In vivo conversions of cerebroside and ceramide in rat brain

KRYSTYNA C. KOPACZYK* and NORMAN S. RADIN

Mental Health Research Institute, University of Michigan, Ann Arbor, Michigan

SUMMARY Lignoceric acid-1-C^{14} was used to make lignoceroyl psychosine (kerasin) and stearic acid-1-C^{14} to make stearoyl sphingosine (ceramide). Emulsions of the labeled sphingolipids were injected directly into the brains of young rats and the resultant lipoidal metabolic products were examined for radioactivity.

Most of the metabolized C^{14} was found in the ester-linked fatty acids, particularly in the acids corresponding to the ones injected. This shows that the lignocerate and stearate were released from amide linkage and incorporated into other lipids. Activity was found also in other fatty acids and cholesterol, showing that the released fatty acids were partially degraded to acetate. Ceramide and sphingomyelin showed relatively high activities, and the fatty acids of these lipids were labeled primarily in the carboxyl group. This suggests that ceramide is formed directly from cerebroside via a galactosidase, and that the ceramide is converted to free fatty acids as well as sphingomyelin. Little activity was found in the hydroxy cerebrosides and sulfatides, suggesting that neither of the injected lipids is directly converted to these glycolipids. Ester-linked lignocerate was found to occur naturally in brain.

KEY WORDS cerebroside · ceramide · brain metabolism · rat · lignoceroyl-1-C^{14} cerebroside · stearoyl-1-C^{14} psychosine · stearoyl-1-C^{14} ceramide · intracranial injection · metabolic conversions · sphingomyelin biosynthesis · cerebroside sulfate

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* National Institutes of Health postdoctoral fellow, 1962–1964. Present address: Institute for Enzyme Research, University of Wisconsin, Madison, Wis.

Abbreviations: B is benzene, C is chloroform, E is diethyl ether, H is hexane (Skellysolve B), M is methanol, W is water. TLC is thin-layer chromatography, GLC is gas–liquid chromatography. Fatty acid abbreviations show first the carbon number, then the number of double bonds.

TURNOVER STUDIES in young rats, using labeled galactose and acetate, have shown that the galactose and fatty acid moieties of brain cerebrosides undergo metabolic conversions (2, 3). The nature of the conversions is still only partly known. It is possible that part of the cerebroside molecules undergo conversion to cerebroside sulfate. The shapes of the turnover curves and the fatty acid distribution curves are consistent with this idea (2, 4, 5). It is likely that the fatty acid residues, once released from the cerebroside molecules, are processed by a variety of enzyme systems: hydroxylation, degradation, chain lengthening, and incorporation into complex lipids. Such conversions have been demonstrated by intracranial injection of labeled fatty acids (6). Attempts to demonstrate by in vitro experiments the existence of enzymes which act on cerebrosides have been disappointing and the claims for a galactosidase rest on tenuous evidence (7–9). In vitro studies of sphingolipid metabolism, with very few exceptions, have been characterized by very low activities and curious specificity properties which must be considered artifacts of solubilization. To gain a better idea of the nature of cerebroside metabolic conversions in brain, we have tried direct injection of labeled cerebroside into rat brains. The conversion products of the labeled cerebroside and of a probable intermediate, ceramide, have been examined.

METHODS

Preparation of Lignoceroyl-1-C^{14} Psychosine (Kerasin)

About 10 mg of lignoceric acid-1-C^{14} was refluxed with 0.4 ml of thionyl chloride for 30 min in a one-piece jacketed test tube capped with a calcium chloride tube.

The solution was then evaporated to dryness under vacuum and a mixture of 25 mg of psychosine sulfate (beef brain), 0.4 ml of tetrahydrofuran, and 0.5 ml of 50% aqueous sodium acetate (trihydrate) was added (10). The reaction mixture was stirred magnetically for 90 min, during which time the kerasin precipitated.

The cerebroside was purified by partitioning several times in a C–M–W system (11), then by TLC on 0.5 mm thick plates of Silica Gel G. The plates were developed with C–M–W–concd ammonia 280:70:6:1 (12), sprayed with bromothymol blue–NaOH (13), and the spots eluted with chloroform and C–M–W 7:7:1 (14) in small columns. The second effluent, which contained most of the C^{14} and bromothymol blue, was washed by partitioning in the above C–M–W system (11). The chloroform phase was evaporated to dryness, yielding 12.1 mg of kerasin (54% of theoretical), specific activity 3.59×10^6 cpm/mg as measured by liquid scintillation.

