METABOLISM OF ESSENTIAL FATTY ACIDS

IV. INCORPORATION OF LINOLEATE INTO ARACHIDONIC ACID* 

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It has recently been demonstrated in this laboratory (1) that arachidonic acid is formed in the body from acetate and a C₁₈ precursor and that, under the conditions of the experiment, linoleic acid is not synthesized from acetate. Spectrophotometric evidence (2, 3) has indicated that administration of linoleate to animals results in an increase in tetrane of the arachidonate type (as measured by alkali isomerization). The experiments reported in this paper were designed to test the hypothesis that the exogenous C₁₈ precursor is linoleic acid and that the linoleate molecule is incorporated in its entirety into arachidonate.

EXPERIMENTAL

Treatment of Animals—Four 200 gm. rats were fed a total of 547 mg. (0.5 mc.) of methyl linoleate-1-C¹⁴ (4). After 4 hours the animals were sacrificed, and the liver, kidney, heart, spleen, and abdominal fat were pooled and frozen at once.

Isolation of higher unsaturated fatty acids as polybromides was carried out essentially as described earlier (1), except that the unsaturated fatty acids were concentrated by the low temperature crystallization (5) instead of the lead-salt technique. Addition of bromine to an ether solution of the unsaturated fatty acid concentrate (6) precipitated the crude octabromide fraction. Crude tetrabromide was precipitated from the ethereal mother liquors (concentrated to 20 ml.) by adding 100 ml. of 60–70° petroleum ether.

Debromination and Reduction of Polybromides—The crude octabromide fraction was treated with zinc dust in acidified n-propyl alcohol as described previously (1, 7). Solids were centrifuged from the reaction mixture, a suspension of 130 mg. of prereduced Adams' catalyst in 35 ml. of n-propyl alcohol was added, and the mixture was hydrogenated at room temperature and atmospheric pressure. When the rate of hydrogen up-

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take slowed down, more (200 mg.) catalyst in suspension was added, and hydrogenation continued until no more hydrogen was taken up. After removal of catalyst, the mixture was saponified to liberate the free fatty acids from the propyl esters formed during the zinc debromination; the crude fatty acids were then chromatographed on silicic acid (see Fig. 1). The semisolid materials from Fractions 12 to 23 of the chromatogram were combined and again hydrogenated over Adams' catalyst in tetrahydrofuran to give 169 mg. of solid saturated fatty acids.

Isolation of Arachidic Acid—As shown earlier (1), saturated fatty acids of chain lengths both greater and less than C\textsubscript{20} are obtained (in addition to

![Figure 1. Chromatography of fatty acids on silicic acid. A 2.8 × 13 cm. column containing 52 gm. of silicic acid was dry packed and activated by prewashing successively with three column volumes each of acetone, ether, and pentane. Fractions of one column volume (48 ml.) were collected. The eluting solvents were pentane (P), ethyl ether in pentane (numbers indicate per cent ether, volume per volume), and acetone (A).](https://www.jbc.org/)

the desired arachidic acid) on reduction of the crude ether-insoluble octabromide fraction. In the present instance, arachidic acid was separated from homologous impurities by the reversed phase partition chromatographic method first described by Howard and Martin (8) and later extended to include fatty acids of C\textsubscript{24} chain length by Silk and Hahn (9); the siliconized Celite used as the stationary phase support was prepared as described by the latter (9). Prior to use, 100 gm. of the siliconized Celite were stirred into 78.8 gm. of mineral oil (Mefford Chemical Corporation, No. 15, viscosity 345 to 55 Saybolt, density 0.875) to give a coarse, homogeneous powder which was suspended in Solvent A-67\textsuperscript{1} and homogenized batchwise in a top drive homogenizer.

\textsuperscript{1} The solvents thus indicated were composed of mineral oil-saturated acetone-water mixtures containing the volume per cent acetone indicated by the number.
The column was a jacketed tube, 3.36 (inner diameter) by 50 cm., constricted at the bottom, fitted with an adjustable flow throttling stop-cock, and temperature-controlled at 35°.

Special precautions were taken during packing to avoid inclusion of air bubbles. The homogenized slurry was heated to about 50° and transferred to the column (half filled with Solvent A-67) by means of a large dropping pipette. (The slight suction used to draw the hot slurry into the pipette was sufficient to remove the air bubbles sticking to the Celite particles; the slurry was then expelled with the pipette tip below the surface of the liquid in the column.) By suitable intermittent draining of solvent from the bottom of the column, packings of convenient flow rate were obtained. Additional Celite slurry was added and compacted in this manner until a column of packed Celite about 31 cm. in height was obtained; 250 ml. of Solvent A-70 were then passed through the column.

