METABOLISM OF ESSENTIAL FATTY ACIDS

INCORPORATION OF ACETATE INTO ARACHIDONIC ACID*

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It has been observed repeatedly (1–5) that the feeding of esters of linoleic acid to animals results in an increase in the arachidonic acid content of their tissues. Proof is lacking, however, that these observations demonstrate the direct conversion of the diene to the tetraene acid; in fact, obscurity typifies present knowledge of the metabolism of linoleic acid and the mechanism of action of the essential fatty acids.

A recent total synthesis (6) and a method of decarboxylation and reconstitution (7) of linoleic acid developed in these laboratories have made possible the tracing of this compound and the products of its metabolism in the animal body. A necessary preliminary project concerned the incorporation of labeled acetate into the polyunsaturated acids. This information was essential for several reasons: first, it was important to show again (8–10) that, under the conditions of the experiment, linoleic acid itself is not synthesized in the animal body; second, it was of interest to test the obvious hypothesis that arachidonic acid is derived from a C_{18} acid by the addition of a 2-carbon fragment; and finally, it was desirable to have available data on the amount of incorporation to be expected in later experiments in which labeled linoleic acid may be partially oxidized to 2-carbon fragments.

EXPERIMENTAL

Treatment of Animals—Seventeen weanling rats were given three daily 0.5 ml. intraperitoneal injections each of a solution containing 0.5 mM of sodium 1-C^{14}-acetate per ml., with an activity of 100 μc. per mM. On the 4th day the animals were sacrificed, and the livers, kidneys, hearts, and abdominal fat deposits were pooled and immediately frozen.

Isolation of Fatty Acid Fractions—The pooled organs were lyophilized, and the dry residue (16.5 gm.) extracted in a Waring blendor with four 150 ml. portions of absolute ethanol. The residue from the alcohol extraction was extracted with ether for 6 hours in a Soxhlet apparatus. The

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combined solutions were concentrated to dryness at reduced pressure and dried *in vacuo*. The residue was made up to 200.0 ml. with petroleum ether (boiling range 60–70°) and duplicate aliquots were evaporated for counting.[1]

The dried residue remaining after evaporation of the bulk of the petroleum ether solution (3.56 gm.) was saponified with 30 ml. of 4 per cent ethanolic KOH by refluxing for 15 minutes and letting the mixture stand overnight at room temperature. The non-saponifiable fraction (0.31 gm.) was isolated for counting. The fatty acid fraction (2.04 gm.) was also counted.

The fatty acids were separated into a saturated and an unsaturated fraction by lead salt fractionation (12); carrier stearic acid was used to "wash out" the active saturated acids from the unsaturated fraction. Approximately 0.9 gm. of saturated fatty acids (including 90.2 mg. of added carrier) with an activity of 36.61 counts per second per mg. (corrected for carrier) and 1.14 gm. of unsaturated acids with an activity of 11.87 counts were obtained (see Table I).

Bromination of Unsaturated Fatty Acids—The total unsaturated fatty acid fraction was brominated at 0° in ethyl ether solution (13). After removal of the insoluble polybromides by centrifugation, the soluble bromides were freed of solvent and the residue was treated with petroleum ether (boiling range 60–70°) to dissolve the dibromide fraction, the crude tetrabromide fraction remaining undissolved. Following preliminary purification, 38.8 mg. of the tetrabromide fraction (m.p. 106–122°) were diluted with 40.9 mg. of inactive tetrabromostearic acid (m.p. 114.7–115.1°), and the mixture was recrystallized eight times from ethylene chloride, each crystallization being followed by a wash with 0.5 ml. of solvent at −10°. The activity of the fraction was assayed after each of the last four crystallizations and had decreased almost to zero after the eighth, as shown in Table I. The final crystallization gave 8.1 mg. of tetrabromostearic acid, m.p. 113–113.5°, with a specific activity of 0.085 counts.

