papers and notes on methodology

Analysis of galactosylsphingosine (psychosine) in the brain[1]

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Abstract A sensitive and specific analytical procedure has been developed for determination of galactosylsphingosine (psychosine) in the brain. The method takes advantage of two unusual properties of psychosine—the strong positive charge and the reactivity of the free amino group. It involves lipid extraction, separation from other lipids on a cation-exchange column (AG-50W), elimination of the last trace of galactosylceramide by silicic acid chromatography, dansylation of psychosine, Florisil and DEAE-Sephadex chromatography, and finally, fluorescent densitometry of dansylated psychosine separated by thin-layer chromatography. The detection limit is 5–10 ng/100 mg brain tissue when the standard procedure is followed exactly. Reliable determination can be made for 50 ng/100 mg or higher in the presence of 200,000-fold excess of other lipids and in the presence of 40,000-fold excess of galactosylceramide. The sensitivity can be increased fivefold by using a larger aliquot for the final determination. This analytical procedure has been successfully applied to demonstrate abnormal accumulation of psychosine in the brain in human, canine, and murine genetic galactosylceramidase deficiencies (globoid cell leukodystrophy).—**Igisu, H., and K. Suzuki.** Analysis of galactosylsphingosine (psychosine) in the brain. *J. Lipid Res.* 1984. **25:** 1000–1006.

Supplementary key words globoid cell leukodystrophy • Krabbe disease • fluorescent densitometry • dansylation • human • dog • mouse

Galactosylsphingosine was first obtained by Thudicum (1) by alkaline hydrolysis of galactolipid of the brain and was named psychosine. In 1960, Cleland and Kennedy (2) demonstrated that microsomes of guinea pig and rat brain contained an enzymatic mechanism to synthesize galactosylsphingosine by galactosylation of sphingosine. A hydrolase in rat brain that catalyzes the reverse reaction was characterized by Miyatake and Suzuki (3). These authors also showed that the activity of galactosylsphingosine galactosyl hydrolase was deficient in tissues of patients with globoid cell leukodystrophy (Krabbe disease) (4). They further showed that this hydrolase was probably the same enzyme as galactosylceramidase (EC 3.2.1.46) (5), which had earlier been shown to be deficient in Krabbe patients (6). Miyatake and Suzuki (4) proposed that galactosylsphingosine might be the cytotoxic compound responsible for the biochemical pathogenesis of Krabbe disease, rather than galactosylceramide, the natural substrate of the genetically deficient enzyme ("the psychosine hypothesis").

Natural occurrence of galactosylsphingosine in normal brain, however, has not been convincingly demonstrated. In 1975, Vanier and Svennerholm (7) reported that there was a small amount of psychosine in normal infant brain and that it was increased at least 10-fold in cerebral tissues of patients with Krabbe disease. In a more recent study, Svennerholm, Vanier, and Månsson (8) reported up to a 100-fold increase of psychosine in cerebral white matter of patients with Krabbe disease over the essentially unmeasurable normal level. Their analytical methodology was a combination of conventional glycolipid isolation procedures that required 5 g

[1] The content of this article was presented in part at the 15th Annual Meeting of the American Society for Neurochemistry, Portland, OR, March 1984, and was published in abstract form (Igisu, H., and K. Suzuki. 1984. Galactosylsphingosine (psychosine) in the brain of twitcher mouse. *Trans. Am. Soc. Neurochem.* **15:** 104).

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of cerebral white matter from patients with Krabbe disease. More sensitive methodology is required to establish the level of psychosine in normal brain. Such methodology is also essential for analysis of psychosine in brains of the recently discovered mouse mutant, twitcher, which is an enzymatically authentic genetic model of human globoid cell leukodystrophy (9). We describe an analytical procedure that involves exhaustive elimination of all interfering lipids and the fluorescence densitometry of dansylated psychosine separated by thin-layer chromatography. The method is suitable for determination of psychosine in 100 mg of brain tissue.

