

# Cerebroside galactosidase of brain

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**ABSTRACT** The galactoside bond in cerebroside was found to be cleaved by an enzyme in rat and pig brain. Emulsified stearoyl- $^{14}\text{C}$  psychosine was used as the substrate and the extent of cleavage was studied by isolating and counting the stearoyl sphingosine (ceramide) formed. The reaction products, ceramide and galactose, were characterized by column and thin-layer chromatography. Cerebroside containing galactose- $^3\text{H}$  was also used to show liberation of galactose. Cholic acid was found to be required for activation of the enzyme, which has a pH optimum of 4.5. Similar cerebroside activity was found in spleen, kidney, and lung of rat; liver and heart showed very slight activity.

The partially purified enzyme from pig brain also formed ceramide from ceramide lactoside, ceramide glucoside, and cerebrononyl psychosine. The enzyme was active toward *o*-nitrophenyl galactoside and could be fractionated by Sephadex chromatography into a fraction active toward the nitrophenyl galactoside only and a fraction active toward both this substrate and ceramide galactoside. Human spleen, normal and Gaucher, exhibited cerebroside activity.

**KEY WORDS** stearoyl- $^{14}\text{C}$  psychosine · cerebrononyl- $^{14}\text{C}$  psychosine · cerebroside · rat organs · pig brain · ceramide · ceramide lactoside · galactosidase · ceramide glucoside · Gaucher spleen · glucosidase

**A**N IN VIVO study in this laboratory with labeled galactose showed that the galactose residue of rat brain cerebroside undergoes turnover (1). The mechanism of the turnover was, however, not determined; the decrease in galactose radioactivity could be due to hydrolytic cleavage or to sulfation to form cerebroside sulfate or to some other conversion reaction. In a later study, in which lignoceroyl- $^{14}\text{C}$  psychosine (kerasin)

Abbreviations: TLC, thin-layer chromatography; GLC, gas-liquid chromatography; C, chloroform; M, methanol; W, water; HOAc, acetic acid; EtOAc, ethyl acetate.

Fatty acids are identified by the carbon number and number of double bonds (e.g., 18:0 is stearic). Ceramide is acyl sphingosine (and related bases); hydroxy ceramide is ceramide in which the fatty acid has a 2-hydroxy group.

was injected into the brains of young rats, highly labeled ceramide was formed (2). Since the  $^{14}\text{C}$  in the ceramide was found to be still in the carboxyl group, it was concluded that the cerebroside was converted directly to ceramide, presumably via a hydrolytic step. This report, previously given in preliminary form (3), describes a hydrolytic enzyme which carries out the postulated reaction.

## METHODS

### Substrates

Stearic acid-1- $^{14}\text{C}$  was converted to cerebroside as part of a previous study (2). One milligram of cerebroside (500,000 cpm) was dried down from solution with 10 mg of Tween 20 (polyoxyethylene sorbitan monolaurate) and 5 mg of G-2159 (polyoxyethylene stearate). To the residue was added 40 mg of sodium cholate (Mann Research Labs., New York, N.Y.) in 4 ml of water and the mixture was agitated in an ultrasonic bath and heated briefly at 70°C to produce a clear emulsion. This method of emulsification was also used with the other lipoidal substrates.

Labeled cerebrin was made and emulsified similarly. DL-Cerebronic acid-1- $^{14}\text{C}$  was prepared from lignoceric acid-1- $^{14}\text{C}$  (4), then acetylated with isopropenyl acetate (5). The acetoxy acid was next treated with thionyl chloride and psychosine (6) and the product was purified by solvent partitioning. Mild alkaline hydrolysis yielded the deacetylated cerebrin, which was purified by TLC (C-M-W 24:7:1). While the acetyl cerebrin gave one spot on TLC, just ahead of kersin, the cerebrin gave two spots, one in the usual cerebrin region and the other ahead ( $R_f$  of cerebrin = 0.58; isomer = 0.63). The two spots were similar in intensity and equally radioactive. Similar results were obtained without the preliminary acetylation, but the yields were lower.

The two cerebrin spots were evidently the diastereoisomers arising from the use of DL-cerebronic acid, the unnatural (L) isomer being the faster moving. This

interpretation was strengthened by synthesizing cerebron from natural cerebronic acid, which yielded only the normal spot.

