Localization of Sialidase in the Plasma Membrane of Rat Liver Cells*

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SUMMARY

Sialidase activity towards ganglioside substrate, including hematoside, paralleled activity of 5'-nucleotidase and was found to be enriched in the plasma membrane fractions (TOUSTER, O., ARONSON, N. N., Jr., DULANEY, J. T., AND HENDRICKSON, M. (1970) J. Cell. Biol. 47, 604) prepared from rat liver cells. Lesser sialidase activity towards ganglioside substrate was found in fractions (LEIGHTON, F., POOLE, B., BEAUPAY, H., BAUDHUIN, P., COFFEY, J. W., FOWLER, S., AND DEDUVE, C. (1968) J. Cell. Biol. 37, 482) containing the lysosomal marker, acid phosphatase. Concentration of gangliosides, and total bound sialic acid per mg of protein, also paralleled the plasma membrane marker, 5'-nucleotidase. Subcellular fractions were prepared from rat liver cells by a recently developed procedure (TOUSTER et al.) which favors the isolation of the plasma membrane fraction, and by an independent method (LEIGHTON et al.) favoring the isolation of lysosomes. These findings indicate that a major fraction of sialidase with activity towards ganglioside substrate is localized in the plasma membrane of the liver cell, and that ganglioside substrate is also localized in this cellular structure.

Mahadevan et al. (1), have presented evidence that sialidase (neuraminidase, N-acetyl-neuraminosyl glycohydrolase, EC 3.2.1.18) is localized in the lysosomes of rat liver and kidney cells. Horvat and Touster (2) have also suggested that sialidase appears to behave as a lysosomal enzyme when isolated from rat liver and from Ehrlich ascites tumor cells. They found that sialidase showed the latency characteristic of lysosomal enzymes although its distribution did not completely parallel that of a lysosomal marker. Tettamanti et al. (3), reported that sialidase from rabbit brain had a bimodal distribution, being localized both in the myelin and in the nerve ending fractions. More recently, Schengrund and Rosenberg (4) have presented evidence that sialidase of steer brains is localized in the neuronal synaptosomal membrane. Öhman et al. (5), have shown that the membrane bound human brain enzyme must deplete an endogenous substrate before it can act on added, exogenous, substrates. In time studies with steer brain sialidase, Schengrund and Rosenberg (4) showed that the enzyme acts upon endogenous substrate immediately, but that there is a time lapse of 15 to 20 min before it will act on added, exogenous, substrate. This finding suggests a possible explanation for the apparent latency observed by Horvat and Touster (2) with rat liver preparations.

The purpose of the present work was to determine the distribution of sialidase in rat liver cells fractionated by the scheme developed recently by TOUSTER et al. (6), for the isolation of plasma membranes, and by the procedure of LEIGHTON et al. (7), which specifically separates lysosomes by increasing their buoyancy through the inclusion of detergent molecules, in order to ascertain whether sialidase may in actuality be localized in comparable cellular structures in the neuron and in the liver cell and yet give apparently diverse patterns of distribution upon subcellular fractionation of these tissues.

MATERIALS

Gangliosides were obtained from bovine brain by the partition-dialysis method (8). Contaminating lipids were removed by elution of the crude gangliosides from a column of Silica Gel H with a discontinuous gradient of chloroform-methanol-water of increasing polarity regulated by increasing the water content. Glucose 6-phosphate, sialyllactose, β-glycerophosphate, and oxidized cytochrome c were obtained from Sigma. Triton WR 1339 was obtained from Ruger Chemical Co., Inc. Phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, sphingomyelin, and lysophosphatidyl choline were obtained from Supelco.

METHODS

Source of Tissue and Preparation of Subcellular Fractions—Livers from female Sprague Dawley rats, weighing 160 to 180 g, which had been fasted overnight, were used in these experiments. Subcellular fractionation of the liver was done by a procedure developed by LEIGHTON et al. (7). In this procedure, the experimental animals received intraperitoneal injections of Triton WR 1339 in physiological 0.9% NaCl solution and the control animals, injections of saline alone. Isolation of the lysosomal and mitochondrial fraction was achieved by the ultracentrifugation procedure of LEIGHTON et al. (7). Subfractionation of the lysosomes from the mitochondrial fraction was done on a discontinu-
ous sucrose gradient (0.4 to 1.2 M) as described by Whittaker et al. (9), for the isolation of synaptosomal membranes.

A second procedure used for subcellular fractionation was that recently developed by Touster et al. (6), for the isolation of plasma membranes.

**Analysis of Subcellular Fractions**—To remove most of the sucrose from the samples used for assays, they were diluted with water and pelleted at 100,000 × g for 60 min and the pellets were homogenized in water.