Essentially as described elsewhere (9), the fatty acid mixture was incorporated into a small amount of the supported stationary phase, transferred to the top of the column as a slurry in Solvent A-50, compacted by draining, and constrained by circles of filter paper. Eluate fractions of 25 ml. were collected with the aid of a fraction cutter and titrated under nitrogen in a cell of the type described by Howard and Martin (8). Fractions 1 through 73 were eluted with Solvent A-70 and Fractions 74 through 99 with Solvent A-75; finally, use of Solvent A-97 collapsed the column by displacing most of the mineral oil. The development (at 10 to 12 minutes per fraction) was followed by titration with 0.025 N alcoholic KOH. The eluate was divided into principal fractions (A through H) according to peaks delineated by the titration values (Fig. 2).

Fraction E, containing the purified arachidic acid, was acidified with sulfuric acid, concentrated to 250 ml. at reduced pressure, and extracted thoroughly with 60–70° petroleum ether. After removal of the solvent from the extract, there remained 93 mg. of residue, which was chromatographed on silicic acid (see Table I) to remove the indicator (strongly adsorbed) and mineral oil (very weakly adsorbed); 52.3 mg. of pure arachidic acid were obtained. Fatty acids from Fractions D (stearic acid, 23 mg.) and G (behenic acid, 33 mg.) were isolated similarly.

Degradation of Arachidic Acid—The Schmidt degradation used earlier (1) was not employed in the present instance, since several 1-carbon degradations were necessary, and the intermediate long chain aliphatic amines produced (in addition to CO₂) could not be converted conveniently to the corresponding carboxylic acids necessary for recycling.

A sequence (10) involving bromination of the saturated fatty acid, hydrolysis of the resulting α-bromo acid to the corresponding α-hydroxy acid, and oxidation of this derivative to carbon dioxide and the next lower satu-
rated fatty acid was investigated. Although a variety of oxidative methods and conditions were tried, none gave products entirely free of lower homologues.

![Graph showing reversed phase chromatography of fatty acids obtained by reduction of ether-insoluble polybromides. The dotted lines indicate blank titers.](http://www.jbc.org)

**Fig. 2.** Reversed phase chromatography of fatty acids obtained by reduction of ether-insoluble polybromides. The dotted lines indicate blank titers.

### Table I

**Chromatographic Purification of Arachidic Acid**

<table>
<thead>
<tr>
<th>Eluent*</th>
<th>Column volumes</th>
<th>Material eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>2</td>
<td>Mineral oil</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>None</td>
</tr>
<tr>
<td>10</td>
<td>6</td>
<td>Fatty acid</td>
</tr>
<tr>
<td>15</td>
<td>2</td>
<td>None</td>
</tr>
<tr>
<td>25</td>
<td>4</td>
<td>&quot;</td>
</tr>
<tr>
<td>50</td>
<td>2</td>
<td>&quot;</td>
</tr>
<tr>
<td>100</td>
<td>2</td>
<td>&quot;</td>
</tr>
</tbody>
</table>

* Per cent (volume per volume) ether in n-pentane.

Because of these objectionable features of other approaches to the stepwise degradation of saturated fatty acids, the procedure of Dauben, Hoerger, and Petersen (11) has been employed in the present study (see Fig. 3). This method involves no oxidation, is readily amenable to recycling, and conveniently yields the removed carbon atom in the form of benzoic acid. The possibility of undegraded fatty acid contaminating the lower homo-
logues was excluded by purification of the isolated intermediates by chromatography on silicic acid.

The purified arachidic acid was diluted to 249.8 mg. (0.801 mM) with carrier (Sapon Laboratories, Inc., b.p. 203–205° at 1 mm., m.p. 73.6–74.8° after recrystallization from petroleum ether) and converted (11) to arachidophenone. The crude product was extracted from the reaction mixture with cyclohexane, and the extract was concentrated, diluted to one column volume with 5 per cent ether in pentane, and chromatographed on silicic acid (see Table II). The purified ketone was nitrosated and rearranged, giving benzoic acid and nonadecanenitrile. After purification by sublimation, the benzoic acid weighed 71 mg. (72.8 per cent based on arachidic acid). The nonadecanoic acid obtained by hydrolysis of the nitrile was purified by chromatography on silicic acid (cf. Fig. 1); yield 162 mg. (67.9 per cent).

Repetition of this series of reactions (starting with nonadecanoic acid) gave stearic acid and benzoic acid containing the 2nd carbon of the original arachidic acid as its carboxy carbon atom and finally margaric acid and benzoic acid containing the 3rd carbon of the original arachidic acid.

**Counting Methods**—The counting data were obtained on a Tracerlab

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**Figure 3.** Dauben degradation reaction sequence

**Table II**

<table>
<thead>
<tr>
<th>Fraction Nos.</th>
<th>Eluent (per cent ether in pentane)</th>
<th>Weight (mg)</th>
<th>Material eluted</th>
</tr>
</thead>
<tbody>
<tr>
<td>1- 6</td>
<td>5</td>
<td>276</td>
<td>Arachidophenone</td>
</tr>
<tr>
<td>7-14</td>
<td>6- 8</td>
<td>20</td>
<td>Arachidic acid</td>
</tr>
<tr>
<td>15-17</td>
<td>12-100</td>
<td>6</td>
<td>Tails</td>
</tr>
</tbody>
</table>
CE-1 liquid scintillation counter. The scintillator solution contained 3.0 gm. of 2,5-diphenyloxazole and 0.050 gm. of 2-(1-naphthyl)-5-phenyloxazole ("scintillation grade," Arapahoe Chemicals, Inc., Boulder, Colorado) in 1 liter of toluene (reagent). The samples were all soluble in this medium. Benzoic acid was weighed and washed into the sample bottles. Fatty acids were transferred into the scintillation solution by dipping a thin glass rod into the melt, dissolving the sample in the solution directly, and weighing the sample by difference.