The ether-insoluble polybromides weighed 0.516 gm. and had a specific activity of 3.36 counts. After washing with warm benzene to remove lower bromides, the residual insoluble material, usually termed "arachidonic octabromide," had an activity of 2.91 counts. Treatment of this material with hot tetrachloroethane resulted in soluble and insoluble fractions, both of which had activities of 2.3 counts. (The benzene-soluble

[1] Counting was performed with a Nuclear Instrument and Chemical Corporation flow counter or a thin end window counter. Samples were prepared according to standard procedures. Fatty acids were plated directly on 1 inch aluminum planchets with or without lens paper (11). $BaCO_3$ was plated on copper disks (3.9973 sq. cm.). All counts were corrected for self-absorption by curves determined for either $BaCO_3$ or fatty acids.

fraction (50.6 mg.) had an activity of 3.29 counts and may be related to the active contaminant of the tetrabromostearic acid fraction.)

Debromination and Reduction of "Octabromide" Fraction—The benzene-insoluble polybromide fraction (234 mg.) was subjected to debromination

TABLE I

Isotope Concentration in Various Fractions

Fraction	Specific activity	
	Counts per sec. per mg.	Counts per sec. per mg. carbon
Total fat	20.32	
" fatty acids	17.97	
Non-saponifiables	29.29	
Saturated fatty acids	36.61*	
Unsaturated fatty acids	11.87	
Tetrabromostearic acid (crude)	3.59	
" " + carrier	1.69†	
" " 5th crystallization	0.328	
" " 6th "	0.204	
" " 7th "	0.126	
" " 8th "	0.085	
Ether-insoluble polybromides	3.36	
Benzene-insoluble polybromides	2.91	
Benzene-soluble polybromides	3.29	
Tetrachloroethane-insoluble polybromides	2.28	
Tetrachloroethane-soluble "	2.33	
"Arachidic acid" (from debromination)	7.11	
" " 6th crystallization	11.73 (0.787)‡	
" " + carrier	6.12 (0.411)†‡	
Arachidic acid from chromatogram	4.82 (0.291)‡	6.27 (0.379)‡
" " + carrier	2.40 (0.144)†‡	3.12 (0.187)†‡
$BaCO_3$, carboxyl carbon of arachidic acid	(0.201)‡	(3.53)‡
Nonadecylamine (crude)	(0.013)‡	(0.016)‡

* Corrected for carrier added during the lead salt fractionation.

† Value calculated from the activity of the original sample and the weights of sample and added carrier.

‡ The values enclosed in parentheses were obtained with a thin (1.9 mg. per cm.) end window tube (see foot-note 5).

with zinc dust (14) in *n*-propyl alcohol suspension at 100° for 11 hours with intermittent addition of 0.1 to 0.2 ml. portions of 12 N HCl. The reaction products were isolated by evaporation of the solvent and were hydrogenated in the presence of 4 per cent palladium on barium sulfate in methanol solution at atmospheric pressure to saturate the olefinic groups and cause hydrogenolysis of any remaining carbon-bromine bonds. The

products of the hydrogenation were freed of catalyst and saponified with alcoholic potassium hydroxide by standing overnight at room temperature under nitrogen, followed by refluxing for 5 hours. The acid was regenerated and rehydrogenated in methanol solution over palladium on barium sulfate. From the methanol solution there were obtained by evaporation 72.5 mg. of saturated, bromine-free acids with an activity of 7.11 counts, melting at 58.5–66.5° (arachidic acid is reported to solidify at 74.35° (15)). Repeated recrystallization raised the melting point to a maximum of 68.5–69.2°; this melting point and x-ray diffraction data[2] indicated that the material was probably a mixture of arachidic and behenic acids. Attempts to resolve this mixture by recrystallization were unsuccessful.