In one of the experiments performed to characterize the enzymatic products, cerebroside labeled with galactose- $^3\text{H}$  was used. This was prepared (details to be presented later) by oxidizing a mixture of beef spinal cord cerebroside with galactose oxidase (7) and reducing the resultant aldehyde with sodium borohydride- $^3\text{H}$ . The product, on hydrolysis with aqueous HCl and partition with C-M 2:1, yielded 98% of its radioactivity in the aqueous (galactose) layer.

Ceramide glucoside (glucocerebroside) was isolated from human Gaucher spleen as described before (8). It showed only two spots in TLC with C-M-W 60:35:8 and one spot with propanol-ammonia-W 160:19:21. In the latter case, the spot moved distinctly faster than the two spots given by ceramide galactoside. The sample exhibited the expected infrared absorption spectrum (9) and yielded the expected amount of glucose on hydrolysis (10).

Ceramide lactoside (cytoside), a gift, was prepared by partial hydrolysis of brain ganglioside and was found to contain the expected proportions of fatty acids and sphingosine.

*o*-Nitrophenyl  $\beta$ -D-galactoside was obtained from Sigma Chemical Company, St. Louis, Mo.

#### *Enzyme Assay*

Incubations were carried out in screw-cap test tubes containing 0.4 ml of cerebroside emulsion (0.1 mg or 50,000 cpm cerebroside), enzyme solution, 0.1 ml of M sodium citrate, pH 4.5, and water to make 1 ml. The capped tube was shaken gently at 37°C for 2 hr, after which 7 ml of C-M 1:1 was added and the suspension was mixed well. The C-M contained 0.1 mg of cerebroside and 0.2 mg of ceramide as carriers to minimize adsorption losses. The precipitated protein was discarded by centrifugation and the extract was washed with 6 ml of water, which removed the buffer. After centrifugation, the lower layer was evaporated to dryness in a stream of nitrogen and the residue was passed through a 5 mm column containing 0.5 g of silica gel (Unisil, Clarkson Chemical Company, Williamsport, Pa.) in 8 ml of C-M 98:2. This solvent eluted the ceramide, which was counted in a scintillator solution containing toluene-absolute ethanol 95:5.

For recovery studies the remaining radioactive cerebroside was eluted with C-M 9:1 and counted in a toluene-ethanol 80:20 mixture. Counting efficiencies were normalized by means of an internal standard.

Enzyme activity toward nitrophenyl galactoside was assayed by a slight modification of the Lederberg method (11). Portions of enzyme were incubated in a

total volume of 1 ml with 5  $\mu$ moles of substrate and 100  $\mu$ moles of sodium citrate, pH 4.5, for 1 hr at 37°C. After addition of 4 ml of 0.25 M sodium carbonate and centrifugation, the nitrophenol color was determined at 420  $\mu$ . The color yield was proportional to the amount of enzyme at all stages of purification.

#### *Other Materials and Methods*

Carrier cerebroside, a mixture of hydroxy and non-hydroxy ceramide galactoside, was prepared from beef spinal cord lipids (12). Carrier ceramide was prepared from this mixture by the periodate method (2, 13). TLC was carried out with Silica Gel G (Brinkmann Instruments Inc., Westbury, N.Y.). Protein concentrations were measured by a biuret method (14).

#### *Preparation of the Enzyme*

Pig brains from the local slaughterhouse were washed free from blood and homogenized in a Waring Blender with 2 volumes of water at 2°C. The insoluble portion was removed by centrifugation for 10 min at 12,000  $\times g$ , then for 3 hr at 70,000  $\times g$ . Acetic acid (0.1 N) was added to the supernatant solution to bring the pH to 5.0 and the mixture was stirred for 15 min, then centrifuged at 12,000  $\times g$  for 15 min. The residue formed an opalescent solution in 0.02 M potassium phosphate (pH 6.9). Powdered ammonium sulfate (Mann Research Labs.) was slowly added to the 28% saturation point. After 15 min the insoluble material was collected by centrifugation and dissolved in 0.01 M phosphate, pH 7.4. One half volume of chilled acetone was then added to the opalescent solution in a freezer room (-15°C) and the mixture was stirred for 15 min. The resultant precipitate was isolated by centrifugation at -10°C, washed with 0.01 M phosphate, pH 7.4, and stored as a fine suspension in the same buffer. All steps were carried out at 0-2°C, except for the last one.

