J. Lipid Research, July, 1962
Volume 3, Number 3

Biosynthesis of the cerebroside odd-numbered fatty acids*

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[Received for publication February 15, 1962]

SUMMARY

Rats were injected with radioactive acetate or propionate and, after four days, the saturated fatty acids were isolated from the brains and the brain cerebrosides. The amounts and specific activities of the individual normal and hydroxy acids were determined. In the rats given propionate, the odd-numbered acids (15:0 to 25:0) had much higher specific activities. In the rats given acetate, the differences were much smaller, with the even-numbered acids (16:0 to 26:0) showing higher specific activities. These data are consistent with earlier work, which indicated that the odd-numbered acids of other organs are derived from propionate and acetate. Comparison of the very long odd-numbered cerebroside acids with the more common ones (15:0 and 17:0) indicated that these are made by a similar synthetic route. Improved techniques are described for the isolation of the fatty acids. Comparison of the cerebroside acids and total brain fatty acids showed that most of the hydroxy acids are present in the cerebrosides. Hydroxystearic acid is a minor component of the hydroxy acid fraction; most of it is not in the cerebrosides. Degradation of the unsaturated acids from cerebrosides of rats given labeled propionate showed that propionate is a specific precursor of these acids also.

Propionic acid appears to be a precursor of various odd-numbered fatty acids, acting as a primer on the ω-end for the condensation of several C_2 units (as malonyl-CoA) upon it. The evidence appears in *in vitro* work with cow udders (1), pigeon liver enzymes (2), and adipose tissue enzymes (3), and in work with mice injected with C^{14}-propionate (4). The feeding of large amounts of tripropionin yields increased deposition of 15:0, 17:0, and 17:1 fatty acids in mice (5).

While the odd-numbered fatty acids constitute a very small fraction of most body fats (about 1% of the total), appreciable amounts are found in the brain cerebrosides (6, 7) and their relative contents increase with age (8). These cerebroside acids are mainly the 23:0, 25:0, 23h:0, and 25h:0 acids.[1] It appeared possible that these unusual acids are made by a different pathway than that used for the shorter odd-numbered acids. To test this possibility, C^{14}-propionate and H^3-acetate were injected into rats, and the total brain saturated acids and the cerebroside acids were isolated and counted. The distribution of activities was consistent with the conclusion that the cerebroside odd-numbered acids and the previously studied odd-numbered acids are made from propionate in a similar fashion.

* Supported in part by PHS Grant B-3192 from the National Institute of Neurological Diseases and Blindness, U. S. Public Health Service.

[1] The abbreviation for stearic acid is 18:0 in accordance with present custom. We have inserted an h to indicate hydroxy acids, such as 24h:0 to indicate cerebronic acid, and a d to indicate the ω-dicarboxylic acids, as in 4d:0 for succinic acid.

EXPERIMENTAL METHODS

Materials and Equipment. Redistilled A.C.S. absolute methanol, U.S.P. chloroform, petroleum ether b.p. 67-68° (Skellysolve B), and 2,2-dimethoxypropane were used. Florisil (60/100 mesh) was activated at 600° for 1 hr. After cooling, 7 ml of water was added to each 100 g of Florisil as described by Carroll (9). Other materials and the gas-liquid chromatographic techniques were as described before (6, 10). The 15:0, 17:0, and 19:0 fatty acids used to check GLC retention times were Eastman Organic Chemicals. The 12d:0 and 14d:0 acids were from Aldrich Chemical Company (Milwaukee 10, Wisconsin). 1-C^{14}-propionate and 2-H^3-acetate were obtained from New England Nuclear Corp. (Boston, Mass.). The latter compound was recovered from the wastes of steroid acetylation reactions.

Animals. Two groups of ten male weanling rats, average weight 70 g, were used. Each rat received a single intraperitoneal injection of radioactive material in isotonic saline. The sodium propionate given to each rat was 60 μc (2.88 mg); the sodium acetate per rat was about 800 μc (0.7 mg). The rats were then kept four days on Rockland Rat Diet and killed by CO_2 anesthesia. The brains were stored at $-20°$ for 1–2 weeks before processing.