### Table III

<table>
<thead>
<tr>
<th>Isotope Concentration in Various Fractions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fraction</td>
</tr>
<tr>
<td>------------------------------------------</td>
</tr>
<tr>
<td>Arachidic acid (C₁₈) (diluted 1:5)</td>
</tr>
<tr>
<td>Benzoic acid (carbon atom 1)</td>
</tr>
<tr>
<td>&quot; &quot; (&quot; &quot; 2)</td>
</tr>
<tr>
<td>&quot; &quot; (&quot; &quot; 3)</td>
</tr>
<tr>
<td>Margaric acid (C₁₇)</td>
</tr>
<tr>
<td>Tetrabromostearic acid</td>
</tr>
<tr>
<td>Behenic acid (C₁₉)</td>
</tr>
</tbody>
</table>

* Corrected to 100 per cent yields.
† Circumstances under which the activity of this substance was determined limit its accuracy to ±15 per cent; accuracy of the rest of the determinations is estimated to be ±4 per cent. Factors affecting measurements obtained with the liquid scintillation counter have been described recently (14).

**RESULTS AND DISCUSSION**

From the activities of the various fractions (Table III) can be calculated the percentage distribution of C¹⁴ in the different portions of the arachidic acid molecule.

\[
\text{% of total activity} = \frac{3}{2} \times \frac{2}{1} \times 74.7
\]

The activity found in the benzoic acid representing carbon atom 3 and the complete lack of activity in the C₁₇ residue provide convincing evidence that the 18-carbon chain of linoleate is incorporated intact into arachidonic acid. Only linoleic acid and its immediate C₁₈ derivatives would present such a distribution of activity. All other fatty acids available for reaction would contain molecules labeled throughout the chain, having been formed from acetate derived in part from the first 2 carbons of the labeled linoleic acid. Ample evidence is available, indicating that these carbon atoms are readily available for biosynthetic reactions. For example, a recent report from this laboratory (12) revealed that, after the
feeding of carboxy-labeled linoleate to mice, label appeared rapidly in the cholesterol isolated from these animals. Furthermore, the high activity in carbon 1 (the carboxy carbon atom of arachidonic acid) attests the fact that the acetate pool available for such reactions contains considerable activity, since it was shown previously (1) that acetate gives rise to the first 2 carbon atoms of arachidonate.

This high specific activity of the carboxy carbon of arachidonic acid derived from carboxy-labeled linoleate is not surprising in view of earlier observations on the activity of CO₂ eliminated following ingestion of methyl linoleate-1-C¹⁴ (12). These experiments demonstrated that the amount of ingested linoleate oxidatively destroyed (presumably via acetate to CO₂) is large compared with that which manages to become incorporated intactly into lipides, the fatty acids of which are apparently not readily accessible to the β oxidation enzyme system; the remarkable stability of this stored linoleate toward oxidative destruction may be deduced from the protracted period required to deplete essential fatty acids stored in tissues of animals maintained on a diet deficient in these substances. The present observations thus indicate that arachidonate is elaborated from such oxidation-resistant linoleate stores, inefficiently augmented by ingested linoleate, and an acetate pool which has rapidly attained a high specific activity as a consequence of the β oxidative catabolism of the bulk of the ingested labeled linoleate. The high activity of the behenic acid is presumably due to the incorporation into this molecule of two active acetates in reactions similar to that bringing about the conversion of the linoleate to arachidonate.

Under the conditions employed, the efficiency of incorporation of linoleate into arachidonate is relatively low, presumably because of the rapid oxidative catabolism of linoleate; the low activity of the petroleum ether-insoluble tetrabromide fraction derived from the residual pool of intact linoleate (about equal to that of the linoleate moiety of arachidonic acid) is of interest in this connection.

Further application of techniques developed in these studies for the elucidation of the nature of the intermediates involved in the conversion of linoleic acid to arachidonic acid in vivo (13) is in progress.

SUMMARY

Arachidonic acid from rats fed methyl linoleate-1-C¹⁴ was isolated as its octabromide, reduced to arachidic acid, and degraded by the non-oxidative procedure of Dauben, Hoerger, and Petersen. The distribution of the label indicates that arachidonate is synthesized in the rat by the condensation of linoleate (or one of its immediate C₁₈ derivatives) with acetate.
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BIBLIOGRAPHY

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