Preparation of Stearoyl-1-C^{14} Sphingosine (Ceramide)

Labeled stearoyl psychosine was prepared as above from stearic acid-1-C^{14} (New England Nuclear Corp. Boston, Mass.) and beef spinal cord psychosine, then converted to ceramide by the method of Carter et al. (15). The ceramide was purified by TLC, using triple development with C–M 95:5, band location with bromothymol blue, elution with C–M 2:1, and partitioning as above. The ceramide weighed 2.6 mg (53% of theoretical, starting from stearic acid) and had a specific activity of 4.3×10^7 cpm/mg.

Administration of Labeled Compounds

A solution of 0.72 mg of labeled kerasin and 7.2 mg of a polyoxyethylene stearate (G-2159) in C–M was evaporated to dryness in a small conical tube. The residue was warmed with 0.2 ml of water, immersed briefly in an ultrasonic bath, and centrifuged to remove air bubbles. The clear emulsion, 0.025 ml or 320,000 cpm/rat, was injected with a Hamilton gas-tight microsyringe into the approximate center of the brain (6). Six 15-day old rats were used, then returned to their mother, and sacrificed in pairs 1, 2, and 4 days later (Group A).

An additional group of six 15-day old rats was injected similarly and sacrificed after 2 days (Group B).

The labeled ceramide was treated similarly, but only 0.163 mg was used. In this case, all six rats were given 8.8×10^5 cpm each and sacrificed 2 days after injection (Group C).

Lipid Isolation Methods

The pooled brains were extracted with C–M 2:1 and the extract was washed with 0.2 volume of 0.1 M KCl and twice with 0.05 M KCl in "Folch pure solvents upper phase" (11). The lipid phase was then evaporated to a small volume with the aid of benzene additions and lyophilized from benzene. The samples were taken up in C–M 3:1 and stored at −20°.

Two basic methods were used for isolating the various lipids, separate samples of total lipids being the starting point for different procedures. One procedure, based on a quantitative method developed in this laboratory,[1] yields the ester-linked fatty acids, cholesterol, ceramides, cerebrosides, and sulfatides. The other procedure, based on the method of Hausheer et al. (16), yields sphingomyelins. In each case, separations were monitored by TLC with the aid of standard lipids, the bromothymol blue spray, and a variety of solvent systems.

In the first procedure, the total lipids were subjected to alkaline transmethylation (17). Following 60 min of mixing at room temperature, the alkali was neutralized with 0.5 M acetic acid and water was added to bring the total amount of water to 0.2 volume. The chloroform layer was evaporated to dryness and the remaining lipid applied to a Florisil column in H–E 99:1. (The Florisil is dried in portions at 600° for 60 min, then deactivated with 8 ml of water per 100 g. It is stored for no more than 3 months after deactivation. For each mg of total lipids, 50 mg of Florisil is used.)

Elution of the column with 99:1 solvent, 20 ml/g Florisil, yielded the methyl esters, which are derived from the ester-linked fatty acids. Cholesterol was eluted with a similar volume of H–E 8:2; ceramides with C–M 95:5; and the remaining glycolipids with C–M 3:1.

This method has the advantage that the methyl esters are uncontaminated with aldehydes or acetals. Moreover, the glycolipids are not contaminated with the ethanolamine phosphatides, which tend to leak into C–M effluents from Florisil columns. Acetic acid rather than a stronger acid is used for the neutralization step to prevent cleavage of the lysoplasmalogens.

In order to isolate the methyl esters of the saturated acids, the ester-linked acid fraction was applied to a silica gel–silver nitrate column (18, 19). The saturated esters were eluted with hexane, using 70 mg of adsorbent per mg of esters, and 30 ml of solvent per g of adsorbent.

Cholesterol was purified by digitonin precipitation and regeneration with pyridine (20).

The ceramides were purified further by TLC as described above, each plate being developed four times with the same solvent.

