Mixed Substrates: trans- and cis-[1-14C]Monounsaturated Fatty Acids with a Fairly Uniform Distribution of Positional Isomers

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Simple methods are described for the preparation of trans-[1-14C]monounsaturated fatty acids and cis-[1-14C]monounsaturated fatty acids, each with a fairly uniform distribution of positional isomers, which can serve as mixed substrates in biochemical studies. Methyl trans-[1-14C]octadecenoates and methyl trans-[1-14C]eicosenoates are prepared by partial catalytic hydrogenation of methyl [1-14C]linolenate and methyl [1-14C]arachidonate, respectively, followed by argentation chromatography of the partially hydrogenated products. Methyl trans-[1-14C]octadecenoates and methyl trans-[1-14C]eicosenoates, thus obtained, yield the corresponding methyl cis-[1-14C]octadecenoates and methyl cis-[1-14C]eicosenoates, respectively, after trans-cis equilibration and argentation chromatography. Hydrolysis of each of these methyl esters yields the fatty acids.

Introduction

Mixtures of a great variety of isomeric monounsaturated fatty acids are known to occur in plants [1], animals [2, 3], humans [4] and dietary fats [5] for humans. In a great number of nutritional and biochemical studies, positional and geometrical isomers of octadecenoic [6—12] and docosanoic [13] acids and their esters have been used as substrates individually — rather than in form of isomeric mixtures. Many of the monounsaturated fatty acids differing in chain-length, position and configuration of the double bond have been synthesized individually [14, 15]. Yet, the synthetic procedures involved are rather tedious and cumbersome, especially if a great number of radioactively labelled isomers are needed. Very few species of radioactively labelled and unlabelled isomeric monounsaturated fatty acids are so far commercially available.

In metabolic studies concerned with the specificity of enzymes for certain species of monounsaturated fatty acids, it might be of advantage to use mixtures of isomeric fatty acids as mixed substrates in a single experiment rather than the pure isomers individually. We wish to report simple methods for the preparation of mixtures of trans-[1-14C]monounsaturated fatty acids and mixtures of cis-[1-14C]-monounsaturated fatty acids, each with a nearly even distribution of positional isomers. The method involves partial catalytic hydrogenation of methyl esters of [1-14C]polyunsaturated fatty acids until the maximum formation of monounsaturated trans-isomers, which are then isolated by argentation chromatography. cis-Isomers are easily prepared from the trans-isomers by isomerization and subsequent argentation chromatography. Hydrolysis of the methyl esters yields the corresponding mixtures of isomeric fatty acids. Alternatively, derivatives, such as alcohols, aldehydes, acetates and alkylglycerols, which can also serve as mixed substrates or reference mixtures, are easily obtained from the methyl esters [16]. Recently we have used the mixtures of isomeric trans-[1-14C]octadecenoic acids and cis-[1-14C]octadecenoic acids, which were prepared by the methods described here, in the study of lipid metabolism in plant cell cultures [17].

Materials and Methods

[1-14C]Linolenic acid, 60 mCi/mmol, and [1-14C]-arachidonic acid, 50 mCi/mmol were obtained from Amersham Buchler GmbH, D-3300 Braunschweig, Germany. These acids, dissolved in diethyl ether-methanol, were converted to their methyl esters using diazomethane [18]. Unlabelled methyl linolenate and methyl arachidonate as well as other methyl esters used as reference compounds, were purchased from Nu-Chek-Prep, Elysian, Minn. 56028, USA. Palladium chloride/barium sulfate containing 10% palladium, triphenol and azobis-
isobutynitrile as well as all other reagents of analytical grade were obtained from E. Merck AG, D-6100 Darmstadt, Germany.

Hydrogenations were carried out in a 20 ml screw-capped reaction tube provided with a magnetic stirrer. The tube was fitted with a Teflon-lined septum, through which two stainless steel needles were inserted to serve as inlet and outlet for hydrogen or nitrogen. Palladium chloride/barium sulfate, 10 mg, was suspended in 4 ml hexane in the reaction tube and reduced to the active catalyst by bubbling pure hydrogen through the reaction mixture under stirring at ambient temperature for 10 min. The reaction tube containing the catalyst suspension was purged with nitrogen. Mixtures of either methyl [1-14C]linolenate, 50 μCi/0.83 μmol, and methyl linolenate, 0.18 mmol, or methyl [1-14C]-arachidonate, 50 μCi/μmol, and methyl arachidonate, 0.15 mmol, were dissolved in 2 ml hexane and added to the reaction tube. Hydrogenation was started by bubbling hydrogen through the reaction mixture under stirring at ambient temperature. At intervals of 2 min, the hydrogenation was interrupted by replacing hydrogen with nitrogen. Partially hydrogenated samples were withdrawn from the supernatant of the reaction mixture and analyzed by radio gas chromatography as follows.

