Highly active enzymatic hydrolysis of galactosylceramide was detected in the murine intestine in confirmation of an earlier report in the rat intestine (Brady, R. O., Gal, A. E., Kanfer, J. N., and Bradley, R. M. (1985) J. Biol. Chem. 240, 3766-3770). Unlike the classical galactosylceramidase (EC 3.2.1.46), which is present in other organs, as well as also in the intestine, this intestinal enzyme was not activated by sodium taurocholate and was inhibited by oleic acid. It was effectively activated by sodium taurodeoxycholate and had a pH optimum of 5.2. This taurodeoxycholate-activated galactosylceramidase did not appear to be present in the brain, liver, kidney, and spleen. Its activity was not deficient in affected twitcher mice, a newly discovered mutant caused by a genetic deficiency of the taurocholate-activated galactosylceramidase. Although it showed a relatively neutral pH optimum, the taurodeoxycholate-activated galactosylceramidase is not a nonlysosomal "neutral" β-galactosidase, because unlike the latter, it was adsorbed to Concanavalin A-Sepharose after solubilization with 0.5% sodium taurocholate and was eluted by α-methylmannoside or α-methylglucoside. The taurodeoxycholate-activated galactosylceramidase could be completely separated from the taurocholate-activated galactosylceramidase and G_{M1}-ganglioside β-galactosidase (EC 3.2.1.23) by octyl-Sepharose hydrophobic chromatography. Thus, the taurodeoxycholate-activated galactosylceramidase localized in the intestine is distinct from the two known glycosphingolipid β-galactosidases and the neutral β-galactosidase.

Two distinct β-galactosidases are known that hydrolyze glycosphingolipids with a terminal β-galactose residue. One is galactosylceramidase (EC 3.2.1.46) which catalyzes hydrolysis of galactosylceramide to ceramide and galactose. It is also active toward galactosylsphingosine (1, 2), monogalactosyldiacylglyceride (3), and lactosylceramide (4-6). The activity of this enzyme is genetically deficient in globoïd cell leukodystrophy occurring in humans (7), dogs (8), and mice (9). The other is G_{M1}-ganglioside β-galactosidase (EC 3.2.1.23), the deficiency of which causes G_{M1}-gangliosidosis (10). It also cleaves the terminal galactose from asialo G_{M1}-ganglioside (11, 12), lactosylceramide (5, 11, 12), and asialofetuin (13). Both β-galactosidases appear to be active toward artificial substrates, such as 4-methylumbelliferyl or p-nitrophenyl β-galactoside, although at different rates (11). To assay activities of these β-galactosidases with natural lipid substrates, bile salts are usually included in the reaction mixtures. The nature and amount of the bile salts are often critically important because certain combinations of the enzyme and substrate exhibit high specificity with respect to the bile salt.

In 1965, Brady et al. (14) described a galactosylceramidase in rat intestine which was activated by sodium cholate. Highly purified preparations were active not only toward p-nitrophenyl β-galactoside but also toward glucosylceramide and p-nitrophenyl β-glucoside. The relationship of this rat intestinal enzyme to the two well defined β-galactosidases has not been clarified. In the present report, we describe presence of a similar galactosylceramidase in murine intestine, an optimized assay procedure with the use of sodium taurodeoxycholate, and some of its properties which clearly indicate that this enzyme is genetically and chromatographically distinct from the other two glycosphingolipid β-galactosidases.

**EXPERIMENTAL PROCEDURES**

**Commercial Materials**—The following materials were obtained from commercial sources as indicated: sodium taurocholate (synthetic), sodium taurodeoxycholate, sodium cholate, sodium deoxycholate, sodium taurochenodeoxycholate, sodium glycodeoxycholate, oleic acid, α-methylmannoside, α-methylglucoside, Triton X-100 (Sigma Chemical Co., St. Louis, MO); galactosylceramide (bovine brain), bovine brain gangliosides, 4-methylumbelliferyl β-galactopyranoside (products of Koch-Light Labs through Research Products International, Elk Grove Village, IL); potassium [3H]borohydride (specific activity, 302 Ci/mol) (New England Nuclear, Boston, MA); galactose oxidase (Worthington Biochemical Corp., Freehold, NJ); Concanavalin A-Sepharose 4B, octyl-Sepharose, DEAE-Sepharose (Pharmacia Fine Chemicals, Piscataway, NJ); Bio-Solv BBS-3 (Beckman Instruments, Fullerton, CA); activated silicic acid (Unisel, Clarkson Chemical Co., Williamsport, PA); AG 1-×8 anion-exchange resin (200 to 400 mesh, acetate form) (Bio-Rad Labs, Richmond, CA).

