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notes on methodology

Specific tritium labeling of cerebrosides at the 3-positions of *erythro*-sphingosine and *threo*-sphingosine

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Summary Cerebrosides containing either *threo*- or *erythro*-[3-^{3}H]sphingosine were synthesized by a new procedure. Glucopyranosyl or galactopyranosyl ceramides were converted to their 3-keto derivatives with 2,3-dichloro-5,6-dicyanobenzoquinone and reduced with ^{3}H-labeled sodium borohydride. The resulting tritiated cerebrosides, which contained *erythro*- and *threo*-sphingosines in the ratio of 84:16, were deacylated with butanol-KOH, and the *erythro*- and *threo*-psychosines were separated by silica gel column chromatography and reacylated with lignoceroyl chloride.

Supplementary key words 3-keto derivative of sphingolipids · *erythro*- and *threo*-psychosines · galactocerebroside · glucocerebroside

Until now, radioactive sphingolipids used as the precursors or substrates of in vivo or in vitro studies have usually been labeled in the fatty acid (1), the carbohydrate (2–5), or the choline moiety (6). Sphingolipids containing labeled dihydrosphingosine have previously been prepared either by catalytic hydrogenation of the sphingolipids with tritium gas (7) or by acylation of [3-^{3}H]dihydrosphingosine (8). Recently, Stoffel and Bister (9) prepared dihydroceramide and dihydrosphingomyelin, both of which contained [3-^{3}H]dihydrosphingosine, by reducing their 3-keto derivatives with ^{3}H-labeled sodium borohydride. They utilized these compounds for metabolic studies in vivo without separating diastereoisomers containing *erythro*- and *threo*-dihydrosphingosines.

Recently, we reported that ceramides,[1] cerebrosides, and sphingomyelins, all of which contain sphingosine, are converted to their 3-keto derivatives by reaction with 2,3-dichloro-5,6-dicyanobenzoquinone (10, 11). The 3-ketosphingolipids can be quantitatively reduced to the original sphingolipids with sodium borohydride (11). This two-step reaction provides a simple procedure for preparing various sphingolipids labeled with ^{3}H at the 3-position of the sphingosine moiety when ^{3}H-labeled sodium borohydride is used for the reduction. Unlike the procedure of Stoffel and Bister (9), the present method yields radioactive sphingolipids rather than dihydrosphingosine. Because the sphingosine-containing sphingolipids are more abundant in mammalian tissues, they may be more suitable for use in metabolic studies (12). This communication describes the preparation of ^{3}H-labeled cerebrosides containing either *erythro*- or *threo*-[3-^{3}H]sphingosine by using the above new procedure.

Materials. Galactocerebrosides were isolated from calf brain (13). Glucocerebrosides were isolated from the spleen (provided by Dr. J. N. Kanfer) of a patient with Gaucher's disease. A mixture of *erythro*- and *threo*-sphingosines was a gift from Dr. R. H. McCluer. Tetrahydrofuran was redistilled over lithium aluminum hydride immediately before use. Tritiated sodium borohydride was purchased from New England Nuclear Corp., Boston, Mass. Sodium borodeuteride was obtained from Alfa Products, Ventron Corp., Beverly, Mass. Thin-layer chromatographic plates precoated with 0.25-mm-thick silica gel G were obtained from Analtech, Newark, Del. Silica gel 60 extra pure (70–230 mesh) and plates precoated with 0.25-mm-thick silica gel F 254 were purchased from EM Laboratories, Elmsford, N.Y. Unisil (a silica gel, 100–200 mesh) was obtained from Clarkson Chemical Co., Williamsport, Pa.

3-Ketocerebrosides. 3-Ketogalactocerebrosides were prepared from 20.5 mg of calf brain cerebrosides by oxidation with 2,3-dichloro-5,6-dicyanobenzoquinone in dioxane (10, 11). The products were isolated by preparative thin-layer chromatography (10). The two bands of 3-ketocerebrosides were eluted together, and the solvents were evaporated; the residue was recrystallized once from acetone–methanol 4:1. The yield of 3-ketogalactocerebrosides was 20.3 mg. 3-Ketoglucocerebroside was similarly prepared; a single band was obtained by preparative thin-layer chromatography.

