Metabolism of Essential Fatty Acids

VIII. ORIGIN OF 5,8,11-ECOSATRIENOIC ACID IN THE FAT-DEFICIENT RAT*

ARMAND J. FULCO AND JAMES F. MEAD

From the Department and Laboratories of Nuclear Medicine and Radiation Biology, School of Medicine, University of California, Los Angeles, California

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The fat deficiency disease in the rat (1), dog (2), and chick (3) is characterized in part by sharply increased concentrations of one or more trienoic acids in the tissues, as indicated by alkali isomerization analyses. Indeed, the increase in conjugatable triene in the mitochondrial fatty acids may be the first noticeable symptom of the onset of fat deficiency (4). The major triene component of the fat deficient rat has been characterized as 5,8,11-eicosatrienoic acid (5). Since the increase in this triene during fat deficiency disease has been found to parallel roughly a decrease in arachidonic (5,8,11,14-eicosatetraenoic) acid, the suggestion has been made that triene was formed by hydrogenation of the double bond in the 14 position of arachidonic acid (6). However, the work to date on the transformations of unsaturated fatty acids in animal tissues would seem to argue against this hypothesis. A more likely possibility is that 5,8,11-eicosatrienoic acid is actually a dehydrogenation product of oleic acid as has been suggested by several workers (7,8). A transformation of this type, involving chain lengthening by two carbons and the introduction of two methylene-interrupted double bonds, is exemplified by the conversion of linoleic to arachidonic acid in the animal body (9).

The experiments reported in this paper were designed to test the hypothesis that oleic acid, in an analogous series of transformations, is converted to 5,8,11-eicosatrienoic acid in the fat-deficient rat.

EXPERIMENTAL

Treatment of Animals.—Nineteen male albino rats, which had been maintained on a fat-free diet (10) from weaning until they reached a growth plateau (17 weeks) were given intraperitoneal injection of 0.2 mc. (0.07 mM) each of sodium-1-C14-acetate in diethylene glycol and adipic acid, on a firebrick support. The columns were of stainless steel, 1/2 inch inner diameter and 5 feet in length, and packed with LAC 446, a polyester of diethylene glycol and adipic acid, on a firebrick support. The carrier gas was helium and the operating temperatures ranged from 140 to 225°, depending on the sample. For quantitative determination of the composition of a mixture, the peaks obtained on a chromatogram were traced, cut out, and weighed. It was found that, with the exception of methyl arachidonate, the area under any peak was directly proportional to the weight of the methyl ester represented by that peak. Methyl arachidonate (Hormel) gave a peak size 20 per cent too small in area when compared with those obtained from known acids.

1 Gas chromatographic analyses were routinely used to determine the composition of various mixtures of mono- and dicarboxylic acids (both saturated and unsaturated) or dicarboxylic acids. The methyl esters were prepared from the acids with diazomethane in ether at 0° and analyzed on an Aerograph Master A-100 gas chromatographic instrument (Wilkens Instrument and Research, Inc.). The columns were of stainless steel, 1/4-inch inner diameter and 6 feet in length, and packed with LAC 446, a polyester of diethylene glycol and adipic acid, on a firebrick support. The carrier gas was helium and the operating temperatures ranged from 140 to 225°, depending on the sample. For quantitative determination of the composition of a mixture, the peaks obtained on a chromatogram were traced, cut out, and weighed. It was found that, with the exception of methyl arachidonate, the area under any peak was directly proportional to the weight of the methyl ester represented by that peak. Methyl arachidonate (Hormel) gave a peak size 20 per cent too small in area when compared to the other components in a known mixture. This may have been due to the presence of nonvolatile oxidation products in the samples of arachidonate used. Table I shows the results of a typical analysis obtained by this method. With unknown mixtures, chromatograph peaks were first identified by comparison with those obtained from known acids.
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Fig. 1. Gas-liquid chromatogram of oleic concentrate methyl esters. 1, laurate; 2, myristate; 3, palmitate and palmitoleate; 4, oleate.

