Identification of an Acyl Donor in Steryl Ester Biosynthesis by Enzyme Preparations from Spinach Leaves

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ABSTRACT

A pathway for steryl ester biosynthesis in acetone powder preparations from spinach (Spinacia oleracea L.) leaves has been elucidated. Free sterol and 1,2-diglyceride were the substrates. Although animals synthesize cholesteryl esters by three distinct biosynthetic pathways, none of these pathways utilizes 1,2-diglyceride as an acyl donor. Phosphatidylincholine, phosphatidic acid, triglyceride, 1,3-diglyceride, 1-monoglyceride, free fatty acid, and fatty acyl-CoA were not acyl donors for spinach leaf steryl ester biosynthesis in our assay system. The unstable 2 isomer of monoglyceride was not tested. It is possible that 1,2-diglyceride and 2-monoglyceride were both acyl donors for spinach leaf steryl ester biosynthesis. Acyl-labeled phosphatidylincholine and acyl-labeled phosphatidylethanolamine were rapidly degraded by acetone powder preparations to 1,2-diglyceride via phosphatic acid. The 1,2-diglycerides were slowly metabolized to monoglycerides, triglycerides, free fatty acids, and steryl esters. The monoglycerides were rapidly degraded to free fatty acids and glycerol.

The acyl donors and the enzymes involved in steryl ester biosynthesis in higher plants have not been identified. In animals cholesterol esterase (10), fatty acyl-CoA-cholesterol acyltransferase (9), and phosphatidylincholine-cholesterol acyltransferase (8) catalyze the esterification of a fatty acid from free fatty acid, fatty acyl-CoA, and phosphatidylincholine, respectively, to free cholesterol, forming steryl esters. Bartlett et al. (1) have recently shown the biosynthetic pathways for fungal steryl ester biosynthesis to be the same as those established for animal choleseryl ester biosynthesis. Kolattukudy (13, 14) has shown that the wax ester biosynthetic pathway in broccoli leaves are mechanistically analogous to the three pathways catalyzed by animals in cholesteryl ester biosynthesis. In both broccoli leaf wax ester biosynthesis and animal cholesteryl ester biosynthesis the acyl donors are free fatty acid, fatty acyl-CoA, and phospholipid. It is not unreasonable to assume, therefore, that higher plants may utilize one or more of these acyl donors in the biosynthesis of steryl esters. Our investigation indicates, however, that spinach leaves employed a unique steryl ester biosynthetic pathway in which 1,2-diglyceride was the acyl donor. Although phospholipids were promising in our initial studies, an extensive product analysis indicated that they were rapidly metabolized by phospholipase D and phosphatidic acid phosphatase to 1,2-diglycerides. These two enzymes are frequently found in plants (11, 17, 18).

MATERIALS AND METHODS

Materials. Sodium [1,2-14C]acetate (55 mCi/mmol) was purchased from International Chemical and Nuclear Corp., Irvine, Calif. [4-14C]Cholesterol (56 mCi/mmol), [16-14C]palmitic acid (18.7 mCi/mmol), and [1-14C]palmitoyl-CoA (57.8 mCi/mmol) were obtained from New England Nuclear. [1-14C]Palmitate-labeled dipalmitin (116 mCi/mmol) and [1-14C]palmitate-labeled monopalmitin (58 mCi/mmol) were obtained from Dhom Products, Ltd., North Hollywood, Calif. Tripalmitin, 1,3-dipalmitin, 1,2-diolein, 1,2-dipalmitin, 1-monopalmitin, palmitoyl-CoA, cholesteryl palmitate, and BSA3 were obtained from Sigma Chemical Company. All other chemical were reagent grade or better. Chloroform, methanol, acetone, toluene, and benzene were glass-distilled. Fresh spinach (Spinacia oleracea L.) was purchased from the local markets.

Acetone Powder Preparation. In most cases, the acetone powder of the 20,000g pellet fraction from spinach leaves was prepared as described previously (6). The acetone powder used in the [1-14C]palmitate-labeled dipalmitin and [1-14C]palmitate-labeled monopalmitin assays was prepared in a slightly different manner. In this case, the homogenizing buffer contained 10 mM MES-NaOH (pH 6); the 20,000g subcellular suspension was added to 120 ml of acetone at 0 C; and the precipitated protein was washed with 120 ml of acetone at 0 C. All other procedures were as described previously (6).