Chromatographic Purification of Arachidic Acid—A modification[3] of the method of Boldingh (16), in which partition chromatography on a rubber column is employed, was used to separate the arachidic acid from higher homologues. Mealorub vulcanized rubber powder (purchased from the Andresen Corporation, Chicago) was extracted with acetone in a Soxhlet apparatus for 24 hours. The rubber was ground in a mortar, and the portion passing through a 20 mesh sieve was stored under methanol. The apparatus consisted of a 750 ml. jacketed, temperature-controlled separatory funnel reservoir connected to a 55 cm. jacketed, temperature-controlled column of 18 mm. inner diameter. An adjustable flow stop-cock led into a fraction cutter similar in design to those commonly used in vacuum distillation, which was flushed continuously with nitrogen and led, via an adapter, into the open portion of a titration device similar to that described by Howard and Martin (17). α-Naphtholphthalein was used as the indicator.

To constitute a column, the rubber powder (18 gm.) was washed thoroughly with acetone and swelled by adding 105 ml. of water in seven portions, with shaking, to the rubber suspended in a mixture of 215 ml. of acetone and 30.7 ml. of Skellysolve E (boiling range 100–140°). The rubber swelled to about 3 to 4 times its original volume and filled the column to a height of 360 mm. when packed by the force of maximal flow rate. It was finally washed with Solvent Mixture A-78.[4]

[2] Schuette and Vogel (15) give the following data: moles per cent of C_{20}, 81.96, 84.47; C_{22}, 18.04, 15.35; solidification point, 68.65°, 69.40°. Mr. G. V. Alexander of the Spectroscopy Section of this Project compared the x-ray diffraction pattern of this acid with that of authentic arachidic acid, for which the long spacings ($c \sin \beta$) were 44.15 A and 48.86 A, respectively, for the α and β forms. The active acid had a long spacing of 49.28 A, which is compatible with that expected for a mixture of 81 per cent arachidic acid (C_{20}) and 19 per cent behenic acid (C_{22}).

[3] The authors are grateful to Dr. J. Boldingh for making available unpublished modifications of his original procedure, which proved more effective in application to the higher fatty acids.

[4] Solvents thus indicated were composed of water-acetone mixtures containing the

The active fatty acid mixture (32.7 mg.) was diluted with 30.1 mg. of inactive arachidic acid and dissolved in 3 ml. of 4:1 acetone-Skellysolve E, placed on the column, and allowed to move into the rubber. 10 ml. of Solvent Mixture A-78, the initial developing solvent, were used to move the sample farther into the column. Any acid which had precipitated was redissolved by local warming with a heat lamp. After equilibration overnight, the solvent mixture was allowed to flow through the column at 1 drop per second at 25.0° ± 0.5°. Development of the chromatogram was followed by titrating successive 10 ml. portions with 0.01 N NaOH, and

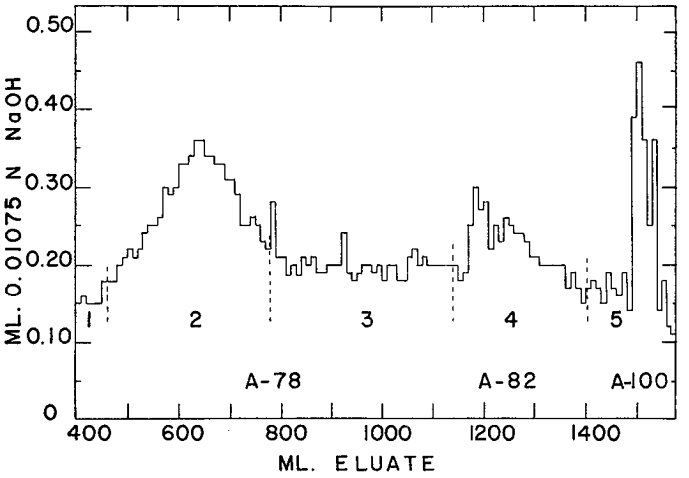

FIG. 1. Chromatographic separation of arachidic and behenic acids on rubber. The fractions are numbered and demarcated by dash lines. The chromatogram was interrupted overnight at the following eluate volumes: 490 ml., 920 ml., 1240 ml.

the eluate was divided into five main fractions according to the acid content maxima thus determined (see Fig. 1).