Table 1
Analysis of standard fatty acid mixture by two chromatographic methods

<table>
<thead>
<tr>
<th>Acid</th>
<th>Mole per cent in mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Gas-liquid</td>
</tr>
<tr>
<td>Myristic</td>
<td>25.6</td>
</tr>
<tr>
<td>Palmitic</td>
<td>26.4</td>
</tr>
<tr>
<td>Stearic</td>
<td>24.4</td>
</tr>
<tr>
<td>Arachidic</td>
<td>23.6</td>
</tr>
</tbody>
</table>

Fig. 2. Gas-liquid chromatograms of the methyl esters of (A) polyunsaturated concentrate and (B) C20 polyunsaturated concentrate obtained by gas-liquid fractionation of the above. Arrows indicate where fraction was cut. 1, C16 monoenoic; 2, C16 monoenoic; 3, C18 dienoic; 4, C18 trienoic; 5, C18 dienoic; 6, C18 trienoic; 7, C20 tetranoic.

Fig. 3. Reversed-phase chromatogram of the C20 polyunsaturated fatty acids. A, tetraenoic acid peak and B, trienoic acid peak.

Permanganate Oxidation of Oleic and Eicosatrienoic Acids.—Since the oleic acid obtained by acetone and ether crystallizations contained chiefly saturated fatty acids as contaminants, it was oxidized directly without further purification. The oxidation procedure was an adaption of that used by Haverkamp Begemann et al. (12). Oleic acid (0.97 gm.) was dissolved in 10.5 gm. of glacial acetic acid and, with mechanical stirring, 2.5 gm. of powdered reagent grade potassium permanganate were added in small portions over a period of 5 hours. After reaction was complete, the mixture was diluted to 50 ml. with water, and potassium metabisulfite was added to reduce manganese dioxide and excess permanganate. The solution was then made strongly acid with sulfuric acid and the mono- and dicarboxylic acids were selectively extracted with pentane and ether, respectively. The methyl esters of the two fractions were then analyzed and purified by gas chromatography (Figs. 4 and 5). Nonanoate and azelate of better than 99 weight per cent purity were obtained by this method.

The oxidation of the eicosatrienoic acid obtained by reversed-phase chromatography was carried out as for oleic acid except that 360 mg. of potassium permanganate were used to oxidize 45 mg. of triene and the reaction was carried out over a 6-hour period. The mono- and dicarboxylic acids obtained from the pentane and ether extraction were selectively extracted with pentane and ether, respectively. The methyl esters by the two fractions were then analyzed and purified by gas chromatography (Figs. 4 and 5). Nonanoate and azelate of better than 99 weight per cent purity were obtained by this method.

The oxidation of the eicosatrienoic acid obtained by reversed-phase chromatography was carried out as for oleic acid except that 360 mg. of potassium permanganate were used to oxidize 45 mg. of triene and the reaction was carried out over a 6-hour period. The mono- and dicarboxylic acids obtained from the pentane and ether extraction were selectively extracted with pentane and ether, respectively. The methyl esters of the two fractions were then analyzed and purified by gas chromatography (Figs. 4 and 5). Nonanoate and azelate of better than 99 weight per cent purity were obtained by this method.

Stepwise Degradations of Hydrogenated Oleic and Trienoic

The aqueous solution (100 ml.) containing the dicarboxylic acids was extracted with eight 100-ml. volumes of ether. However, the dicarboxylic acids containing 6 or less carbon atoms were incompletely extracted and corrections had to be applied to obtain accurate analytical results. A study of the ether-aqueous phase partition coefficients with the use of a model system of known composition indicated that the extracted reaction mixture still contained 5.25 per cent total adipic, 23.4 per cent of the total glutaric, 29.1 per cent of the total succinic, and essentially all of the malonic acids.