N-*Acetyl*[*3-*^{3}*H*]*sphingosine β-galactosides* (*containing a mixture of* threo- *and* erythro-*sphingosines*). The above

[1] Trivial names used: *erythro*- or *threo*-sphingosine, D-(+)-*erythro*-4-*trans*-sphingenine or its *threo*-diastereoisomer (absolute configuration of *erythro*-sphingosine is [2*S*, 3*R*] and that of the *threo* isomer is [2*S*, 3*S*]); ceramide, *N*-acylsphingosine; *erythro*- or *threo*-psychosine, 1-*O*-galactopyranosyl (or glucopyranosyl) *erythro*- or *threo*-sphingosine; galacto- or glucocerebroside, *N*-acylpsychosine; 3-ketosphingolipids, sphingolipids containing 3-dehydrosphingosine.

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3-ketogalactocerebrosides were treated with 2.0 mg of tritiated sodium borohydride (138.9 Ci/mole) by using tetrahydrofuran as the solvent as described previously (11). The yield was 19.4 mg (99.2% of theoretical value); the specific activity was 38.2 Ci/mole.

N-*Acyl*[*3-^{3}H*]*sphingosine β-glucoside.* 3-Ketoglucocerebroside was reduced with tritiated sodium borohydride (250 Ci/mole) in a similar way. The product had a specific activity of 52.4 Ci/mole.

Erythro-[*3-^{3}H*]*sphingosine β-galactoside and* threo-[*3-^{3}H*]*sphingosine β-galactoside.* The ^{3}H-labeled galactocerebrosides were saponified with 5 ml of 90% *n*-butanol containing 350 mg of KOH according to the procedure of Radin (14). The product was purified by chromatography on a column containing 1 g of Unisil. The column was eluted successively with 30 ml of chloroform, 30 ml of chloroform–methanol 4:1, 30 ml of chloroform–methanol 2:1, 50 ml of chloroform–methanol 1:4, and 30 ml of methanol. The psychosine obtained from the last three fractions was further fractionated into galactosyl *erythro*-[3-^{3}H]sphingosine and its *threo* diastereoisomer on a 0.6 cm × 60 cm column packed with silica gel 60. Chloroform–methanol–concentrated ammonium hydroxide 14:5:1 was used for the elution of the column, with a flow rate of 0.5 ml/min, as described by Raghavan, Mumford, and Kanfer (15) for the isolation of glucosylsphingosine. 1-ml fractions were collected and analyzed for psychosine content by thin-layer chromatography using both ninhydrin and Molisch's reagent for detection. Fractions 48–56 contained *erythro*-psychosine, fractions 66–82 contained *threo*-psychosine, and fractions 57–65 contained both diastereoisomers.

N-*Lignoceroyl* erythro-[*3-^{3}H*]*sphingosine β-galactoside.* Fractions 48–56 were pooled, and after removal of the solvent the residue was reacted with lignoceroyl chloride (16). The product was purified by preparative thin-layer chromatography as described previously (10).

N-*Lignoceroyl* threo-[*3-^{3}H*]*sphingosine β-galactoside.* Fractions 66–82 were pooled and reacted with lignoceroyl chloride, and the product was purified as described above.

Methanolysis of cerebrosides. Cerebrosides were heated with 0.5 N methanolic HCl (17), and the liberated fatty acid methyl esters were extracted with hexane. The remaining methanolic solution was first neutralized with silver carbonate and then made basic with dilute aqueous NaOH solution. Free sphingosine bases were extracted with ether. The final aqueous solution was evaporated to dryness under nitrogen, and the amount and the ^{3}H content of hexose were determined. The hexane and ether extracts were combined and fractionated by Unisil column chromatography to obtain fatty acid methyl esters and sphingosines (18).

Determination of radioactivity. Radioactivity was measured with a Tri-Carb liquid scintillation spectrophotometer model 3820 or 3880 (Packard Instrument Co., Downers Grove, Ill.). Cerebrosides, fatty acid methyl esters, and sphingosines were dissolved in a toluene-based scintillation mixture containing 5% ethanol. Radioactivity of psychosine and hexose was determined in a toluene–Triton X-100-based scintillation mixture (19). [^{3}H]Toluene (New England Nuclear) was used as the internal standard.