EXPERIMENTAL PROCEDURE

Commercial materials

The sources of commercial materials were as follows: galactosylsphingosine and phosphatidylcholine (egg) (Supelco, Inc., Bellefonte, PA); dansyl chloride, fluorescamine, cupric acetate, phosphatidylserine, phosphatidylethanolamine, and triethanolamine (Sigma Chemical Co., St. Louis, MO); DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, NJ); silicic acid (Unisil, Clarkson Chemical Co., Williamsport, PA); cation-exchange resin, AG-50W (hydrogen form), and Econo-column (Bio-Rad Labs., Rockville Center, NY); high-performance thin-layer chromatographic plates (silica gel 60, 10 × 20 cm) manufactured by E. Merck (American Scientific Products, Rochester, NY); and Sep-Pak cartridge (C-18 and Florisil) (Waters Associates, Milford, MA). The authenticity of the galactosylsphingosine from Supelco, Inc. was tested in the following manner. It co-chromatographed in thin-layer chromatography in solvents II and VI (Table 1) with tritium-labeled galactosylsphingosine prepared in our laboratory from labeled galactosylceramide by alkaline degradation (3). Acid hydrolysis yielded sphingosine and galactose as tested by thin-layer chromatography.

Standard analytical procedures

All column chromatography was done with disposable polypropylene columns (0.8 × 4 cm with a 10-ml reservoir). Glass wool was not used since it was found to contribute to fluorescent contamination. Evaporation of solvents was done under a stream of nitrogen at 37°C (before the dansylation step) and at 25°C (after dansylation).

Tissue extraction. Brain was weighed in a preweighed test tube, 1 ml of cold water was added, and the tissue was homogenized with the PT10-35 Polytron homogenizer (Brinkmann Instr., Westbury, NY) equipped with a PT10ST probe generator at a setting of 4 for 10 sec twice. The volume of the homogenate was measured and an aliquot of 0.1 ml was removed for determination of protein and galactosylceramidase activity when needed. The homogenate was extracted with the addition of 10 volumes of chloroform–methanol 2:1 (v/v) and homogenization. The mixture was diluted by further addition of the same volume of the chloroform–methanol mixture and was homogenized once more. The homogenate was filtered through a sintered glass funnel (medium porosity) under low vacuum. A portion of the filtrate, equivalent to 100 mg of brain tissue, was used for subsequent analysis of galactosylsphingosine. For each analytical run, at least four aliquots of normal brain lipid extract were set up simultaneously with the sample(s) to be analyzed. Different amounts of standard galactosylsphingosine were added and these normal brain extracts with the added standard were carried through the procedure together with the samples.

Cation-exchange chromatography. AG-50WX8 resin, 200–400 mesh, was converted to the sodium form in order to avoid lowering of pH during the procedure. Simple treatment of the commercial resin with 1 N NaOH gave unsatisfactory results and the following procedure was adopted. One hundred and fifty grams of resin in hydrogen form was placed in a large Büchner funnel and washed successively with 3 N HCl (3 l), water (3 l), 10% NaCl in water (1.5 l), water (1.5 l), 3 N HCl (0.5 l), water (0.8 l), 10% NaCl in water (0.6 l), water (0.6 l), 10% NaCl in water (0.9 l), water (2.5 l), and finally methanol (0.9 l). The resin was then kept in methanol. Each batch of resin thus prepared was tested in the following manner before use. The resin was packed to a column of 1-ml bed volume, washed with 20 ml of methanol, and then with 7.5 ml of methanol–aqueous 0.4 M $CaCl_2$ (3:1, v/v). If the pH of the final eluate was below 3.0, the batch was not used. This precaution was necessary to avoid hydrolysis of trace amounts of galactosylceramide. A column was packed to a bed volume of 2 ml and washed with 40 ml of methanol. The filtered and dried brain lipid extract was suspended in 3 ml of methanol and applied to the column. The tube was washed twice with 1.5 ml of methanol and both washings were also applied to the column. The column was then washed successively with 9 ml of methanol, 30 ml of chloroform–methanol (2:1, v/v), and 15 ml of methanol. Psychosine was adsorbed under these conditions and was subsequently eluted with 15 ml of methanol–0.4 M $CaCl_2$ in water 3:1 (v/v).

