoxycholate (0.05 mg) and sodium acetate (10  $\mu$ mol, pH 5.0) in 0.1 ml for 36 h at 37°C. After partitioning, the

evaporated digest was incubated with jackbean  $\beta$ -*N*-acetylhexosaminidase (0.2 U) and sodium citrate (5  $\mu$ mol, pH 5.0) for 36 h at 37°C. After each step, the digest was applied to a silica gel plate.

## 3. RESULTS AND DISCUSSION

The plasma membrane was isolated from rat liver homogenate according to [7] and identified by enrichment of plasma membrane markers (5'-nucleotidase, ATPase and alkaline phosphatase) and elimination of markers for other membrane components (cytochrome oxidase for mitochondria, glucose 6-phosphatase for microsomes, catalase for peroxisomes, acid phosphatase and  $\beta$ -*N*-acetylglucosaminidase for lysosomes and sialyltransferase for Golgi) (table 1). When this preparation was assayed for sialidase activity at pH 4.5, mixed gangliosides (bovine brain),

Table 1  
Ganglioside-sialidase and several marker enzyme activities in rat liver plasma membrane

Enzymes	Specific activity	Relative specific activity	Recovery
5'-Nucleotidase	36.8 $\pm$ 3.5	27.5 $\pm$ 4.3	14.7 $\pm$ 1.4
Mg <sup>2+</sup> -ATPase	45.8 $\pm$ 1.9	8.5 $\pm$ 0.9	6.0 $\pm$ 0.2
Alkaline phosphatase	3.4 $\pm$ 0.5	17.8 $\pm$ 1.9	11.1 $\pm$ 0.8
Cytochrome oxidase		0.61 $\pm$ 0.21	0.68 $\pm$ 0.15
Acid phosphatase		1.22 $\pm$ 0.12	0.67 $\pm$ 0.17
<i>N</i> -Acetylglucosaminidase		0.60 $\pm$ 0.21	0.35 $\pm$ 0.13
Catalase		0.81 $\pm$ 0.13	0.39 $\pm$ 0.07
Glucose-6-phosphatase		0.98 $\pm$ 0.02	0.59 $\pm$ 0.03
Sialyltransferase		0.87 $\pm$ 0.08	0.44 $\pm$ 0.05
GM <sub>1</sub> -sialidase	0.66 $\pm$ 0.17		
GM <sub>2</sub> -sialidase	2.58 $\pm$ 0.22	15.2 $\pm$ 2.1	5.5 $\pm$ 0.4
GM <sub>3</sub> -sialidase	12.7 $\pm$ 1.6	16.1 $\pm$ 1.1	9.4 $\pm$ 1.1
GD <sub>1a</sub> -sialidase	12.9 $\pm$ 2.0	14.7 $\pm$ 0.5	9.2 $\pm$ 0.4

The plasma membrane fraction as well as the homogenate was assayed for the enzymes indicated. For sialidase assay, [<sup>14</sup>C]gangliosides served as substrate. Values are means  $\pm$  SE of 3–7 experiments. Specific activities for the plasma membrane are expressed in  $\mu$ mol (5'-nucleotidase, ATPase and alkaline phosphatase) or nmol (sialidase)/h per mg. Relative specific activity is the specific activity for the plasma membrane fraction/specific activity for the whole homogenate. Recovery: percent recovery of the homogenate activity in the plasma membrane fraction