## RESULTS

#### *Purification of the Pig Brain Enzyme*

Table 1 lists the results of one purification run, together with the assay results obtained with nitrophenyl galactoside. The results show that a modest degree of purification was achieved, about 3-fold on the basis of the initial extract. In other runs, as much as 5-fold purification was obtained, as well as higher yields of enzyme. Because of the large amount of endogenous cerebroside in whole brain, no attempt was made to assess the extent of purification based on the total homogenate.

As indicated above, the enzyme became insoluble as a result of the acetone treatment. Attempts to solubilize the enzyme in buffers of different pH or ionic strengths

TABLE 1 CEREBSIDE GALACTOSIDASE PURIFICATION

Step	Total Protein	Specific Activity* CRB	Yield†	Specific Activity* NPG
	mg		%	
Aqueous extract	1080	0.94	(100)	111
pH 5 precipitate	576	1.43	80	94
Ammonium sulfate precipitate	204	2.06	41	118
Acetone precipitate	60	2.86	17	60

CRB, cerebroside (stearoyl-1-<sup>14</sup>C psychosine); NPG, *o*-nitrophenyl  $\beta$ -galactoside.

\*  $\mu$ moles hydrolyzed per hour per milligram of protein. In the standard cerebroside incubation, 1  $\mu$ mole of hydrolyzed cerebroside yields about 890 cpm in the ceramide.

† Yield based on cerebrosidase activity.

failed. Other purification attempts, such as the use of alumina C<sub>7</sub> gel or fractionation on DEAE columns, gave either no increase in specific activity or large losses in activity. Since the enzyme showed maximal activity at pH 4.5, at which point the enzyme is insoluble at any purification stage, we decided that it would not be unreasonable to carry out these early investigations with the insoluble preparation obtained from the acetone step.

#### Properties of the Enzyme

The optimal pH was about 4.5, as shown in Fig. 1. Under these conditions it was shown that the enzyme, cholic acid, and the isotopic substrate all precipitate.

The enzyme's activity was found to be fairly linear with increasing amounts of enzyme (Fig. 2) and with time over a period of 3 hr (Fig. 3).

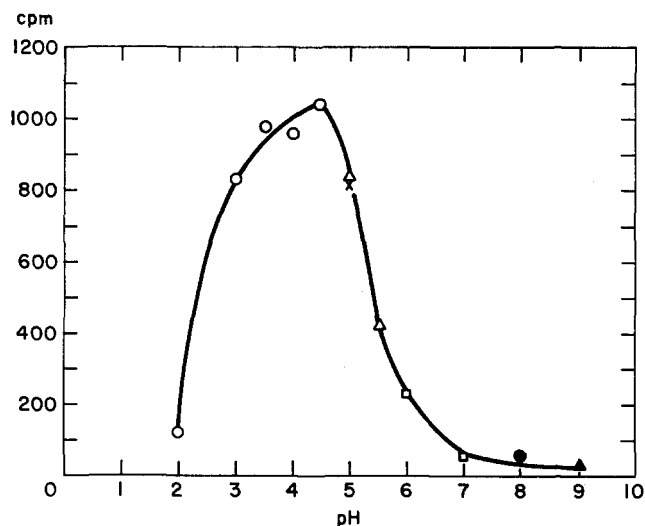


FIG. 1. Relative hydrolysis rates toward stearoyl psychosine by pig brain enzyme as a function of pH. O, citrate buffer; X, acetate;  $\Delta$ , citrate-phosphate;  $\square$ , phosphate;  $\bullet$ , Tris-HCl;  $\blacktriangle$ , glycine-NaOH. Ordinates represent activity in ceramide isolated from incubation mixture.

