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Studies on Plasma Membranes

XX. Sialidase in Hepatic Plasma Membranes

A. Visser and P. Emmelot

Department of Biochemistry, Antoni van Leeuwenhoek-Huis,
The Netherlands Cancer Institute, Amsterdam, The Netherlands

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Summary. Plasma membranes and lysosomes were isolated from rat liver and assayed for sialidase activity with gangliosides and sialyllactose as substrates. Plasma membrane and lysosomal activities differed in substrate preference, heat stability, inhibition by Cu^{2+}, K_m values and pH dependence. It is concluded that plasma membranes and lysosomes contain different sialidases. Hepatoma plasma membranes also exhibited sialidase activity towards the two substrates.

Sialic acid is a constituent of glycoproteins and gangliosides in plasma membranes, and has been implicated in structural [1], adhesive [5, 14] and functional [6, 31] contact relations between cells. Its content may be controlled by the activities of sialyl transferring and cleaving enzymes. The generally decreased sialic acid content of plasma membranes of virally transformed cells [7, 10, 20, 21, 34], and the shift in ganglioside pattern to smaller homologues in these cells [4, 11, 12] have been attributed to the observed decreases in intracellular sialyl transferase activities. However, plasma membranes and cell surfaces also contain sialyl transferase activities using exogenous or endogenous *a*-sialoglycoprotein or peptides (liver [22], normal and transformed fibroblasts [2, 33], blood platelets [3]) and lactosyl ceramide (neuroblastoma plasma membranes [28]) as acceptors. Intercellular glycosyl [23], i.e. galactosyl [24] and sialyl [3] transfer, has been proposed to function in mediating adhesive and "recognition" contacts between cells. If sialidase would be present in the cell surface, it could create acceptor sites for sialyl transferase. This tandem of enzymes might thus, or otherwise, act in contact reactions between cells, by locally and temporarily changing sialic acid densities in cell surfaces.

In the present investigation we studied whether hepatic plasma membranes contained sialidase activity towards gangliosides and sialyllactose. Very recently, Schengrund *et al.* [26] also reported on the localization of sialidase in rat liver plasma membranes. The present paper, which is based on independent discovery of the enzyme in liver plasma membranes, lists various properties of plasma membrane sialidase and shows that the latter differs from lysosomal sialidase.

Materials and Methods

Young adult rats, mostly F_1 (U × R-Amsterdam) males, were used. Plasma membranes were isolated from liver and rat hepatoma-484A as previously described [8, 9]. The plasma membrane preparations were of established purity according to general electron-microscopic, chemical and enzymic criteria. Lysosomes were prepared by the method of Leighton *et al.* [15] as used by Tulsiani and Carubelli [30]. Sialyllactose was isolated from cow colostrum, purified and identified according to Öhman and Hygstedt [18, 19]. All other reagents were from commercial sources and of analytical pure grade. β-Glucuronidase [29] and acid phosphatase were assayed with 0.01 M *p*-nitrophenyl glucuronide and 0.015 M *p*-nitrophenyl phosphate as substrates, respectively, in 0.05 M sodium acetate buffer of pH 4.0; incubation in a total volume of 0.4 ml for 1 to 10 min at 37 °C depending on reaction rates. Reactions were stopped by adding 2.8 ml of 0.1 N NaOH and *p*-nitrophenol was read at 400 nm. Standard incubations were made with amounts of plasma membranes and lysosomes corresponding to 200 to 400 µg protein, measured according to Lowry *et al.* [16], and 200 µg bovine brain gangliosides (Sigma, containing ca. 25% bound sialic acid) or 0.25 µmole sialyllactose [17] in 0.4 ml 0.05 M sodium acetate buffer of pH 4.4 (unless indicated otherwise; in some experiments 1 mg bovine albumin—Fraction V, Sigma—was also added). The reaction mixtures were shaken at 150 to 170 strokes per min for 1 to 3 hr at 37 °C. Reaction of plasma membranes was stopped by addition of periodate reagent [32] or sometimes by keeping the reaction mixture for 3 min at 100 °C. Lysosomal reaction was stopped by freezing at −40 °C. After thawing, the reaction mixture was purified over Dowex Cl^- (1 × 8 cm) columns eluted with water to remove neutral sugars followed by 0.1 N NaCl. The sialic acid-containing fractions were freeze-dried and sialic acid was assayed by the thiobarbituric acid method of Warren [32], using N-acetylneuraminic acid as standard. Values were corrected for blanks incubated in the absence of substrate, the latter being added at 0 °C after incubation, immediately followed by termination of the reaction as indicated. For measurement of K_m values plasma membranes or lysosomes corresponding to 300 µg protein were incubated for 1 hr with six concentrations of sialyllactose over the range of 33 to 200 µg. For depletion of endogenous substrate the membranes were preincubated in 0.05 M acetate buffer at pH 4.4 for 75 min at 37 °C, followed by two washings with 1 mM $NaHCO_3$. All measurements were made in duplicate or triplicate. Acetate buffers served in the pH range studied.