A Hewlett-Packard Gas Chromatograph 5750 G (Hewlett Packard, San Diego, California, USA) equipped with a flame ionization detector was used in combination with a Packard Gas Proportional Counter, Model 894 (Packard Instrument Company, Inc., Downers Grove, Ill., USA). A glass column (1.8 m x 4 mm) was packed with 10% EGSS-X on Gas-Chrom P, 100 – 120 mesh (Applied Science Laboratories Inc., State College, Pa. 16801, USA) and held at 175 °C. Helium (85 ml/min) served as carrier gas; the effluent carrier gas was divided by a stream splitter, 5:1, connected to the proportional flow counter and flame ionization detector, respectively. The methyl esters of 14C labelled fatty acids were identified by comparing their retention times with those of reference compounds. The proportions of the various radioactively labelled components in each mixture were calculated as percentage area of the respective peak, as measured by triangulation.

Hydrogenation of polyunsaturated methyl esters was continued until a maximum proportion of monounsaturated methyl esters was formed.

Samples of the partially hydrogenated methyl esters were fractionated into trans- and cis-isomers by argentation chromatography [19] on a layer of silica gel containing 20% silver nitrate. The plates were developed twice with hexane:diethyl ether (9:1) at room temperature. The distribution of radioactivity on the thin-layer chromatograms was determined by means of a Berthold TLC Scanner LB 2760 (BF-Vertriebsgesellschaft, D-7547 Wildbach, Germany). The radioactive zones corresponding to methyl esters of trans- and cis-monounsaturated fatty acids were scraped off the plates and the methyl esters were eluted with ether, saturated with water. Both fractions of methyl esters were analyzed by radio gas chromatography, as described above.

Aliquots of all trans- and cis-methyl ester fractions were taken in scintillation vials, 10 ml of “Aquadas-2” (NEN Chemicals GmbH, D-6072 Dreieich, Germany) was added and radioactivity determined in a Packard Tri-Carb C.2425 Liquid Scintillation Counter.

Fractions of methyl trans-[1-14C]octadecenoates and methyl trans-[1-14C]eicosanoates obtained by partial hydrogenation of methyl linolenate and methyl arachidonate, respectively, were isomerized using thiophenol and azobisisobutynitrile [20]. The isomerized products were fractionated into trans- and cis-monounsaturated methyl esters by argentation thin-layer chromatography as described before.

Aliquots of trans- and cis-monounsaturated methyl esters, ca. 100 μg (ca. 0.2 μCi), were dissolved in pentane and ozonized at −70 °C using a Supelco Micro-Ozonizer (Supelco, Inc., Bellefonte, Pa. 16823, USA) [21]. The reaction products were evaporated to dryness at room temperature, dissolved in 40 μl carbon disulfide, and the ozonides were reduced with 1 – 3 mg of triphenylphosphine. After standing at room temperature for 30 min, the mixture of aldehydes and aldesters was analyzed by radio gas chromatography, using a column, 1.8 m x 4 mm, packed with a 5:1 mixture of 10% OV-17 and 3% OV-1, each on Gas-Chrom Q, 100 – 120 mesh (Applied Science Laboratories Inc.). The temperature was programmed from 70 °C to 270 °C, 4 °C/min. Identification of fragments was made by comparison with the retention times of aldehydes, obtained by ozonolysis of monounsaturated methyl esters of different chain-lengths and known positions of double bonds. Quantitative results were obtained.
from peak areas of [1-\(^{14}\)C] aldehydes, measured by triangulation. As an example, Fig. 1 shows a radio gas chromatogram of the ozonolysis products of methyl trans-[1-\(^{14}\)C] octadecenoates, obtained by partial hydrogenation of methyl [1-\(^{14}\)C] linolenate. The trans- and cis-[1-\(^{14}\)C] monounsaturated fatty acids were prepared by hydrolysis of the corresponding methyl esters [22].

**Results and Discussion**

Partial hydrogenation of polyunsaturated fatty acids and their alkyl or glyceryl esters using metal catalysts is known to yield mixtures of trans- and cis-monounsaturated fatty acids or the corresponding esters having double bonds widely distributed throughout the hydrocarbon chain [23]. Based on this fact, we have attempted to develop a simple method for the preparation of mixtures of positional isomers of trans- and cis-[1-\(^{14}\)C] monounsaturated fatty acids with uniform distribution of isomers. The method involves partial hydrogenation of methyl esters of radioactively labelled polyunsaturated fatty acids, that are commercially available.

Methyl [1-\(^{14}\)C]linolenate, 50 \(\mu\)Ci/0.18 mmol, after hydrogenation for 6 min, yields a product containing approximately 80% methyl octadecenoates, 15% methyl octadecanoate and 5% methyl octadecadienoates. This reaction product is fractionated into methyl trans- and cis-octadecenoates by argentation chromatography. The methyl trans-octadecenoates contain ca. 45% of the radioactivity which was present in methyl linolenate. The fraction containing methyl cis-octadecenoates is found to contain ca. 15% of the label from methyl linolenate. The composition of positional isomers in methyl trans- and cis-[1-\(^{14}\)C] octadecenoates, determined by reductive ozonolysis and radio gas chromatography, is given in Table I. It is evident that the methyl trans-octadecenoates are composed of positional isomers ranging from 6 to 16 with a fairly uniform distribution from 9 to 15. The methyl cis-octadecenoates contain each of the positional isomers ranging from 8 to 15, however, the distribution of these isomers is highly nonuniform; pronounced maxima are found at 9, 12 and 15 isomers.