The glycolipids were separated further by TLC, using 0.5 mm thick plates, 6 mg of lipid per plate, and a single development with C–M–W 24:7:1 (21). Elution and

[1] Y. Kishimoto, W. E. Davies, and N. S. Radin, paper in preparation.

washing were performed as with the synthetic cerebrosides.

In the second procedure, a sample of total lipids was applied to a silica gel column[2] (90 mg/mg lipid) in C–M 3:1. The less polar lipids were eluted with the same solvent (20 ml/g adsorbent) and the sphingomyelin with methanol (10 ml/g). The main contaminant of the sphingomyelin, lecithin, was removed by transmethylation as above and chromatography on another silica gel column (175 mg/mg lipid). The methyl esters were eluted with C–M 2:1 and the sphingolipid again with methanol.

Isolation of the Sphingolipid Fatty Acids

When the free fatty acids were desired, the lipids were cleaved with KOH–propylene glycol and the acids separated on a silica gel column (22). The unsubstituted acids were eluted with B–E 99:1 and the hydroxy acids with B–E 6:1.

When the methyl esters were desired, the lipids were cleaved with methanolic HCl and the esters separated on a Florisil column (23).

The ganglioside fatty acids were isolated from the aqueous washings of the lipids from the ceramide-injected rats. The method[3] involves mild saponification to remove the traces of ester-linked lipids, acid methanolysis to cleave the gangliosides, and purification of the methyl esters by TLC.

The saturated esters from the glycolipids were obtained by chromatography on silica gel–silver nitrate columns. The individual esters were then isolated by programmed temperature GLC with an SE-30 column (24). Decarboxylation of individual or mixed unsubstituted fatty acids was carried out with the azide reaction (3). The hydroxy acids were decarboxylated with permanganate–benzene–acetic acid (3).

Other Materials and Methods

All solvents were redistilled from a glass still: U.S.P. chloroform, hexane, A.C.S. methanol, and benzene. Absolute ether was distilled from KOH pellets as needed. Tetrahydrofuran was distilled from KOH and stored at −20°.

Infrared spectra were obtained from 5 mm pellets, 0.25 mg of sample in 25 mg of KBr, prepared by evaporation from C–M while grinding (25). A Perkin-Elmer Infracord Model 237 spectrophotometer was used with a beam condenser.

Samples were counted by liquid scintillation in a solution of scintillators in ethanol–toluene 5:95. All samples were re-counted after addition of an internal standard and corrected accordingly.

Characterization of Compounds

Because of the high specific activities of the synthetic sphingolipids, the infrared spectra were determined with the products of trial runs made with nonradioactive material. The spectra corresponded closely to those previously reported for highly purified samples (26), but the hydroxyl peak of both cerebrosides was slightly split into two peaks (2.93 and 3.02 μ). Yokoyama and Yamakawa (27) have observed a similar double peak in naturally occurring kerasin. It is possible that the failure of other workers to observe this is due to the use of spectrophotometers with poorer resolution in this region, or to the use of mixed, homologous cerebrosides. Our instrument does not show the splitting with natural mixtures of cerebrosides, but it clearly shows the small peak at 3.39 μ for carbon–carbon unsaturation in all sphingolipids. Shapiro et al. (28) observed a split hydroxyl peak with synthetic sphingomyelin and attributed it to an ionic, hydrogen-bonded hydroxyl group.

All of our cerebroside preparations show a slight, flat peak at about 5.8 μ, which could indicate, in the case of the synthetic samples, the presence of a small amount of esterified fatty acid. There is the possibility that some of the labeled acyl chloride reacted with one of the psychosine hydroxyl groups, although this is very unlikely in the presence of concentrated sodium acetate. To check this possibility, a portion of the labeled kerasin was treated with about 2 N NaOH in W–M at 38° for 3 hr. This treatment had previously been shown to have little effect on cerebrosides (23). The mixture was acidified and extracted with chloroform, and the lipids were fractionated on a silica gel column. The fatty acid effluent (which included 24:0 carrier) contained only 0.26% of the starting activity in this fraction.

Examination by TLC of 190 μg of labeled kerasin showed a trace spot at the solvent front and a heavy spot corresponding to the fast-moving cerebroside spot found with natural mixtures of cerebrosides. The solvent front contained 0.05% of the activity applied, but tests with different solvents and carriers showed that this contaminant was not lignoceric acid or ceramide. The remainder of the plate contained no radioactivity.

