Metabolism of Labeled Isomeric Octadecenoates
by the Laying Hen

T.L. MOUNTS, E.A. EMKEN, W.K. ROHWEDDER and H.J. DUTTON,
Northern Regional Research Laboratory, Peoria, Illinois 61604

ABSTRACT

Discrimination between octadecenoic acid isomers by the laying hen has been studied using tritium \((^3\text{H})\), carbon-14 \((^14\text{C})\) and deuterium \((^d)\) labeled oleate and elaidate esters. Hydrogen isotopes were positioned at the double bond, whereas \(^{14}\text{C}\) was located in the \(1,\) carboxyl carbon. The egg acted as a biological trap, providing an automatic daily biopsy with which to study the metabolism of the fed isomers. Monitoring the incorporation of isomers was facilitated by dual label feeding experiments, and \(^3\text{H}/^{14}\text{C}\), \(d_2/d_0\) and \(d_2/d_1\) ratios were determined on the isomeric mixtures fed, on the total egg lipids extracted and on the isolated neutral lipid and phospholipid fractions. Comparison of isotopic ratios of the fed mixture and of the lipid fractions provided an evaluation of discrimination by the hen during the transport of isomeric octadecenoates into the egg lipids. Radioactive and stable isotope ratios determined for the neutral lipid indicated a preferential incorporation of the \(\text{cis}\) isomer. Stable isotope ratios determined for the phospholipid showed that the \(\text{trans}\) isomer is preferentially incorporated. The \(^3\text{H}/^{14}\text{C}\) ratios for the phospholipid recovered in each experiment increased greatly whichever isomer was labeled with \(^3\text{H}\), indicating an elimination of the \(^{14}\text{C}\)-label. Gas liquid radiochromatographic separation of the methyl esters from the neutral lipids and phospholipids showed that the isotopic labels were present almost exclusively in the octadecenoic acid constituent.

INTRODUCTION

Geometric as well as positional isomers of unsaturated fatty acids are formed during the catalytic hydrogenation of vegetable oils to produce commercial oils and shortenings \((1)\). Investigations of the metabolism of \(\text{trans}\) mono-

enoic acids, principally elaidic acid, have given conflicting results. Transfer of \(\text{trans}\) fatty acids across placental membranes, amniotic and allantoic, has been reported by McConnell and Sinclair \((2)\) with rats, Ono and Fredrickson \((3)\) with rats and dogs, Kaufmann and Mankel \((4)\) with hens, Billek \((5)\) with rats and dogs and Le Breton and Le Marchal \((6)\) with rats and hens. Johnston, Johnson and Kummerow \((7)\) with rats and Sgoutas and Kummerow \((8)\) with humans have reported the nontransfer of \(\text{trans}\) fatty acids.

In all the cited investigations that report transfer across the membranes, the rate of incorporation of elaidic acid was of the same order of magnitude as for oleic acid. Although most of the investigators cited above did not compare the incorporation of \(\text{trans}\) fatty acids into the triglyceride and phosphatide fractions of the lipid, Kaufmann and Mankel \((4)\) reported that the \(\text{trans}\) unsaturated fatty acid content of the triglyceride was always greater than that in the corresponding phosphatides.

In investigations considering metabolic uses of \(\text{trans}\) fatty acids other than transfer across placental membranes, Coots \((9)\) reported that elaidic acid showed a slightly greater incorporation into the lymph phospholipid fraction than did oleic acid. Sinclair \((10,11)\) reported a similar tendency for elaidic acid to be incorporated into the phospholipid fraction of blood and other tissues.

In the present investigation, mixtures of isotopically labeled oleic and elaidic acid esters were fed to a laying hen. Each geometric isomer was labeled with tritium \((^3\text{H})\), radiocarbon \((^{14}\text{C})\) or deuterium \((^d)\). The labeled isomers were fed in pairs or groups, with each individual labeled differently, to permit measurements of ratios of \(^3\text{H}/^{14}\text{C}\) and ratios of \(d_2/d_0\) or \(d_2/d_1\), or both. The egg served as a "biological trap" yielding an automatic daily biopsy with which to study the metabolism of the isomer in the hen. Relative rates of transfer of isomers across membranes and their incorporation into lipids were indicated by the isotopic ratios determined for the fed mixture, the total egg lipid, the neutral lipid and the phospholipids. The dual label design improves the precision and the accuracy of experiments because exogenous fatty acid metabolism is not confused with the endogenous.