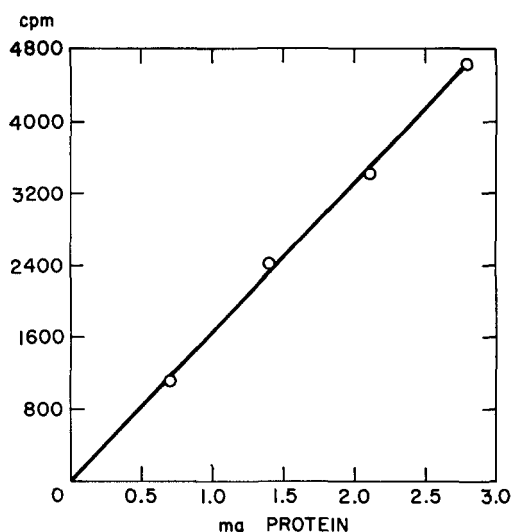


FIG. 2. Hydrolytic activity of pig brain enzyme as a function of enzyme concentration. All other assay conditions held constant.

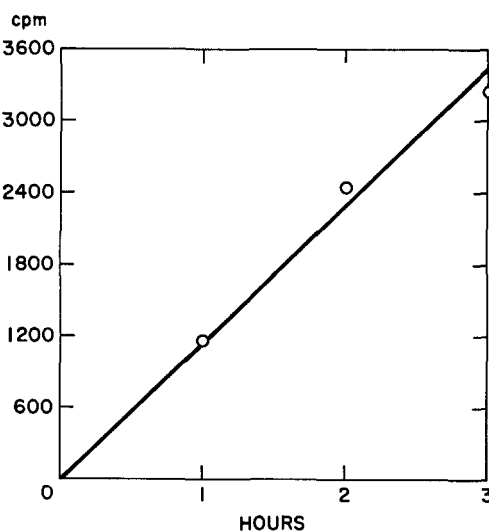


FIG. 3. Hydrolysis of 18:0 cerebroside as a function of time. Assay conditions as in text.

Increasing the concentration of radioactive substrate did not give the usual type of curve (Fig. 4). In this study the amounts of detergents and cholate were kept constant, making the ratio of substrate:detergent variable. This may have caused the distortion in the early part of the curve.

The enzyme was found to be very heat labile; all its activity was lost after 5 min heating at 50°C. However, it was quite stable in the refrigerator, for at least 10 days. It was stable when stored for 2 hr at 4°C at pH 5, but somewhat unstable at pH 9 and higher. High activity was observed in human Gaucher and normal spleen which had been frozen for many months. In this case the spleen was homogenized with water and the supernatant solution from high speed centrifugation

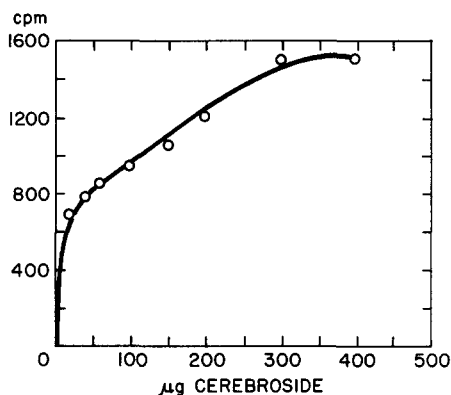


FIG. 4. Hydrolytic activity of pig brain enzyme as a function of cerebroside concentration. All other components as in standard assay.

was used directly. When frozen brain was used as the enzyme source, however, the high speed supernatant fluid was cloudy, evidently because of contamination with released lipids. In this case the enzyme activity was low, probably because of excessive dilution of the radioactive cerebroside with endogenous cerebroside.

The reversibility of the cerebroside cleavage was tested by incubating 0.1 mg of nonradioactive cerebroside with 250,000 cpm of galactose-1-<sup>14</sup>C (specific activity 1 μc/mg) for 2 hr. The remaining cerebroside was isolated and found to contain no activity.

#### Characterization of the Products

For this purpose a 10-fold scale incubation was carried out with stearoyl-<sup>14</sup>C psychosine (one-half the usual specific activity) for 4 hr. One incubation tube contained the purified enzyme, another contained the substrate with heat-denatured enzyme, and a third contained the active enzyme but no substrate. After incubation each mixture was lyophilized, taken up in 1 ml of water, and extracted with 7 ml of C-M 1:1. The extract was transferred to a centrifuge tube and the incubation tube washed again with water and C-M. The pooled extracts were centrifuged to remove protein and the supernatant solution was washed with water. The aqueous layer was washed with chloroform, which was then added to the chloroform layer. The aqueous layer contained the galactose, buffer, and a small portion of the detergents.