3 The aqueous solution (100 ml.) containing the dicarboxylic acids was extracted with eight 100-ml. volumes of ether. However, the dicarboxylic acids containing 6 or less carbon atoms were incompletely extracted and corrections had to be applied to obtain accurate analytical results. A study of the ether-aqueous phase partition coefficients with the use of a model system of known composition indicated that the extracted reaction mixture still contained 5.25 per cent total adipic, 23.4 per cent of the total glutaric, 29.1 per cent of the total succinic, and essentially all of the malonic acids.
Acids—A sample of the active oleic acid was hydrogenated over 5 per cent palladium on charcoal and the resulting stearic acid was freed from homologues by reversed-phase chromatography. The purified stearic acid (91.5 mg.) was diluted to 221 mg. with inactive stearic acid and was degraded by the method of Dauben et al. (9, 13) to margaric acid and benzoic acid, the carboxy carbon of which represented carbon atom 1 of the original oleic acid.

Similarly, hydrogenated trienoic acid was freed from traces of stearic acid by reversed-phase chromatography. The purified arachidic acid (16.5 mg.) was diluted to 326 mg. with inactive arachidic and degraded to margaric acid and three successive samples of benzoic acid; the carboxy carbons of which represented carbon atoms 1, 2, and 3, respectively, of the original trienoic acid. After removal of the first two carbons of arachidic acid, a sample of the resulting stearic was purified and saved for counting.

RESULTS AND DISCUSSION

Table II shows the relative amounts of the mono- and dicarboxylic acids resulting from oxidation of oleic acid. Nonanoic acid was derived only from oleic acid and hence is a measure of the amount of oleic acid in the original mixture. On the other hand, heptanoic acid was derived from the terminal 7 carbons of both palmitoleic acid, present as an impurity in the original mixture, and 11-octadecenoic acid, a positional isomer of oleic acid. The mole percent of undecanedioic acid in the dicarboxylic acid fraction fixes the amount of 11-octadecenoic acid in the original mixture and, by difference, also allows us to calculate the amount of palmitoleic acid that was present. Table III gives the composition of unsaturated acids in the original mixture. For counting, the highly purified nonanoic and azelate (Figs. 4B and 5B) were used.

Tables IV and V reveal the relative amounts of the mono- and dicarboxylic acids resulting from the oxidation of eicosatrienoic acid. Bearing in mind that the original eicosatrienoic acid mixture contained about 90 per cent eicosatrienoic acid with eicos-
dienoic and oleic acids being the only detectable contaminants, we can readily deduce the structure and relative composition of the acids in the original mixture. The only monocarboxylic acids arising from the oxidation of the eicosatrienoic acid mixture were nonanoic and heptanoic acids. Nonanoic acid would arise from oleic and 5,8,11-eicosatrienoic acids and from 8,11-eicosadienoic acid if present. Heptanoic acid could arise from 10,13-eicosadienoic acid or from 7,10,13-eicosatrienoic acid. The presence of 10,13-eicosadienoic acid is ruled out, however, since no sebacic (C₁₂-dicarboxylic) acid is found among the oxidation products. On the other hand, we do find pimelic (C₆-dicarboxylic) acid in an amount corresponding almost exactly with the amount of heptanoic acid. This, then, indicates the presence of 7,10,13-eicosatrienoic acid in the original mixture. The eicosadienoic acid must have been the 8,11 compound, a conclusion which is confirmed by finding the expected amount of suberic (C₉-dicarboxylic) acid. It will be noted that oleic acid, 8,11-eicosadienoic acid, and 5,8,11-eicosatrienoic acid all give nonanoic acid on oxidation, but that each gives rise to a different dicarboxylic acid. Thus the relative amounts of azelaic, suberic, and glutaric acids allow us to calculate the amounts of oleic, 8,11-eicosadienoic, and 5,8,11-eicosatrienoic acids, respectively, in the original mixture. The fact that the total mole per cent of azelaic, suberic, and glutaric acids (92.0 per cent) corresponds almost exactly with the mole per cent of nonanoic acid (92.7 per cent) further substantiates the validity of this reasoning. The composition of the original mixture is shown in Table VI.