**Radioactive Substrates**—Bovine brain galactosylceramide was labeled with tritium at the terminal galactose moiety by the galactose oxidase-sodium borohydride procedure (15), followed by purification through a silicic acid column (16). G_{M1}-ganglioside was prepared from a bovine brain ganglioside mixture by neuraminidase treatment followed by a DEAE-Sephadex column according to the method of Ando and Yu (17). It was labeled at the terminal galactose with the same principle as for galactosylceramide with modifications necessitated by the hydrophilic nature of the ganglioside (18). The labeled G_{M1}-ganglioside was purified once more by the DEAE-Sephadex column chromatography. The radioactive substrates were diluted with nonradioactive compounds to specific activity of 500 to 700 cpm/nmol for enzyme assays.

**Enzyme Source**—The C57BL/6J strain of mice was used for all experiments. The twitcher mutant, which is caused by a genetic deficiency of galactosylceramidase, also occurs in the same strain. The twitcher mutant was first discovered at the Jackson Laboratory.
Bar Harbor, ME. Frozen organs of affected mice were sent to New York from the colony maintained in the Department of Neuropathology, The Institute of Neurology, The National Hospital, London. Removed organs were processed either fresh or within 1 month of frozen storage at −20°C. Only 40- or 41-day-old mice were used for this series of study. A portion of the intestine approximately 3 cm distal to the pylorus and 2 cm proximal to the cecum was slit longitudinally and washed thoroughly in 0.85% NaCl. Approximately 2 g of brain or small intestine was homogenized in 19 volumes (w/v) of ice-cold 10 mM sodium phosphate buffer, pH 7.2, containing 50 mM NaCl and 0.02% sodium azide in a hand-operated Dounce homogenizer (Kontes Glass Co., Vineland, NJ). The homogenate was centrifuged at 10,000 × g for 30 min, and the supernatant was discarded. The pellet was resuspended by homogenization in the same buffer but with additional 0.5% sodium taurodeoxycholate. After 1 h at 4°C, the homogenate was centrifuged at 105,000 × g for 60 min. The supernatant which contained the extracted enzyme was applied to a Concanavalin A-Sepharose column (0.7 × 5.3 cm). The column was washed with the same buffer and then the adsorbed enzyme was eluted at room temperature with additional 10% α-methylmannoside or a-methylglucoside and 0.5% NaCl. The eluate was dialyzed extensively against 10 mM sodium phosphate buffer, pH 7.2, for at least 48 h with frequent changes of the buffer. The retentate was then applied to an octyl-Sepharose column (1.0 × 6.3 cm). After washing with the same buffer, a linear gradient of Triton X-100 (0 to 1%) was introduced to the elution medium. Activity of the intestinal taurodeoxycholate-activated galactosylceramidase was not absorbed to this column. The taurocholate-activated galactosylceramidase was adsorbed and was eluted with approximately 0.1% Triton X-100. The fractions containing the enzymatic activity was pooled and used as the enzyme source.

**Assay Procedures**—The reaction mixture for mouse brain galactosylceramidase was essentially that of Tanaka and Suzuki (11) except that the amount of [3H]galactosylceramide was reduced from 60 to 25 μg and the pH of the buffer was 5.5. It contained, in the final volume of 0.2 ml, 25 μg of the labeled substrate, 0.5 mg of sodium taurocholate, 50 μg of oleic acid, 0.1 ml of 0.1/0.2 M sodium citrate/phosphate buffer, pH 5.5, and 0.1 ml of the enzyme source. This assay was referred to as the taurocholate system.