Acetone-Ether Powder Preparation. The acetone-ether powder of the 20,000g pellet fraction from spinach leaves was prepared as described previously (7), except the dried acetone powder was resuspended in water instead of 100 mM MES-NaOH (pH 6).

[16-14C]Palmitic Acid Reaction Mixtures. The reaction mixtures were the same as those described for the [14C]acyl-labeled phosphatidylcholine reaction mixtures, except the assays contained 28,800 cpm [16-14C]palmitic acid (18.7 mCi/mmol).

[1-14C]Palmitoyl-CoA Reaction Mixtures. The unlabeled cholesterol was added to the acetone powder in acetone at 0 C. The acetone powder was dried under vacuum at 0 C before the enzyme-cholesterol mixture was homogenized to uniformity in cold 100 mM MES-NaOH (pH 6) with a Potter-Elvehjem homogenizer. The reaction mixtures contained 44,000 cpm [1-14C]palmitoyl-CoA (57.8 mCi/mmol). All other reaction mixture components and experimental procedures were as described for the [14C]acyl-labeled phosphatidylcholine reaction mixtures.

[1-14C]Acyl-labeled Phosphatidylcholine Reaction Mixtures. The labeled phosphatidylcholine and unlabeled cholesterol were added

3 Abbreviations: ASG: acylated steryl glucoside; BSA: essentially fatty acid-free bovine albumin; DG: diglyceride (dicylglycerol); EP: ethyl palmitate; FFA: free fatty acid; MG: monoglyceride (monoaoylglycerol); O: origin; PA: phosphatidic acid; PC: phosphatidylcholine; PE: phosphatidylethanolamine; SE: steryl ester; TG: triglyceride (triaoylglycerol).
to the acetone powder in diethyl ether at 0 C. The acetone powder was
dried under vacuum at 0 C before the enzyme-substrate
mixture was homogenized to uniformity in cold 100 mm MES-
NaOH (pH 6) with a Potter-Elvehjem homogenizer. The reaction
mixtures contained 34,000 dpm [14C]acyl-labeled phosphatidyl-
choline (0.66 mCi/mmoll, 0.2 mmole cholesterol, 100 mm MES-
NaOH (pH 6), 10 mg of acetone powder of the 20,000 g pellet
fraction (3 mg of protein), and water to make a final volume of 1
ml. The reaction was initiated by adding 3 ml of CHCl3-
CH3OH (1:2, v/v). Whenever enzymic catalysis was evident in the
0-min incubation, this background was subtracted from the
metabolites.

[14C]Acyl-labeled 1,2-Diglyceride Reaction Mixtures. The re-
action mixtures were the same as those described for the [14C]acyl-
labeled phosphatidylcholine reaction mixtures, except the assays
contained 20,000 dpm [14C]acyl-labeled 1,2-diglyceride (<0.66
mCi/mmoll).

[1-14C] Palmitate-labeled Dipalmitin and [1-14C] Palmitate-
labelled Monopalmitin Reaction Mixtures. The reaction mixtures
were the same as those described for the [14C]acyl-labeled phos-
phatidylcholine reaction mixtures, except the assays contained
either 100,000 cpm [1-14C]palmitate-labeled dipalmitin (116
mCi/mmoll) or 100,000 cpm [1-14C]palmitate-labeled monopal-
mitin (58 mCi/mmoll).

Extraction, Isolation, and Counting of Acyl-labeled Lipids
(Method A). The lipids were extracted according to the method of
Bligh and Dyer (2) and separated into individual components by
TLC. The lipid samples were applied in small aliquots of CHCl3
to silica Gel G TLC plates that had been activated at 110 C for
1 hr. Neutral lipids were resolved by developing the plates in
hexane-diethyl ether-acetic acid (70:30:1, v/v). Polar lipids were
resolved by developing the plates in chloroform-methanol-acetic
acid-water (85:15:3:2, v/v). The radioactive areas were detected by
autoradiography and identified by their co-chromatography with
standards (cholesterol, palmitate, tripalmitin, palmitic acid,
1,2-dipalmitin, 1-monopalmitin, methyl stearate, phosphatidyl-
choline, phosphatidylycholine, and phosphatidic acid). The
standards were detected by spraying with 1% (w/v) iodine in
methanol. The radioactive areas were scraped into scintillation
vials, 10 ml of liquid scintillation fluid (10 g of PPO + 0.6 g of
POPOP + 2 liters of toluene) were added to each vial, and
the samples were counted for 10 min in a Nuclear-Chicago 720 series
liquid scintillation system. Quenching was corrected by the channel
ratio method.