The synthetic ceramide was examined similarly. The nonradioactive ceramide yielded a spectrum similar to that from a sample isolated from beef lung, and much like that found with ceramide made from sphingomyelin (26). However, the published spectrum shows a peak at 12.5 μ which we do not find.

The radioactive ceramide was examined by triple-development TLC, using a solvent system different from

[2] Unisil, Clarkson Chemical Co., Williamsport, Pa.
[3] Y. Kishimoto and N. S. Radin, paper in preparation.

that used in the purification: chloroform–acetic acid 95:5 (13). Only one spot was visible with 100 μg. When ceramide was made from beef spinal cord cerebrosides, a strong second spot was observed below the usual ceramide spot. This is evidently ceramide containing hydroxy fatty acids. The infrared spectrum of this preparation resembles that of beef spinal cord cerebrosides, but lacks the 12.8 μ peak characteristic of cerebrosides. The main differences between synthetic and spinal cord-derived ceramides are: the latter has a more rounded hydroxyl peak; it shows only a single peak in the 6.1 μ region; the twin peaks at 9.09 and 9.56 μ are replaced in the latter by a heavy, diffuse peak in between. The differences probably are due to the presence of the 2-OH group in the spinal cord preparation.

The ceramide isolated from the injected rats gave the expected infrared spectrum except for a distinct ester peak (5.75 μ). The sample was treated with alcoholic KOH at 80° (29), acidified, extracted with chloroform, and purified by passage through a Florisil column. Only 6% of the radioactivity was lost by the saponification, so the presence of the contaminant did not appreciably affect the findings.

The sphingomyelin isolated from the injected rats gave the expected spectrum (26). Examination by TLC showed a major spot with a trace of slower material. This could be lysophosphatidal choline, formed by the alkaline treatment of the crude sphingomyelin, which must have been contaminated by phosphatidal choline (and its hydrogenated analogue). Such contamination would not influence our results, as the fatty acids were released by alkaline treatment.

RESULTS

Total Lipids

From the data in Table 1, it appears that much of the injected sphingolipid was metabolized to nonlipids or eliminated from the brain. Judging by the time course with the kerasin-injected rats, the process was gradual. However, it is possible that there was some leakage from the brain shortly after the injection.

Ester-Linked Fatty Acids and Cholesterol

Two days after injection of kerasin, 2.84% of the injected activity was found in the fatty acids and 0.32% in the cholesterol (Group B); the corresponding values for the ceramide-injected rats were 8.67 and 0.17% (Table 3). The compositions of the fatty acids isolated by GLC (Table 2) are significantly different. In Group B, where the initial label was in lignoceric acid, most of the activity was found in palmitic acid, secondarily in lignoceric acid. In the ceramide group, where the label was in stearic acid, this acid contained most of the C^{14}.

TABLE 1 ACTIVITY FOUND IN TOTAL LIPIDS* OF BRAIN AFTER INJECTION OF LABELED SPHINGOLIPIDS

Rat Group	Brain Weight	Lipid Weight	Specific Activity	Recovered Activity†
	g/rat	*mg/rat*	*cpm/mg*	%
(A) Kerasin-injected				
1 day	1.3	72	1,960	44
2 days	1.4	78	1,430	38
4 days	1.3	81	1,140	28
(C) Ceramide-injected				
2 days	1.4	81	1,430	13

* After removal of the ganglioside fraction.
† Compared with activity injected.

Of incidental interest is the observation that lignoceric acid occurs as an ester-linked fatty acid in brain. The concentration is very low, only about 0.2% of the saturated ester-linked acids. The 24:0 peak was observed by GLC on diethylene glycol succinate polymer with flame ionization detector. Peaks corresponding to 23:0 and 22:0 were also distinctly visible. The peaks were seen also in the saturated ester-linked acids of uninjected rats, showing they were not artifactually derived from the injected lignoceroyl psychosine. We have previously reported the isolation of nervonic acid (24:1) from pig brain esters (30).