C-18 reverse phase chromatography. Polypropylene disposable pipette tips (a large (Rainin C-5000) and a small (Rainin RT-20)) were connected to the top and the bottom, respectively, of the Sep-Pak C-18 cartridge, which was then placed on a vacuum filtration apparatus (Fisher Scientific, Springfield, NJ) so that elution could be made by suction. Before the application of samples,

the cartridge was washed with methanol (10 ml), chloroform–methanol 1:1 (v/v), (20 ml), methanol (10 ml), and methanol–water 1:1 (v/v), (10 ml). To the eluate from the AG-50W column (approximately 15 ml) was added 17 ml of water. The mixture was then applied to the Sep-Pak C-18 cartridge. The eluate was collected and applied to the cartridge once more to assure complete adsorption of lipids. After the cartridge was washed with 100 ml of water, lipids were eluted with 2 ml of methanol and 15 ml of chloroform–methanol 2:1 (v/v). Egg phosphatidylcholine, 0.5 mg, was added to the combined eluate which was then dried.

Silicic acid column chromatography. Unisil was suspended in chloroform and packed to a column of 1.0 ml bed volume. The column was pre-washed with 15 ml of chloroform and 10 ml of chloroform–methanol 9:1 (v/v). The sample was dissolved in 3 ml of chloroform–methanol 9:1 (v/v) and applied to the column. Two washings of the sample tube with the same solvent mixture (1.5 ml each) were also applied to the column. The column was washed with 9 ml of the same solvent mixture, and the eluate was discarded. The remaining lipids were eluted with 15 ml of methanol and dried.

Dansylation. The dansylation of galactosylsphingosine was done according to Huang (10) with some modifications. The sample was quantitatively transferred to a screw-capped test tube (16 × 125 mm) with a small amount of chloroform–methanol 2:1 (v/v) and dried. Dansyl chloride was dissolved in methanol at 15 mg/ml, and 0.3 ml was added to the dried sample. The contents were vigorously mixed with a vortex mixer. While the test tube was being shaken, 0.4 ml of 1 M $NaHCO_3$–NaOH (pH 10) was slowly added. The tube was closed with a Teflon-lined cap and the contents were sonicated in a water-bath type sonicator for 15 sec. The tube was then shaken gently at room temperature for 2.5 hr. During this period, the tube was vigorously shaken once with a vortex mixer. After the reaction, the content was neutralized with the addition of 0.3 ml of 2 N HCl. Ten ml of chloroform–methanol 2:1 (v/v) and 1.4 ml of water were added and the tube was shaken. After brief centrifugation, the upper layer was discarded. The lower phase was washed three times with the addition of chloroform–methanol–water 3:48:47 (by vol), and dried.

Sep-Pak Florisil cartridge chromatography. The physical set-up of the Sep-Pak Florisil cartridge was as described for the Sep-Pak C-18 cartridge above. After the cartridge was washed with 15 ml of chloroform, the sample dissolved in 5 ml of chloroform was added. Two 5-ml washings of the sample tube were also applied to the cartridge. The cartridge was washed with 75–80 ml of chloroform to remove free dansyl chloride. Dansylated psychosine was then eluted with 30 ml of acetone–methanol 9:1 (v/v) and dried.

DEAE-Sephadex column chromatography. The acetate form of DEAE-Sephadex was prepared according to Ledeen, Yu, and Eng (11), suspended in chloroform–methanol–water 30:60:8 (by vol), and packed to a column of 1-ml bed volume. The column was washed with at least 40 ml of the same solvent mixture. The sample was dissolved in 1.5 ml of the same solvent and applied to the column. Two 1.5-ml washings of the sample tube were also applied to the column. Unadsorbed material was collected by elution with an additional 10.5 ml of the same solvent, and dried.

Thin-layer chromatography. The sample was quantitatively transferred to a small test tube or a tapered vial with chloroform–methanol 2:1 (v/v), and dried. It was dissolved in 25–50 μl of ice-cold chloroform–methanol 2:1 (v/v), while the tube was kept on ice. This was to avoid evaporation of the small volume of the solvent. A one-fifth or one-tenth aliquot of the sample was then spotted on a silica gel 60 high-performance thin-layer plate as a 5-mm-wide streak. The control samples of normal brain lipid extract with added standard psychosine were always placed on the same thin-layer plate as the test samples. The plate was used without activation. Development was in a solvent system of chloroform–methanol–water 65:15:2 (by vol). After development, the plate was briefly dried and 10% triethanolamine in chloroform was sprayed to "enhance and stabilize" the fluorescence (12).