Isolation of the Cerebrosides. The previously described method for cerebroside isolation (6) was modified slightly to speed the procedure and reduce the danger of splashing during vacuum evaporations. The essential steps are (*1*) extraction and drying of total lipids, (*2*) passage through Florisil to remove cholesterol and phospholipids, and (*3*) saponification and ion exchange to remove ester impurities.

The brains were pooled and extracted with 20 volumes of chloroform-methanol 2:1 (11). In the case of the C^{14} rats, $^1/_{11}$ of the filtrate was used for isolation of the total saturated fatty acids. The crude extract was mixed with toluene and evaporated to a small volume, and the remaining chloroform and methanol were removed by two additional evaporations with 50 ml portions of benzene. The remaining water in the extract was then removed by adding 50 ml of benzene and lyophilizing.

The use of moistened Florisil (9) allowed us to use smaller volumes of solvents for elution. For each gram of Florisil, 7 ml of absolute ether was used to elute the cholesterol and 25 ml of chloroform-methanol 4:1 was used to elute the cerebrosides.

After the saponification step in methanol, the mixture was neutralized with 3 N HCl and enough water was added to produce a final concentration of 50% methanol. Two volumes of chloroform per volume of methanol were added, the suspension was centrifuged, and the lower layer was evaporated to dryness with the addition of toluene to prevent splashing (12). Following the ion-exchange step, we lyophilized the cerebrosides with benzene as described above. The galactose contents of similarly processed samples were 20.2–21.5% (13). The yield of cerebrosides was 9.55 mg per brain in the group of rats given acetate and 9.67 mg per brain in the group of rats given propionate, in good agreement with our previous results (8).

Isolation of Fatty Acids from Cerebrosides. The cerebrosides were hydrolyzed by refluxing for 8 hr with 20 ml of methanol and 4 ml of concentrated HCl. Boiling chips and a magnetic stirrer were used together to prevent bumping. After cooling, 24 ml of water was added and the esters and free acids were extracted with 3 × 20 ml of ether. The combined ether extracts were mixed in a screw-cap centrifuge bottle with 30 ml water, 30 ml toluene, 6 ml ethanol, and 0.6 g KCl. The mixture was centrifuged briefly, and the upper layer was evaporated to dryness in vacuum. The use of the above materials in the described ratios prevented emulsion formation and precipitation at the interface (serious problems with samples rich in the hydroxy fatty acids) and allowed the vacuum evaporation to take place without splashing.

The residue was esterified with dimethoxypropane (14) and purified with a 1.5-g Florisil column (5 mm inside diameter) by eluting with 50 ml of Skellysolve-absolute ether (9:1). The column retained some contaminating material (derived from the sphingosine) that interfered with the subsequent removal of unsaturated esters. The dried esters were heated in a screw-cap test tube for 2 hr at 66° with 4.5 ml of methanol and 300 mg of mercuric acetate (6). The reaction mixture was then evaporated to dryness together with 50 ml of Skellysolve and 0.5 g of Florisil. The solvents were removed by evaporating to a small volume in a rotary evaporator, then blowing in nitrogen at room temperature. The entire mixture was then transferred with Skellysolve B to a 2.5-g Florisil column (7 mm diam) packed in the same solvent, and the normal saturated esters were eluted with 100 ml of Skellysolve. The hydroxy saturated esters were next eluted with 100 ml of 9:1 solvent (6). In the case of the samples prepared from propionate-injected rats, the unsaturated normal plus hydroxy esters were also eluted out.

The hydroxy saturated esters were converted to methyl ethers as before (6), but, to make sure that radioactive non-ether impurities were absent, we purified the ethers by passage through a 1-g Florisil column with Skellysolve-absolute ether (99:1). The column retained about 5% of the material.

