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# Biosynthesis of nervonic acid and its homologues from carboxyl-labeled oleic acid*

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## SUMMARY

An emulsion of carboxyl-labeled potassium oleate was injected directly into the brains of a group of rats, and 24 hr later the rats were sacrificed. The brain sphingolipids were isolated, and from these, the individual normal unsaturated acids. The major acid, nervonic, was cleaved by ozonolysis and also by decarboxylation. Virtually no activity was found in the chain portion from the $\omega$-end, while 74% of the activity was found in the carboxyl group. From these and other data, it would appear that very little oleate entered the cells that make the sphingolipid acids and that much of it was degraded to acetate. The labeled acetate thus formed was used to lengthen shorter acids (endogenous 18:1, 20:1, and 22:1). Apparently, there are pools of metabolically active unsaturated acids of various chain lengths, which can be incorporated into the sphingolipids or lengthened.

Previous work (1, 2) and an accompanying paper (3) from this laboratory have shown that the very long saturated fatty acids of brain sphingolipids are made from the shorter acids by a 2-carbon chain-lengthening mechanism, as well as by a 1-carbon chain-*shortening* mechanism (4). As the result of a structural study of the isomers of the unsaturated sphingolipid acids (5), we concluded that the same two enzyme systems act on the unsaturated acids as well. Fulco and Mead (6) have suggested, on the basis of an experiment with labeled acetate, that nervonic acid (24:1) is formed by chain lengthening of oleic acid (18:1). This paper describes a test of this possibility in which labeled oleic acid was injected directly into the brains of rats and various sphingolipid unsaturated acids were isolated, counted, and degraded chemically.

## METHODS AND MATERIALS

Oleic acid-1-$C^{14}$ (5.5 mc/mmole)[1] was purified with a silica gel column to remove possible polar impurities (7). The acid was converted to the potassium salt, emulsified with a nonionic detergent, and injected intracranially into six 56-g rats (3). Each portion injected had a volume of 20 $\mu$l and contained 80 $\mu$g of oleic acid. The rats were killed after 24 hr, and the brain lipids were immediately extracted. The normal sphingolipid acids were isolated as the methyl esters as described before (3).

*Isolation of the Individual Unsaturated Esters.* It was expected that the unsaturated normal esters would contain an appreciable amount of radioactivity in the 18:1, which might trail disturbingly into the 24:1 fraction when GLC[2] was used for isolation. To eliminate this problem, we took advantage of the different mobilities shown by esters of differing chain lengths on a silver nitrate–silica gel column (5). Before chromatography, we added as carrier some 24:2 previously isolated from pig brain. The column effluents were analyzed by GLC with a flame ionization detector and DEGS[2] column. A nervonate-rich fraction was obtained with SkB–benzene 9:1, and an oleate-rich fraction with an 8:2 mixture of the same solvents.[2] The former fraction contained only a small

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[1] Radiopurity 99+%, from Applied Science Laboratories, Inc., State College, Pa.

[2] Abbreviations used: GLC, gas-liquid chromatography; TLC, thin-layer chromatography; SkB, Skellysolve B (a commercial hexane); SE-30, a silicone polymer; DEGS, a polymer made from diethylene glycol and succinic acid; EtOH, ethanol; PPO, 2,5-diphenyloxazole; POPOP, 1,4-di-2-(5-phenyloxazolyl)-benzene. In fatty acid abbreviations, the first number indicates the chain length; the second number, the number of double bonds; the superscripts, the positions of the double bonds.

amount of 18:1 while the latter contained only traces of the longer monoenes.

The nervonate-rich fraction and the dienes were separated into the individual esters by GLC with a SE-30[2] column.

*Ozonolysis of Methyl Nervonate.* About 1 mg of the isolated 24:1 was subjected to oxidative ozonolysis (5), and a small portion of the products was analyzed by GLC with a DEGS column to determine the relative amounts of the positional isomers. The remainder was mixed with the ozonolysis products from 3 mg of pig brain 24:1 as carrier, and the mono- and diesters were isolated by GLC with an SE-30 column. The GLC run was programmed between 50° and 290° during 94 min. The 7:0 and 9:0 esters were collected in a U-tube containing glass wool and were cooled with dry ice, while the longer esters were collected with the aid of Millipore filters (8).