For assays of the taurodeoxycholate-activated galactosylceramidase, the reaction mixture contained, in the final volume of 0.2 ml, 50 μg of the labeled galactosylceramide, 0.5 mg of sodium taurodeoxycholate, 0.1/0.2 M sodium citrate/phosphate buffer, pH 5.2, and 0.1 ml of the enzyme source. This reaction system was referred to as the taurodeoxycholate system. In all assays, solutions of the substrate, bile salt, and, when present, oleic acid, were dried together in screw-capped tubes (13 × 100 mm) under a stream of nitrogen. The mixture was dispersed with the addition of the buffer and sonication in a water bath-type ultrasonicator, and the enzyme source was added just prior to incubation. The incubation was for 60 min at 37°C with gentle shaking. The enzymatically liberated [3H]-galactose was determined as described previously (5).

The assay mixture for Gαγ-galactoside β-galactosidase was according to our standard procedure (19). The determination of the liberated [3H]-galactose was modified as follows. The reaction was terminated by the addition of 1 ml of chloroform/methanol (2:1, v/v) and vigorous shaking. After brief centrifugation, the clear upper phase was transferred to a centrifuge tube which contained approximately 1.5 ml of AG-1 anion exchange resin in the acetate form. After addition of 1.5 ml of water and shaking, the tube was centrifuged at 1,000 × g for 5 min. The radioactivity of the supernatant was determined on an aliquot in the same way as for the galactosylceramidase assay.

**4-Methylumbelliferyl β-galactosidase activity was assayed according to the method of Ho and O'Brien (20).**

All assays were carried out in duplicate and the sample counts were corrected for appropriate blank counts. The protein contents of the enzyme sources were determined by the method of Lowry et al. (21) with bovine serum albumin as the standard.

**RESULTS**

**Effect of pH, Bile Salts, and Oleic Acid**—With whole homogenate of normal mouse intestine as the enzyme source, effects of pH and the nature of bile salts on hydrolysis of galactosylceramide were examined (Fig. 1). A few findings, dramatically different from those in the brain, were noted. Sodium taurocholate which is an excellent activator for the brain galactosylceramidase was the least effective to stimulate intestinal galactosylceramidase activity either with or without additional oleic acid. Other bile salts, most notably sodium taurodeoxycholate, were far more effective. The pH optima with other bile salts were higher than that with taurocholate. At the optimum pH, the activity determined with sodium taurodeoxycholate was 1 order of magnitude greater than that with sodium taurocholate which gave activity in the range similar to those of the galactosylceramidase in other organs.

The activation by sodium taurodeoxycholate was concentration-dependent (Fig. 2). Unlike the taurocholate-activated galactosylceramidase, oleic acid was inhibitory to the taurodeoxycholate-activated galactosylceramidase (Fig. 3). The standard taurodeoxycholate assay system for the intestinal galactosylceramidase described under "Experimental Procedures" was developed based on these findings.

**Properties of the Reaction**—With the taurodeoxycholate system and intestinal homogenate or solubilized and partially...
purified preparations as the enzyme source, the reaction was linear for up to 1 h of incubation and was also linear with respect to the enzyme source up to 20 μg of protein/tube. The apparent $K_m$ values for the substrate were $6.9 \times 10^{-3} \text{M}$ for whole homogenate and $1.2 \times 10^{-3} \text{M}$ for the partially purified preparation. While a lower $K_m$ value for whole homogenate seemed unusual, the finding was consistent in several repeated experiments.

**Intestinal Galactosylceramidase in Twitcher Mouse**—In order to test the possibility that the taurodeoxycholate-activated intestinal galactosylceramidase was genetically distinct from the taurocholate-activated galactosylceramidase, whole homogenates of the brain and intestine from twitcher mice, a mutant with a genetic deficiency of the taurocholate-activated galactosylceramidase, whole homogenates and 1.2 mg protein per tube were examined in both of the assay systems (Table I). With the taurocholate system, the genetic status of each mouse could be readily established with brain homogenate. The affected mice showed galactosylceramide-cleaving activity of only 1% of the controls. Although less clear cut quantitatively, qualitatively identical results were obtained in the intestinal homogenates assayed with the taurocholate system. When the taurodeoxycholate system was used, the activities in control brains were substantially lower than those in the taurocholate system. The activities in the brains of the affected mice were higher than those in the taurocholate system but were still much lower than the control. In contrast, the taurodeoxycholate system gave intestinal galactosylceramidase-cleaving activities in control mice more than 50 times those with taurocholate. Furthermore, the activities in the affected mice were generally higher than the control activities. Examinations of the liver, kidney, and spleen showed that these systemic organs were qualitatively identical to the brain with respect to their galactosylceramidase-cleaving activities determined with the two assay systems (Table II). These findings indicated clearly that the murine intestine contained at least two enzymes capable of hydrolyzing galactosylceramide; one activated by taurocholate and common with other organs, and the other activated most effectively by taurodeoxycholate. The galactosylceramidase activated by taurodeoxycholate is genetically distinct from the taurocholate-activated galactosylceramidase which is genetically deficient in globoid cell leukodystrophy, including the twitcher mouse, and this enzyme is practically absent in the brain, liver, kidney, and spleen.