Table VII gives the activities of various acetone and ether fractions and Table VIII gives the activities of the various oxidation and degradation products of oleic and 5,8,11-eicosatrienoic acids. It should be noted that the arachidic acid which was degraded presumably arose from a mixture containing 87.3 per cent 5,8,11-eicosatrienoic acid, 5.2 per cent 8,11-eicosadienoic acid, and 7.5 per cent 7,10,13-eicosatrienoic acid. The 8,11-eicosadienoic acid and 5,8,11-eicosatrienoic acid should both have the

<table>
<thead>
<tr>
<th>Acid</th>
<th>Carbon atoms</th>
<th>Mole per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Actual</td>
</tr>
<tr>
<td>Decanoic</td>
<td>10</td>
<td>trace</td>
</tr>
<tr>
<td>Nonanoic</td>
<td>9</td>
<td>82.8</td>
</tr>
<tr>
<td>Octanoic</td>
<td>8</td>
<td>9.0</td>
</tr>
<tr>
<td>Heptanoic</td>
<td>7</td>
<td>7.5</td>
</tr>
<tr>
<td>Hexanoic</td>
<td>6</td>
<td>0.7</td>
</tr>
</tbody>
</table>

Table V

Dicarboxylic acids resulting from oxidation of trienoic concentrate

<table>
<thead>
<tr>
<th>Acid</th>
<th>Carbon atoms</th>
<th>Mole per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Actual</td>
</tr>
<tr>
<td>Azelaic</td>
<td>9</td>
<td>5.5</td>
</tr>
<tr>
<td>Suberic</td>
<td>8</td>
<td>4.9</td>
</tr>
<tr>
<td>Pimelic</td>
<td>7</td>
<td>7.1</td>
</tr>
<tr>
<td>Adipic</td>
<td>6</td>
<td>4.0</td>
</tr>
<tr>
<td>Glutaric</td>
<td>5</td>
<td>70.8</td>
</tr>
<tr>
<td>Succinic</td>
<td>4</td>
<td>11.7</td>
</tr>
</tbody>
</table>

* Adipic acid was shown by control experiments to be an artifact arising from the polyadipate packing of the gas chromatographic column. Hence it was disregarded in calculating the mole percentage of the other dicarboxylic acids.

Table VI

Composition of trienoic concentrate

<table>
<thead>
<tr>
<th>Acid</th>
<th>Mole per cent</th>
</tr>
</thead>
<tbody>
<tr>
<td>5,8,11-Eicosatrienoic</td>
<td>81-82.5</td>
</tr>
<tr>
<td>8,11-Eicosadienoic</td>
<td>4.8-5.0</td>
</tr>
<tr>
<td>7,10,13-Eicosatrienoic</td>
<td>7.1-7.3</td>
</tr>
<tr>
<td>Olieo (6 octadecenoic)</td>
<td>5.4-5.6</td>
</tr>
</tbody>
</table>

Table VII

C¹⁴ activity of various acetone and ether fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>D.p.s. per mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total fatty acids</td>
<td>98.2</td>
</tr>
<tr>
<td>F-1 (saturated plus oleic)</td>
<td>117.2</td>
</tr>
<tr>
<td>F-2 (unsaturated)</td>
<td>61.7</td>
</tr>
<tr>
<td>F-3 (polyunsaturated plus palmitoleic)</td>
<td>58.7</td>
</tr>
<tr>
<td>Olieo (crude)</td>
<td>57.4</td>
</tr>
</tbody>
</table>

* The counting of all samples was performed with a Traceerlab CE-1 liquid scintillation counter as described previously (14).
same label distribution, however, since presumably both arise from the same precursor. For practical purposes, then, the distribution of label in the arachidic acid can be considered as reflecting the actual label distribution in 5,8,11-eicosatrienoic acid. The small amount of the 7,10,13 isomer present in the original mixture would not be expected to change significantly the label distribution in the arachidic acid from that derived solely from 5,8,11-eicosatrienoic acid.

Fig. 7 shows the relative distribution of label in oleic acid and in the terminal 18 carbons of 5,8,11-eicosatrienoic acid.