*Recovery of Esters from Counting Solution.* All samples were counted by liquid scintillation, using toluene containing PPO and dimethyl-POPOP[2] as scintillators (8). In the case of 20:1 and 22:1, the entire samples were counted and had to be recovered for the decarboxylation analysis. The counting solutions were evaporated to dryness, the residues were taken up in 5 ml of SkB–benzene 7:3, and the sample was added to a column of 1.5 g silica gel packed in Skellysolve B. Elution with 30 ml of the 7:3 solvent yielded all of the methyl esters, leaving the scintillators on the column.

*Decarboxylation of the Individual Esters.* About 5 mg each of 20:1[11] and 22:1[13] (both furnished through the courtesy of the Lipid Program of the National Institutes of Health) was added to the recovered esters as carrier. A similar amount of 24:1 ester from pig brain was added to 1 mg of isolated radioactive 24:1. The esters were hydrogenated by a micromodification of the method of Brown and Brown (9), in which the catalyst and hydrogen are generated in situ. The reaction vessel consisted of a test tube, 13 mm o.d. and 60 mm long, with a capillary side arm bent to dip into a mercury well. To the vessel was added 200 μl of absolute EtOH[2], 20 μl of 0.2 M chloroplatinic acid in absolute EtOH, and a small Teflon-covered magnetic bar. The test tube was closed with a serum stopper, the side arm was lowered into the mercury slightly, and the inside air was replaced with nitrogen by blowing in the gas through a hypodermic needle inserted through the stopper. By means of microsyringes,[3] 100 μl of 1 M sodium borohydride in absolute EtOH–2 M aqueous NaOH 19:1, 30 μl of acetic acid, and the methyl ester (in 30 μl of absolute EtOH) were injected into the tube successively. An additional 100 μl of borohydride solution was added dropwise during the following 30 min. After an additional 15 min of stirring, the hydrogen was replaced with nitrogen and the platinum was removed by filtration. Ether was used to complete the transfer. After a wash with water, the filtrate was evaporated to dryness.

Analysis by GLC of the resultant 24:0 ester showed that the hydrogenation was complete and that ethanolysis (10) had not occurred. Incidentally, this hydrogenation supported the structural identification of the three positional isomers (5), for only one product was formed.

The hydrogenated esters were saponified and decarboxylated by the Schmidt reaction (1).

[3] Hamilton Company, Inc., Whittier, Calif.

## RESULTS

*Primary Fractionation Steps.* Of the injected dose of labeled oleate, only 0.4% of the $C^{14}$ was found in the sphingolipid fraction and 10% in the other lipids. Most of the activity in the former mixture appeared in the ester fraction. Of this fraction, 12,450 cpm were found in the hydroxy esters (saturated and unsaturated) and 32,910 cpm in the normal esters. The silica gel column separated the latter into a saturated fraction (10,030 cpm), another fraction that might be a saturated dimethyl acetal fraction (1,400 cpm), and unsaturated fractions. The diunsaturated ester added as carrier proved on GLC to contain negligible activity.

The ability of the silver nitrate–silica gel combination to isolate a fairly distinct fraction following the saturated esters is potentially useful for other studies. In our isolation procedure, it is likely that the brain plasmalogens yield aldehydes, which are converted at least in part to dimethyl acetals by the dimethoxypropane esterification step. This fraction yielded a single spot in TLC,[2] just below the normal esters.

*Distribution of $C^{14}$ in the Nervonate-Rich Fraction.* Table 1 shows the results of GLC separation of the

TABLE 1. AMOUNTS AND RADIOACTIVITIES OF FATTY ACIDS OF NERVONATE-RICH FRACTION

| Acid Isolated | Amount per Brain | Radioactivity | |
|---|---|---|---|
| | | Total | Specific |
| | *μmole* | *cpm** | *cpm/μmole* |
| 18:1 | 0.074 | 1,370 | 3,680 |
| 20:1 | 0.025 | 860 | 6,760 |
| 22:1 | 0.090 | 440 | 960 |
| 24:1 | 1.57 | 14,310 | 1,790 |
| 26:1 | 0.10 | 203 | 410 |

* Radioactivity actually found in the fraction isolated by GLC.