Fraction 2, containing the bulk of the arachidic acid, was concentrated, acidified, and extracted with benzene. The benzene solution was washed with water and evaporated to dryness at reduced pressure. After three crystallizations from acetonitrile, the residue yielded 18.6 mg. of arachidic acid, m.p. 73.0–73.8° and specific activity 0.291 counts.[5]

volume per cent acetone indicated by the number and saturated with Skellysolve E; Solvent Mixture A-100 (pure acetone) was not saturated with Skellysolve; it extracts the hydrocarbon solvent from the rubber and collapses the column.

[5] Possibly because of vapor pressure, counting rates obtained from a given sample of directly plated lipide material in flow and end window counters are not in the same ratio as is observed with plated barium carbonate (12 for our counters). Therefore the important measurements of the final degradation products of arachidonic acid were made on both counters, the end window data being used in the final comparison as in this case.

Decarboxylation of Arachidic Acid—The Schmidt degradation (18, 19) was used in preference to the more commonly used Borodin degradation, since in our hands the former reaction proved simpler and gave consistently better yields.

The active arachidic acid (diluted with inactive material to a total of 37.0 mg. or 0.118 mM) was dissolved with warming in 0.6 ml. of 100 per cent H_2SO_4 (3 parts of concentrated H_2SO_4 to 1 part of 20 per cent fuming H_2SO_4) in the reaction flask, and, after cooling to 0°, 14.7 mg. (0.226 mM) of sodium azide were added. The reaction vessel was connected to the system (which had been flushed with nitrogen), 10 ml. of CO_2-free 1.2 N NaOH were placed in the second trap (19), and the reaction flask was warmed slowly with a heat lamp while being stirred with a magnetic stirrer, reaching 65° in 30 minutes. The system was maintained at 65° for another 30 minutes, whereupon it was swept with nitrogen at 130 ml. per minute for 20 minutes. The carbon dioxide absorbed in the alkali trap was precipitated as $BaCO_3$ (18 mg.), which was found to have an activity of 3.53 counts per second per mg. of carbon (corrected for a 2.2 mg. blank). Barium carbonate having an activity of 3.78 counts per second per mg. of carbon would be expected on the basis of all the activity of the arachidic acid sample being located in its carboxyl group. The alkyl residue of arachidic acid was isolated in crude form from the reaction mixture as nonadecylamine and was found to have an activity of 0.016 counts per second per mg. of carbon.

DISCUSSION

From the data presented above, it appears that no significant synthesis of linoleic acid occurs in weanling rats under the conditions employed in this experiment. This confirms the work of Bernhard and Schoenheimer (8) with deuterium.

Arachidonic acid is evidently formed from an exogenous C_{18} precursor (presumably derived from linoleic acid) by the addition of a C_2 fragment derived from acetate. In the present instance, in which carboxyl-labeled acetate was injected, the label appeared almost exclusively in the carboxyl group of the arachidonic acid. In future studies, the nature and derivation of the C_{18} portion of the molecule will be considered.

One further point of interest remains to be considered. It is apparent from our results that the so called "octabromide" fraction derived by treatment of the ether-insoluble polybromides with hot benzene is certainly not pure arachidonic acid octabromide and that a significant amount of at least one C_{22} polyunsaturated acid derivative is present in the fraction. The nature of this acid will be the subject of future investigation in this laboratory.

SUMMARY

By the use of carboxyl-labeled acetate, it has been shown that acetic acid is not incorporated into the linoleic acid of the lipides of weanling rats to an appreciable extent. Arachidonic acid, on the other hand, is derived from acetate and (presumably) an exogenous C_{18} precursor.

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