The chloroform layer was evaporated to dryness and the residue partitioned between C-M and water; the latter removed much of the detergent. To the lower layer was added 0.2 mg of stearic acid as carrier, the solvent was removed, and the residue was fractionated on a silica gel column to yield fatty acids, ceramide, and cerebroside. The activities and method are shown in Table 2. It can be seen that the blank value for ceramide activity was quite low (0.07% of the substrate appeared in the ceramide fraction in the control in-

cubation). In contrast, the ceramide fraction from the incubation with enzyme contained 9.5% of the substrate activity. The enzyme incubation also yielded a small amount of activity in the free fatty acid fraction, possibly because of the presence of some ceramidase (15) acting on the enzymatically produced ceramide.

The ceramide fraction was characterized further by TLC of a portion with C-HOAc 96:4, carrier ceramide being added first. All of the activity applied to the plate was found in the nonhydroxy ceramide spot.

Another portion of ceramide was purified on a Florisil column, which yields ceramide when eluted with C-M 95:5 (2). This material was examined by TLC without added carrier and showed the major spot to have an *R<sub>f</sub>* equal to that of standard nonhydroxy ceramide in two different solvents: C-M 96:4 and C-HOAc 96:4. Again all the radioactivity was found in the ceramide spot. In the case of the enzyme incubated without substrate, small spots, which evidently arose from cerebrosides contaminating the enzyme, were found for nonhydroxy and hydroxy ceramides. A C-M extract of purified enzyme gave spots, on TLC, corresponding to nonhydroxy and hydroxy cerebrosides.

The other product, galactose, was characterized by stirring the aqueous layer with a mixture of ion-exchange resins (Amberlite MB3), then packing the suspension into a column and eluting with water. This removed the ionic contaminants. The dry solids from the solution were examined by TLC on microcrystalline cellulose (16) with the developing solvent pyridine-EtOAc-W-HOAc 5:5:3:1 (17). This system separates a variety of monosaccharides, although the separation of glucose from galactose is incomplete with a single solvent pass. Treatment of the plate with aminodiphenyl oxalate showed the presence of a spot corresponding to galactose in position and color. The spot intensity, compared with that of standard spots, indicated that about 20 μg was present; the amount calculated on the basis of the ceramide activity (Table 2) was 24 μg. No galactose was obtained with the boiled enzyme

TABLE 2 CHARACTERIZATION OF THE LIPID PRODUCTS OF ENZYMATIC CEREBROSIDE CLEAVAGE

Column Fractions	Radioactivity in Incubated Sample	Radioactivity in Heat-Denatured Enzyme Sample
	<i>cpm</i>	<i>cpm</i>
Free fatty acids	695	0
Ceramides	26,510	180
Cerebrosides	250,700	276,000
Recovered <sup>14</sup> C	277,905	276,180

The incubation lipids were applied to a 3.5 g silica gel column and eluted with 50 ml each of benzene-ether 99:1 (fatty acids), C-M 98:2 (ceramides), and C-M 92:8 (cerebrosides).

TABLE 3 EFFECT OF DETERGENTS ON ENZYMIC CLEAVAGE OF CEREBROSIDE

Incubation Medium	Ceramide Activity
Complete system	cpm*
Less Tween 20	2441
“ G-2159	1492
“ Na cholate	2205
“ Tween and G-2159	58
Complete system	1433
Less cholate	1920
“ “ + deoxycholate 4 mg	22
“ “ + taurocholate “	72
“ “ + K oleate “	3197
	446

\* Corrected for no-enzyme blank (100 cpm).

control, but a faint spot was seen in the case of enzyme incubated without substrate. This, like the ceramides from this incubation, must have come from endogenous cerebroside. No other spot was visible on the plate.