Results

Plasma membranes were isolated from dilute liver homogenates prepared in 1 mM $NaHCO_3$ (1:20, w/v) [8]. Next to the residual nuclear fraction, a 20,000 × *g* fraction (obtained by centrifugation for 20 min) and a

Table 1. β-Glucuronidase and acid phosphatase activities of rat liver plasma membranes and lysosomes

Enzyme source	β-Glucuronidase (μmoles *p*-nitrophenol × mg^{-1} protein × hr^{-1})	Nitrophenyl phosphatase
Plasma membranes	0.063	5.8
Lysosomes	9.2	63.0
Homogenate	0.42	8.1

Assay at pH 4.0. Typical experiment. Homogenate of liver in 0.25 M sucrose (1 : 5 w/v).

105,000 × *g* pellet and supernatant (from 60-min centrifugation) were also prepared from the same homogenate. Under the conditions employed, the homogenate and the 105,000 × *g* pellet and supernatant showed questionable sialidase activity with gangliosides as substrate. The specific enzyme activity of purified plasma membranes, nuclear fraction (possibly containing some plasma membranes) and 20,000 × *g* fraction (containing mitochondria and lysosomes) amounted to 9.5, 1.5 and 0.9 nmoles sialic acid released × mg^{-1} protein × hr^{-1}.

In the following experiments lysosomes were isolated according to the slightly modified method of Leighton *et al.* [15]. Electron-microscopy showed the lysosomal fraction to satisfy the morphological criteria of these organelles. The two "markers," β-glucuronidase and acid phosphatase (substrate: *p*-nitrophenyl phosphate), were markedly enriched in lysosomes relative to the homogenate in terms of specific enzyme activity (Table 1). By comparison, plasma membranes exhibited negligible or small enzyme activities.

Sialidase Activities towards Gangliosides, Sialyllactose and Endogenous Substrate

Since a soluble sialidase is known to occur in the liver cytosol [30], all experiments were carried out with plasma membranes extracted with 0.15 M NaCl at 0 °C in order to remove any contaminating cytoplasmic proteins and enzymes. No significant sialidase activity could be demonstrated in the saline-soluble fraction obtained from the membranes using gangliosides or sialyllactose as substrates.

As illustrated in Table 2 about 60% more sialic acid was released from gangliosides (200 μg/0.4 ml) than from sialyllactose (0.25 μmole) by liver plasma membranes at pH 4.4. Under similar conditions, the opposite sub-

Table 2. Sialidase activities of rat liver plasma membranes and lysosomes and the effect of Cu^{2+}

Substrate	Addition	Plasma Membranes (nmoles sialic acid × mg^{-1} protein × hr^{-1})	Lysosomes (nmoles sialic acid × mg^{-1} protein × hr^{-1})
Gangliosides	–	11.9 ± 2.8 (8)	5.0 ± 1.5 (5)
Sialyllactose	–	7.4 ± 2.8 (11)	44.3 ± 10.6 (6)
		Percent of initial activity	
Gangliosides	1 mM Cu^{2+}	0.0 (3)	4, 30, 45
Sialyllactose	1 mM Cu^{2+}	7.0 ± 3.9 (4)	95 ± 7.5 (4)

Assay at pH 4.4. Averages with number of experiments between parentheses, corrected for release of endogenous sialic acid.

strate preference was demonstrated by the lysosomes which were less than one-half as active as the plasma membranes towards gangliosides, but very active towards sialyllactose.