Extraction, Isolation, and Counting of Acyl-labeled Lipids
(Method B). This method was used only for the [1-14C]palmitate-
labeled dipalmitin and [1-14C]palmitate-labeled monopalmitin as-
lays. The lipids were extracted by a modified Bligh and Dyer
method (2) in which 1 ml of CHCl3 and 0.8 ml of 2 N KCl in 0.5
m K-phosphate at pH 7.3 were added to the 4 ml of monophasic
CHCl3-CH3OH-H2O (1:2:1, v/v) solution. This gave a distinct
biphasic CHCl3:CH3OH-H2O (2:2:1, v/v) solution. The CHCl3
phase was removed, dried under vacuum, and redissolved in a
small aliquot of CHCl3. The lipids were separated by one-dimen-
sional silica Gel G TLC using a two-step development system as
described by Skipski and Barclay (20). The first development
was done in isopropanol ether-acetic acid (96:4, v/v), and the second
development was done in hexane-diethyl ether-acetic acid
(90:10:1, v/v). All other procedures were as described in method
A, except the samples were counted for 10 min in a Beckman LS-
230 liquid scintillation system.

[4-14C] Cholesterol Reaction Mixtures. The [4-14C] cholesterol
and unlabeled cholesterol were added to the acetone powder in
acetone at 0 C. The acetone powder was dried under vacuum at
0 C before the enzyme-substrate mixture was homogenized to
uniformity in cold 100 mm MES-NaOH (pH 6) with a Potter-
Elvehjem homogenizer. The reaction mixtures contained 44,400
dpm [4-14C]cholesterol (50 mCi/mmoll), and the incubation time
was either 20 or 60 min. All other reaction mixtures components
and experimental procedures were as described for the [14C]acyl-
labeled phosphatidylcholine reaction mixtures. The [4-14C]choles-
teryl esters were extracted, isolated, and counted as described
previously (6).

AgNO3 TLC. The biosynthesized [4-14C]cholesterol esters were
separated into different fatty acyl groups depending upon their
degree of unsaturation by AgNO3 TLC as described previously
(7).

Preparation of [14C] Acyl-labeled Phosphatidylcholine and [14C] Acyl-
labeled Phosphatidylethanolamine. [14C] Acyl-labeled phos-
phatidylcholine and [14C] acyl-labeled phosphatidylethanolamine
were biosynthesized as outlined by Galliard (5), using 87 mCi of
sodium [1-14C]acetate (55 mCi/mmoll) as the starting material
and 27 g of potato discs from White Rose potato tubers as the
enzyme source. The labeled phospholipids were partially purified
by DEAE-cellulose column chromatography as described by
Galliard (5). The glycolipid contaminants were removed by silicic
acid chromatography as outlined by Vorbeck and Marinetti (23).
The purified [14C] acyl-labeled phosphatidylcholine (0.66
mCi/mmoll) and [14C] acyl-labeled phosphatidylethanolamine
(0.27 mCi/mmoll) gave single autoradiography spots when the
Silica Gel TLC plates were developed in chloroform-methanol-
acetic acid-water (34:6:4:1, v/v) or chloroform-methanol-acetic
acid-water (25:15:2:4, v/v).

Preparation of [14C] Acyl-labeled 1,2-Diglyceride. The [14C]acyl-
labeled 1,2-diglyceride was prepared by incubating 50 mg of
acetone powder preparation with 170,000 dpm [14C]acyl-labeled
phosphatidylcholine (0.66 mCi/mmoll) for 10 min. The reaction
mixtures were as described for the [14C] acyl-labeled phosphatidyl-
choline assays. The [14C] acyl-labeled 1,2-diglyceride was purified
by preparative Silica Gel G TLC using hexane-diethyl ether-acetic
acid (70:30:1, v/v) as the solvent system. The labeled 1,2-diglycer-
ides were scraped onto filter paper and eluted with CHCl3 into
a drying flask. The sample was then dried under vacuum and
dissolved in a small aliquot of CHCl3-CH3OH (9:1, v/v). Silica
Gel TLC in hexane-diethyl ether-acetic acid (70:30:1, v/v)
indicated that the labeled 1,2-diglycerides were 89% pure.

