J. Lipid Research, January, 1962
Volume 3, Number 1

Biosynthesis of linoleic acid from 1-C^{14}-*cis*-2-octenoic acid by the laying hen*

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[Received for publication June 28, 1961]

SUMMARY

A white leghorn hen was given 0.2 mc of 1-C^{14}-*cis*-2-octenoic acid in one 1.05-g dose, and five eggs laid subsequently were collected. Linoleic acid isolated from the eggs, both as the tetrabromide and by preparative gas-liquid chromatography, was found to be radioactive. This was proven to be octadeca-9,12-dienoic acid by characterizing the products of oxidation—caproic and azelaic acids, and 3 CO_2. The caproic acid, which was isolated by gas-liquid chromatography from periodate-permanganate oxidation of the labeled linoleic acid, was found to be inactive. The carbon 11 of the linoleic acid was isolated as $BaCO_3$ after oxidation with $KMnO_4$ in acetic acid and was found to be over 4.3 times as radioactive as the active azelaic acid carbons. It was concluded that the hen synthesized octadeca-9,12-dienoic acid from the 1-C^{14}-*cis*-2-octenoic acid.

In previous studies in this laboratory, it was observed (1) that the laying hen cannot synthesize linoleic acid *in toto* from 1-C^{14}-acetate. The studies of Mead and his group (2) point clearly both to the mechanism of alternating dehydrogenation and addition of acetate to linoleic acid for the production of arachidonic, and to oleic acid for the production of eicosa-5,8,11-trienoic acids. The same laboratory has recently reported, however, that 1-C^{14}-octadeca-*cis*-12-enoic acid serves inefficiently, if at all, as a precursor of linoleic acid in the rat (3). It is thus evident that a double bond cannot be added to the 9 position of octadeca-*cis*-12-enoic acid. However, it is possible that a much shorter acid with a double bond between the sixth and seventh carbons from the distal end can be extended into linoleic acid. The simplest acid possible with this potential is *cis*-2-octenoic acid. The present study demonstrates that this, in fact, takes place.

EXPERIMENTAL PROCEDURE

Preparation of 1-C^{14}-cis-2-octenoic Acid. This labeled acid was prepared by the method of Knight and Diamond (4). To an anhydrous ether solution of ethyl magnesium bromide, made from 1.25 g of magnesium and 5.75 g of ethyl bromide, was added 5 g of 1-heptyne over a period of 30 minutes. The solution was refluxed for about 2 hours. Four millicuries of radioactive barium carbonate (10.0134 g) was treated with sulfuric acid, and the $C^{14}O_2$ generated was introduced over the ethereal Grignard solution at −15°. The reaction mixture was hydrolyzed with ice and hydrochloric acid, and the product was extracted with benzene. It was dried and distilled to yield 1.9 g (27% yield) of 1-C^{14}-2-octynoic acid (b.p. 111°–114° at 2 mm). The poor yield of 2-octynoic acid was due to an accidental loss. A yield of 52% had been obtained from a run with unlabeled materials. Semihydrogenation of the above with Raney Nickel Catalyst in benzene gave 1.6 g of radioactive 1-C^{14}-*cis*-2-octenoic acid (b.p. 98–102° at 2 mm).

Since preparation of this material, D. R. Howton, Organic Chemistry Section, University of California Medical Center, Los Angeles, California, in a personal communication, presented data showing that the reduction of 2-octynoic acid results in mixtures of octenoic and octanoic acids plus some unreacted octynoic acid. This has since been confirmed in this laboratory. The presence of the labeled by-products in no way changes the conclusions of this study. Octanoic acid could not be the precursor of the labeled linoleic acid. Conceivably octynoic acid could be but, if so, it would have to be biologically reduced to the *cis*-2-octenoic acid first. Also, since the position of the C^{14} in the linoleic acid was demonstrated, the

* Supported in part by grants from the U.S. Atomic Energy Commission and the Robert A. Welch Foundation.

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dienoic acid could have originated from the 1-C^{14}-labeled eight-carbon precursor only and not from any contaminant.

Production of Labeled Egg Fatty Acids. A white leghorn hen in full production was placed on a low fat diet for 6 weeks in order to reduce the amount of linoleic acid in the egg lipids to a minimum. The low fat diet contained the following constituents per kilogram: 610.40 g cerelose, 226.75 g Drackett protein,[1] 30.00 g wood pulp, 14.50 g vitamin mixture, 87.70 g mineral mixture, and 0.66 mg choline chloride.[2] During the next 15 days a triglyceride mixture made up of equal amounts of myristic and lauric acids was added to the low fat diet at the 10% level in the hope that it would minimize the utilization of the labeled 2-octenoic acid for energy but stimulate the demand for, and therefore the synthesis of, linoleic acid. On the day of the test, the hen was given 0.2 mc (1.05 g) of the 1-C^{14}-*cis*-2-octenoic in one capsule. The first five eggs subsequently laid were collected.