When incubated at pH 4.4, plasma membranes agglutinate and tend to adhere to the glass surface. Since this was prevented by the gangliosides but not by sialyllactose, the incubation tubes were always vigorously shaken to minimize possible negative effects. Albumin (1 mg/0.4 ml) counteracted membrane agglutination and prevented adherence to glass occurring at low pH, but it did not affect the cleavage of sialic acid from sialyllactose (or gangliosides) by the plasma membranes. Lysosomes do not visibly agglutinate at pH 4.4, but these particles are very labile below pH 5.0 [13] and the disruption of their membranes, which was also evident from the clearance of the lysosomal suspensions during the present incubations, has been shown to unmask enzyme activity. It is therefore unlikely that the substrate preference of the organelles is governed by the accessibility of substrates to enzymes. Sialic acid release from gangliosides was but little affected by Triton X-100; i.e., 0.05, 0.1 and 0.2% causing 0 to 20% stimulation, and 0 to 10% and 25% inhibition of plasmamembrane enzyme activity, respectively. Although added Triton WR 1339 (used to isolate lysosomes) at 0.2% inhibited plasma membrane sialidase towards gangliosides for some 30%, sialic acid release from gangliosides by a combination of plasma membranes and lysosomes proceeded additively.

Sialidase in cell fractions shows activity towards added and endogenous substrates [27]. Sialic acid release from added gangliosides or sialyllactose by plasma membranes proceeded linearly from minute 50 (endogenous sialic

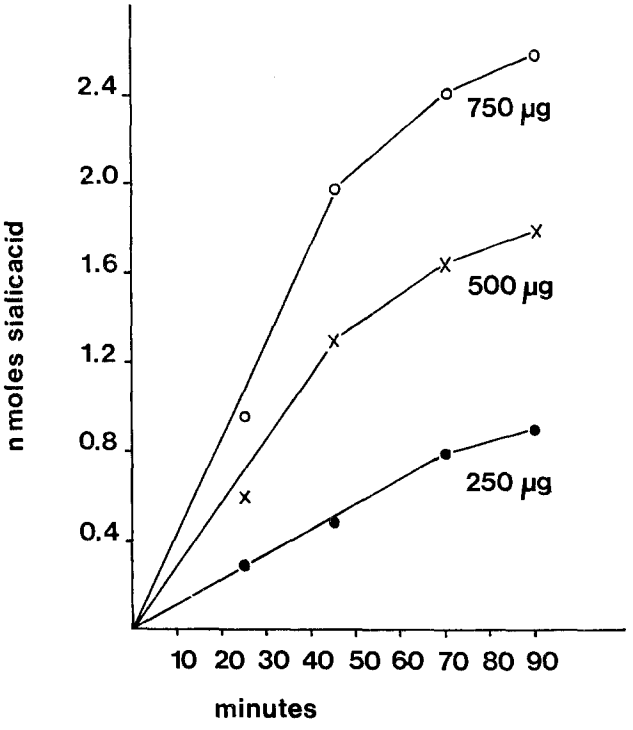

Fig. 1. Sialic acid release from endogenous substrate as a function of plasma membrane concentration

acid release largely terminated) to 180 after starting incubation at pH 4.4, at a rate of 0.2 nmole × mg^{-1} protein × min^{-1} for gangliosides. The rate of sialic acid cleavage from endogenous plasma membrane substrate measured during the first 45 min of incubation (Fig. 1) proceeded slower, i.e. about 0.06 nmole sialic acid being released × mg^{-1} protein × min^{-1} for each of three membrane concentrations studied corresponding to 0.25, 0.50 and 0.75 mg protein. When measured in the presence of albumin to counteract membrane agglutination, the latter value amounted to 0.09 nmole sialic acid mg^{-1} protein × min^{-1} during the first 25 min, after which the reaction slowed down appreciably at about the same rate for the three membrane preparations. Since these kinetics show that the reaction rate is not dependent on membrane concentration, an intramembrane, rather than an intermembrane reaction between enzyme and endogenous substrate would be involved. Triton X-100 (0.1 and 0.2%) reduced the sialic acid release from endogenous substrate to 50% of the original value.