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\(^1\)Presented at the AOCs Meeting, Houston, May 1971.

EXPERIMENTAL PROCEDURES

Materials

Oleic acid-1-14C was purchased from Amersham-Searle Co., Des Plaines, Ill. Specific activity was 57.8 mc/mM, <1.0% trans. Oleic acid was esterified using diazomethane. A single peak was indicated by a radiochromatographic scan of thin layer chromatographic (TLC) plate.

Tritiated water (HTO) was purchased from Amersham-Searle Co. Specific activity was 3.6 C/mM.

Oleic acid: The urea-half soap crystallization procedure (12) was used to prepare 110 g of oleic acid from 780 g of olive oil. The purified oleic acid contained less than 0.5% palmitic acid and less than 0.5% linoleic acid impurities.

Stearolic acid: Oleic acid (20 g) was brominated and dehydrohalogenated at 200°C using KOH in ethylene glycol. The reaction mixture was diluted with H2O, acidified and extracted twice with petroleum ether (PE). Crystallization of the PE extract from ethanol-water at 4°C yielded 10.5 g of pure stearolic acid.

Methyl oleate 9(10)-d3: Stearolic acid was esterified with diazomethane. One hundred milligrams of copper chromite catalyst (Harshaw Chemical Co.) was stirred with 16 µl of HTO and H2 gas at atmospheric pressure for 1 hr at 195°C. Approximately 1.0 g of methyl stearolate was injected into the reaction vessel through a rubber septum. Stirring was continued for 2 hr while H2 was maintained at atmospheric pressure. The product was filtered through a medium sintered disk funnel. Gas liquid radiochromatography (GLRC) (13) gave mass and radioactivity tracings which coincided and which were the same as a methyl oleate standard. Ozonolysis-pyrolysis (14) of the product yielded radioactive fragments identified by GLRC as 9-aldehyde and 9-aldehyde ester. 1R analysis indicated that the product contained less than 1% trans isomer. Such stereospecific hydrogenation of stearolate with copper catalysts was reported by Kontala (15). Approximately 95% of the tritium was located at the double bond position as determined by bromination, dehydrohalogenation and assay of the hydrogenation product.

Radioactivity of the product was assayed by dissolving weighed samples in 10 ml of toluene scintillation solvent [4 g of 2,5-diphenyl oxazole (PPO) per liter] using a Beckman LS-250 scintillation counter. The specific activity was determined to be 5.27 mc/mM.

Ethyl oleate-9(10)-d1: Ethyl oleate-d1 was prepared from methyl stearolate according to the following procedure (16). A 250 ml, three-necked, round-bottomed flask was equipped with thermometer, dropping funnel and drying tube. The glassware was thoroughly flame-dried before being used. Sodium borohydride (2.05 g) was mixed with 40 ml of diglyme freshly distilled from LiAlH4 and cooled to ca. 3°C. Next 15.2 ml of 2-methyl-2-butene was mixed with 25 ml of dry diglyme and added to the sodium borohydride. After cooling to ca. 0°C, 9.2 ml of boron trifluoride-etherate (172 mmole) in 20 ml of dry diglyme was added over a period of 20 min while holding the reaction temperature at ca. 10°C. The mixture was then stirred at 3°C for 3 hr. Methyl stearolate (15.2 g) dissolved in 20 ml of diglyme was added during a 10 min period, stirred at 0°C for 1 hr and stirred at room temperature for 3 hr. Next the solution was cooled to 3°C and 15 ml of deuterioacetic acid was added at a rate of 1 ml per min. The solution was stirred at 3°C for 3 hr and the mixture diluted with 600 ml of ice water and extracted three times with petroleum ether (PE). The PE extract was washed with NaHCO3, then H2O and dried over Na2SO4 and the solvent evaporated. After standing several days at -25°C, the methyl oleate-9(10)-d1 became cloudy and a small amount of white precipitate was filtered out. The sample was transesterified using freshly prepared sodium ethoxide and distilled at 138°C under 0.1 mm pressure of Hg. Ethyl oleate-9(10)-d1 was obtained.