Fluorescence densitometry. Before densitometry, the plate was observed under ultraviolet light to ascertain proper development. Intensity of fluorescent bands was quantitated by fluorescent densitometry with a Shimadzu CS-910 densitometer, equipped with an attachment for fluorometry and a CR-1B data processor. The excitation wavelength of the mercury lamp was set at 315 nm, and an interference filter of 550 nm was used. The "height" of the beam was set at 7 mm and the "width" at 0.8 mm. Scanning was done in the linear mode at 24 mm/min. The linearizer was not used. For each plate, the "slope test" was carried out and the half value of the result was used to assure detection and calculation of even very small peaks by the integrator.

Calculation of the results. The "standard curve" was constructed for each thin-layer plate from the results obtained for the series of normal brain samples with added authentic galactosylsphingosine and was used to quantitate the amounts in the test samples. The amounts of the standard were chosen so that the amounts in the test samples fell within the range of the standard. The final results for the test samples were therefore automatically corrected for the loss during the procedure.

RESULTS AND DISCUSSION

The goal of this investigation was to determine the level of galactosylsphingosine in the brain of a mutant mouse, the twitcher, which weighs 100–400 mg depending on the stage of development. Two most important considerations in developing the analytical methodology were high sensitivity and high specificity. We aimed at reliable determination of 50 ng of psychosine in 100 mg of brain tissue. This required satisfactory recovery of small amounts of psychosine throughout the analytical steps and the final sensitivity sufficient for 10 ng of psychosine. With the use of standard psychosine it was readily established that fluorescent densitometry of dansylated psychosine separated by thin-layer chromatography provides the necessary sensitivity. The second requirement for high specificity proved to be more difficult to satisfy. One hundred mg of brain contains approximately 10 mg of total lipid, including up to 2 mg of galactosylceramide. Thus, if 50 ng of psychosine is present in the mixture, the ratio of psychosine to total lipid is $1:2 \times 10^5$ and that of galactosylsphingosine to galactosylceramide is $1:4 \times 10^4$. Even a minute amount of inadvertent hydrolysis of galactosylceramide to galactosylsphingosine would invalidate the results, and other lipids must be reduced enough so as not to interfere with the final determination.

The standard procedure described in the Procedure section satisfies these requirements. **Fig. 1** gives the densitometric tracings when varying amounts of galactosylsphingosine were added to a normal brain lipid mixture and carried through the entire procedure. The method can reliably determine 50 ng of psychosine in 100 mg of brain. Linearity of the fluorescent densitometry of dansylated psychosine on the thin-layer plate was excellent, allowing reliable quantitative determination within a relatively wide range (**Fig. 2**). Elimination of other lipids and absence of inadvertent hydrolysis of galactosylceramide was assured by the results of the consistent absence of psychosine in normal human, canine, and murine brains, except for rare instances when it was barely detectable. When very small amounts of galactosylsphingosine were added to the lipid mixtures from 100 mg of normal brain and were carried through the procedure, the limit of detectability could be estimated at 5–10 ng/100 mg of brain. Since only one-fifth of the total sample was used for the final thin-layer chromatography, the sensitivity of the procedure would actually be fivefold higher, if the sample was quantitatively applied to the thin-layer plate. Recovery of psychosine through the procedure was estimated to be approximately 50% by running known amounts of tritium-labeled psychosine through the entire procedure.