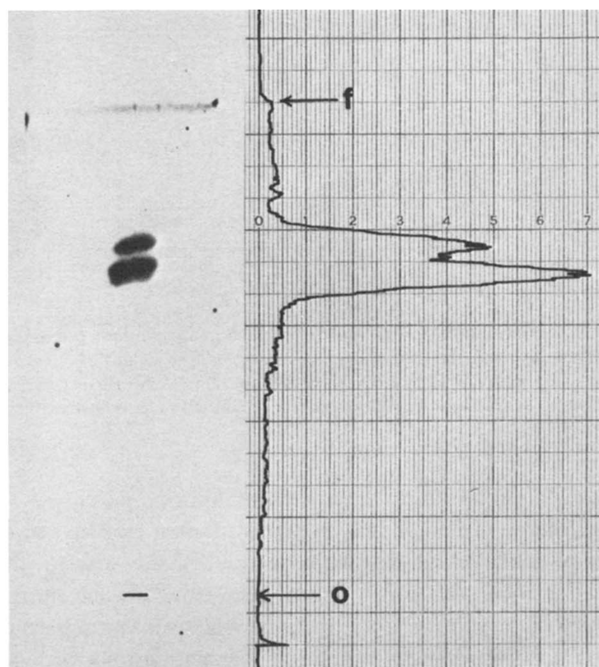

Fig. 1. Thin-layer chromatogram and radioscan of galactocerebrosides prepared by the reduction of 3-ketogalactocerebrosides with tritiated sodium borohydride. The ^{3}H-labeled galactocerebroside was chromatographed on a silica gel G plate with chloroform–methanol–water 65:25:4 (by vol) The lipids were visualized after the plate was exposed to iodine vapor. Arrows indicate the origin (*o*) and the solvent front (*f*).

Results. Reduction of 3-ketocerebrosides with tritiated sodium borohydride was quantitative. **Fig. 1** shows the thin-layer chromatogram of the product from 3-ketogalactocerebroside and its radioscanning by a Varian/Berthold radioscanner (Varian Associates, Palo Alto, Calif.). The specific activity of the radioactive cerebrosides was 38.2 Ci/mole. Similar results were obtained with the reduction of 3-ketoglucocerebrosides. The specific activity of this cerebroside was 52.4 Ci/mole. The reduction of 3-ketocerebrosides produced a racemic mixture containing (3*S*)- and (3*R*)-hydroxyl groups (9, 11) and thus a mixture of diastereoisomers of cerebrosides containing *erythro*- and *threo*-sphingosines. Separation of these diastereoisomers of galactocerebrosides was unsuccessful and, in order to separate these isomers, the cerebrosides were first saponified with butanolic KOH (14). Under these conditions, 78% of the radioactivity in the cerebrosides was recovered in psychosine, 13% in unreacted cerebrosides, and 8% in sphingosine. Thin-layer chromatography–radioscanning of the psychosine indicated that it was a mixture of *erythro* and

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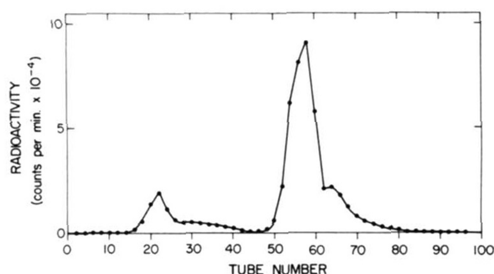

Fig. 2. Distribution of radioactivity in silica gel 60 column chromatography of the crude ^{3}H-labeled psychosine. See text for details of the procedure. The peak at fractions 16–30 was found to be sphingosine, and the two peaks in fractions 48–80 were *erythro-* and *threo-*psychosines in the order of elution.

TABLE 1. Distribution of radioactivity in ^{3}H-labeled cerebrosides

		Lignoceroyl *erythro*-[3-^{3}H]-Sphingosine β-Galactoside		Lignoceroyl *threo*-[3-^{3}H]-Sphingosine β-Galactoside	
		cpm	%	*cpm*	%
Before methanolysis		650,900	(100)	402,900	(100)
After methanolysis	Fatty acids	4,035	(0.6)	2,898	(0.7)
	Sphingosines	636,170	(99.4)	393,387	(99.1)
	Hexose	192	(0.0)	715	(0.2)

threo diastereoisomers in a ratio of approximately 84:16. Such stereoselectivity was previously noted for the reduction of 3-ketodihydrosphingosine synthesized in vitro (20) and for 3-ketodihydrosphingomyelin (9). Because the recovery of psychosine from the plate was poor, we separated these diastereoisomers by column chromatography with silica gel 60 extra pure (**Fig. 2**). Out of 794 mCi of the crude psychosine applied, 39% was recovered in the *erythro*-psychosine fraction, 8% in the *threo* isomer fraction, 50% in the fraction containing both isomers, and 4% in other fractions; 75% of the total radioactivity was recovered.