Methyl [1-\(^{14}\)C] arachidonate, 50 \(\mu\)Ci/0.15 mmol, after 10 min of hydrogenation, yields a product containing approximately 60% methyl eicosanoates, 25% methyl eicosanoate and 15% methyl eicosadienoates. Argentation chromatography of this product yields methyl trans-eicosanoates and methyl cis-eicosanoates containing ca. 25% and 15%, respectively, of the radioactivity present in methyl arachidonate. The composition of mixtures of positional isomers in methyl trans- and cis-[1-\(^{14}\)C] eicosanoates is also included in Table I. It is evident that the methyl trans-eicosanoates are composed of positional isomers ranging from 4 to 16 with a fairly uniform distribution from 5 to 15, whereas the positional isomers in the mixture of methyl
Table I. Percentage composition of positional isomers of methyl esters of trans- and cis-[1-14C]monounsaturated fatty acids obtained via trans-cis equilibration after partial hydrogenation.

<table>
<thead>
<tr>
<th>Positional isomer (A)</th>
<th>Methyl [1-14C]octadecenoates</th>
<th>Methyl [1-14C]eicosenoates</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>trans a</td>
<td>cis a</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>2</td>
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<tr>
<td>5</td>
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<td>13</td>
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<tr>
<td>16</td>
<td>5</td>
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</tr>
</tbody>
</table>

a via partial hydrogenation.
b via trans-cis equilibration after partial hydrogenation.

cis-eicosenoates show pronounced maxima at A5, A8, A11 and A14.

The results presented show that methyl esters of trans-[1-14C]monounsaturated fatty acids with fairly uniform distribution of positional isomers are conveniently prepared in good yields by partial hydrogenation of labelled polyunsaturated methyl esters and subsequent argentation chromatography. In order to assess the reproducibility of this method, unlabelled methyl linolenate and arachidonate were partially hydrogenated until levels ranging from 10 to 30% saturated methyl esters were attained. Methyl trans-octadecenoates and methyl trans-eicosenoates, isolated from the partially hydrogenated products, were analyzed by reductive ozonolysis and gas chromatography [24]. It was found, that although the yield of trans-monounsaturated methyl esters varied with the degree of hydrogenation, the distribution of positional isomers in methyl trans-octadecenoates and methyl trans-eicosenoates was independent of the degree of hydrogenation. Thus, it is evident, that the fairly uniform pattern of distribution of positional isomers in trans-[1-14C]-monounsaturated methyl esters, obtained by partial hydrogenation of polyunsaturated methyl esters, can be easily reproduced.

trans-cis Isomerization of unsaturated methyl esters by free radicals is known to occur without migration of double bonds [20, 25]. We have applied this principle for the preparation of uniformly distributed mixtures of positional isomers of cis-[1-14C]monounsaturated fatty acids from methyl esters of trans-[1-14C]monounsaturated fatty acids, that are obtained by partial hydrogenation, as described above.

Methyl trans-[1-14C]octadecenoates, 10 µCi/0.03 mmol, prepared by partial hydrogenation and argentation chromatography, as described above, yield, on isomerization using thiophenol in the presence of azobisisobutylnitrile [20], an equilibrium mixture containing 20% methyl cis-octadecenoates. Separation by argentation chromatography yields methyl cis-[1-14C]octadecenoates representing ca. 18% of the label from methyl trans-[1-14C]octadecenoates. Similarly, methyl trans-[1-14C]eicosenoates, 5 µCi/0.015 mmol, yield, after isomerization and argentation chromatography, the methyl cis-[1-14C]-eicosenoates containing ca. 16% of the label from the starting material. The composition of positional isomers in methyl cis-[1-14C]octadecenoates and methyl cis-[1-14C]eicosenoates, obtained via trans-cis equilibration, is given in Table I. It is evident that the methyl cis-octadecenoates contain positional isomers ranging from A9 to A15 in a fairly uniform distribution. Similarly, the methyl cis-eicosenoates contain positional isomers ranging from A5 to A15 in a fairly uniform distribution. The results show that methyl esters of cis-[1-14C]monounsaturated fatty acids with fairly even distribution of positional isomers are conveniently prepared via trans-cis equilibration of the corresponding trans-isomers, prepared by partial hydrogenation of polyunsaturated methyl esters.

The methyl trans- and cis-octadecenoates as well as eicosenoates yield the corresponding fatty acids on hydrolysis. Reesterification with diazomethane and subsequent analysis by reductive ozonolysis and radio gas chromatography shows that the isomeric composition of the methyl esters is not altered by hydrolysis.