Analytical Method. Protein was determined by the method of
Lowry et al. (15).

RESULTS

[16-14C] Palmitic Acid. [16-14C] Palmitic acid was not incor-
porated into steryl esters when incubated with a spinach leaf acetone
powder preparation (data not shown). Autoradiography analysis of
the thin layer chromatograms of the lipid extract revealed that
palmitic acid was the only labeled lipid in these assays. Control
experiments indicated that this acetone powder preparation was
able to incorporate [4-14C] cholesterol into [4-14C] cholesteryl ester.

[1-14C] Palmitoyl-CoA. A small amount of label from [1-14C]
palmitoyl-CoA was incorporated into cholesteryl [1-14C] palmitate,
as shown in Table 1. It is not clear, however, if the label was
directly or indirectly incorporated into steryl ester. The data
indicate that free fatty acid was the most highly labeled product,
followed by diglyceride. Phosphatidylcholine and phosphatidyl-
ethanolamine were not significantly labeled in the 120-min incu-
bation (Table 1) nor in shorter incubation times (data not shown).

The MG + ASG? + X assignment in Table 1 is a combination of
at least three lipids that were not successfully resolved on the
thin layer chromatograms. Additional TLC indicated that MG
was a very minor component, accounting for only 3% of the label
in MG + ASG? + X. ASG accounted for 51% of the label, but its
Table 1. Product Analysis for the Metabolism of [1-14C]Palmitoyl-CoA

| Lipid | cpm | %
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PC</td>
<td>0</td>
<td>0.0</td>
</tr>
<tr>
<td>PE</td>
<td>39</td>
<td>0.1</td>
</tr>
<tr>
<td>X1</td>
<td>184</td>
<td>0.6</td>
</tr>
<tr>
<td>MC + ASC + X</td>
<td>3,750</td>
<td>11.0</td>
</tr>
<tr>
<td>X2</td>
<td>633</td>
<td>1.1</td>
</tr>
<tr>
<td>DG</td>
<td>4,750</td>
<td>15.7</td>
</tr>
<tr>
<td>FFA</td>
<td>20,100</td>
<td>66.3</td>
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<tr>
<td>TG</td>
<td>591</td>
<td>1.9</td>
</tr>
<tr>
<td>SE</td>
<td>695</td>
<td>2.3</td>
</tr>
</tbody>
</table>

Table 1. Product Analysis for the Metabolism of [1-14C]Palmitoyl-CoA

Reaction mixtures were as described under Materials and Methods. Incubation time was 120 min. Polar lipids (PC, PE, X1, and MC + ASC + X) and neutral lipids (X2, DG, FFA, TG, and SE) were extracted, isolated, and counted as described for acyl-labeled lipids under method A of Materials and Methods. Samples were counted in a Beckman LS-230 liquid scintillation system. X1: a polar lipid that chromatographed just above PE in the polar solvent system; X2: a polar lipid that chromatographed just below DG in the neutral solvent system.

Fig. 1. [14C]Acyl-labeled phosphatidylcholine time course. Reaction mixtures were as described under “Materials and Methods.” Reactions were terminated by adding 0.15 ml of 80% trichloroacetic acid, followed by 3 ml of CHCl3-CH3OH (1:2, v/v). Acyl-labeled lipids were extracted, isolated, and counted as described under method A of “Materials and Methods.”

Identification is tentative. The remaining 46% has not been identified.

Steryl ester biosynthesis was relatively insensitive to 5 mg of BSA when cholesteral was the labeled precursor but was almost completely inhibited by it when palmitoyl-CoA was the labeled precursor (data not shown). Serum albumin tightly binds long chain fatty acids (21) and assumingly binds the CoA derivatives of fatty acids as well (12, 22). The binding of palmitoyl-CoA by BSA would make it unavailable to enzymatic attack.

[14C]Acyl-Labeled Phospholipids. Figure 1 is a time course for the degradation of [14C]acyl-labeled phosphatidylcholine in a spinach leaf acetone powder preparation. Labeled phosphatidic acid was detected but only when the protein was precipitated by either trichloroacetic acid, as in the case of Figure 1, or by boiling methanol (data not shown). Control experiments indicated that trichloroacetic acid was not hydrolyzing phosphatidylcholine to phosphatidic acid nor to 1,2-diglyceride. Furthermore, the labeled phosphatidylcholine was not degraded if the acetone powder preparation had been boiled for 5 min or omitted from the reaction mixture, indicating that enzymatic reactions were involved.