In order to demonstrate the position of the labeling and the configuration of the sphingosine moiety of the two ^{3}H-labeled psychosines, another preparation of 3-ketogalactocerebrosides was reduced with sodium borodeuteride (with deuterium content of more than 98%) and saponified, and the resulting ^{2}H-labeled psychosines were separated into the *erythro* and *threo* diastereoisomers under the conditions described above. **Fig. 3** shows the thin-layer chromatogram

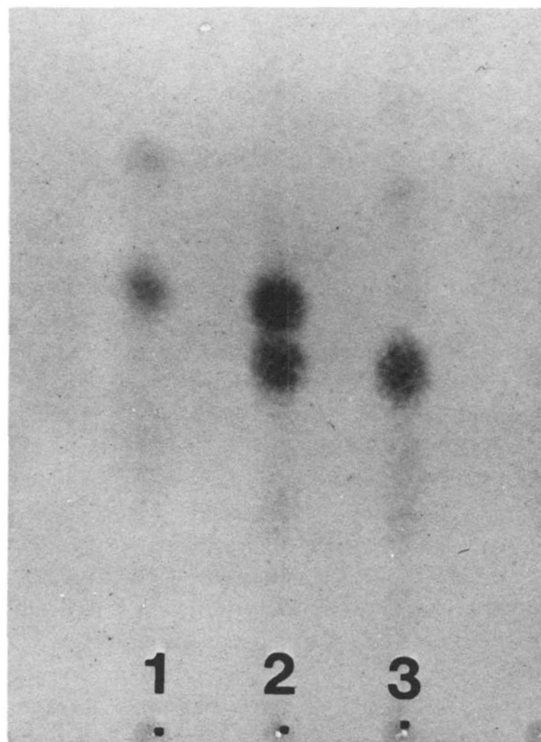

Fig. 3. Thin-layer chromatogram of *erythro-* and *threo-*psychosines before and after separation by column chromatography. Chloroform–methanol–concd ammonia 14:5:1 (by vol) was the developing solvent. *1, erythro*-psychosine fraction; *2,* mixture of *erythro-* and *threo-*psychosines prior to column chromatography; *3, threo-*psychosine fraction.

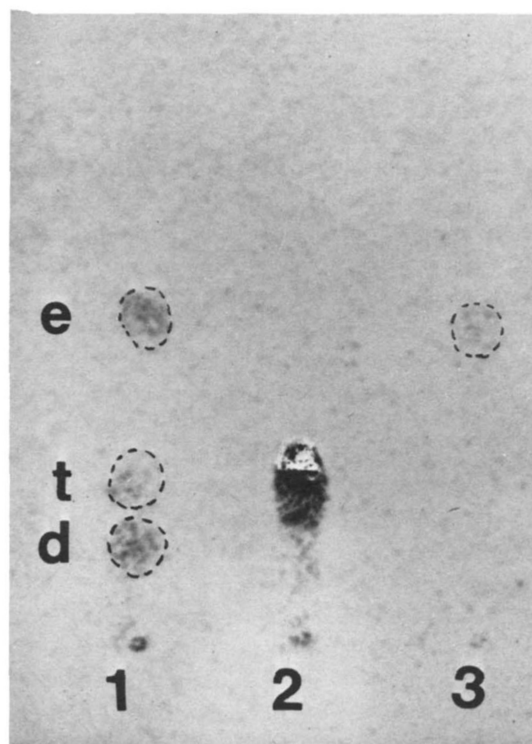

Fig. 4. Thin-layer chromatogram of sphingosine preparations. *1,* mixture of *erythro-*sphingosine (*e*), *threo-*sphingosine (*t*), and dihydrosphingosine (*d*); *2,* sphingosine from the psychosine that gave the lower spot in Fig. 3; *3,* sphingosine from the psychosine that gave the upper spot in Fig. 3. The silica gel F 254 plate was developed with chloroform–methanol–2 N NH_4OH 40:10:1 (by vol) and visualized by the H_2SO_4 charring method.

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of the *erythro-* and *threo-*psychosines thus obtained. Both diastereoisomers were further hydrolyzed with anhydrous methanolic HCl as described by Taketomi and Kawamura (21), and the sphingosine bases were examined by thin-layer chromatography as described by Sambasivarao and McCluer (22). The results demonstrated that the base obtained from *erythro-*psychosine (the one with the higher R_f on thin-layer chromatography) was *erythro-*sphingosine and that from *threo-*psychosine (the one with the lower R_f) was *threo-*sphingosine (**Fig. 4**). Both sphingosines were converted to *N*-acetyl-*O*-trimethylsilyl ethers (23) and examined by gas-liquid chromatography-mass spectrometry as described previously (24). The spectra of both sphingosines were identical with that of *N*-acetyl[3-^{2}H]sphingosine trimethylsilyl ether reported by Gaver and Sweeley (25).