Hepatoma Plasma Membranes

Plasma membranes isolated [9] from rat hepatoma-484A released 12.3 ± 2.3 and 11.1 ± 2.0 nmoles sialic acid $\times$ mg^{-1} protein $\times$ hr^{-1} from gangliosides and sialyllactose, respectively.

Some Properties of Liver Plasma Membrane and Lysosomal Sialidases

Effect of $CuCl_2$. Cu^{2+} has been found to inhibit the soluble sialidase of liver acting on sialyllactose but not to affect the corresponding lysosomal activity [30]. The latter result was confirmed here (Table 2) and it was further demonstrated that 10^{-3} M Cu^{2+} strongly inhibited the plasma membrane enzyme. Plasma membrane sialidase acting on gangliosides was also reduced to zero by Cu^{2+} both at pH 4.4 and 6.0. The corresponding lysosomal activity at pH 4.4 was variously inhibited. The release of endogenous sialic acid from plasma membrane was inhibited by Cu^{2+} for at least 50%.

Effect of Li^+ and EDTA. LiCl has been reported to inhibit the sialidase activity of rat liver lysosomes appreciably, but to have no effect on the soluble sialidase of rat liver [30]. Preincubation of plasma membranes with 0.20 M LiCl in acetate buffer of pH 4.4 or 6.0 at 0 °C for 30 to 60 min and assay of sialic acid release from gangliosides at a final concentration of 0.15 M LiCl, showed an inhibition of enzyme activity of 28% at pH 4.4 and of 35% at pH 6.0. (LiCl prevented the agglutination of the plasma membranes at pH 4.4.)

Preincubation of plasma membranes with 2×10^{-4} M EDTA for 1 hr at 0 °C in bidistilled water did not inhibited but rather tended to increase the subsequent release of sialic acid from gangiosides (to about 130%). Preincubation alone did not affect enzyme activity. The plasma membrane enzyme is apparently not dependent on divalent cations for activity.

K_m Values. These were calculated for sialyllactose as substrate at pH 4.4 with the use of Lineweaver-Burke plots. The kinetic data on plasma membrane sialidase was obtained under two different conditions. First, blanks (without added sialyllactose) were included and the sialic acid released from endogenous substrate was subtracted from values measured in the series with added sialyllactose. Due to the release of endogenous sialic acid the calculated release from added substrate at low substrate concentrations was inaccurate, K_m values of $6.24 \pm 0.87 \times 10^{-4}$ M being thus obtained in four experiments. Alternatively, plasma membranes were first preincubated for

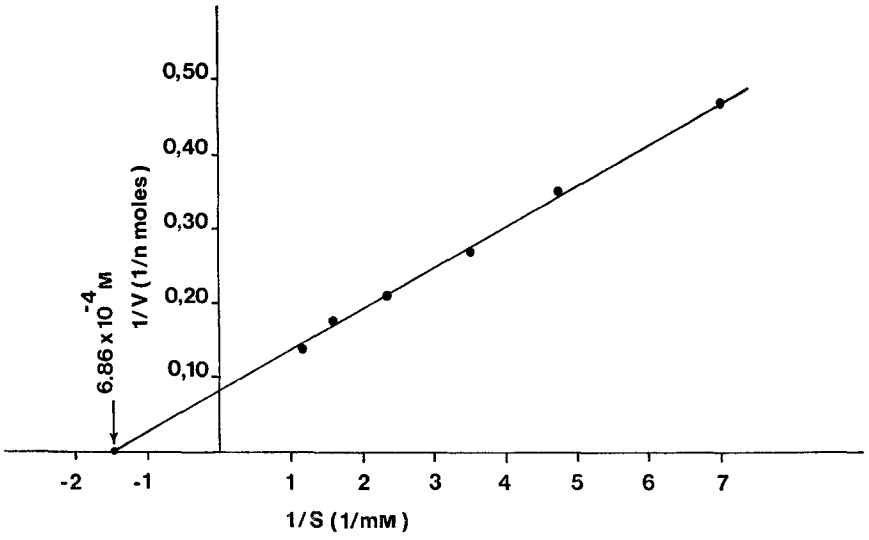

Fig. 2. Lineweaver-Burke plot for determining K_m value of sialyl cleavage from sialyllactose by plasma membranes previously incubated to deplete endogenous substrate