Isolation of Total Fatty Acids from Brain. The remaining $^1/_{11}$ of the C^{14}-lipids was evaporated to dryness by lyophilizing, then cleaved with methanol-HCl as above. In this case, it was necessary to remove nonsaponifiable material before proceeding further. The mixture of fatty acids and esters was heated 2 hr at 80° in a screw-cap test tube with 20 ml of ethanol and 1 ml of aqueous 40% KOH (w/v) (15). Five ml of water was added to the tube, and the nonsaponifiable lipids were removed with 10, 10, and 5 ml of Skellysolve. The combined hexane extracts were backwashed with 10 ml of water, which was added to the alcoholic layer. The acids were liberated with HCl and extracted with ether, and the ether layer was washed with water, using the toluene-alcohol-KCl mixture described above. The acids were then treated

like the cerebroside acids. The acid cleavage step was used before the saponification step because the sphingolipid acids appear to be relatively stable to alkali.

The weights of normal saturated esters per rat brain were 0.92, 0.81, and 23.62 mg for the cerebrosides in the propionate group of rats, cerebrosides in the acetate group of rats, and total brain, respectively. The corresponding values for the hydroxy saturated esters were 2.32, 2.26, and 2.86 mg per brain.

Gas-Liquid Chromatography. The weighed esters were transferred with Skellysolve to a 13 mm (outside diameter) screw-cap test tube that had been pulled down to a conical shape. The solvent was removed with a nitrogen stream, the tube was weighed to correct for the volume of remaining solvent, and the residue was dissolved in xylene, about 0.01 ml per mg of esters. The column, silicone SE-30, and the instrument have been described before (10). An almost linear temperature program between 185° and 290° was used, and the helium flow rate was about 130 ml/min. Injector and collector temperatures were 300°. Since the detector cell was close to the column, inside the oven, the base line shifted during each run, but this did not interfere with triangulation measurements or the detection of peaks. The individual esters were collected with a tube attached to a Millipore filter, which trapped the fog (10). When the amount of a component was too small to be seen in the recording (<10 μg), collection was made anyway on the basis of retention times observed with standards. By using standards, we found that an almost straight line was obtained in the plot of retention time *vs.* carbon number on linear-graph paper.

All samples were counted by liquid scintillation in a Packard TriCarb counter, which yielded a background of 31 cpm for tritium (using polyethylene vials) and 22 cpm for C^{14}. Recovery tests were made in several runs, by counting an additional aliquot of the xylene solution. The recoveries were 93.8, 95.6, 98.6, and 98.6%. Collections were made for 10–15 min after elution of the 26: 0 peaks, but very little additional activity came off.

RESULTS AND DISCUSSION

In Tables 1, 2, and 3 are shown the values found for the individual fatty acids. Where a peak was too small to see or measure, approximate limits for the amount and specific activity were calculated. No corrections were made for the slight amount of trailing found in our gas chromatograph, as this was negligible in every case but three. The tables show the data from

TABLE 1. RADIOACTIVITY AND AMOUNT OF FATTY ACIDS IN THE TOTAL BRAIN OF RATS INJECTED WITH 1-C^{14}-PROPIONATE

	Normal Acids			Hydroxy Acids		
Acid*	Radioactivity†	Specific Activity	Total Amount per Brain	Radioactivity†	Specific Activity	Total Amount per Brain
	cpm	*cpm/μmole*	*μmoles*	*cpm*	*cpm/μmole*	*μmoles*
14:0	2	24	0.59			
15:0	303	6165	0.34			
16:0	116	21	39.1	4	39	0.13
br 17:0	50	930	0.33			
17:0	715	9201	0.54	15	825	0.02
br 18:0				9	77	0.11
18:0	99	18	37.7	26	84	0.31
br 19:0	16	270	0.30			
19:0	53	>1650	<0.22	10‡	137‡	0.09‡
20:0	8	64	0.85	9	75	0.09
21:0	14	>470	<0.21	49	945	0.04
22:0	4	31	0.90	42	15	1.72
23:0	22	>810	<0.19	364	1000	0.44
24:0	4	18	1.56	90	22	3.88
25:0	10	>400	<0.17	94	699	0.13
26:0	9	>360	<0.16	27	40	0.16

* To save space in the tables, the same abbreviation is used for the normal and hydroxy acids.