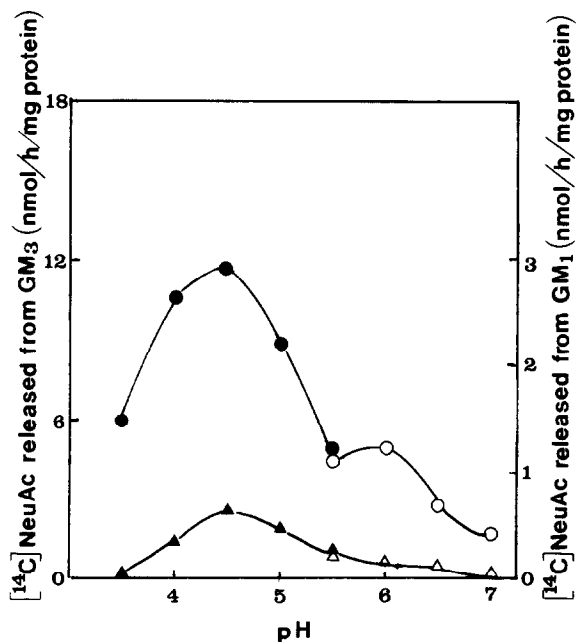


Fig. 1. Effect of pH on plasma membrane sialidase. The enzyme was assayed with  $[^{14}\text{C}]\text{GM}_3$  (●, ○) or  $[^{14}\text{C}]\text{GM}_1$  (▲, △) as substrate at pH values obtained with 50 mM sodium acetate (●, ▲) or sodium cacodylate (○, △).

(2→3)sialyllactose and orosomucoid were hydrolyzed at relative rates of 100, 60 and 3, respectively. This preference for gangliosides distinguishes the membrane sialidase from other sialidases, since the best substrate for intralysosomal and cytosolic sialidase is sialyllactose [1,2]. The activities of the plasma membrane preparation towards individual gangliosides were determined by using  $[^{14}\text{C}]\text{NeuAc}$ - and  $[^3\text{H}]\text{sphingosine}$ -labeled substrates. The results with  $^{14}\text{C}$ -substrate reported in table 1 indicate that the plasma membrane sialidase is capable of removing sialic acid from  $\text{GD}_{1a}$ ,  $\text{GM}_3$  and  $\text{GM}_2$ . Almost the same results were obtained using  $^3\text{H}$ -substrate (not shown). Comparison of these activities with those for various marker enzymes in relative specific activity

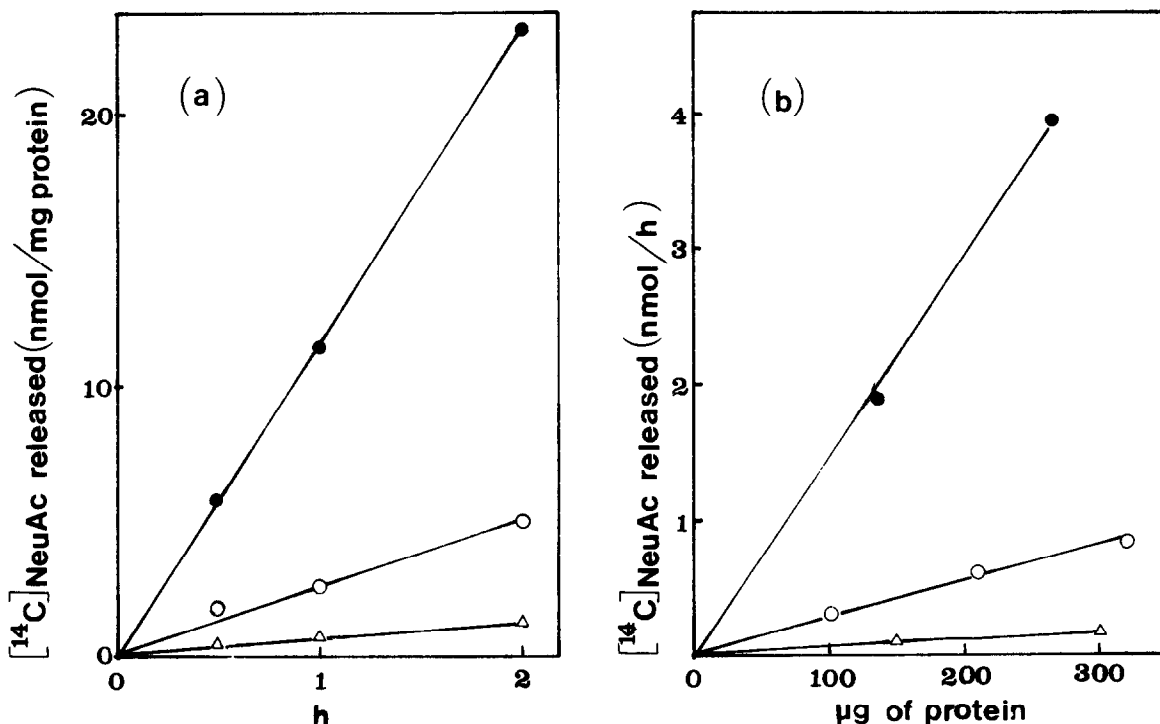


Fig. 2. Effect of incubation time (a) and enzyme concentration (b) on plasma membrane sialidase reaction.  $[^{14}\text{C}]\text{GM}_3$  (●),  $[^{14}\text{C}]\text{GM}_2$  (○) or  $[^{14}\text{C}]\text{GM}_1$  (△) was used as substrate.