To characterize the galactose more thoroughly, we incubated <sup>3</sup>H-labeled cerebroside in the standard way, then added carrier galactose and glucose (5 mg each), and deionized the aqueous layer. The two sugars were isolated as the borate complexes by ion-exchange (18), converted to the free sugars (19), and counted in a water-dioxane-naphthalene-xylene-Cellosolve scintillation solution (20). The sugar-containing fractions were located by colorimetric analysis with anthrone (21). All the radioactivity that was recovered from the borate column was found to be in the galactose fraction. This amounted to 95% of the activity in the aqueous layer following deionization, or 3.6% of the substrate activity. The activity in the aqueous layer from the incubation with heat-denatured enzyme was only 0.05%.

#### Activators and Inhibitors

Cholic acid was found to be almost essential for enzymic activity (Table 3). The optimum concentration of sodium cholate was found to be 4 mg/ml, a surprisingly high concentration. Less activity was obtained with cholic acid from another supplier; the Mann product is specified as being “enzyme grade.”

The second part of Table 3 shows that cholate is not specifically essential, taurocholate being even better. It is particularly significant that taurocholate does not precipitate at pH 4.5, so that the activating effect of cholate cannot be due to its precipitation when the buffer is added.

Several metal ions were tested at concentrations of  $1-5 \times 10^{-3}$  M (Mg<sup>++</sup>, Mn<sup>++</sup>, Cu<sup>++</sup>, Li<sup>+</sup>, Ca<sup>++</sup>, Fe<sup>+++</sup>, molybdate, and Zn<sup>++</sup>); no appreciable effect on enzyme activity was observed. Mercaptoethanol, 0.01 M, also had no effect but  $2 \times 10^{-4}$  M *p*-hydroxymercuribenzoate

reduced the activity by 91%. The chelator, EDTA, showed no inhibitory effect at 0.01 M.

#### Enzyme Specificity

As the data in Table 1 show, the enzyme preparation at all stages in its purification showed activity toward *o*-nitrophenyl  $\beta$ -galactoside. However, as the purification progressed there was a 2-fold drop in specific activity toward this substrate while the activity toward cerebroside increased 3-fold. This suggests that two galactosidases are present in the enzyme preparation. However, incubation of stearoyl-<sup>14</sup>C psychosine in the standard system together with 20  $\mu$ moles of nitrophenyl galactoside resulted in 73% inhibition. The same amount of lactose did not inhibit the cerebroside activity.

Additional separation of the two galactosidase activities was accomplished with a column of Sephadex G-100 (Pharmacia Fine Chemicals Inc., New Market, N.J.). The packing was  $2.54 \times 35$  cm, in 0.02 M potassium phosphate, pH 7.5, and the enzyme preparation from the pH 5 precipitation step was applied in the same buffer containing 3% sucrose. After elution with the buffer, portions were assayed with cerebroside and nitrophenyl galactoside and compared for relative activities. The observed ratios of activities (nitrophenyl galactoside:cerebroside) for selected successive fractions were 21, 48, 360, 710 and 590. The last fractions had very little activity toward cerebroside but were still quite active toward the unnatural substrate.

The nature of the fatty acid residue in the cerebroside substrate does not seem to be a critical factor. Both D- and L-cerebron were hydrolyzed at about the same rate as 18:0 cerebroside. A mixture of nonradioactive cerebroside gave rise to hydroxy and nonhydroxy ceramides, identified by TLC.

Emulsions of ceramide glucoside and ceramide lactoside were examined as substrates under the standard conditions scaled-up 6-fold. The enzyme was also incubated alone and with stearoyl-<sup>14</sup>C psychosine. The 98:2 C-M effluent from silica gel chromatography was examined by TLC and found to contain ceramide in each as the major component. The ceramide spot was faint in the sample from the enzyme incubated alone, but strong for the other three samples. Evidently the pig brain preparation contained activity toward the glucoside link in ceramide glucoside and toward the galactoside link in ceramide lactoside.