† Radioactivity actually found in the fractions isolated by gas chromatography.

‡ These numbers include a small peak apparently due to a branched 20:0 acid.

TABLE 2. RADIOACTIVITY AND AMOUNT OF FATTY ACIDS IN THE BRAIN CEREBROSIDES OF RATS INJECTED WITH 1-C^{14}-PROPIONATE*

	Normal Acids			Hydroxy Acids		
Acid	Radioactivity	Specific Activity	Total Amount per Brain	Radioactivity	Specific Activity	Total Amount per Brain
	cpm	*cpm/μmole*	*μmoles*	*cpm*	*cpm/μmole*	*μmoles*
14:0	1	>24	<0.01			
15:0	1	>25	<0.01			
16:0	1	11	0.03			
17:0	40	>1140	<0.01			
18:0	20	10	0.69	8	89	0.07
19:0	84	>2630	<0.01	6	110	0.04
20:0	10	20	0.18	6	43	0.09
21:0	49	>1660	<0.01	42	>1550	<0.02
22:0	18	15	0.44	34	16	1.51
23:0	256	1064	0.08	601	1166	0.38
24:0	56	18	1.06	102	22	3.40
25:0	124	860	0.05	120	1026	0.08
26:0	8	32	0.09	10	52	0.14

* See footnotes to Table 1.

TABLE 3. RADIOACTIVITY AND AMOUNT OF FATTY ACIDS IN THE BRAIN CEREBROSIDES OF RATS INJECTED WITH H^3-ACETATE*

Acid	Normal Acids			Hydroxy Acids		
	Radioactivity	Specific Activity	Total Amount per Brain	Radioactivity	Specific Activity	Total Amount per Brain
	cpm	*cpm/μmole*	*μmoles*	*cpm*	*cpm/μmole*	*μmoles*
16:0	16	590	0.02			
17:0	5	>140	<0.01			
18:0	780	499	0.49	24	303	0.07
19:0	17	>530	<0.01	60†	750†	0.08†
20:0	343	666	0.16	84	740	0.10
21:0	20	>660	<0.01	14	>460	<0.03
22:0	616	487	0.40	802	527	1.40
23:0	84	317	0.08	126	333	0.36
24:0	1734	524	1.03	2134	603	3.30
25:0	72	535	0.04	48	439	0.09
26:0	161	614	0.08	134	818	0.14

* See footnotes to Table 1.

† Includes a small amount of a branched 20h:0 acid.

single chromatographic runs; similar results were obtained in the duplicate runs made with the normal acids.

Specific Activities. It may be seen from Table 1, for propionate-injected rats, that the odd-numbered fatty acids of the total lipids have much higher specific activities than the even-numbered acids. The highest specific activities are found in the 17:0 and 15:0 acids, which had been shown in other tissues to be the major products of biosynthesis from propionate. However, even the longer odd-numbered acids (19:0, 21:0, 23:0, 25:0), which constitute too small a fraction of the total to be detected, have very high specific activities. Two extra peaks appear in the normal and in the hydroxy acid chromatograms; these are presumably branched chain acids. It seems likely that much of the radioactivity found in these acids arises from incomplete resolution from the nearby odd-numbered acids. This explanation may also apply to the 26:0 acid. It is curious that branching seems to occur in the 17 and 19 normal acids and in the 18 and 20 hydroxy acids. However, the identifications are admittedly tenuous; we know only that these are not unsaturated or ordinary saturated acids.

The distribution of activities in the hydroxy acids in Table 1 is similar, except that the contrast is not as great. The most common odd-numbered acid, 23h:0, has the highest specific activity, but unexpectedly high specific activities are found in trace acids, 17h:0 and 21h:0.

