Intracellular Location and Properties of Bovine Brain Sialidase*

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SUMMARY

Intracellular distribution of the sialidase of adult bovine brain gray matter was determined. The sialidase was found to be concentrated in the synaptosome fraction, and when the latter was disrupted and further fractionated, sialidase activity was found to be concentrated in the synaptosomal membrane. Sialidase in all cell fractions showed activity toward an intrinsic endogenous substrate, as well as toward added disialo- and trisialo-gangliosides, and sialyllactose. $K_m$ values for the sialidase in an acetone powder preparation from gray matter were $8 \times 10^{-5}$ and $2 \times 10^{-5}$ M for gangliosides and sialyllactose, respectively.

There was an unequivocal lag phase of about 15 min between the time of addition of exogenous substrate and the time the sialidase measurably acted on it. The enzyme was found to act on the intrinsic endogenous substrate during the lag phase. Thin layer chromatography showed monosialo-, disialo-, and trisialo-gangliosides to be present in all cell fractions having sialidase activity. Analysis of the active fractions after incubation at 37° for 75 min without added exogenous substrate showed that monosialo-ganglioside, the end product of enzyme action on the intrinsic ganglioside substrate, remained associated with the membrane fragments.

Sialidase (neuraminidase, $N$-acytlyneuraminosyl glycohydrodase, EC 3.2.1.18) of nervous tissue (1) was first isolated in a somewhat purified form from calf brain by Leibovitz and Gatt (2) who found the enzyme to be particulate, to have a pH optimum of 4.4, and to hydrolyze disialo- and trisialo-gangliosides but not sialyllactose. Intracellular distribution of sialidase has been studied in rabbit and rat liver preparations (3, 4). Liver sialidase was found to have properties characteristic of a lysosomal enzyme. Tettamanti et al. (5), in a preliminary report on the properties of sialidase from rabbit brain, concluded that there were two particulate sialidases, one localized in the myelin fraction and the other in the nerve ending fraction. Both enzymes were active toward ganglioside substrates, but only sialidase in the nerve ending fraction was found to act on sialyllactose.

The purpose of this work was to determine the intracellular distribution of sialidase in gray matter of bovine brain, and to compare the nature of enzymatic action on endogenous substrate (6) with that on added exogenous substrates. The results obtained indicate that bovine brain sialidase is a presynaptic membrane component and that it occurs together with an intrinsic, highly reactive native substrate.

* This work was supported by Grant NS 08258 from the National Institute of Neurological Diseases and Stroke, United States Public Health Service.

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assayed without preliminary incubation were warmed with added substrate at 37° for 90 min. Previously incubated samples were first warmed without exogenous substrate for 75 min at 37°, pelleted, and diluted to the desired concentration buffer. The samples were then incubated with added substrate for 90 min at 37°. The reaction was stopped by adjusting the pH to 7 with 0.1 N sodium hydroxide. The samples were frozen and stored at −20° until analyzed; at which time they were thawed and run through Dowex 1-X10 columns, as described (4), to remove chromogenic substances which interfered with the accurate determination of free sialic acid by the Warren thiobarbituric acid procedure (11). Recovery was consistently 75% of added free sialic acid. Fig. 1 shows the “Warren” color obtained for the enzyme sample, before and after chromatographic purification, and for pure sialic acid. The extensive contamination by spurious chromogens in the analysis for free sialic acid, and their essentially complete removal by chromatographic treatment of the assay mixture, can be seen.

**Succinate Dehydrogenase**—Succinate dehydrogenase was determined by measuring the rate of oxidation of succinate to fumarate, linked to reduction of cytochrome c, by the following modification of the method of Green, Mehl, and Kohout (12). The assay mixture contained 0.03 mmole of phosphate buffer, pH 7.4, 0.5 μmole of potassium cyanide, 0.135 mmole of potassium succinate, an amount of enzyme to cause an absorbance change of about 0.05 per min, and water to give a 2.9-ml volume. The assay mixture was warmed at 38° for 10 min, transferred to a 3-ml absorption cell, and the reaction started by addition of 0.1 ml of cytochrome c solution (1.5 mg of cytochrome c per 0.1 ml water). The reaction was followed at 550 nm in a Gilford recording spectrophotometer.

**Lactate Dehydrogenase**—The procedure described by Kornberg (13) was used to determine lactate dehydrogenase activity.

**Acid Phosphatase**—This was determined by the procedure of Gianetto and deDuve (14) with p-nitrophenylphosphate as substrate. Enzyme and substrate blanks were run as controls. Released p-nitrophenol was assayed colorimetrically by the method of Fiske and SubbaRow (15).

**Glucosaminidase**—Glucosaminidase was assayed by the procedure of Frohwein and Gatt (16), with p-nitrophenyl-N-acetyl-β-D-glucosaminide as substrate.

**Identification of Gangliosides in Cell Fractions**—Lipids were extracted with chloroform-methanol (2:1, v/v). The extract was concentrated, and spotted on silica gel thin layer chromatography plates which were developed in chloroform-methanol-water (60:35:8) (17). The ganglioside spots were visualized by use of resorcinol spray (18) and identified by their relative mobilities.

