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In vivo conversion of labeled fatty acids to the sphingolipid fatty acids in rat brain*

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SUMMARY

Emulsions of carboxyl-labeled palmitate, stearate, lignocerate, and DL-cerebronate were injected into the brains of weanling rats. After various time intervals, the rats were sacrificed, and the saturated fatty acids of the brain sphingolipids were isolated and decarboxylated. All four acids had been incorporated into the sphingolipids. The palmitate and stearate gave rise to radioactive lignocerate and cerebronate in which the carboxyl groups were relatively nonradioactive, indicating the latter acids are made by chain lengthening of the shorter acids. The lignocerate-C^{14} gave rise to labeled cerebronate in which the carboxyl group contained nearly all the activity, showing that the normal acid is converted fairly directly into the hydroxy acid. Some radioactivity was found also in the shorter acids, apparently by the intermediate degradation of lignocerate to acetate. The injected cerebronate-C^{14} underwent little or no conversion to normal acids.

The cerebrosides of the mammalian nervous system contain very long-chain normal and 2-hydroxy fatty acids (C_{20} and C_{26}) as well as the more common fatty acids (C_{16}–C_{18}). As the result of a study with labeled acetate (2), we concluded that the very long acids are made in the brain by a chain-lengthening mechanism from pre-existing shorter acids rather than by a de novo synthesis from acetate. This hypothesis was tested by injecting labeled fatty acids directly into rat brains and isolating the sphingolipid acids. This experiment also allowed us to determine whether the normal and hydroxy acids are interconverted.

MATERIALS AND METHODS

Most of the materials and equipment were described before (2, 3). *Preparation of* DL-*Cerebronic Acid-1-*C^{14}. Lignoceric acid-1-C^{14} (2.5 mg) was converted to the acid chloride by refluxing 30 min with 100 μl $SOCl_2$ in a small test tube sealed to a condenser. The acid chloride was converted to the 2-bromo compound by adding 7 μl of bromine and 43 μl of $SOCl_2$ and refluxing 120 min (4, 5). The excess reagents were evaporated off, and the bromo compound was hydrolyzed by stirring 48 hr at 100° with 1 ml of 8% KOH (6). The cerebronic acid was esterified with dimethoxypropane (7) and purified by TLC, using silica gel G and SkB–absolute ether 85:15 (8).[1] The ester was visualized with bromothymol blue (9), eluted with ether, and purified on a Florisil column (3). The free acid was regenerated by saponification and a trace of colored material was removed by means of a silicic acid column[2] (10).

The final yield was 40% of theoretical. Appreciable amounts of another acid formed as a by-product; it was apparently α,β-unsaturated lignoceric acid, judging by its behavior in TLC and GLC.[1]

Purification of the C^{14} *Fatty Acids.* The palmitic acid-1-C^{14} (2.06 mc/mmole)[3] and stearic acid-1-C^{14} (14.7 mc/mmole)[4] were esterified and freed of polar impurities with a Florisil column (3). Homologous radioactive esters were removed by programmed GLC on an SE-30 column (11). The free acids were regenerated and purified by elution with benzene from a silicic acid column.

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[1] Abbreviations used: SkB, Skellysolve B (a commercial hexane); TLC, thin-layer chromatography; GLC, gas-liquid chromatography; MeOH, methanol; and h in a numerical abbreviation of a fatty acid indicates a 2-hydroxy acid.

[2] Unisil silicic acid, Clarkson Chemical Co., Inc., Williamsport, Penna.

[3] From Tracerlab, Waltham, Mass.

[4] From New England Nuclear Corp., Boston, Mass.

Lignoceric acid-1-C^{14} (2 mc/mmole) was prepared by Dr. John R. Allen by condensing $KC^{14}N$ with 1-bromotricosane[5] and saponifying the nitrile. Polar impurities were removed (8 years later) by silicic acid chromatography.

Intracerebral Injection. The acids were converted to soaps by dissolving them in chloroform–methanol in a small conical tube, then adding the calculated amount of NaOH or KOH in MeOH.[1] The weights of acids used were 1.7 mg (16:0), 0.34 mg (18:0), 1.6 mg (24:0), and 1.0 mg (24h:0).[1] In the case of palmitate and stearate, the sodium soap suspension was evaporated to dryness and agitated in a warm ultrasonic bath with 0.25 ml of 25% bovine albumin solution. In the case of the longer acids, the potassium soap suspension was evaporated to dryness together with 8 mg of a nonionic detergent.[6] The residue was heated at 100° with 0.25 ml water, with intermittent agitation in an ultrasonic bath. The shorter acids yielded turbid suspensions, but the longer ones yielded clear emulsions.