Table 2 shows similar data for the cerebroside acids of the same rats. Because of the relatively low contents of palmitic and stearic acids, we were able to obtain chromatographic recordings that show the rarer normal acids more distinctly and to give more explicit values for their specific activities. The distribution of activities is qualitatively similar to that found with the total brain acids. The cerebrosides contain negligible amounts of the 16:0, 17h:0, and 16h:0 acids, and the 26:0 has a somewhat lower specific activity than the whole brain 26:0.

The presence of appreciable amounts of activity in the even-number acids has been observed by others (1, 4) and is presumably due to the oxidation of C^{14}-propionate and fixation of the resulting $C^{14}O_2$ by β-methylcrotonyl-CoA to form β-methylglutaconyl-CoA. This, after hydration, is cleaved to acetoacetate and then to acetate-1-C^{14} (16).

The data in Tables 1 and 2 are in themselves insufficient to show that propionate is the specific precursor of the odd-numbered fatty acids. One explanation that could be offered for the results is that the even-numbered acids undergo turnover, while the odd-numbered acids are not broken down at all. Thus, after a sufficient time interval after injection, the even-numbered acids would have lost a good deal of their activity while the odd-numbered acids were still gaining in activity from the radioactive acetate derived from breakdown of the even-numbered acids. Explanations based on turnover rates could be checked by administration of labeled acetate, which is the major precursor of odd- and even-numbered fatty acids.

Table 3 shows the data for acetate, using rats killed after the same time interval. The figures are for cerebroside acids and should be compared with those in Table 2. The specific activities are rather similar for all the acids, with the even-numbered acids showing higher values than most of the adjacent odd-numbered acids. (The activities shown for the 19:0, 21:0, and 25h:0 acids are too high because of trailing from the earlier homologs, which amounted in test runs to 1–2%.) The results show that the propionate data cannot be accounted for on the basis of differences in turnover and that propionate must play the same specific role in the biosynthesis of the shorter and longer odd-numbered acids. The possibility that the odd-numbered acids are derived by chain-shortening (17, 18) of the adjacent even-numbered homologs seems to be ruled out.

The unexpected accumulation of the odd-numbered cerebroside acids that occurs with age (8) may be due to a lack of breakdown of the odd- (but not even-) numbered acids or to an increasing availability of

propionate in the cerebroside-synthesizing cells. While ingested propionate is burnt rapidly (5), the ingestion of odd-numbered acids does seem to increase the deposition of odd-numbered acids in at least part of the body (5, 19, 31). It may therefore be that propionate formed endogenously could in certain cells be utilized directly for formation of odd-numbered acids.

The finding of Fulco and Mead with rats injected with C^{14}-acetate (18) that lignoceric acid (24:0) had sixteen times the specific activity of cerebronic acid (24h:0) is in marked contrast to our results. The difference may be due to their use of younger rats, or to the use of rather impure cerebrosides, or to the different injection schedule. From their degradation data, they concluded that lignoceric and cerebronic acids are probably made by chain-lengthening of stearic acid. It may then be assumed that the odd-numbered acids (23:0, 23h:0, etc.) are made similarly by chain-lengthening of the 17:0 acid. In this connection, it is interesting to note the finding of very high amounts of 15:0 and 17:0 acids in spinal fluids (20, 30).

The Unsaturated Cerebroside Acids. The distribution of chain lengths in the unsaturated cerebroside acids is somewhat different from that found in the saturated acids (6, 7). Another point of dissimilarity is found in the distribution of C^{14} in the carbon chains of lignoceric and nervonic acids (18). It therefore seemed possible that the odd-numbered unsaturated acids differed in not being made from propionate. This point was tested by collecting the unsaturated acids from the cerebrosides of the propionate-injected rats. A 5-mg portion was cleaved at the double bond by ozonolysis following a method based on the procedure of Ackman et al. (21).