### Table I

| Genetic status | Brain* | Intestine |
|---------------|--------|----------|
| Taurocholate system | Taurodeoxycholate system | Taurocholate system | Taurodeoxycholate system |
| **Affected** | nmol/h/mg protein | nmol/h/mg protein | nmol/h/mg protein | nmol/h/mg protein |
| 1 | 0.04 | 0.4 | 0.3 | 191 |
| 2 | 0.04 | 0.2 | 2.2 | 180 |
| **Heterozygote** | 2.4 | 0.8 | 1.4 | 178 |
| Control | 1 | 3.2 | 13 | 120 |
| 2 | 4.5 | 1.1 | 1.9 | 98 |
| 3 | 4.6 | 1.5 | 1.5 | 94 |

*The pH of the taurodeoxycholate system for the brain homogenate was 4.5, as determined by preliminary experiments.*

### Table II

| Organ and systems | Twilight (n = 1) | Control (n = 1) |
|-------------------|-----------------|----------------|
| Brain             | Taurocholate | 0.08 | 4.8 |
|                   | Taurodeoxycholate | 0.0 | 2.0 |
| Liver             | Taurocholate | 0.1 | 3.2 |
|                   | Taurodeoxycholate | 0.5 | 2.4 |
| Kidney            | Taurocholate | 0.3 | 25.4 |
|                   | Taurodeoxycholate | 1.2 | 5.6 |
| Spleen            | Taurocholate | 0.2 | 2.8 |
|                   | Taurodeoxycholate | 0.7 | 1.9 |
| Intestine         | Taurocholate | 0.3 | 2.2 |
|                   | Taurodeoxycholate | 193 | 120 |

**Fig. 3.** Effect of oleic acid on the intestinal taurodeoxycholate-activated galactosylceramidase. The activities were determined by the standard taurodeoxycholate system described in the text except that varying amounts of oleic acid were added as indicated. Each data point represents the average of duplicate determinations in a representative experiment. The results are expressed in per cent of the standard activity without oleic acid.

**Fig. 4.** Octyl-Sepharose chromatography of mouse intestinal galactosylceramidases. The dialyzed post-Concanavalin A preparation containing 1.4 mg of protein was applied to an octyl-Sepharose column and eluted as described in the text. O--O, taurocholate-activated galactosylceramidase (1 activity unit = 50 cpn/tube); ×--×, taurocholate-activated galactosylceramidase (1 activity unit = 2,000 cpn/tube); ×--×, Gb-ganglioside β-galactosidase (1 activity unit = 250 cpn/tube); Δ--Δ, 4-methylumbelliferone, β-galactosidase (1 activity unit = 5 nmol/tube). All incubations were for 1 h. Although not shown in order to save space, the gradient elution continued to 1% Triton X-100 but no further enzymatic activities were eluted.
Octyl-Sepharose Chromatography—The octyl-Sepharose hydrophobic column chromatography clearly separated the taurodeoxycholate-activated galactosylceramidase from the taurocholate-activated galactosylceramidase and GMI-ganglioside β-galactosidase (Fig. 4). The fraction unadsorbed by octyl-Sepharose contained the taurodeoxycholate-activated galactosylceramidase but contained little taurocholate-activated galactosylceramidase activity and no activity of GMI-ganglioside β-galactosidase. The activities of the latter two enzymes were adsorbed to the column and required 0.1% or higher concentrations of Triton X-100 for elution. Both adsorbed and unadsorbed fractions were active toward 4-methylumbelliferyl β-galactoside. The unadsorbed enzyme fraction was absent when brain tissue was subjected to the same purification procedure and finally to the octyl-Sepharose column chromatography (Fig. 5).