FIG. 1. Incorporation of 3H- and 14C-labeled fatty esters into egg lipid.
TABLE I

Labeled Ester Mixtures Fed to Hen

<table>
<thead>
<tr>
<th>Feeding</th>
<th>Labeled esters</th>
<th>Total µc</th>
<th>Isotopic ratios</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Oleate-9,10-3H + oleate-1-14C</td>
<td>924.3 482.0</td>
<td>d2/d1 1.93</td>
</tr>
<tr>
<td>2</td>
<td>Elaidate-9,10-3H + oleate-1-14C</td>
<td>830.0 113.0</td>
<td>d2/d1 7.31</td>
</tr>
<tr>
<td>3</td>
<td>Elaidate-9,10-3H + oleate-1-14C</td>
<td>375.0 500.0</td>
<td>d2/d1 0.75 1.00</td>
</tr>
<tr>
<td></td>
<td>Oleate-9,(10)-d</td>
<td>521.2 176.8</td>
<td>d2/d1 2.95 1.00</td>
</tr>
<tr>
<td>4</td>
<td>Oleate-9,10-3H + elaidate-1-14C</td>
<td>830.0 113.0</td>
<td>d2/d1 7.31</td>
</tr>
</tbody>
</table>

a Methyl esters.

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TABLE III
Radiochemical Analysis, Egg Phospholipids

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fed esters</th>
<th>Specific activity</th>
<th>Isotopic ratios</th>
<th>Recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>$\mu$c/mg $\times 10^3$</td>
<td>$^3$H/$^1$C</td>
<td>phospholipid</td>
</tr>
<tr>
<td>1</td>
<td>Oleate-9,10-$^3$H + oleate-1-$^1$C</td>
<td>38.01 1.38</td>
<td>1.93</td>
<td>27.59</td>
</tr>
<tr>
<td>2</td>
<td>Elaidate-9,10-$^2$H + oleate-1-$^1$C</td>
<td>17.88 1.08</td>
<td>7.31</td>
<td>16.55</td>
</tr>
<tr>
<td>3</td>
<td>Elaidate-9,10-$^3$H + oleate-1-$^1$C</td>
<td>4.37 4.99</td>
<td>0.75</td>
<td>0.88</td>
</tr>
<tr>
<td>4</td>
<td>Oleate-9,10-$^3$H + elaidate-1-$^1$C</td>
<td>5.29 0.32</td>
<td>2.95</td>
<td>16.59</td>
</tr>
</tbody>
</table>

* Methyl esters.

from the residual cis monoene by silver-ion exchange resin chromatography (21). The effluent was monitored by a flow-through cell accessory to the Beckman LS 250. The cell was packed with europium-activated calcium fluoride as the scintillator. Recovery of trans monoene was approximately 60%.

Animal Experiment

In the absence of elaborate metabolism chambers, an adequate area for maintenance of a white leghorn hen was devised. A wire cage fitted with a feeding trough and watering device was mounted above a stainless steel tray in a laboratory hood with continuous airflow. The tray was equipped with a drainage tube for easy recovery of radioactive feces or their disposal after monitoring by water flushing. The hen was maintained on a diet of "Critic" laying mash (Citric Mills, Inc., Beardstown, Ill.) throughout the experiments.

Mixtures of isomers were prepared from the $^1$C and $^3$H radioactive esters alone and from the deuterium as well as $^1$C and $^3$H labeled esters as shown in Table I. Approximately 50 mg of the radioactive esters were administered in a 150 μl gelatin capsule placed in the gullet of the hen. Approximately 6 g of the mixed isotopes were force-fed using a 5 ml syringe tightly inserted into a 5-in. segment of Teflon tubing. The syring plus fatty esters was tared, and the esters were administered by inserting the tubing approximately 4 in. down the hen's gullet in order to bypass the trachea.

Eggs laid subsequent to the feeding of the labeled isomers were collected. With each egg the yolk was separated from the white, and the egg lipid was extracted by stirring the yolk with a 50:50 mixture of chloroform:methanol for 1 hr. Coagulated protein was allowed to settle, and the lipid solution was decanted. The residue was washed with the solvent and filtered. This filtrate was added to the previously decanted lipid solution, the solvent was evaporated, and the weight of total lipids was determined. Egg lipids were fractionated by silicic acid chromatography (22) using chloroform:benzene (70:30) followed by 100% methanol as eluting solvents. Fractions collected were analyzed by TLC on Silica Gel G with chloroform:benzene (70:30) solvent. Neutral lipid and phospholipid were isolated free of other constituents. These lipid fractions were used to determine the ratio of incorporation of isomers.