Sphingomyelins

The sphingomyelins contained a significant fraction of the injected radioactivity. In the first three groups given kerasin, the sphingomyelins contained 1.3, 2.1, and 2.2% of the injected material. In the ceramide rats, 1.3% of the injected activity was found in sphingomyelins (Table 3).

The individual fatty acids of the sphingomyelins were isolated from the kerasin rats, Group A. At 2 days, 95.2% of the radioactivity was in the lignocerate; at 4 days, 94.3%. Since it is possible that this high proportion merely reflects a particularly rapid synthesis of the lignocerate member of the sphingomyelin family at the particular age studied, we examined the distribution of C^{14} in the molecules by decarboxylation of the total fatty

TABLE 2 DISTRIBUTION OF C^{14} IN THE SATURATED ESTER-LINKED FATTY ACIDS AFTER INJECTION OF LABELED SPHINGOLIPIDS

Kerasin-injected Rats (Group B)		Ceramide-injected Rats (Group C)	
Fatty Acid	% of Total	Fatty Acid	% of Total
0 – 15:0	3.6	0 – 15:0	0.3
16:0	46.6	16:0 – 17:0	9.6
17:0 – 23:0	21.3	18:0	72.1
24:0	28.6	19:0 – 23:0	7.3
		24:0	10.7

TABLE 3 RECOVERIES IN LIPID FRACTIONS OF INJECTED RADIOACTIVITY AFTER 2 DAYS

	Kerasin-Injected (Group B)	Ceramide-Injected (Group C)
	%	%
Ester-linked fatty acids	2.84	8.67
Cholesterol	0.32	0.17
Ceramides	0.45	—
Sphingomyelins	1.63	1.3
Cerebrosides	—	0.26
Cerebroside hydroxy acids	1.07	—
Sulfatides	0.09	0.05
Ganglioside fatty acids	—	0.13
Total lipids (minus gangliosides)	38	13

acid mixture. The relative carboxyl activities were 92.5, 90.3, and 89.0% at the three time points. (Recoveries of C^{14}, including the fatty amine formed by decarboxylation, were about 97%.)

Ceramides

In the case of the 2-day kerasin rats (Group B), 0.45% of the injected lipid activity was found in this fraction. This sphingolipid, suggested as the precursor of the other sphingolipids (5), had the highest specific activity of all the lipids isolated, except for the kerasin. Its specific activity was about 5700 cpm/μmole and the ester-linked fatty acids about 660 cpm/μmole. Upon decarboxylation of the unsubstituted saturated acids of the ceramides, all of the activity was found in the carboxyl group.

Ganglioside Fatty Acids

The methyl esters of the gangliosides from the ceramide-injected rats contained 0.13% of the injected activity. GLC of the saturated esters showed primarily 16:0, 18:0, and 20:0 acids, the stearate peak being by far the major one. The distribution of activity within the three acids showed 15.4% in the 16:0, 62.4% in the 18:0, and 22.2% in the 20:0. Decarboxylation of the 18:0 acid showed that 96.3% of the C^{14} was in the carboxyl group.

Cerebrosides and Sulfatides

The sulfatides from both groups of rats contained rather little activity. With the 2-day kerasin rats (Group B), 0.09% of the injected activity was found; with the ceramide rats, 0.05%.

The kerasin from the kerasin-injected rats was, of course, highly radioactive. To test the possibility that direct hydroxylation of the kerasin lignocerate takes place, the hydroxy acids of the hydroxylated cerebrosides were isolated. They contained 1.07% of the injected activity, an appreciable fraction, but only 8.4% of this activity was in the carboxyl group.

The cerebrosides from the ceramide-injected rats contained 0.26% of the injected activity. Comparison of the hydroxy and unsubstituted fatty acids in this fraction showed about equal activities in the two groups.

A summary of the major findings is shown in Table 3.

DISCUSSION

The Main Routes of Kerasin and Ceramide Metabolism

The data reveal that the injected compounds primarily underwent degradative breakdown. The activity in cholesterol indicates that the fatty acid moieties, 24:0 and 18:0, were released and degraded to acetate. Conversion of acetate to fatty acids explains the relatively high activities found in 16:0 and 20:0 of the ester-linked acids and gangliosides. It also explains the similar activities in cerebrosides with hydroxylated and unsubstituted fatty acids, the small amount of activity in the sulfatides, and the low activity in the carboxyl group of the cerebroside hydroxy acids.