Tritiated *erythro-* and *threo-*galactocerebrosides were prepared from the tritiated *erythro-* and *threo-*psychosines, respectively, by reacting with lignoceroyl chloride. Specific activities, as calculated from galactose contents, in *erythro-* and *threo-*galactocerebrosides were 34.0 Ci/mole and 34.6 Ci/mole, respectively. Decline in the specific activities of both cerebrosides in the above procedure may have been due to the exchange of ^{3}H with ^{1}H during alkaline treatment. On thin-layer plates containing silica gel G developed with chloroform-methanol-water 24:7:1 and with chloroform-methanol-2 N NH_4OH 40:10:1, both isomers had R_f values identical with that of authentic lignoceroyl sphingosine β-galactoside (16).

Both radioactive cerebrosides were subjected to methanolysis, and the distribution of radioactivities in the component fatty acid methyl esters, sphingosines, and galactose was examined (**Table 1**). As expected, essentially all of the radioactivity was recovered in sphingosines. The small amounts of radioactivity observed in the fatty acid fractions of both cerebrosides may have been due to contamination by sphingosine during the process of fractionation.

Gaver and Sweeley (25) inferred that 1,4 addition occurs partially in the sodium borohydride reduction of *N*-acetyl-3-ketosphingosine. If 1,4 addition of 3-ketocerebroside had occurred during the reaction, one would expect that cerebroside containing [3,5-^{3}H]dihydrosphingosine would be formed. In order to examine whether this was the case, the ^{3}H-labeled *erythro*-cerebroside (299,085 cpm) was mixed with 1 mg of nonradioactive *N*-lignoceroyl psychosine and subjected to oxidation with dichlorodicyanobenzoquinone (11). Cerebroside containing dihydrosphingosine is not oxidized by this treatment (10). The product was purified by column chromatography on Unisil by first eluting with chloroform and then with chloroform-methanol 4:1. The latter fraction, which contained both cerebrosides and 3-ketocerebroside, contained 2503 cpm. This result indicates that more than 99% of the radioactivity was lost by the oxidation; in other words, 99% of the radioactivity was in the 3-position of sphingosine. An additional 1 mg of nonradioactive *N*-lignoceroyl psychosine was added to the reaction product, and 3-ketocerebroside and cerebroside were separated from each other by preparative thin-layer chromatography. The determination of ^{3}H in these two compounds demonstrated that all radioactivity in the reaction product was in unreacted cerebroside that contained dihydrosphingosine.

Discussion. The present procedure, which describes the synthesis of cerebrosides labeled in the sphingosine moiety, could be used for the preparation of various other sphingolipids. Preparation of the 3-keto derivatives of ceramides, sphingomyelin, and lactosyl ceramide[2] has been reported previously (10, 11), and Fujino and Negishi (26) have reported saponification of sphingomyelin to sphingosine phosphorylcholine and further separation of *erythro* and *threo* diastereoisomers by thin-layer chromatography. These findings suggest that radioactive ceramides, sphingomyelins, and lactosyl ceramides could be prepared by the same procedure. Conceivably, this list may also include other sphingolipids, such as globosides and gangliosides. By acylating ^{3}H-labeled nonacylated sphingolipids with ^{14}C-labeled fatty acyl chloride, one could also readily synthesize double-labeled sphingolipids that would be particularly useful in in vivo metabolic studies.

More than 99% of the ^{3}H incorporated in the cerebroside was shown to be at the C-3 position of the sphingosine moiety. Approximately 0.9% of the radioactivity appears to have been added by 1,4 addition to form cerebroside containing [3,5-^{3}H]dihydrosphingosine. The smaller degree of 1,4 addition in this case compared with that observed by Gaver and Sweeley (25) may have been due to the different solvents that were used for the reduction or steric hindrance by the lignoceroyl or galactose moiety (27).

The authors are grateful to Drs. Klaus Biemann and Charles Hignite of the Massachusetts Institute of Technology for obtaining the mass spectral data. The skillful assistance of Mr. James Davenport is also gratefully acknowledged. These studies were supported in part by grants NS 11899, NS 10741, NS 10473, HD 05515, and HD 04147 from the National Institutes of Health, U.S. Public Health Service.

Manuscript received 19 December 1974; accepted 8 April 1975.

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