There was a rapid disappearance of label from phosphatidylcholine and a rapid incorporation of label into 1,2-diglyceride (Fig. 1). Only a trace of 1,3-diglyceride was detected. Label also appeared in steryl ester and free fatty acid but less rapidly than into 1,2-diglyceride. After the label in phosphatidylcholine had become depleted, the label in 1,2-diglyceride slowly disappeared, suggesting that 1,2-diglyceride may have been responsible for the continued incorporation of label into steryl ester and free fatty acid. A preliminary investigation with [14C]acyl-labeled phosphatidylethanolamine indicated that a similar product distribution was obtained with this phospholipid.

Small amounts of labeled triglycerides and monoglycerides were also detected in the labeled phosphatidylcholine reaction mixtures (data not shown). Labeled lysophosphatidylcholine was not detected in these assays.

[14C]Acyl-labeled 1,2-Diglyceride. Figure 2 compares the rate of steryl ester biosynthesis in a [14C]acyl-labeled phosphatidylcholine assay with an assay containing only the labeled 1,2-diglyceride that was generated in a [14C]acyl-labeled phosphatidylcholine assay. The rate of steryl ester biosynthesis in Figure 2 was the same whether phosphatidylcholine or 1,2-diglyceride was the labeled precursor. All of the fatty acids that were esterified to cholesterol in the phosphatidylcholine assays, therefore, came either directly from 1,2-diglyceride or from a catabolite of 1,2-diglyceride, such as monoglyceride, and little, if any, came directly from either phosphatidylcholine or phosphatidic acid.

The disappearance of label from 1,2-diglyceride was accompanied by the appearance of label in steryl ester and free fatty acid (Fig. 3). Steryl ester was the most heavily labeled product throughout the time course. Monoglycerides and triglycerides were also detected (data not shown) but were minor components.

[1-14C]Palmitate-labeled Dipalmitin and [1-14C]Palmitate-labeled Monopalmitin. Table 2 shows a product analysis for the metabolism of [1-14C]palmitate-labeled dipalmitin and [1-14C]palmitate-labeled monopalmitin. Free fatty acid, monoglyceride, triglyceride, and steryl ester were all major products in the metabolism of dipalmitin. Label, however, was more rapidly incorporated into free fatty acid and monoglyceride than into triglyceride and steryl ester. Monopalmitin was rapidly degraded into free fatty acid and glycerol. In this case, free fatty acid and diglyceride were the major products, whereas triglyceride and steryl ester were minor products. Monopalmitin was more susceptible to degradation than was dipalmitin.

Fig. 2. Incorporation of label from [14C]acyl-labeled phosphatidylcholine and [14C]acyl-labeled 1,2-diglyceride into steryl ester. Reaction mixtures were as described under “Materials and Methods.” After 10 min of incubation, [14C]acyl-labeled phosphatidylcholine assays contained 14,000 dpm (■) and 19,000 dpm 1,2-diglyceride (○). In [14C]acyl-labeled 1,2-diglyceride assays, 20,000 dpm 1,2-diglyceride (○) from a 10-min [14C]acyl-labeled phosphatidylcholine assay was added to 10 mg of acetone powder. Acetone powders used in all of these assays were different aliquots from the same acetone powder preparation. In order to make a valid comparison between [14C]acyl-labeled phosphatidylcholine and [14C]acyl-labeled 1,2-diglyceride assays, only the rate of steryl ester biosynthesis after the first 10 min of incubation in the [14C]acyl-labeled phosphatidylcholine assays was plotted. Label in steryl esters were isolated by silica gel TLC in hexane-diethyl ether-acetic acid (70:30:1, v/v), detected by autoradiography, and counted as described for acyl-labeled lipids under method A of “Materials and Methods.” [14C]Acyl-labeled phosphatidylcholine assays (■, ○), [14C]acyl-labeled 1,2-diglyceride assays (○).
FIG. 3. [14C]Acyl-labeled 1,2-diglyceride time course. Reaction mixtures were as described under “Materials and Methods.” Acyl-labeled lipids were extracted, isolated, and counted as described under method A of “Materials and Methods.”