Glucose 6-phosphatase was determined by a slight modification of the method of Hiers et al. (10). Enzyme blanks and enzyme plus β-glycerophosphate blanks were used as controls to correct for phosphate released from the enzyme sample or from the substrate by acid phosphatase activity. Released phosphate was determined by the method of Fiske and SubbaRow (11).

β-Nuclease was determined according to the procedure of Touster et al. (6).

Acid phosphatase was assayed according to the method of Gianetto and deDuve (12) with β-glycerophosphate as substrate. Substrate and enzyme blanks were run as controls. Released phosphate was assayed colorimetrically by the method of Fiske and SubbaRow (11).

Succinate dehydrogenase was determined as described in a previous paper (4).

Sialidase activity towards added, exogenous, substrate (added mixed disialo- and trisialogangliosides, or added sialyllactose) was determined as described in an earlier paper (4) except that the pH for the assay was 4.2, and only 200 μg of sialylactose per ml of incubation mixture were used. Available endogenous substrate was determined by incubating the membrane preparation under similar conditions for 90 min, but with no ganglioside added. This time was adequate for release of all sialic acid available to the sialidase.

Free sialic acid was determined using the thiobarbituric acid procedure developed by Warren (13) as modified by Aminoff (14). Bound sialic acid was determined by the procedure of Jourdian et al. (15) with the following modification. No 3-butanol was added. Instead, the blue-green color obtained was extracted into 3 ml of isooamyl alcohol, and the optical density was determined. This served to eliminate errors in optical density measurements due to turbidity in some samples.

Protein concentration was determined by the procedure of Lowry et al. (16).

Based upon known protein content, samples of the various subcellular fractions were taken for lipid extraction. Lipids were extracted with chloroform-methanol (2:1, v/v). The extract was evaporated to dryness and taken up in 1 ml of chloroform-methanol (2:1, v/v). Of each sample, 10 μl were applied to Silica Gel G thin layer chromatography plates and developed in chloroform-methanol-water (60:35:8, v/v/v) (17). Appropriate reference standards were run. The spots were visualized with iodine and resorcinol spray (18), and the spots were identified by their relative mobilities.

**RESULTS**

**Identification of Subcellular Fractions and Distribution of Sialidase Activity**—The lysosomal fractions obtained with the Triton WR 1339 procedure (7) were identified by monitoring acid phosphatase enrichment. It was found after separation on a discontinuous sucrose gradient that the highest specific activity for acid phosphatase in the control preparation was spread over the 1.0 to 1.4 M sucrose bands. In the Triton-injected preparation, the highest specific activity was obtained in the 0.6 to 1.0 M bands. Sialidase showed its highest specific activity in the 1.0 M band in the control, and its location was unchanged upon Triton treatment, as can be seen in Table I.

The subcellular fractions obtained by the procedure of Touster et al. (6), were initially identified using their characterization: P1, crude nuclear; P2, lysosomal-mitochondrial; P3, microsomal; P4, nuclear; and LP4, erythrocytic. P4 and LP4 were derived from P1. The subfractions came from centrifugation of P3 in a discontinuous sucrose density gradient, and the subfractions are from a similar treatment of P4. Subsequent identification was made by following glucose 6-phosphatase, β-nuclease, acid phosphatase, and succinic dehydrogenase activities. The distributions of the relative specific activities of these enzymes paralleled the identifications made by Touster et al. (6), and are shown in Table II. On the basis of total activities, 54% of the total sialidase activity towards added ganglioside substrate was in P3, 20% in P4, and 20% in P5; LP4 contained no measurable sialidase activity. P5 contained 51% of the total acid phosphatase activity and 88% of the total succinic dehydrogenase. LP4 contained 20% of the acid phosphatase activity and 9% of the succinic dehydrogenase activity. Therefore, by the use of P3 and P4 for further fractionation, 70% of the lysosomal marker and essentially all of the mitochondrial enzyme marker are removed, while only 26% of the total sialidase activity is lost.

**Sialidase activity towards endogenous substrate** paralleled that of the plasma membrane marker β-Nuclease. Sialidase activity towards added, exogenous, ganglioside substrate also paralleled the β-Nuclease activity most closely as is shown in Table III. Activity towards sialyllactose, a relatively poor substrate for the membrane-bound enzyme, was observed, but appeared somewhat more diffusely.
it was found that approximately 35\% of the residues were susceptible to cleavage. Thin layer chromatography of the lipid extract from M2 which was tested for sialic acid content, it was found to contain 27\% of the total sialic acid. When M2 and N2 were tested for total sialic acid released, it was found that approximately 35\% of the residues were susceptible to cleavage. Thin layer chromatography of the lipid extract from the membranes after endogenous sialidase action showed full loss of the hematoside, and only monosialo-ganglioside remained, as shown in Fig. 2. Therefore, practically all of the susceptible sialyl residues of the gangliosides were cleaved, but only a small fraction of the nonlipid sialyl residues.