To confirm the conclusion that the nonhydroxy ceramide spots were indeed derived from the incubated substrates, we eluted the spots from the silica gel plate, cleaved the acids by methanolysis (22), and examined the fatty acid esters by GLC on diethylene glycol succinate polyester. The results of the analyses are listed in Table 4. Line 1 (enzyme blank) shows a typical as-

TABLE 4 FATTY ACIDS IN THE CERAMIDES FORMED BY INCUBATING GLYCOLIPIDS WITH PIG BRAIN GLYCOSIDASE

Substrate	Relative Amounts of Fatty Acids						
	14:0	16:0	18:0	20:0	22:0	23:0	24:0
	% of total						
None added	6	14	39	5	15	5	15
18:0 cerebroside	4	8	62	3	9	3	12
Ceramide glucoside	2	17	11	4	24	8	35
Ceramide lactoside	5	11	59	4	8	4	9
Ceramide glucoside (nonincubated)	—	17	3	4	26	8	38

Minor peaks were not included in the calculations, except for the original ceramide glucoside (4% 24:1).

sortment of acids characteristic of brain cerebroside except for the absence of unsaturated acids and a relatively high amount of the shorter acids. Line 2 shows the expected increase in 18:0, at the expense of the relative amounts of the other acids.

Line 3 of Table 4 shows an increase in the relative amounts of 16:0, 22:0, 23:0, and 24:0. Analysis of the original substrate (bottom line) shows these are the major acids of glucocerebroside, in confirmation of the report of Marinetti, Ford, and Stotz (23). Glucocerebroside from spleen also contains some 24:1 (24) but the ceramide from this experiment did not show a peak for this acid.

The data for ceramide lactoside (line 4) are much like those for the radioactive 18:0 cerebroside, which is to be expected as the cytoside was made from ganglioside. Brain ganglioside contains 18:0 as the predominant acid.

#### Activity of the Galactosidase in Various Organs

Several organs of 250-g rats were homogenized in 9 volumes of 0.25 M sucrose, then centrifuged for 1 hr at 100,000  $\times g$ . The supernatant solution was tested for activity toward stearoyl-<sup>14</sup>C psychosine by the standard procedure. The results (Table 5) show that activity toward cerebroside occurs in brain, kidney, spleen, and lung, but there is little activity in liver and heart. Since kinetic studies were not made in this survey, the comparisons are rough. It can be seen that the activity was proportional to amount of extract only in the first three samples.

## DISCUSSION

#### Relation to Glycolipid Breakdown

This study establishes the presence in brain of an enzyme which can cleave galactocerebrosides to form ceramide and galactose. The enzyme complements the ceramidase found by Gatt (15), which continues the process of

cerebroside breakdown to form fatty acids and sphingosine. The combination of the two enzymes accounts for the descending portions of the turnover curves observed for the galactose (1) and fatty acid (25) residues of cerebroside. The cerebroside galactosidase also accounts for the presence of nonhydroxy and hydroxy ceramides in brain (26).

This study also establishes the presence of enzymes in the supernatant fraction of brain homogenates which can cleave the carbohydrate bond in ceramide glucoside and ceramide lactoside to form ceramide. Although these lipids do not occur in appreciable amounts in brain, they are probably intermediates in ganglioside breakdown and thus the above enzymes account for the turnover curves observed for the galactose (1) and fatty acid (27) residues of ganglioside. The glucosidase activity also explains the finding by Kanfer (28) that intracranially administered ceramide glucoside is converted to ceramide in the rat.

#### Enzyme Distribution

In some respects the distribution of activity toward cerebroside found in rat organs (Table 5) follows the distribution of ceramide galactoside. The two most active organs were kidney and spleen. The former has recently been shown to contain ceramide galactoside (29, 30) and the latter has been shown to be able to make this lipid (31), although the galactoside is not present in detectable concentrations in human spleen (10). Ceramide galactoside has been found in bovine spleen (29) and the possibility exists that it is made and destroyed normally in all spleens, the level usually being too low for detection.

The low activity detected in the brain extract is due in part to dilution of the radioactive substrate by the endogenous cerebroside in the extract. Ceramide is relatively concentrated in lung and the possibility exists that it arises by action of the galactosidase on cerebroside. Liver, which ranked very low in ability to

TABLE 5 DISTRIBUTION OF CEREBROSIDE-HYDROLYZING ACTIVITY IN RAT ORGANS

Organ	Ceramide Activity	
	0.2 ml	0.5 ml
	<i>cpm</i>	
Kidney	994	2466
Spleen	825	2144
Lung	299	699
Brain	208	362
Heart	133	189
Liver	129	199

Incubation conditions as in text. Each tissue extract was assayed at two levels, 0.2 and 0.5 ml of enzyme. Activities have been corrected for the no-enzyme blank (100 cpm).

hydrolyze ceramide galactoside, is known to lack this lipid (32).