**RESULTS**

**Identification of Subcellular Fractions**—Fractions were initially identified by sedimentation properties with Whittaker’s characterization (19). The identifications were: P₃, the crude nuclear pellet; P₂, the crude mitochondrial pellet (small myelin and membrane fragments, synaptosomes, and mitochondria); and P₁, microsomes. The crude mitochondrial fraction after subfractionation on a discontinuous sucrose gradient gave Fractions 0.32 P₂ which had small myelin fragments; 0.8 P₂, synaptosomes; and 1.2 P₂, mitochondria. The numbers refer to the molality of the sucrose layers in which the subfraction bands were isolated.
Disialo- and Trisialogangliosides

**TABLE I**

Sialidase activity in subfractions of hypo-osmotically ruptured synaptosomes

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total activity</th>
<th>Relative specific activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Sialyllactose</td>
<td>Exogenous ganglioside</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Synaptic vesicles</td>
<td>5.4</td>
<td>2.6</td>
</tr>
<tr>
<td>Synaptic membranes</td>
<td>16.1</td>
<td>13.9</td>
</tr>
<tr>
<td>Partially ruptured membranes</td>
<td>20.0</td>
<td>24.4</td>
</tr>
<tr>
<td>Pellet</td>
<td>24.4</td>
<td>21.4</td>
</tr>
</tbody>
</table>

a Percentage of total activity divided by percentage of total protein for each fraction.

b For assay conditions, see Methods.

c The numbers refer to the molarity of sucrose in the discontinuous gradient in which the sedimented bands were obtained.

discontinuous sucrose gradient, the greatest amount of activity was found in Fraction 0.8 P3, which forms a band between 0.8 and 1.2 M sucrose in the gradient. Fig. 2 also shows that where there is activity toward endogenous substrate, there is also activity toward added exogenous disialo- and trisialogangliosides, and sialyllactose. The $K_m$ values determined for the sialidase in the aqueous phase, decreasing to a minimal

**TABLE II**

Distribution of acid phosphatase, glucosaminidase, and succinate dehydrogenase in cell fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Acid phosphatase</th>
<th>Glucosaminidase</th>
<th>Succinate dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total activity</td>
<td>Relative specific activity</td>
<td>Total activity</td>
</tr>
<tr>
<td>P1</td>
<td>26</td>
<td>1.13</td>
<td>36</td>
</tr>
<tr>
<td>P2</td>
<td>69</td>
<td>0.99</td>
<td>61</td>
</tr>
<tr>
<td>0.52 P2</td>
<td>6</td>
<td>0.27</td>
<td>7</td>
</tr>
<tr>
<td>0.8 P3</td>
<td>16</td>
<td>0.34</td>
<td>32</td>
</tr>
<tr>
<td>1.2 P3</td>
<td>78</td>
<td>2.57</td>
<td>61</td>
</tr>
<tr>
<td>P3</td>
<td>5</td>
<td>0.70</td>
<td>2</td>
</tr>
</tbody>
</table>

For assay conditions, see Methods.

After rupture of the synaptosomes in water and centrifugation of the lysate on a second discontinuous sucrose gradient, the sialidase activity was recovered primarily in the fractions containing synaptosomal membranes and also in a fraction containing incompletely ruptured synaptosomes (Table I).
The monosialogangliosides were not further susceptible to sialidase action (2), which pelleted from 1.2 M sucrose while the sialidase was concentrated in a separate fraction which banded between the 0.6-0.8 and 0.8-1.0 M sucrose layers. Koenig (21) points out that for a certain metabolically induced environmental conditions, the enzyme conceivably might be activated, thereby changing the normal ganglioside pattern of the presynaptic membrane with respect to sialic acid content. With altered conditions, an activation of sialyl transferase which appears to be located in a neuronal subfraction (22) could mediate a resynthesis of the ganglioside complex in the presynaptic membrane.

There is no clear lag of about 15 min before the enzyme starts to act on the exogenous substrate. This observation seems to indicate that either the enzyme is initially saturated with substrate in the membrane structure, and must act on this substrate first before sites become available for added substrate, or alternatively, it may take time for sufficient exogenous substrate to penetrate the enzymatically active membrane. When fractions were incubated without exogenous substrate, centrifuged, the pellets extracted with chloroform-methanol (2:1, v/v), and the extracted lipids determined by thin layer chromatography, monosialogangliosides (the end product of ganglioside degradation by sialidase) with a trace of disialogangliosides were found to remain associated with the membranes in all cellular fractions. Our observation that the location of endogenous activity coincides with exogenous activity, the latter demonstrable after preliminary incubation, and that the product of the reaction, monosialoganglioside, remains attached to the membrane, would seem to indicate a structural as well as a functional role for the sialidase-ganglioside complex in the presynaptic membrane. Under certain metabolically induced environmental conditions, the enzyme conceivably might be activated, thereby changing the normal ganglioside pattern of the presynaptic membrane with respect to sialic acid content. With altered conditions, an activation of sialyl transferase which appears to be located in a nerveonal subfraction (22) could mediate a resynthesis of oligosialogangliosides. Studies are in progress to test the sialidase-active presynaptic membrane preparations for such sialyl transferase activity.

Acknowledgment—We gratefully acknowledge the technical assistance of Mrs. Doris S. Jensen.

REFERENCES

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