and percentage yield further suggests that in rat liver, GD<sub>1a</sub>, GM<sub>3</sub> and GM<sub>2</sub> are hydrolyzed mainly if not entirely in the plasma membrane. We were unable to compute the percentage yield of the GM<sub>1</sub>-hydrolyzing activity since in the homogenate, GM<sub>1</sub> was converted to GM<sub>2</sub>. This conversion was not detectable in the plasma membrane preparation as demonstrated below, but GM<sub>1</sub> was hydrolyzed at a significant rate (table 1). As shown in fig.1, both the GM<sub>3</sub>- and GM<sub>1</sub>-hydrolyzing activities were maximum at pH 4.5 though the curve for GM<sub>3</sub> had a shoulder at pH 6.0. Bile salts and Triton X-100 were not essential to these activities, but they stimulated the reactions to variable extents.

Although Brady and his co-workers once reported GM<sub>2</sub>-hydrolyzing activity in the intestine [16] and heart [20] of the rat, accumulated

evidence suggests that mammalian sialidases lack the activities towards GM<sub>1</sub> and GM<sub>2</sub> [1,4-6]. Additional experiments were therefore carried out to confirm that the plasma membrane sialidase actually attacks GM<sub>1</sub> and GM<sub>2</sub>. Fig.2 shows that the hydrolyses of GM<sub>1</sub>, GM<sub>2</sub> and GM<sub>3</sub> are all linear against incubation time and enzyme concentration even though the reactions with GM<sub>1</sub> and GM<sub>2</sub> were very slow. In the experiment shown in fig.3a, [<sup>3</sup>H]GM<sub>1</sub> and [<sup>3</sup>H]GM<sub>2</sub> were incubated with the plasma membrane preparation and the <sup>3</sup>H-products formed were analyzed by thin-layer chromatography and fluorography. When [<sup>3</sup>H]GM<sub>1</sub> was the substrate, a major spot corresponding to authentic asialo-GM<sub>1</sub> (lane 1) was obtained (lane 6). Likewise, we were able to identify asialo-GM<sub>2</sub> as the desialylation product of GM<sub>2</sub> (cf. lanes 8,2). These asialo spots were not