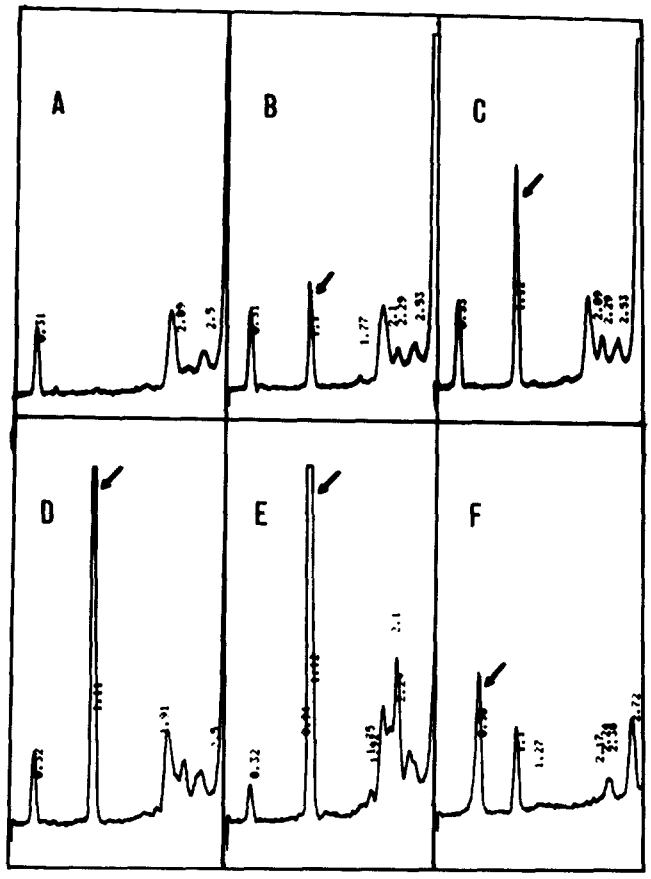

Fig. 1. Fluorescent densitometric tracings of thin-layer chromatograms of dansylated psychosine. Known amounts of standard psychosine were added to lipid extracts of 69-year-old normal human brain, each equivalent to 100 mg of tissue (A, no addition; B, 0.25 μg; C, 0.5 μg; D, 1 μg; E, 2 μg; F, 0.5 μg) and were processed through the entire analytical procedure. Aliquots of 1/10 were applied to the plates. Therefore, these peaks represent 1/10 of the original amounts added to the samples. The left end of each panel is the origin and the right end is the solvent front. A through E were run under the standard thin-layer chromatographic conditions (system V in Table 1), while a borate-impregnated plate was used for F (system V1). Note in A the absence of psychosine in normal brain lipid extract and the interference-free baseline in the area of the psychosine peak. The off-the-scale peaks in D and E could be accurately determined by the integrator. As expected, dansylated psychosine shows reduced mobility in F.

The calculated results, however, were automatically corrected for the loss since standard galactosylsphingosine was included at the beginning and carried through the procedure. Similarly, the variability of the analytical results was estimated to be within ±10%.

Evaluation of analytical steps

The key steps of the entire procedure take advantage of the strong positive charge and the reactivity of the free amino group of galactosylsphingosine. Few lipids are as strongly positively charged as psychosine which

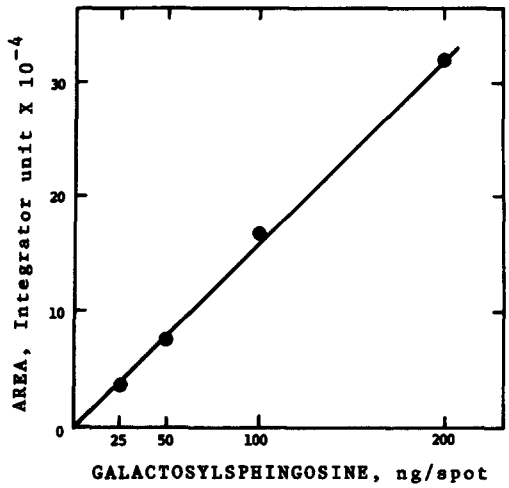

Fig. 2. The standard curve obtained from the peaks shown in Fig. 1. In each series of analysis, such standard curves were constructed by carrying through a series of normal brain samples with added psychosine and were used for calculation of the sample values.

could thus be separated from bulk of other lipids by the strong cation-exchange chromatography. The reactivity of the amino group allowed easy and quantitative introduction of the fluorescent group.

Cation-exchange chromatography. This step efficiently eliminated most of brain lipids. It was important to apply the sample in methanol. When chloroform and/or water was present in the solvent, varying amounts of psychosine passed the column unadsorbed. The size of the total lipid sample and the bed volume of the column were also important for efficient exchange of psychosine. When 10 μg of psychosine, labeled with tritium on the galactose residue (3, 13), was added to the lipid extract from 100 mg of brain and passed the AG-50W column of 2-ml bed volume in methanol (the standard condition), less than 8% of psychosine passed through the column. Once psychosine was adsorbed to the resin, however, the column could be exhaustively washed with any combination of chloroform, methanol, and water without displacing psychosine. Neither sodium nor potassium was very effective in eluting psychosine from the column: less than 40% of adsorbed galactosylsphingosine could be eluted with 15 ml of chloroform–methanol–water 3:47:48 (by vol) containing either 0.2 M Na_2HPO_4 or 0.2 M KCl. Under the standard condition of 15 ml methanol–0.4 M $CaCl_2$ (3:1, v/v), 85–98% of adsorbed psychosine could be eluted off the column.