Radiochemical analysis. Dual isotope assay was performed using the three-channel liquid scintillation counter. This instrument is designed to give a counting efficiency of 80% for

TABLE IV
Stable Isotope Analysis, Egg Lipids

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Fed esters</th>
<th>Isomeric ratios (E1/E0)</th>
<th>Recovered neutral lipid</th>
<th>Recovered phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>Elaidate-9,10-d$_2$ + oleate-9,(10)-d$_1$</td>
<td>1.00 0.82</td>
<td>1.17</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Elaidate-d$_0$ + oleate-9,10-d$_2$</td>
<td>1.00 0.56</td>
<td>1.09</td>
<td></td>
</tr>
</tbody>
</table>

* Ethyl esters.
The lipids were transesterified by the sodium methoxide procedure (23) and the methyl esters analyzed by the gas-liquid chromatography-liquid scintillation counting (GLC-LSC) procedure described by Thomas and Dutton (24).

Stable isotope analysis. An Aerograph Autoprep (Model No. A-700) was used to isolate ca. 10–15 mg of monoene from each fraction for IR and MS analysis. Baseline separation of components was achieved using a sample size of 12 to 15 μl on a 10 ft x 3/8 in. aluminum column packed with 10% EGSS-X on Chromosorb P, with a column temperature of 180°C, and a helium flow rate of 80 ml/min was used.

IR analysis for elaidate-d0 was accomplished using a Perkin-Elmer 621 grating IR spectrometer. The procedure used was essentially the same as described by Allen (25) except for two differences: a 50 μl, 0.5 cm micro cell was used rather than the normal IR cell, and the ratio of absorbance at 10.4 vs. 9.2 μ was plotted against the per cent trans instead of the absorbance ratio 10.5 vs. 8.6 μ. A standard curve was prepared for 1–20% trans using known mixtures of oleate and elaidate. Values from three analyses were averaged, but inherent errors by this procedure were ± 0.4% at low trans values.

Monoenes separated by preparative GLC were reduced to methyl stearate using anhydrous hydrazine (26) and the per cent monodeutero stearate or dideutero stearate, or both, in these samples was determined by MS. Hydrazine was used to insure that there was no hydrogen-deuterium exchange during reduction. Isolation and reduction of the monoene improves the sensitivity of the mass spectrometric analysis by (1) increasing the per cent deuterium in the sample analyzed by about three times, (2) eliminating interference of traces of stearate-d0 in the determination of oleate-d0 by analyzing for stearate-d2 and (3) being more sensitive and reproducible for stearate than for oleate. The deuterated samples were analyzed six times and the average values at masses 299 (MeSt-d1) and 300 (MeSt-d2) were calculated. These values were corrected for the contribution of methyl stearate d0 to the mass 299 and 300 peaks due to the natural 13C abundance. Highly purified methyl stearate-d0 was run before and after analysis of the deuterated methyl stearate to obtain values for the mass 299 and 300 peaks.

RESULTS AND DISCUSSION

Liquid scintillation assay of the total lipids extracted from the eggs showed that the radioactive content increased rapidly until the fifth day and then decreased to background by the thirteenth day after force feeding (Fig. 1). MS analysis of egg lipids recovered during stable isotope experiments confirmed this incorporation pattern. As described by Card and Nesheim (27), the hen develops several yolks simultaneously varying in size from ca. 640 mm in diameter. During any one 24 hr period the yolk increases about 4 mm in diameter. Thus the labeled isomers are incorporated into lipid of several yolks at the same time. The amount of incorporation is a function of the over-all size of the yolk at the time the labeled material is fed; that is, the larger the yolk the greater is the incorporation of labeled isomers. An average of 3.0 g of egg lipid was recovered from each egg, consisting of about 90% neutral lipid and 10% phospholipid. Approximately 6% of the total radioactivity administered orally was recovered in the egg lipids. The 3H/14C ratio of the total lipid was about the same as that for the mixture fed, indicating that the cis and trans isomers are transferred across membranes at the same rate.

Only low levels of radioactivity were detected in the feces; the remainder of the radioactive material is assumed to be respired or deposited in the depot fat. Coots (9) has reported the respiration of almost 70% of radioactivity within 51 hr of its administration to rats. Approximately 6% of the deuterium fed to the hen was also recovered in the egg lipid.