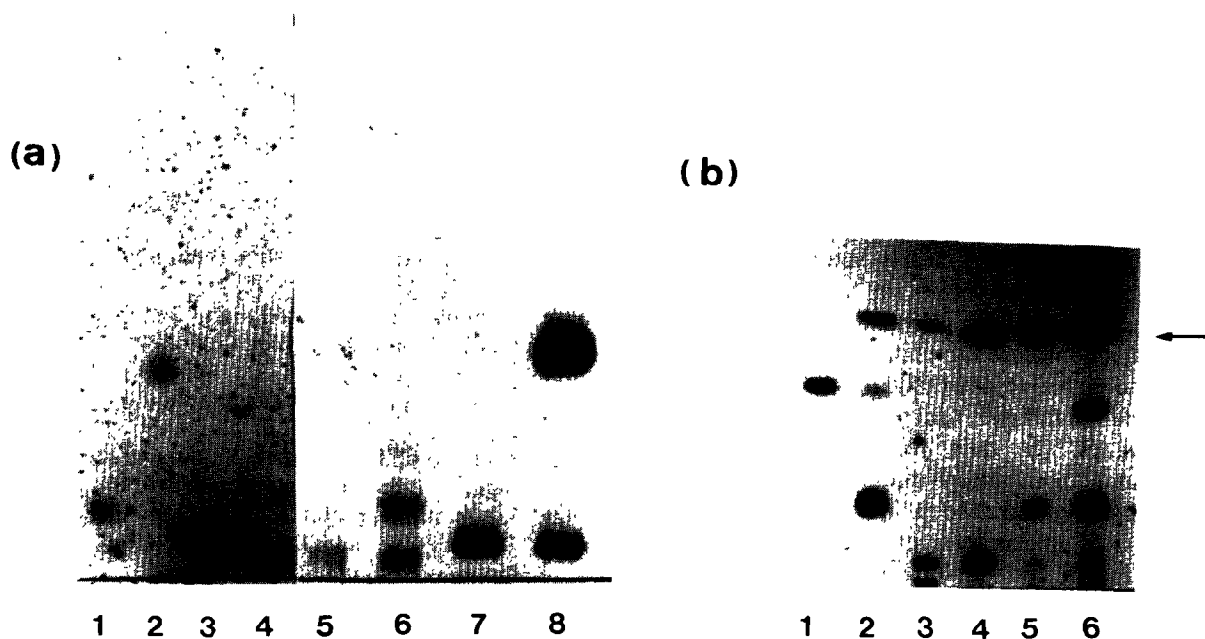


Fig.3. Identification of the products in plasma membrane sialidase reactions. (a) Thin-layer chromatography of the reaction product with [<sup>3</sup>H]sphingosine-labeled GM<sub>1</sub> or GM<sub>2</sub> as substrate. The plate was developed with chloroform-methanol-water (65:25:4) as described in the text. <sup>3</sup>H-labeled compounds were detected by fluorography [19] and standards were visualized with anthrone reagent. Lanes: 1, asialo-GM<sub>1</sub>; 2, asialo-GM<sub>2</sub>; 3, GM<sub>2</sub>; 4, GM<sub>1</sub>; 5, GM<sub>1</sub> + boiled plasma membrane; 6, GM<sub>1</sub> + plasma membrane; 7, GM<sub>2</sub> + boiled plasma membrane; 8, GM<sub>2</sub> + plasma membrane. (b) The reaction product with GM<sub>1</sub> as substrate was sequentially digested with  $\beta$ -galactosidase and  $\beta$ -N-acetylhexosaminidase as described in the text. The plate was developed with chloroform-methanol-water (65:25:4) and then stained with anthrone. Lanes: 1, lactosyl ceramide; 2, asialo-GM<sub>2</sub> + lactosylceramide; 3, GM<sub>1</sub> + asialo-GM<sub>1</sub>; 4, original product (from GM<sub>1</sub>); 5, after digestion with  $\beta$ -galactosidase; 6, after digestion with  $\beta$ -N-acetylhexosaminidase.

The arrow indicates deoxycholate included in the reaction mixture.

detectable when the previously boiled plasma membrane preparation was employed (lanes 5,7). Under these conditions, formation of GM<sub>2</sub> and GM<sub>3</sub> from GM<sub>1</sub> and of GM<sub>3</sub> from GM<sub>2</sub> was negligible (see fig.3a). The results obtained were similar when chloroform-methanol-water (60:35:8) was used as the solvent to achieve better resolution of gangliosides (not shown). It should be noted that the plasma membrane fraction exhibited low  $\beta$ -galactosidase and  $\beta$ -N-acetylgalactosaminidase activities with the respective 4-MU-glycosides as substrate. These indicate that the plasma membrane fraction possesses sialidase which removes sialic acid from GM<sub>1</sub> and/or GM<sub>2</sub> directly, and not with the mediation of  $\beta$ -galactosidase and  $\beta$ -N-acetylgalactosaminidase. To confirm further the identity of the desialylation product of GM<sub>1</sub>, it was scraped off the plate and subjected to stepwise digestion with exogenous  $\beta$ -galactosidase followed by  $\beta$ -N-acetylhexosaminidase. As demonstrated in fig.3b, the products of the first and second digestions coincided with asialo-GM<sub>2</sub> and lactosylceramide, respectively. From the observations shown in fig.3a,b, it is evident that the major desialylation product formed from GM<sub>1</sub> by the plasma membrane sialidase was asialo-GM<sub>1</sub>.

The present results clearly show that rat liver plasma membrane removes sialic acid from not only GD<sub>1a</sub> and GM<sub>3</sub> but also GM<sub>2</sub> and GM<sub>1</sub>, which have been considered to resist hydrolysis by mammalian sialidase [1,4-6]. Although it is likely that a single sialidase desialylates all of these gangliosides, the data for the pH effect and subcellular distribution may suggest that more than a single sialidase is involved in attacking gangliosides in rat liver plasma membrane. The solubilization and purification of these activities are now in progress.

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