The fatty acids of the injected compounds are evidently released in a form suitable for incorporation into complex lipids, for the ester-linked 24:0 from the kerasin rats and the ester-linked 18:0 from the ceramide rats had high activities (Table 2).

The labeled lignocerate could have been released from the injected cerebroside by direct hydrolysis, via an amidase, or after preliminary attack by a galactosidase. In the former case, psychosine (sphingosine galactose) would be the other product; in the latter, ceramide would be formed, then hydrolyzed to lignocerate. The evidence from this experiment supports the intermediate formation of ceramide. First, the ceramide in the brains of the kerasin-injected rats had a high specific activity and all of the C^{14} was in the originally labeled position. Second, ceramide does indeed undergo rapid hydrolytic cleavage in brain, for the labeled ceramide disappeared faster from the brains (Table 1), and its labeled stearate was found in ester linkage (Table 2) and gangliosides. Third, the relatively high activity found in sphingomyelins from both labeled compounds is in agreement with the hypothesis that ceramide is an intermediate in cerebroside breakdown. The high relative carboxyl activity in the sphingomyelin fatty acids strongly indicates direct formation from ceramide (31).

The low activity in the cerebroside sulfate after cerebroside injection indicates that under the conditions used there was little or no direct sulfation of kerasin. The amount of activity found was too low for an accurate measurement of the relative carboxyl activity in the sulfatide fatty acids. The possibility that ceramides are precursors of cerebrosides also seems remote, for the activity found in the cerebrosides of the ceramide-injected rats was distributed similarly between the hydroxy

and nonhydroxy fatty acids. If a direct reaction with a galactose donor had taken place, one would expect to find the activity primarily in the nonhydroxy fatty acids. The distribution actually found resembles that produced when labeled acetate is injected (3). It may be concluded that cerebroside synthesis takes place in the manner indicated by in vitro experiments: sphingosine → psychosine → cerebroside (32, 33).

The role of ceramide in *ganglioside* synthesis is not entirely clear from our results. The finding of much C^{14} in the carboxyl group of ganglioside stearate points to direct utilization of the injected stearoyl sphingosine. However, the large contribution of labeled stearoyl sphingosine to ester-linked stearate (Table 2) indicates that ample quantities of stearate-1-C^{14} were present in the lipid pools of these rats and that this stearate could have been used for ganglioside synthesis. It is tempting, however, in view of the differences in brain distribution and composition of gangliosides and the other glycosphingolipids, to propose that the biosynthetic routes are basically different as well. Thus, it might be that in ganglioside synthesis, ceramide is first formed from sphingosine and the sugar moieties are added subsequently.

Comparison with In Vitro Sphingolipid Studies

The rapid cleavage observed with labeled ceramide is consistent with Gatt's report of an active ceramidase in brain (34). While he found no activity with lignoceroyl dihydrosphingosine and we found evidence that its dehydro analogue is an intermediate in lignoceroyl psychosine breakdown, the difference may be attributable to a difference in methods of emulsification or duration of exposure to the enzymes.

The appreciable formation of sphingomyelin in our kerasin rats, in which the activity rose with time, is consistent with the general outlines of the study in chicken liver by Sribney and Kennedy (31). Since our labeled stearoyl sphingosine was converted to sphingomyelin, it is likely that a similar enzyme operates in whole brain and that it acts on the normally occurring ceramide.

Gatt (34) has shown that the brain ceramidase preparation also synthesizes ceramide from sphingosine and free fatty acid, and Sribney has given a preliminary report of ceramide formation with acyl CoA instead of free fatty acid (35). Thus it would appear that brain can make ceramide by synthetic pathways as well as by hydrolysis of cerebroside and sphingomyelin (36).

The lignoceric acid (sp. act. 8×10^6 cpm/mg) was prepared in this laboratory by Drs. John R. Allen and Y. C. Liu. The beef brain psychosine sulfate was kindly prepared and furnished by Dr. Bernard Kaufman, University of Michigan. The detergent, G-2159, was the gift of Atlas Chemical Industries, Inc., Wilmington, Del. The ceramide sample, for comparison, was kindly furnished by Dr. H. E. Carter, University of Illinois.

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