The methyl esters were dissolved in 0.6 ml of chloroform-methanol 1:2 in a 1-piece test tube-reflux condenser. A slow stream of ozonized oxygen was bubbled through the solution, which was shaken at 0° until absorption of ozone seemed complete (about 4 min). The solvents were removed under vacuum and 200 μl of 88% formic acid and 60 μl of 30% hydrogen peroxide were added to the residue. The mixture was heated 1 hr at 100° with cold water in the condenser section and a mixture of carrier acids was added (about 1 mg each of 8:0, 9:0, 10:0, 10d:0, 12d:0, and 14d:0 acids). The acids were transferred to a distilling flask with toluene and water and distilled under slight vacuum with periodic additions of portions of water. This procedure separated the mono- and dicarboxylic acids. The distillation was stopped when 25 ml of distillate had been collected.

The distillate and residue were prepared for counting by extracting with 3 × 10 ml of ether, washing the combined ether solutions with 6 × 10 ml of water (to remove the formic acid), and evaporating to dryness together with 100 μl of Primene (22). The Primene, a primary long-chain amine, forms toluene-soluble salts which can be counted by liquid scintillation. It was found that the monocarboxylic acids, which are derived from the ω-end of the unsaturated fatty acids, contained 680 cpm and the dicarboxylic acids contained 252 cpm. The recovery of activity in the two fractions was only 78%, but trial runs with methyl nervonate had shown that the ozonolysis is complete, that the dicarboxylic acid fraction is obtained in 100% yield, and that there is some loss in the monocarboxylic portion. By using this information to correct the observed activities, we found that 79% of the activity in the unsaturated acids is in the ω-end and that the specific activity per carbon atom of monocarboxylic acids is roughly six times that of the other carbon atoms. Much of the radioactivity in the unsaturated acids may be presumed to be in the 24:1 and 24h:1 acids, which are the major unsaturated acids, and these may be presumed to be labeled largely in the carboxyl end (18). The finding that most of the activity is actually in the ω-end of the fatty acid mixture is therefore interpreted to mean that the odd-numbered unsaturated acids derive their ω-end primarily from propionate. Then the odd-numbered saturated and unsaturated acids have a common origin in propionate.

Amounts of the Individual Fatty Acids. The data in Tables 1 to 3 offer some interesting points. The values for the amounts of the individual cerebroside acids correspond well with those found in earlier work (8). Comparison of the total *brain* hydroxy acids with the total *cerebroside* hydroxy acids (Table 1 *vs.* Table 2) shows that most of the hydroxy acids are to be found in the cerebrosides. The cerebroside sulfate fraction, discarded in cerebroside isolation, may contain the remaining hydroxy acids (23, 24). The main difference appears in the lack of the shorter acids in the cerebrosides (16h:0, 17h:0, and a branched acid) and the relatively large amount of 18h:0 in the noncerebroside fraction. We had previously noted the presence of 16h:0 and 18h:0 in crude cerebrosides (6), while relatively large amounts of 18h:0 acid seem to be present in spinal cord cerebrosides (25), sheep brain (26), human and chicken brain (27), and crude young rat brain cerebrosides (18). Whether the short hydroxy acids center in cerebroside sulfate or another lipid remains to be determined.

A similar comparison of the saturated normal acids of brain and cerebrosides shows a greater difference, in part because lignoceric acid occurs in appreciable quantities in sphingomyelin (28) as well as in cerebro-

side sulfate (24). Apparently, the very long saturated normal acids do not occur in the ester-linked form (29).

Comparison of the total saturated acids of brain shows that palmitic and stearic acids are by far the most common, but it is interesting to note that the hydroxy acids, 24h:0 and 22h:0, are the next most common. Examination of the odd-numbered acids reveals that 15:0 and 17:0 predominate over the longer acids in the normal series, but 23h:0 is the major acid in the hydroxy series.

We are indebted to Dr. Richard S. Kowalczyk for his kind gift of a large amount of labeled acetic acid.

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