TABLE 2. RADIOACTIVITY IN OZONOLYSIS FRAGMENTS OF 24:1

| Acid Fragment | Radioactivity | |
|---|---|---|
| | Total | Relative Specific* |
| | *cpm* | |
| 7:0 | 0 | 0.00 |
| 9:0 | 18 | 0.08 |
| 15d:0 | 3,450 | 7.1 |
| 17d:0 | 123 | 3.5 |

* Total activity/carbon number/cm² area of GLC peak from DEGS column.

individual esters in the nervonate fraction. It can be seen that all the esters contained radioactivity and that the 24:1 fraction contained most of the activity. The next largest amount of activity was in the 18:1. Because of the relatively large amount of 24:1 in sphingolipids, the radioactive 24:1 synthesized during the 1-day "incubation" period was diluted considerably, as shown in the specific activity column of the table.

The purity of the isolated 24:1 was checked with GLC (DEGS column) and TLC. The former method showed only one peak, while the latter disclosed two faint, nonradioactive spots.

The gas chromatogram of the nervonate-rich fraction showed peaks corresponding to the odd-numbered acids, 19:1 to 25:1, but they were each contaminated by a peak immediately following the ester (on the SE-30 column). The double peaks contained relatively little activity.

The amount of activity in the 18:1 of the nervonate-rich fraction is somewhat more than in the oleate-rich fraction, which contained only 500 cpm. Thus it can be seen that there would have been no difficulty in GLC if both fractions had been pooled.

*Distribution of Activity in the Ozonolysis Fragments.* Table 2 lists the activities in the four fragments found. The "d" in the abbreviation indicates an $\alpha,\omega$-dicarboxylic acid, which is derived from the portion of the original molecule on the carboxyl side of the double bond. The 7:0 and 17d:0 are derived from the 24:1[17] isomer, and the other two acids are derived from the 24:1[15] isomer. We also found some 24:1[16] in pig brain sphingolipids (5), but none could be detected here.

Of interest is the finding that virtually no activity was present in the $\omega$-end of the nervonate. The more plentiful isomer, 24:1[15], had much more activity, although the specific activity was only doubled.

Thin-layer chromatography of the isolated 15d:0 showed only one spot, which contained all the activity applied to the plate.

*Decarboxylation of the Hydrogenated Acids.* Table 3 shows the results of decarboxylating the acids derived from 20:1, 22:1, and 24:1. In contrast to the analogous experiment with saturated acids (3), a large portion of the radioactivity in the samples was found in the carboxyl group. Recovery of activity in the $CO_2$ and the fatty amine resulting from the degradation was quite good.

TABLE 3. RADIOACTIVITY IN DECARBOXYLATION PRODUCTS OF MONOENOIC ACIDS

| Acid Degraded | Radioactivity | | | |
|---|---|---|---|---|
| | Before Degradation (A) | $CO_2$ from Carboxyl (B) | Amine from Rest of Chain | Ratio B/A |
| | *cpm* | *cpm* | *cpm* | % |
| 20:1 | 832 | 713 | 122 | 86 |
| 22:1 | 413 | 220 | 219 | 53 |
| 24:1 | 5,372 | 3,947 | 1,383 | 74 |

## DISCUSSION

Consideration of the results reported above leads us to suggest the following hypotheses. (1) The injected oleate was oxidized very rapidly to carboxyl-labeled acetate. (2) There are metabolically active pools of 16:1, 18:1, 20:1, 22:1, and 24:1 acids in the cells making sphingolipids. (3) The unsaturated acids in these pools can be lengthened by condensation with one or more acetate residues, or incorporated into sphingolipids.