We also tried extensively an alternate approach for separation of psychosine from other lipids—its partitioning into the methanol–water phase at acid pH—but were unsuccessful in obtaining satisfactory results.

Reverse phase chromatography. The Sep-Pak C-18 cartridge was useful for recovering galactosylsphingosine from the eluate containing methanol, water, and a large amount of salt, as it was for recovery of gangliosides from the aqueous phase (14). With 10 μg of tritium-labeled psychosine, more than 92% of psychosine could be recovered from the AG-50W eluate with excellent removal of the salt.

Silicic acid chromatography. This step was included in order to eliminate the last trace of galactosylceramide from the mixture to avoid its inadvertent hydrolysis to galactosylsphingosine under the alkaline condition of dansylation. This precautionary step may not be essential since no detectable hydrolysis of galactosylceramide to psychosine occurred when standard galactosylceramide was carried through from the step of dansylation.

Dansylation. The yield of dansylated psychosine was dependent on the amount of dansyl chloride in the reaction mixture. While the required amount of dansyl chloride was the same, the yield was higher when the reaction mixture contained other brain lipids than when only standard psychosine was dansylated (**Fig. 3**). When individual lipids were tested, presence of cholesterol did not improve the yield but both phosphatidylcholine and phosphatidylserine did. Since phosphatidylcholine of high purity is readily available and since it is not dansylated to interfere with subsequent procedures, 0.5 mg of phosphatidylcholine was chosen as the additional ingredient in the dansylation procedure (**Fig. 4**). Unreacted dansyl chloride and dansylated phosphatidylserine and phosphatidylethanolamine were eliminated through the two subsequent steps, the Florisil Sep-Pak and the DEAE-Sephadex column. The DEAE-Sephadex was particularly useful for elimination of the last traces of the dansylated

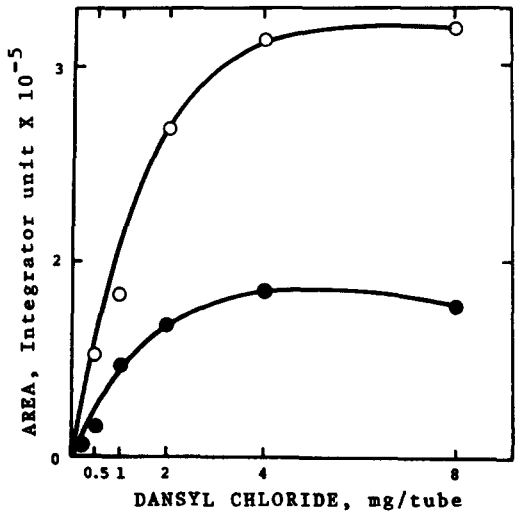

Fig. 3. Effect of the amount of dansyl chloride on the yield of dansylated psychosine. Standard psychosine, 400 ng, was dansylated with various amounts of dansyl chloride, as indicated. The reaction conditions were as described in the text; ● —— ●, dansylation done in the absence of added lipid; ○ —— ○, dansylation done in the presence of additional 0.5 mg of mouse brain total lipid.

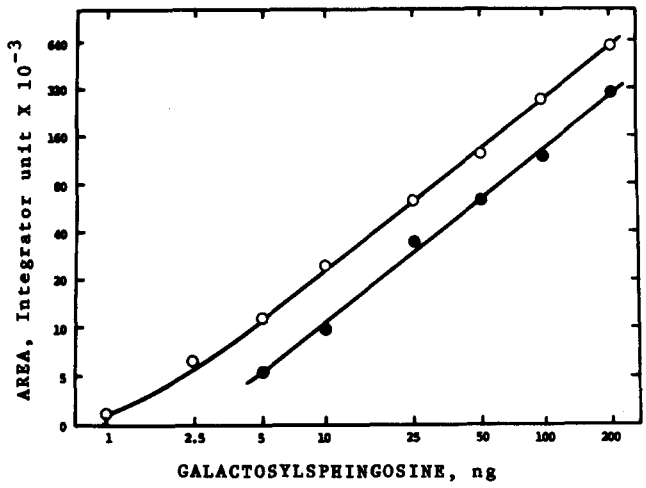

Fig. 4. Dansylation of psychosine with or without added phosphatidylcholine. Various amounts of standard galactosylsphingosine were dansylated under the standard conditions, except that 0.5 mg of phosphatidylcholine was present during the reaction in one series. Similar to Fig. 3, the yield of dansylated psychosine in the presence of added lipid was approximately twice that in its absence. Note that both scales are logarithmic in this figure; ● —— ●, without added phosphatidylcholine; ○ —— ○, with 0.5 mg phosphatidylcholine.