Analyses of the Egg Lipids. The yolk of each egg was mixed in a homogenizer with 10 volumes of chloroform. The chloroform solution was centrifuged, decanted, dried over anhydrous Na_2SO_4, and filtered; the chloroform was removed with a slow stream of nitrogen under reduced pressure. Aliquots of this solution, or lipids obtained from it, were used for the determinations described below. This simple extraction procedure has been shown in this laboratory to be satisfactory for the preparation of representative lipid mixtures from egg yolks.

The first egg was used for trial assays. The specific radioactivity of the cholesterol in each of the remaining four eggs was determined by precipitation as the tomatinide according to the procedure of Kabara *et al.* (5), and the activity of the tomatinide was measured in a liquid scintillation spectrometer.[3] As described by Kabara (5), 100 mg of the lipids was saponified with 1 ml of a 50% solution (w/v) of KOH in absolute methanol, and the mixture was made just acidic to phenolphthalein with 10% acetic acid. The cholesterol was then precipitated with 5 ml of 1% tomatine solution (1 g of crystalline tomatine in alcohol, water, and glacial acetic acid, 55:44:1 [v/v]). The tomatinide was dissolved in glacial acetic acid, and the radioactivity was counted in a liquid scintillation spectrometer. The amount of the cholesterol in the glacial acetic acid solution was determined by the method of Zak *et al.* (6).

Two hundred milligram aliquots of the total lipids of each of the four eggs were saponified with a 4% solution (w/v) of KOH in 95% ethanol, the nonsaponifiable matter was removed, the mixed fatty acids were isolated, and the specific radioactivity of the fatty acids was determined.

In order to determine the radioactivity of the linoleic acid, about 200 mg of the fatty acids of each egg was brominated in ether solution according to Brown and Frankel (7). The ether insoluble hexa- and polybromides were filtered and discarded, and the ether filtrate was evaporated. About 10 ml of petroleum ether was added to the residue; the mixture was allowed to stand in the refrigerator overnight at 5°. The tetrabromostearic acid, thus crystallized, was washed with petroleum ether, recrystallized twice from 80% acetone (m.p. 114°–115°), and its radioactivity determined by liquid scintillation spectrometry.

In order to obtain enough polybromides for degradation studies, the fatty acids from the four eggs were pooled. Both the tetrabromide and gas-liquid chromatographic techniques were used to isolate the linoleic acid. The polybromides from 5 g of the mixed fatty acids were isolated as described above. A quantity of 75 mg of the tetrabromide was debrominated to obtain linoleic acid according to the method of Mathews *et al.* (8). The bromide was dissolved in 20 ml of methanol and refluxed for 1 hour with 200 mg of zinc dust. The zinc was removed and the pure fatty acid was isolated after acidification with hydrochloric acid.

The linoleic acid thus isolated (from the tetrabromide) was oxidized with periodate-permanganate solution (9) to determine if the unlabeled fragment of the 2-octenoic acid constituted the last six carbon atoms. Thirty milligrams of linoleic acid was oxidized, and the fragments were steam-distilled into 20 ml of 1 N NaOH solution with the aid of a slow stream of nitrogen. The steam-distilled fatty acids were liberated from their alkaline salts by acidification, extracted with ether, and converted into their methyl esters with diazomethane. The methyl esters were analyzed in a gas chromatographic apparatus using a 6-foot, 1/4-inch column packed with 10% silicone high vacuum grease on Chromosorb W (60 to 80 mesh), with an ionization detector at a temperature of 100° and a flow rate of 60 ml of argon per minute. The methyl esters were found to consist of almost 100% methyl caproate and a trace of methyl heptanoate. The caproate was collected and assayed for its level of radioactivity.

After the above procedure was completed, a preparative gas-liquid chromatographic apparatus[4] became

[1] Drackett assay Protein C-1, obtained from Archer Daviel Midland and Company, Cincinnati, Ohio.

[2] Added as an aqueous solution of 20 mg per 100 ml.

[3] Packard Instrument Company, Inc., LaGrange, Illinois.

[4] Megachrom, Beckman Instrument Company, Fullerton, California.

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available. In one preparation, eight 250-mg quantities of the mixed methyl esters of the egg fatty acids were chromatographed.[5] After a second pass through the apparatus, 39.8 mg of 97.8% pure methyl linoleate was obtained. This was oxidized with permanganate-periodate solution, and the volatile products were steam-distilled. In this case, the nonvolatile dicarboxylic acids were also chromatographed on 10% silicone grease at 190° and were found to consist of almost 94% azelaic acid. The caproic and azelaic acids were collected in toluene and their radioactivities were determined by liquid scintillation spectrometry.

From a second preparation, 64 mg of linoleic acid isolated by chromatography was oxidized with potassium permanganate in acetic acid (10). Under these conditions, carbons 10, 11, and 12 of linoleic acid are converted to CO_2. The $C^{14}O_2$ was collected in 10% NaOH under conditions that excluded contaminating carbonate and was isolated as $BaC^{14}O_3$. A 92.7% recovery was achieved. Radioactivity was determined in a gas flow counter and by liquid scintillation spectrometry.