### *Is There a Specific Cerebrosidase?*

Since we did not purify either the galactosidase or glucosidase sufficiently to separate all known activities, no definite answer to this question can be offered. However, several findings lend support to the idea that a cerebroside galactosidase exists. (a) The substrate occurs near the enzyme. In brain the enzyme is found in the supernatant fluid and galactocerebroside is found there also, as well as in other subcellular regions (33, 34). (b) Partial separation of activity toward cerebroside and nitrophenyl galactoside was achieved. Certain fractions which were very low in cerebroside activity nevertheless still contained appreciable activity toward the artificial substrate. The inhibitory effect of the artificial substrate on cerebroside cleavage need not represent competition between two substrates. Lactose was not inhibitory. (c) The distribution of activity toward cerebroside in different organs differs in some respects from the distribution of activity toward nitrophenylgalactoside (35) or bromonaphthylgalactoside (36). Liver has high activity toward the unnatural substrates, but low activity toward galactocerebroside (Table 5). (d) Brady, Kanfer, and Shapiro (37) have succeeded in separating the enzymes in spleen which cleave ceramide glucoside and galactoside. A similar separation has been accomplished by Gatt and Rapport (38) with a galactosidase (active toward cytolipin) and a glucosidase (active against ceramide glucoside). These enzymes were obtained from a mitochondrial fraction of rat brain and resemble our preparation in being greatly activated by cholate. (e) At least four galactosidases, active toward nitrophenyl galactoside, have been found in some rat organs (39). It is likely that a variety of glycosidases exist in each organ, each specific toward one or a few natural substrates, but *all* active toward certain unnatural substrates, such as nitrophenyl glycosides.

### *The Bile Acid Activation*

It is interesting that the ceramidase and sphingoglycolipidases of brain are highly activated by cholic acid. This increases the plausibility of the suggestion that these enzymes are operating in a normal sequence of steps in the living animal. Although neither cholic nor taurocholic acid is known to occur in brain, an equivalent substance may be present. Intracisternal injection of cholate or taurocholate can produce demyelination *in vivo* (40) and the possibility must now be considered that the effect is the result of activation of sphingolipid-hydrolyzing enzymes.

The effect of the bile acids cannot be explained as due to liberation of hydrolases from lysosomes, since we found the effect also in sucrose and water extracts following high-speed centrifugation.

### *Comparison with Other Glycosidases*

Like other galactosidases, the cerebroside-cleaving enzyme has a low optimal pH. It is likely that the other galactosidases, when assayed at their optimal pH, are also insoluble and that heterogeneity of the incubation system has an important influence on the reaction rates.

A number of reports have appeared describing a cerebroside galactosidase in brain (41–43), but it is not clear whether adequate blanks were run. Because of this important question, the conflicting statements about the enzyme properties, and the nonspecificity of the assays used, these reports may be of little value.

A kidney preparation, recently described by Sandhoff, Pilz, and Jatzkewitz, has been found to hydrolyze ceramide tetrasaccharide to a series of ceramide saccharides and ceramide (44). Thus this enzyme mixture too contains glucosidase and galactosidase activity. Like the brain galactosidase, the kidney enzyme has an optimum pH of 4.5, but it is found in the crude mitochondria instead of cell supernatant fluid. As Table 5 shows, we found galactosidase activity in the kidney supernatant fraction.

A  $\beta$ -galactosidase, active toward a fluorogenic substrate, has been studied in microscopic samples of rat cerebellum (45). Its activity in white matter rises with age during the period of active myelination, then drops considerably, which suggests it may be involved in cerebroside metabolism. The galactosidase activity in the external granular layer and molecular layer of cerebellum (gray matter) does not change appreciably with age. Perhaps the activity in the gray region reflects the metabolism of ganglioside, a galactolipid concentrated in gray matter.

A recently described cerebroside galactosidase of intestine (46) resembles the brain enzyme in requiring a very high cholate concentration for activation, but occurs in the rapidly sedimenting cell particles. Despite a high degree of purification, the intestinal enzyme was found to be active toward nitrophenyl glycosides, as well as other ceramide glycosides.

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