**Lipid Composition.**—The general lipid pattern is shown in Fig. 1. The lipids, excluding gangliosides, appeared to be qualitatively similar in all of the subfractions. While the concentration of phospholipids seemed to be the same in different samples, the ganglioside concentration, including hematoside, was greatest (compared with protein content as well as to phospholipid content) in both of the plasma membrane fractions (Mz and Nz) as may be seen in Fig. 1. The sialic acid composition in the different subfractions on a per mg of protein basis parallels the ganglioside concentrations, including hematoside, was greatest in Nz, both of which contain increased amounts of ganglioside. For assay conditions, see "Methods."

**Endogenous sialidase activity was measured with respect to total enzymatically releasable membrane-bound sialic acid per mg of protein.**

**DISCUSSION**

Initial results on the fractionation of liver cells from Triton-treated rats showed that, upon discontinuous sucrose density gradient ultracentrifugation, bands with highest specific activity for sialidase are essentially the same for Triton-treated rats as for control animals, but the highest specific activity for acid phosphatase differed in location. This suggests that sialidase activity in the liver is not primarily located in the lysosomes.
FIG. 1 (left). Thin layer chromatogram of the lipids extracted from subcellular fractions prepared from rat liver cells. P1 is the crude nuclear pellet; P2, the lysosomal-mitochondrial pellet; P3, the microsomal pellet; S1 the supernatant above P1 centrifuged at 100,000 × g, 60 min; P4, nuclei; and LP4, red cells. P4 and LP4 are derived from P1, and S1 is the supernatant centrifuged at 100,000 × g, 60 min. The M subfractions are from centrifugation of P3 in a discontinuous sucrose density gradient, and the N subfractions are from a similar treatment of P4 (6). PE, PS, PC, C, and G refer to phosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, cerebrosides, and gangliosides taken as standards. G to the right of the picture indicates the region in which gangliosides, including hematoside, are found.

Note that the gangliosides appear most clearly in fractions N2 and M4, the plasma membrane fractions. The dense spot near the origin is due to sucrose which obscures trisialoganglioside and was not completely removed even though each sample was pelleted from dilute sucrose, taken up in water, and repelleted before lipid extraction. The hematoside, monosialo-, and disialoganglioside spots are clearly seen.

FIG. 2 (right). Thin layer chromatogram of the gangliosides extracted from the plasma membrane fraction of rat liver cells. Column 1 (1) shows the ganglioside pattern of the membrane, as isolated; Column 2 (2) shows the ganglioside pattern after action of plasma membrane sialidase. H, hematoside; M, monosialoganglioside; D, disialoganglioside; and S, sucrose.

Available to the sialidase paralleled 5'-nucleotidase distribution far more closely than that of glucose 6-phosphatase. The loss of the hematoside spot and that of the slow moving gangliosides showed that the sialidase was able to act on these endogenous substrates, leaving only monosialoganglioside. Since 27% of the total sialic acid is lipid bound, and only 35% of the total sialic acid is cleaved, the sialidase appears to act primarily on the lipid substrate and only to a small degree on glycoprotein substrate. Determination of total sialic acid present in each fraction, per mg of protein, showed a greater sialic acid content in the...
plasma membrane fractions (M1 and N2) than in the other fractions (see Table IV). This finding is in agreement with patterns obtained by thin layer chromatography which showed gangliosides, including hematoside, to be concentrated in the plasma membrane fractions, and also agrees with the suggestion of Renkonen et al. (19) that gangliosides may be used as a plasma membrane marker.

Exogenous sialidase activity toward ganglioside substrate paralleled that of 5'-nucleotidase in the nuclear subfractions and was spread over two subfractions in the microsomes rather than banding in a single fraction. This was the largest difference between the distribution of sialidase and that of Y-nucleotidase that was observed. These results indicate that sialidase is localized in the plasma membrane of the cell and that, along with it, there is localized a sialic acid-containing substrate. In studies done with bovine brain sialidase (4), sialidase was found to be localized in the synaptic membrane. In brain, the specific activity of sialidase towards added, exogenous, substrate also followed that of 5'-nucleotidase closely. Therefore, it seems that sialidase which is active towards disialo- and trisialogangliosides generally is localized along with an endogenous substrate in the plasma membrane of mammalian cells.

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