The azelaic acid, resulting from the above oxidation with potassium permanganate in acetic acid, was degraded by the method of Dauben (11). The 1 and 9, and the 2 and 8 carbon atoms were isolated as benzoic acid, and their radioactivities were determined.

RESULTS AND DISCUSSION

The radioactivities of cholesterol, linoleic acid (calculated from the radioactivity of its tetrabromide), and total fatty acids from the individual eggs are presented in Table 1.

The results show that the linoleic acid was labeled and that the specific radioactivity, though of low level in the first eggs laid, increased in succeeding eggs; in the fourth egg, laid on the eighth day, the level equalled that of the other fatty acids (340 dpm per mg). It is apparent that the linoleic acid was synthesized from the 1-C^{14}-*cis*-2-octenoic acid, since the previous study (1) had shown that labeled acetate alone does not give rise to labeled linoleic acid.

Confidence that linoleic acid was synthesized from the 2-octenoic acid was strengthened by the observation that there was no radioactivity in the terminal six carbon atoms of the radioactive linoleic acid (324 dpm per mg) prepared from the active tetrabromide of the pooled eggs. These six carbon atoms were isolated as caproic acid. The absence of radioactivity in the caproic acid strongly suggests that the linoleic acid was built up from the 1-C^{14}-*cis*-2-octenoic acid by addition of acetate from a labeled pool. The activity of the acetate pool is presumed to have originated from β-oxidation of some of the 1-C^{14}-*cis*-2-octenoic acid.

The volatile acids resulting from the oxidation of the linoleic acid isolated by gas-liquid chromatography consisted almost entirely of caproic acid and only a trace of heptanoic. Again the caproic acid contained no radioactivity. The nonvolatile fraction was found to consist of 93.6% azelaic, 6.0% suberic, and 0.4% adipic acids. Thus, there was possibly a small amount of octadeca-8,11-dienoic acid present.

The specific radioactivity of the methyl linoleate isolated by gas-liquid chromatography was 91,140 dpm per mmole and that of the dimethyl azelate obtained from it was 43,416 dpm per mmole. By calculation, the specific activity of each of the five active azelaic acid carbons was 8,683 dpm per mmole; that of the carbon 11 of the linoleic acid, 47,724 dpm per mmole. The carbon 11 was therefore 5.5 times as active as the proximal carbons.

The activity of the mixed carbons 10, 11, and 12, isolated as $BaC^{14}O_3$ and determined both by the gas flow counter and scintillation spectrometry, was 12,672 and 12,465 dpm per mmole, respectively. An average of these two values, assuming that only carbon 11 was radioactive, would give a specific radioactivity for this carbon of 37,708 dpm per mmole. This is somewhat less than that calculated above (47,724 dpm per mmole) but is still 4.3 times the activity of the labeled carbons in the proximal end of the molecule.

The benzoic acid resulting from degradation of the 1 and 9 carbons of the isolated azelaic acid (m.p. 120°–121°) had a specific radioactivity of 70.7 dpm per mg, corresponding to a specific radioactivity of 8,616 dpm per mmole of the carbon atom. This compares, within

TABLE 1. SPECIFIC RADIOACTIVITIES OF TOTAL FATTY ACIDS, CHOLESTEROL, AND LINOLEIC ACID OF THE EGG LIPID

Egg No.	Day No.	Total Fatty Acids* (dpm/mg)	Cholesterol (dpm/mg)	Linoleic Acid (dpm/mg)
1	4			54
2	5	389	832	168
3	7	437	1106	389
4	8	327	1127	340
5	9	212	776	217

* Including linoleic acid.

[5] Eight 6-foot, 5/8 inch i.d. columns, in parallel, packed with 20% (w/w) ethylene glycol succinate on 40 to 60 mesh Johns-Manville C-22 Silocel firebrick was used. The inlet pressure was 10 lb, and the back pressure was 1 lb of helium. The filament current was 250 ma. The temperature of the katherometer detector and discharge outlet was 255°, and that of the column, 185°.

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the limits of experimental error, to the value of 8,683 dpm per mmole given above as calculated from the specific radioactivity of the isolated azelaic acid, assuming that only the 1, 3, 5, 7, and 9 carbons were radioactive.

The benzoic acid from the 2 and 8 carbon atoms of the azelaic acid had no radioactivity, definitely proving that the proximal 10 carbon atoms of the linoleic acid were derived by β-oxidation of the 1-C^{14}-*cis*-2-octenoic acid as well as by the octanoic and unchanged octynoic diluted in the acetate pool.

Thus, the products of oxidation (caproic acid, azelaic acid, and 3 moles of CO_2) demonstrate that octadeca-9,12-dienoic acid was the dienoic acid isolated. The absence of radioactivity in the distal six carbon atoms and the high radioactive level in carbon 11 as compared to that in the 1, 3, 5, 7, and 9 carbon atoms prove that, in fact, carbon 11 had been synthesized from 1-C^{14}-*cis*-2-octenoic acid.

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