It would appear that very little of the injected oleate entered the cells which make sphingolipids. The total activity found in the isolated 18:1 (Table 1) was unexpectedly low, especially when compared with the results when labeled saturated acids were injected (3). However, there is little 18:1 in the sphingolipids, so that the specific activity of the isolated 18:1 was fairly high; it may be that *some* labeled oleate was directly incorporated.

Rapid oxidation of injected oleate would be expected to give rise to highly labeled acetate-1-$C^{14}$. It must be this acetate that was used for chain lengthening, for a large fraction of the $C^{14}$ in the longer acids was in the carboxyl group (Table 3). This result, like the one mentioned above, contrasts strongly with the experiment with labeled 16:0 and 18:0, in which very little $C^{14}$ was found in the carboxyl group of the longer saturated acids.

If the radioactive acetate were used to lengthen endogenous oleate in a direct, multistep elongation, one would expect the nervonate to contain its radioactivity equally distributed among carbon atoms

number 1, 3, and 5. The carboxyl relative activity would then be 1/3 or 33%. Since 74% of the activity was found in the carboxyl atom, it would appear that dilution with nonradioactive 20:1 and 22:1 pools occurred during the elongation. A relatively large proportion of the nervonate must have been formed from moderately radioactive 22:1 and highly radioactive acetate.

The isolated 20:1 acid had even more of its $C^{14}$ in the carboxyl group, so the precursor oleate must have been largely preformed.

The isolated 22:1 acid had a lower specific activity than its assumed precursor, 20:1, from which it may be concluded that it is synthesized more slowly. Because of the slower synthesis, a greater proportion of the precursor acid had time to become radioactive. This explains the observation that only 53% of the 22:1 activity was in the carboxyl.

Further evidence for the considerable degradation of injected oleate comes from the fairly high activity found in the saturated acids in the sphingolipids. Presumably these acids are made from acetate rather than by direct hydrogenation, for the body as a whole hydrogenates oleate only very slightly (11, 12).

The finding that intracranially injected oleate was oxidized much faster than palmitate or stearate could mean that the difference in emulsifying agents introduced a difference in the relative rates of the lengthening and degradative processes. The unsaturated $C^{14}$ acid was emulsified with a detergent, while the saturated acids were dispersed with albumin.

When labeled oleate and stearate were compared in whole animals (fed the methyl esters), the former was found to undergo oxidation much faster (13). Moreover, when rats were fed linoleic acid-1-$C^{14}$, results similar to ours were obtained: the arachidonic acid (isolated as arachidic acid) proved to have most of its activity in the carboxyl group, and the tissue linoleic acid was found to have little activity (14). It is now well established that the *poly*enoic acids can be lengthened in the body (12).

The ozonolysis experiment with nervonate (Table 3) showed that some activity was present in the rarer $24:1^{17}$ isomer. Because of the location of the double bond, it may be inferred that this acid was made by chain elongation of palmitoleic acid (with labeled acetate).

The striking differences we found with labeled saturated and unsaturated acids are paralleled also in the study with labeled acetate by Fulco and Mead (6). They too found a somewhat higher carboxyl relative activity in the nervonate than in lignocerate and suggested the former is made from oleate. Another distinct difference, noted in our first study (15), is in the distribution of chain lengths of the cerebroside saturated and unsaturated acids.

Because of the small extent of conversion of the injected oleate to nervonate and the high fraction of the $C^{14}$ in the nervonate carboxyl group, we examined the possibility that this was the result of trace contamination of the labeled oleate with nervonate-1-$C^{14}$. The original purified oleic acid-1-$C^{14}$ (350,000 cpm) was mixed with nonradioactive carrier acids, 16:0, 18:1, 20:1, 22:1, and 24:1, and esterified. The esters were fractionated as before with silica gel and GLC, and the isolated nervonate was found to contain 30 cpm (0.009% of the total). This activity was the result of tailing in the gas chromatograph, so contamination of the oleate with nervonate must have been negligible. The nervonate isolated from the rats contained about 0.11% of the injected dose.

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