A 50-μl Hamilton syringe with sealed 24-gauge needle was used to inject 20–25 μl of sample. Weanling rats were anesthetized lightly with ether, and the syringe needle was inserted through the top center of the skull, about 5 mm deep. The rats seemed to recover quickly and appeared normal for the remainder of the experiment.

At definite time intervals, the rats were anesthetized with CO_2 and the brains were removed, pooled, and stored at −20° before extraction.

Isolation of the Sphingolipids. The total brain lipids, after lyophilization from benzene, were treated with one of the mild alkali systems of Hübscher, Hawthorne, and Kemp (12). For each gram of lipid, 25 ml of chloroform–methanol 2:1 was added, then 12 ml chloroform and 6 ml of 0.5 *N* methanolic NaOH. Following 30 min of mixing, the pH was brought to 1 with HCl. The mixture was stirred another 60 min to cleave the alkenyl ether linkage, and water was added to bring the total volume up to 11 ml.

After centrifugation, the upper layer was removed, and the lipid layer was washed twice with "Folch upper layer" (13). The solvent was evaporated off, and a concentrated solution of the lipids in chloroform–methanol was evaporated together with 1 g silicic acid, using nitrogen. The powder was slurried with chloroform and applied to a column of silicic acid (7 g/g of total brain lipid) packed in chloroform. The cholesterol, methyl esters (from phosphoglycerides), and aldehydes were eluted with chloroform (20 ml/g of packing). The sphingolipids were eluted with chloroform–methanol 1:1 (20 ml/g) and MeOH (10 ml/g). Thin-layer chromatography with chloroform-methanol-water 24:7:1 (14) showed that the chloroform-methanol fraction contained cerebrosides, cerebroside sulfate, and a little sphingomyelin. The last effluent contained sphingomyelin together with a small amount of a lipid appearing just below the main spot. Perhaps this is cyclic acetal formed from lysoplasmalogen (15). Ganglioside was presumably discarded in the aqueous washes after the cleavage reactions.

Isolation of the Sphingolipid Saturated Fatty Acids. The fatty acids were liberated with KOH–propylene glycol (10) and the methyl esters separated into two groups: normal saturated and hydroxy saturated, using the mercuric acetate adducts and Florisil (3). The purity of each fraction was checked by TLC, which revealed the presence of some radioactive impurities as well as a nonradioactive impurity in the normal esters (derived from the use of undistilled ether). Where the impurities seemed appreciable in quantity, the esters were purified by larger scale TLC. The bromothymol blue used for visualization was not extracted from the powder by ether.

The individual fatty esters were isolated by GLC with an SE-30 column (11). The hydroxy acids were first acetylated with isopropenyl acetate (2). After a portion of the isolated esters was counted, the remainder was saponified and decarboxylated by the Schmidt azide reaction or with permanganate–acetic acid (2). In each case, the degradation product, as well as the CO_2, was counted to insure that the decarboxylation had proceeded normally.

In the case of some of the rats, cerebrosides were isolated instead of sphingolipids, and the acids were isolated similarly. Florisil, mild saponification, and ion exchange were used (3).

Validity of the Data. Interpretation of the results requires the assumption that contamination of the injected and isolated compounds is negligible, for many of the conversion reactions took place to a very small extent and radioactive trace impurities could be misleading. While the decarboxylation and varied isolation steps were good checks against contamination problems, it seemed wise to purify the starting materials rigidly. As a check on the final purity, portions of the carboxyl-labeled acids were mixed with nonradioactive beef spinal cord sphingolipids (16), and the normal and hydroxy ester fractions were isolated as described in this paper. With 16:0 and 18:0, 0.2–0.3% of the C^{14}

[5] Prepared by Dr. Y. C. Liu from nonradioactive lignoceric acid by the Hunsdiecker reaction.

[6] A polyoxyethylene stearate, G-2159, Atlas Chemical Industries, Inc., Wilmington, Del. This detergent has a hydrophile-lipophile balance rating of 18.8, which is higher than that of the more commonly used Tween 20 (16.7).