glycerophospholipids, because, after dansylation, psychosine was neutral while the phospholipids were negatively charged, as also indicated by the greater thin-layer chromatographic mobility in an acidic solvent system.

We also examined fluorescamine as the fluorescent group instead of the dansyl group. Although the derivatization reaction was faster, the product appeared less stable than the dansylated compound and was more difficult to separate cleanly from other interfering materials by thin-layer chromatography.

Thin-layer chromatography. Several solvent systems were evaluated for the final thin-layer chromatography–fluorescent densitometry (**Table 1**). Solvent V was found to be optimal in giving a sharp band of dansylated psychosine and clear separation from traces of interfering fluorescent materials which remained in the final mixture. When borate-impregnated plates, prepared as described previously (15), were used, mobility of dansylated psychosine was greatly reduced as expected due to the terminal galactose residue (Fig. 1). When the thin-layer plate was sprayed with 10% triethanolamine in chloroform, covered with a glass plate, and stored in darkness, the intensity of fluorescence decreased by 30% in 7 days, and approximately 50–60% in 2 months. Since the linearity of fluorescence with respect to the amount of dansylated psychosine was maintained, the densitometric analysis was possible for 2 months after preparation of the plate, provided that the plate was stored in the dark covered with another plate.

The conditions in two steps in the standard procedure were arrived at empirically and we do not understand the exact mechanisms: necessary application of galactosylsphingosine to the AG-50W column in *methanol* and not in a mixture of chloroform and methanol, and the increased yield of dansylated psychosine in the presence of lecithin.

One might question the wisdom of developing an analytical methodology for a compound that does not exist in normal brain. However, the method was essential

TABLE 1. Thin-layer chromatographic mobility of dansylated lipids

Solvent System	Relative Mobility[a]		
	Psychosine	Phosphatidyl-ethanolamine	Phosphatidyl-serine
I. Chloroform–methanol–water (65:25:4)	0.54	0.52	0.21
II. Chloroform–methanol–conc. ammonia (65:25:5)	0.35[b]		
III. Chloroform–methanol–acetone–acetic acid (100:20:40:10)	0.30	0.51	0.17
IV. Chloroform–methanol–acetone (80:16:4)	0.22	0.15	0.05
V. Chloroform–methanol–water (65:15:2)	0.42	0.38	0.17
VI. Same as II with borate-impregnated plate[c]	0.23	0.49	0.22

[a] Mobility relative to the solvent front.
[b] Poorly separated from interfering materials.
[c] The borate-impregnated plate was prepared as described previously (15). In all others, the plates were used directly without any pretreatment. Paper lining of the developing chamber was used in solvents I, III, IV, and V.

in order for us to examine possible abnormal accumulation of galactosylsphingosine in the genetic galactosylceramidase deficiency states in man, dog, and particularly, in mouse, which would allow well-controlled, statistically more meaningful analyses throughout the course of the disease, if a sufficiently sensitive methodology were available. With the method described here, we have been able to demonstrate rapid and progressive accumulation of psychosine in the brain of the twitcher mouse mutant, as well as its predominant accumulation in the white matter in affected human and canine brains (16). These findings provided a strong support for the psychosine hypothesis as the biochemical pathogenetic mechanism in globoid cell leukodystrophy (Krabbe disease) (4).

Although the present method was developed specifically for galactosylsphingosine, the principle should be equally applicable to compounds of similar nature, such as sphingosine, glucosylsphingosine, lactosylsphingosine, etc. Appropriate modifications may well be required for these compounds at individual steps.

This investigation was supported by research grants NS-10885, NS-19321, NS-03356, and HD-01799 from the United States Public Health Service.

Manuscript received 6 April 1984.

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