DISCUSSION

The substrate specificities of the two genetically distinct lysosomal acidic β-galactosidases have been extensively investigated (22). Most of known glycosphingolipids with terminal β-galactose residue are substrates for one or the other of the two β-galactosidases. On the other hand, another genetically unrelated β-galactosidase, nonlysosomal neutral β-galactosidase, has been much less well characterized (23). The general consensus is that natural substrates for the neutral β-galactosidase are not known, although hydrolysis of lactosylceramide by human hepatic neutral β-galactosidase has been reported (24). In view of these more recent developments, the earlier report of Brady et al. (14) on an intestinal glycosidase which was active toward both galactosylceramide and glucosylceramide was of interest. The present study was intended to confirm the presence of the galactosylceramidase in the rodent intestine and then to examine its relationship to other known β-galactosidases.

The results clearly demonstrated the presence of a very active glycosidase in the murine intestine which hydrolyzes galactosylceramide. This enzyme was distinct from either of the two acid β-galactosidases and also from the neutral β-galactosidase. This enzyme was present in the murine intestine in addition to the classical galactosylceramidases. Although both enzymes were active on galactosylceramide, they were different from each other in their properties. The classical galactosylceramidase was effectively activated by sodium taurocholate but the other enzyme was almost totally inactive in the presence of taurocholate and required taurodeoxycholate for maximum activation. The taurocholate-activated galactosylceramidase was adsorbed to octyl-Sepharose and required Triton X-100 for elution while the taurodeoxycholate-activated enzyme was not adsorbed under the same experimental conditions. Thus, the taurocholate-activated and the taurodeoxycholate-activated galactosylceramidase activities were almost completely separated from each other by the octyl-Sepharose chromatography. Most importantly, the activities of the taurodeoxycholate-activated galactosylceramidase were not correlated with the genetic status of the twitcher mice, a mutation caused by deficiency of the taurocholate-activated galactosylceramidase. Even in the intestine, the affected mice could be distinguished when assays were done with taurocholate as the activator, an indication that the taurocholate-activated enzyme in the intestine is the same enzyme as that in the brain. The taurodeoxycholate-activated galactosylceramidase was not present in other organs tested.

The taurodeoxycholate-activated galactosylceramidase had a pH optimum more neutral (5.2) than that of the taurocholate-activated enzyme. However, it is not the same enzyme as the neutral β-galactosidase. The neutral β-galactosidase does not bind to Concanavalin A (25) and does not hydrolyze GM1-ganglioside β-galactosidase.

We believe the murine intestinal taurodeoxycholate-activated galactosylceramidase is similar to the rat intestinal galactosylceramidase reported by Brady et al. (14). They used sodium cholate as the activator. Although cholate was an effective activator also for the murine intestinal enzyme, we elected to use sodium taurodeoxycholate because of the poor solubility of cholate at pH below 5.5 and the consequent wider variations in the assay results. Brady et al. (14) reported that their enzyme was active also toward p-nitrophényl β-glucoside and glucosylceramide. Leese and Semenza (26) presented strong evidence that the enzyme reported by Brady et al. (14) might be identical with phlorizin hydrolase. Although detailed substrate specificity studies must be carried out, our enzyme preparations are not yet sufficiently pure to yield rigorous and unambiguous results.

The physiological function of the taurodeoxycholate-activated galactosylceramidase in the intestine is a matter of speculation at this time. Unlike in other organs, such as the brain, in which bile salts are not present at anywhere near the concentration necessary for activation of sphingolipid hydrolases in vitro, relatively high concentrations of bile salts are present in the intestinal lumen and, therefore, can act as the physiological activator of the enzyme. Very little galactosylceramide is present in the intestinal tissue and it is likely that some other compounds may be the physiological substrates of the enzyme. Very little galactosylceramide is present in the intestinal tissue and it is likely that some other compounds may be the physiological substrates of the enzyme. Substrate specificity studies with more purified enzyme preparations would be required to answer this question.

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