TABLE 1. DISTRIBUTION OF RADIOACTIVITY IN THE NORMAL SATURATED SPHINGOLIPID ACIDS OF RAT BRAIN

Sphingolipid Acid Isolated	C^{14} Acid Injected			Relative Amount of Each Isolated Acid
	16:0	18:0	24:0	
	cpm/brain	*cpm/brain*	*cpm/brain*	%
16:0	3,739	379	587	3.6
18:0	2,790	10,533	404	54.3
19:0	61	149	63	<0.1
20:0	689	3,975	209	4.6
21:0	51	134	115	<0.1
22:0	852	3,895	280	9.2
23:0	63	140	268	1.1
24:0	1,327	3,783	26,380	23.1
25:0	43	90	250	0.7
26:0	93	131	411	1.3

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN THE HYDROXY SATURATED SPHINGOLIPID ACIDS OF RAT BRAIN

Sphingolipid Acid Isolated	Radioative Acid Injected				Relative Amount of Each Isolated Acid
	16:0	18:0	24:0	24h:0	
	cpm/brain	*cpm/brain*	*cpm/brain*	*cpm/brain*	%
22h:0	1,329	1,391	223	212	26.5
23h:0	71	97	93	137	5.9
24h:0	2,018	1,766	2,884	25,347	59.1
25h:0	46	32	45	122	1.8
26h:0	79	86	96	92	2.0

was found in the hydroxy fraction, probably due to trailing from the Florisil column. Cerebronic acid was found to be free from normal acids, while the labeled lignoceric acid yielded 1% of the activity in the hydroxy fraction.

It seemed likely that the polar impurities in the 24:0 were an assortment of hydroxy acids arising from radiolysis, so a portion of the polar fraction was saponified and oxidized with permanganate–acetic acid. (This degradation yields CO_2 from 2-hydroxy acids.) One-seventh of the radioactivity appeared in the resultant CO_2, so contamination by cerebronic acid was about 0.14%.

Gas-liquid chromatography of the above major fractions showed that the palmitate and stearate yielded 16:0 and 18:0 peaks containing about 98% of the total activity, which is the best we have ever found with our chromatograph. The C_{24} acids were found to be contaminated with about 2.5% of the C_{22} acids and somewhat smaller amounts of the shorter acids.

RESULTS

Basic Data. The weight of the rats used ranged between 50 and 60 g. The palmitate and stearate injected rats (ten in each group) were sacrificed 24 hr after injection. Half of the rats given lignocerate and cerebronate were killed after 2 days (5–6 in each group) and half were killed after 9 days. In the case of the latter, only the cerebrosides were isolated. The rats given cerebronate lost weight for 2–3 days after injection, then grew fairly normally. At the end of the 9-day period, their average weight was 104 g while that of the lignocerate group was 131 g. However the yields of saturated cerebroside esters were similar for the two groups: 0.78 mg/brain for the normal esters and 2.5 mg/brain for the hydroxy esters. In the case of the sphingolipids, the yields were 2.0–2.2 mg/brain (normal) and 2.0–2.1 mg/brain (hydroxy).

The incorporation of radioactivity from the injected acids into the saturated sphingolipid acids was 0.7, 1.5, 2.8, and 3.4% for the 16:0, 18:0, 24:0, and 24h:0 acids, respectively. The incorporation of the 24:0 acid activity into the cerebroside saturated acids was 0.62% of the injected dose; with the 24h:0-C^{14}, 0.045% was incorporated. If only the D form of the labeled cerebronate was incorporated, the figures for the percentage incorporation should be doubled.

Distribution of Activity in the Individual Acids. Tables 1 and 2 list the total activity per brain in each normal and hydroxy acid isolated from the sphingolipids. No data are shown for the normal acids of the 24h:0-injected rats as very little activity was found in this group of acids. The relative amount of each acid in the two groups, calculated from the GLC curves, is shown in the last column of each table.

The following points should be noted about Table 1: (1) the largest amount of activity appears in the acid corresponding to the one injected; (2) the odd-numbered acids are somewhat less radioactive than the adjacent homologues; (3) with the exception of C_{26}, even-numbered acids longer than the injected acid are more radioactive than those that are shorter.

From Table 2, it is evident that 24h:0 is more radioactive than any other hydroxy acid, and this feature is more prominent in the rats given 24:0 and 24h:0.

Table 3 shows the corresponding data for the *cerebroside* acids from the rats given 24:0. The findings are similar to those in the first two tables. It may be noted that the weight distribution in the cerebrosides is similar to that in the sphingolipids in the case of the hydroxy acids, but the sphingolipids contain much more 18:0. The former observation is due to the fact that the 2-hydroxy acids of brain occur entirely or almost entirely

TABLE 3. DISTRIBUTION OF RADIOACTIVITY IN THE CEREBROSIDE SATURATED FATTY ACIDS FROM RATS INJECTED WITH LIGNOCERIC ACID-1-C^{14}

Normal Fatty Acids			Hydroxy Fatty Acids		
Acid Isolated	Activity	Relative Amount	Acid Isolated	Activity	Relative Amount
	cpm/brain	%		*cpm/brain*	%
16:0	21	2.1	22h:0	213	23.7
18:0	85	21.2	23h:0	94	7.1
19:0	3	0.1	24h:0	3,513	61.6
20:0	31	7.2	25h:0	52	1.8
21:0	9	0.4	26h:0	111	2.4
22:0	49	15.8			
23:0	21	3.5			
24:0	1,437	44.2			
25:0	25	2.2			
26:0	69	3.3			

in the cerebrosides and sulfatides (17). The latter observation reflects the high content of 18:0 in sphingomyelin.