75 min at pH 4.4 and 37 °C without substrate in order to deplete the endogenous substrate. The K_m value for sialyllactose now amounted to 6.86×10^{-4} M in two experiments (Fig. 2). Parenthetically, the preincubation at 37 °C did not impair the plasma membrane enzyme, whereas a similar treatment severely inhibited the corresponding lysosomal activity towards sialyllactose (*cf.* [30]). Thus, plasma membrane sialidase activity towards sialyllactose was not and that towards gangliosides was inhibited for 16%, following 60-min preincubation at 37 °C, whereas the lysosomal activity towards sialyllactose was inhibited for at least 50%. This difference in stability marks a clear distinction between the two enzyme activities.

Using sialyllactose, the K_m value for lysosomal sialidase was $1.27 \pm 0.13 \times 10^{-3}$ M, published values amounting to 1.05×10^{-3} M (rat kidney lysosomes [17]), 1.98×10^{-3} M (rat liver lysosomes [13]), 2×10^{-3} M (bovine brain preparation [27]), and 3.96×10^{-4} M (soluble liver enzyme [30]).

Thus, the K_m values for plasma membrane and lysosomal sialidase are significantly different, substantiating the conclusion to be drawn from the differential effects of Cu^{2+} and preincubation, that sialyllactose is hydrolyzed by different enzymes in plasma membranes and lysosomes.

pH Dependence. Sialic acid cleavage from gangliosides and sialyllactose by plasma membranes as a function of the pH during incubation is illustrated

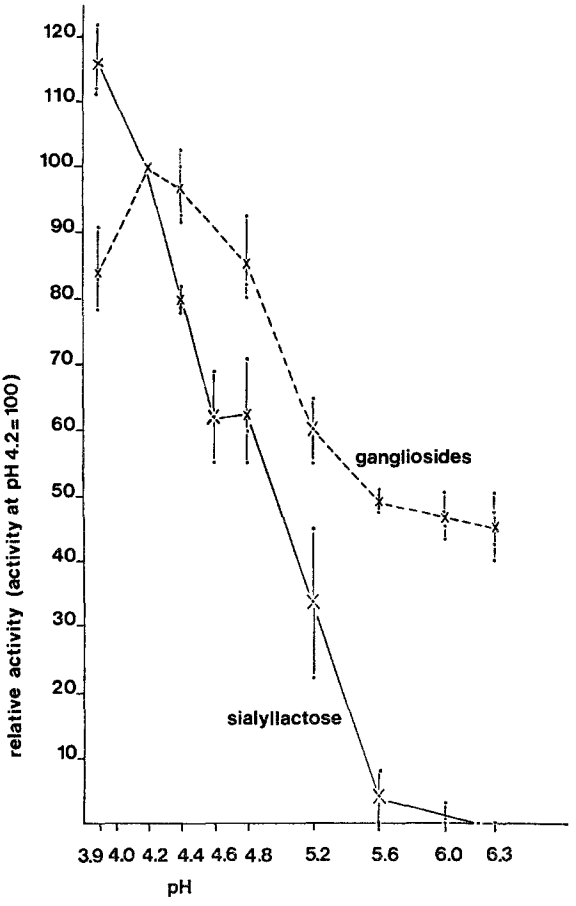

Fig. 3. Sialic acid release from gangliosides and sialyllactose by plasma membranes as a function of pH (acetate buffers)

Table 3. Sialidase activities of liver plasma membranes in 0.01 M Tris-maleate buffers of pH 4.6 and 7.4