The radiochemical analysis of the neutral lipid fractions in each experiment is presented in Table II, and the corresponding analysis for the phospholipid fraction is presented in Table III.

In all radioactively labeled acids the trifluor was located at the double bond, whereas the carboxyl group carbon was labeled with 14C. Oleate-1-14C and oleate-9,10-3H were fed in Experiment I to determine if the position of the label has any effect on the 3H/14C ratio of the incorporated fatty ester. The ratio determined for the neutral lipid was the same as that of the sample fed; indicating that during incorporation of oleate into this lipid fraction there is no isotopic discrimination or loss. However the 3H/14C ratio determined for the phospholipid is about 14 times as great as that fed, showing that 14C has been lost. The data indicate that incorporation of the fed methyl oleate into phospholipid involves a metabolic reaction that is not involved in the incorporation into the neutral lipid. Therefore previous conclusions drawn from metabolic data, which were based on a comparison of rates of incorpo-
FIG. 2. Gas liquid chromatography-liquid scintillation counting of methyl esters of fatty acids from the (a) neutral lipids and (b) phospholipid fractions. The column was 11% EGSS-X (10 ft X 1/4 in. aluminum) operated at 175°C with a helium flow rate of 30 ml/min. Major peaks are (1) palmitate, (2) palmitoleate, (3) stearate, (4) oleate, (5) linoleate and (6) arachidonate.

![Gas liquid chromatogram](image)

ration of 1-14C labeled fatty acids into neutral lipids and phospholipids, should be reevaluated.

When the trans isomer labeled with 3H was fed in mixture with 14C-cis isomer (Experiments 2 and 3, Table II), the 3H/14C ratio of the neutral lipid decreased, signifying a partial preferential incorporation of the cis isomer. With the labeling reversed—that is 3H-cis and 14C-trans (Experiment 4, Table II)—the neutral lipid 3H/14C ratio was found to increase, again a preferential incorporation of the cis isomer. This preference was also shown by the results of the dual-labeled, stable isotope experiments (Table IV).

Owing to the apparent elimination of 14C during decarboxylation reactions leading to the incorporation of the fed esters into the phospholipid, radiochemical analysis was misleading in determining the ratio of isomers incorporated. Therefore this isomeric ratio was determined by use of a stable isotope. Deuterium located at the 9,10 positions in the carbon chain is not subject to loss due to decarboxylation-recarboxylation. These results, shown in Table IV, indicated that there was a partial, preferential incorporation of the trans isomer in the phospholipid.

The tritium specific activity (Table II and...
III) of the phospholipid was consistently higher than that of the neutral lipid. This result would be expected since dilution of the incorporated radioactivity with inactive material is much less in the phospholipid which comprises only 10% of the total lipid of the egg.

Curves for the GLC-LSC analysis of the methyl esters prepared from the neutral lipid (curve A) and the phospholipid (curve B) are presented in Figure 2. The plot of the radiochemical data superimposed on the thenal conductivity curve shows that the 3H- and 14C-labels are almost exclusively associated with the octadecenoic acid constituent in each lipid fraction. MS analysis of isolated fatty esters from each lipid fraction showed that the deuterium label was also associated entirely with the octadecenoic acid constituent.

Although incorporation of the fed esters into the neutral lipid appears to involve hydrolysis and direct acylation, the reactions leading to incorporation into the phospholipid appear to be more complex. Double dual-labeled experiments reveal that although the fatty acid molecule is partially degraded (as indicated by the loss of 14C-carboxyl carbon) the degraded molecule can be elongated to C18 and incorporated into the phospholipid (as indicated by the appearance of tritium and deuterium in the octadecenoic acid constituent only). This observation is not inconsistent with Lynen's (28) description of the fatty acid cycle an elongation of the partially degraded molecule by condensation with malonyl thioester and subsequent incorporation into the phospholipid.

The present experiments have shown the applicability of the dual-labeled isotope technique for elucidation of metabolic reactions. The hen has been shown to transfer cis and trans isomers across membranes at about the same rate and to preferentially incorporate the cis isomer in the neutral lipid and the trans isomer in the phospholipid. Incorporation into the phospholipid involves a reaction that results in the loss of the carboxyl carbon. Deuterium isotope techniques developed for these experiments should find application to human nutrition studies in which radiochemicals cannot be used.

REFERENCES