Relative Activity in the Carboxyl Group. If a fatty acid molecule is made by a single enzyme system by de novo synthesis from carboxyl-labeled acetate, the radioactivity should be uniformly distributed in all the odd-numbered carbon atoms. Thus, for 16:0, the carboxyl relative activity should be $^1/_8$ or 12.5%; for 24:0, it should be $^1/_{12}$ or 8.3%. Any deviation from such a ratio would indicate a different route of biosynthesis.

In Table 4, the carboxyl relative activity (rounded off to the nearest percentage) is shown for various samples of normal acids. The activity in the sample actually degraded is shown to indicate the scale of activity used. It may be seen that nearly all the C^{14} is in the carboxyl group in those acids corresponding to the acid injected. Another finding is that the acids made from *longer* radioactive acids (lines 2, 5, and 6) contain more activity in the carboxyl group than is predicted by de novo synthesis, while the acids made from *shorter* labeled acids (lines 3, 7, 8, 9, and 10) contain relatively little activity in the carboxyl carbon.

Similar data for the hydroxy acids are shown in Table 5. The 22h:0 and 24h:0 from rats given short acids contain very little activity in the carboxyl group. In contrast, the 24h:0 in the rats given labeled 24:0 and 24h:0 shows very high carboxyl relative activity.

DISCUSSION

Direct Incorporation of Fatty Acids into Sphingolipids. The data in the tables indicate that the brain can incorporate a variety of normal and hydroxy acids into the sphingolipids, including cerebrosides. This is shown

TABLE 4. CARBOXYL RELATIVE ACTIVITY IN THE NORMAL SPHINGOLIPID ACIDS*

Acid Degraded	C^{14} Acid Injected	Activity of Degraded Sample	Carboxyl Relative Activity
		cpm	%
16:0	16:0	3,352	96
	18:0	330	15
18:0	16:0	2,767	3
	18:0	9,705	97
	24:0	229	30
	24:0†	149†	25†
22:0	16:0	780	5
	18:0	3,412	3
24:0	16:0	1,035	4
	18:0	3,473	3
	24:0	5,870	97
	24:0†	1,824†	92†

* Carboxyl relative activity to be expected from de novo synthesis from carboxyl-labeled acetate: 16:0-12.5%; 18:0-11.1%; 22:0-9.1%; 24:0-8.3%.

† These acids obtained from the cerebrosides.

in Tables 1, 2, and 3, where we see that the sphingolipid acid having the highest activity is the one that was injected. The relationship is especially notable in the case of palmitate (line 1, Table 1), as the *specific* activity is extremely high in the rats given labeled palmitate. Gatt (18) has also reported finding direct incorporation of palmitate into the cerebrosides in a similar experiment.

The decarboxylation data of Tables 4 and 5 likewise show that direct incorporation occurs. The carboxyl relative activities are close to 100% in those acids corresponding to the ones injected, indicating that most of the labeled sphingolipid acid in these cases was derived from the exogenous carboxyl-labeled acid.

TABLE 5. CARBOXYL RELATIVE ACTIVITY IN THE HYDROXY SPHINGOLIPID ACIDS*

Acid Degraded	C^{14} Acid Injected	Activity of Degraded Sample	Carboxyl Relative Activity
		cpm	%
22h:0	16:0	1,390	4
	18:0	1,263	3
24h:0	16:0	1,650	3
	18:0	1,748	3
	24:0	1,379	94
	24:0*	2,086†	89
	24h:0	7,179	99

* Carboxyl relative activity to be expected from de novo synthesis from carboxyl-labeled acetate: 22h:0-9.1%; 24h:0-8.3%.

† This acid sample isolated from the cerebrosides.

While direct injection into the brain cannot be considered "physiological," it seems likely that the observed incorporation reflects metabolism by the brain rather than by another part of the body. This conclusion is drawn from the observed relatively high degrees of incorporation, the high retention of carboxyl labeling, the rarity of occurrence of cerebrosides in the extraneural organs, and the poor absorption of large molecules through the blood-brain barrier.