Substrate	pH	nmoles sialic acid × mg^{-1} protein × hr^{-1}
Gangliosides	4.6	17.9
Gangliosides	7.4	7.0
Sialyllactose	4.6	5.2
Sialyllactose	7.4	0.3

in Fig. 3. With gangliosides as substrate the pH optimum was at 4.2 (~4.4), and at pH 6.3 sialic acid cleavage still proceeded at nearly one-half of the optimal rate. At pH 7.4 (not illustrated for acetate buffer) the enzyme still

showed 25 to 30% of its optimal activity towards added gangliosides and some 40% towards endogenous substrate; results obtained in 0.01 M Tris-maleate buffer are listed in Table 3. At pH 6.0 and higher no sialic acid was released from sialyllactose by plasma membranes. The sialic acid release from sialyllactose increased about linearly with pH decreasing from 5.6 to 3.9 interrupted by a shoulder at pH 4.6 to 4.8. No spontaneous hydrolysis of sialyllactose occurred at pH $\leqq$ 4.4.

The activity of lysosomal sialidase with sialyllactose and gangliosides as substrate was checked at pH 4.2 (= 100%), pH 5.0 (25 to 50%), pH 5.6 (5%) and pH 6.0 (0%), and at these pH's resembled published lysosomal values [13, 17] obtained with sialyllactose, and that illustrated in Fig. 3 for plasma membrane sialidase acting on sialyllactose.

Discussion

Sialyl cleavage from sialyllactose or gangliosides by plasma membranes and lysosomes has been found to differ in respect of (a) reaction rates, (b) stability of the enzymic process, (c) inhibition by Cu^{2+}, (d) K_m values, and (e) pH dependence. In view of the differences in properties (b, c and d) displayed between the sialidase activities of plasma membranes and lysosomes acting on sialyllactose, it is evident that the plasma membrane activity is not due to lysosomal contamination. Thus, plasma membrane sialidase acting on sialyllactose differs from the corresponding lysosomal enzyme, but resembles to some extent the soluble sialidase of liver as studied by Tulsiani and Carubelli [30]. However, it should be pointed out that the plasma membrane enzyme is tightly bound to the membranes resisting washing with physiological saline. This leads to the conclusion that this enzyme is a genuine constituent of the plasma membrane.

Present data are not sufficient to further differentiate between enzyme activities. Although a marked difference in pH-dependence of sialic acid cleavage from the two substrates by plasma membranes was observed, we consider this as hardly sufficient to decide if two different enzymes are present in plasma membranes. Accordingly, we conclude without specifying substrates that plasma membrane and lysosomal sialidases differ.

It is of interest that the plasma membrane sialidase is still markedly active towards gangliosides at neutral pH. If this activity of isolated plasma membranes reflects *in situ* membrane activity, the question as to the physiological significance of the enzyme arises. A possible role has been outlined in the Introduction of this paper.

According to Schengrund *et al.* [26] the endogenous substrate of the liver plasma-membrane sialidase is ganglioside.* It is not known whether liver plasma membranes contain sialyl transferase activity catalyzing ganglioside formation. However, plasma membranes from mouse neuroblastoma cells contain such activity leading to the formation of hematoside from endogenous and exogenous lactosylceramide [28]. It is not unlikely that such or related activity is also present in plasma membranes of other cells. Physiological signals changing the relative activities of sialyl transfer and cleavage would then create locally and temporarily different sialic acid densities in plasma membrane gangliosides, which might affect membrane behavior. Since ganglioside patterns of "normal" cells change as a function of the growth cycle whereas those of transformed and malignant cells do not [12, 25], sialyl transfer and cleavage could differ quantitatively or be differently controlled in the surfaces of the latter as compared with the former cells.

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* *Note Added in Proof*: Sialyl lactosylceramide (hematoside) which is the main ganglioside present in the hepatoma plasma membranes (P. Emmelot, 1973, *Europ. J. Cancer* **9**:319) is absent after incubation of the membranes in acetate buffer of pH 4.4 for 2 hr at 37 °C as studied by thin-layer chromatography.

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