FIG. 7. Distribution of activity in oleic acid and in the terminal 18 carbons of 5,8,11-eicosatrienoic acid.

The polyunsaturated fatty acids from male fat-deficient rats given injections of acetate-1-14C were concentrated by low temperature crystallization from acetone, and 5,8,11-eicosatrienoic acid was isolated from the concentrate by gas-liquid and liquid-liquid partition chromatography. Part of the triene was oxidized at the double bonds to yield pelargonic, malonic, and glutaric acids. Another sample was hydrogenated and degraded one carbon at a time to margaric acid. By similar methods, oleic acid was isolated and samples were oxidized and degraded. The oxidation and degradation products from trienoic and oleic acids were purified and counted, and the activities of various portions of the two molecules were compared. The results show that oleic acid is the precursor for 5,8,11-eicosatrienoic acid in the fat-deficient rat. The intermediates in this process are at present unknown but the pathway recently proposed by Mead (15) seems the most likely, especially since evidence for the occurrence of one of the hypothetical intermediates, 8,11-eicosadienoic acid, has been presented (Table VI). Furthermore, if the conversion of oleic to eicosatrienoic acid is analogous to the conversion of linoleic to arachidonic acid, then we would expect formation of C15 dienoic rather than a C20 monoenoic acid to be the first step in the process. The possible routes of biosynthesis of 5,8,11-eicosatrienoic acid from oleic acid are shown in Fig. 8 with the pathway favored by the authors indicated by the heavy arrows.

A further point of interest is to be found in Table VI. The finding of heptanoic acid as 7.3 per cent of the monocarboxylic acids derived by oxidation of the C18 triene fractions and of pimelic acid as 7.1 per cent of the dicarboxylic acids from the same source is strong evidence for the existence of 7,10,13-eicosatrienoic acid in the fat-deficient rat. The terminal double bond in this acid is located in the same position as that of palmitoleic acid, a major fatty acid in the fat-deficient rat (16). With the knowledge that oleic acid can serve, in the fat-deficient animal, as a source of polyunsaturated acids, the likelihood that palmitoleic acid could function similarly is strengthened. Thus, it now appears evident that the polyunsaturated acids of the animal body are not necessarily the products of exogenous di- and trienoic acids, but can be synthesized from the monounsaturated acids and, ultimately, from acetate. Whether cis-12-octadecenoic acid can, under certain conditions, serve as a source of linoleic acid, will be revealed by further experiments.

SUMMARY

The polyunsaturated fatty acids from male fat-deficient rats given injections of acetate-1-14C were concentrated by low temperature crystallization from acetone, and 5,8,11-eicosatrienoic acid was isolated from the concentrate by gas-liquid and liquid-liquid partition chromatography. Part of the triene was oxidized at the double bonds to yield pelargonic, malonic, and glutaric acids. Another sample was hydrogenated and degraded one carbon at a time to margaric acid. By similar methods, oleic acid was isolated and samples were oxidized and degraded. The oxidation and degradation products from trienoic and oleic acids were purified and counted, and the activities of various portions of the two molecules were compared. The results show that oleic acid is the precursor for 5,8,11-eicosatrienoic acid in the fat-deficient rat. A probable intermediate in this transformation, 8,11-eicosadienoic acid, was also found as well as a small amount of a second triene, 7,10,13-eicosatrienoic acid, which presumably is formed from palmitoleic acid.

\[
CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH
\]

Oleic acid

\[
CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad +2C \quad -2H
\]

11-eicosenoic

\[
CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad +2C
\]

6,9-octadecadienoic

\[
CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad -2H
\]

8,11-eicosadienoic

\[
CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad +2C
\]

3,6,9-octadecatrienoic

\[
CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad CH_3-(CH_2)_7-CH=CH-(CH_2)_7-COOH \quad 5,8,11-eicosatrienoic acid
\]

Fig. 8. Possible pathways from oleic acid to 5,8,11-eicosatrienoic acid.
REFERENCES

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