Interconversion of the Normal and Hydroxy Acids. The conversion of lignoceric to cerebronic acid is shown by the relatively high amount of radioactivity in the latter (Tables 2 and 3, right side), and the high degree of retention of activity in the carboxyl group (Table 5, lines 5 and 6). This finding is supported also by our turnover study with labeled acetate (2), from which we concluded that the cerebroside-bound 24:0 and 24h:0 have a common precursor. Presumably this precursor is 24:0, the free acid or the CoA derivative. The lignoceric acid could be directly hydroxylated in the α-position, or first dehydrogenated in the α,β-position and then hydrated. Presumably the other hydroxy acids are also made directly from the corresponding normal acids, saturated and unsaturated.

The reverse reaction, reduction of 24h:0 to 24:0, apparently does not occur to a comparable extent. The cerebroside acids from the cerebronate-injected rats contained 8% of their activity in the normal acids and 92% in the hydroxy acids. The sphingolipid acids from the cerebronate-injected acids contained even less activity in the normal saturated acids (0.2%). In contrast, the lignocerate-injected rats contained 69% of the incorporated activity in the cerebroside hydroxy acids. Too little activity was found in the cerebronate-derived normal acids to permit isolation and degradation.

Degradation of the Injected Acids. The finding that acids shorter than the injected acids were radioactive indicates that the latter were degraded, presumably by β-oxidation, to yield carboxyl-labeled acetate. As we and others (2, 3, 19, 20) have shown, brain utilizes acetate to synthesize the odd- and even-numbered sphingolipid acids.

Our acetate turnover study (2) had shown that the cerebroside acids undergo metabolic conversion, but it could not be determined if this conversion involved breakdown (as opposed to sulfation to form cerebroside sulfate, or interconversion between normal and hydroxy acids). It has recently been shown (21, 22) that the C_{24} acids of total lipid are lost from demyelinating areas of brain in multiple sclerosis. It may be inferred that the acids are attacked by the ordinary fatty acid degradation system. In our experiments, much of the injected radioactivity could not be accounted for in the brain lipids, perhaps because of degradation to acetate and then CO_2. However, in a similar experiment with labeled 24:0, Gatt (18) found no formation of radioactive CO_2.

From the data in Table 2, it would appear that labeled cerebronate gave rise to shorter and longer labeled acids. However, the observed activities (2% of the total) probably arose from contamination in the original injected acid as well as in the GLC isolation step. The α-degradation system (23, 24) yields radioactive CO_2, not acetate.

The Chain-Lengthening Reaction. Examination of the carboxyl relative activities in Tables 4 and 5 shows that only palmitate, derived from labeled stearate, has a value close to the theoretical for de novo synthesis (15% vs 12.5%). Our study with labeled acetate had yielded closer values (12.2–12.9%), and the difference in the present experiment is very likely due to slight contamination during GLC by the neighboring stearate, which was much more radioactive and labeled almost entirely in the carboxyl group. Thus this experiment too supports the in vitro evidence that palmitate is the primary product of acetate condensation.

The various low carboxyl relative activities shown in Tables 4 and 5 (3–5%) indicate that the injected acids are lengthened by addition of slightly radioactive acetate moieties. The low values should be contrasted with the high values found, at similar time points, when carboxyl-labeled acetate was injected (2). It is apparent that stearate, as well as palmitate, can be lengthened and the products hydroxylated. The degradation data of Mead and Levis (23), as pointed out earlier (2), support the possibility that 22:0 can be lengthened. The data for the unsaturated sphingolipid acids in an accompanying paper (25) indicate that 18:1, 20:1, and 22:1 can also be lengthened. It seems likely that the chain-lengthening enzymes of brain are not very specific and that pools of acids of intermediate chain length exist in brain.

The stearate isolated from the lignocerate-injected rats (Table 4, lines 5 and 6) shows a high carboxyl relative activity, apparently the result of condensation of nonradioactive 16:0 and moderately radioactive acetate (from degradation of the 24:0). However, the high carboxyl activity could also be due to the presence of a trace amount of carboxyl-labeled stearate in the original radioactive lignocerate.

The acids *longer* than the injected acids are derived from the highly radioactive injected acid lengthened with slightly radioactive acetate. The acids *shorter* than the injected acids are made only from slightly radioactive acetate. This explains why the shorter

(even-numbered) acids are less radioactive than the longer ones (Tables 1 and 3). The palmitate and stearate from lignocerate-injected rats contain fairly high amounts of activity, but this is not surprising in view of the relatively large amounts present in brain.

From previous work, we may conclude that the activity in the odd-numbered acids arises in two ways: by lengthening of propionate with labeled acetate, and by α-degradation of the labeled even-numbered acids.

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