Table II. Product Analysis for the Metabolism of [1-14C]Palmitate-labeled Dipalmitin and [1-14C]Palmitate-labeled Monopalmitin

<table>
<thead>
<tr>
<th>Labeled Lipids</th>
<th>Dipalmitin Assays</th>
<th>Monopalmitin Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm</td>
<td>%</td>
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<tr>
<td>0</td>
<td>527</td>
<td>0.7</td>
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<tr>
<td>X1</td>
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<tr>
<td>MG</td>
<td>2,270</td>
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<tr>
<td>DC</td>
<td>66,400</td>
<td>83.5</td>
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<td>FFA</td>
<td>6,620</td>
<td>8.3</td>
</tr>
<tr>
<td>TG</td>
<td>1,660</td>
<td>2.1</td>
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<td>EP</td>
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<td>0.2</td>
</tr>
<tr>
<td>SE</td>
<td>1,400</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*14C]Palmitate-labeled dipalmitin and [1-14C]labeled monopalmitin were synthesized chemically from glycerol and [1-14C]palmitoyl chloride by Dom Products Ltd. Since blocking groups were not used in their synthesis, dipalmitin was a mixture of the 1,2 and 1,3 isomers, and monopalmitin was a mixture of the 1,2 and 2,3 isomers. The 1,2 isomer of dipalmitin contained equal amounts of two isomers that were mirror images of each other (enantiomers). The 1 isomer of monopalmitin also contained equal amounts of two isomers that were mirror images of each other. An equilibrium mixture of diglycerides contains about 60% of the 1,2 isomer and about 40% of the 1,3 isomer (16, 19), whereas an equilibrium mixture of monoglycerides contains about 90% of the 1 isomer and about 10% of the 2 isomer (16, 19).

Figure 4 compares the ability of [1-14C]palmitate-labeled dipalmitin and [1-14C]palmitate-labeled monopalmitin to serve as acyl donors in steryl ester biosynthesis. Dipalmitin was a better acyl donor than was monopalmitin. At 5 min there was six times as much steryl ester in the dipalmitin assays as in the monopalmitin assays and at 80 min there was three times as much.

Stimulation of [4-14C]Cholesteryl Ester Biosynthesis by Unlabeled Acyl Donors. Table III tests the ability of various unlabeled acyl lipids to stimulate the incorporation of [4-14C]cholesterol into [4-14C]cholesterol ester in a spinach leaf acetone-ether powder preparation. The concentration of each acyl lipid was 0.2 mM. Table III shows that: (a) 1,2-diolein and 1,2-dipalmitin stimulated steryl ester biosynthesis; (b) 1,2-diolein stimulated steryl ester biosynthesis more extensively than did 1,2-dipalmitin; (c) 1-monopalmitin and tripalmitin did not stimulate steryl ester biosynthesis; (d) 1,3-dipalmitin and palmitoyl acid stimulated steryl ester biosynthesis only slightly; (e) palmitoyl CoA inhibited steryl ester biosynthesis; (f) PC and PE stimulated steryl ester biosynthesis the most effectively of all the acyl lipids tested; and (g) PE stimulated steryl ester biosynthesis more extensively than did PC.

Table IV compares the effect of 1,2-diolein and 1,2-dipalmitin on the fatty acid composition of [4-14C]cholesterol esters. The control in Table IV indicates that the predominant steryl ester biosynthesized in a spinach leaf acetone powder preparation contained saturated fatty acids. There was a dramatic increase in monounsaturated fatty acids, however, when 1,2-diolein was added to the incubation mixtures. The 1,2-diolein stimulation of steryl ester biosynthesis, therefore, was primarily due to an increased incorporation of oleate from 1,2-diolein into [4-14C]cholesterol oleate. A certain amount of nonspecific activation may have occurred, however, since there was a slight increase in the amount of steryl esters with saturated fatty acids in the 1,2-diolein incubation.

DISCUSSION

The results show that some of the label from palmitoyl-CoA was incorporated into steryl ester. The inability of BSA to inhibit

FIG. 4. Incorporation of label from [1-14C]palmitate-labeled dipalmitin and [1-14C]palmitate-labeled monopalmitin into steryl esters. Reaction mixtures were as described under “Materials and Methods.” Acyl-labeled steryl esters were extracted, isolated, and counted as described under method B of “Materials and Methods.”

Table III. Effect of Various Lipids on [4-14C]Cholesteryl Ester Biosynthesis

<table>
<thead>
<tr>
<th>Exp. No.</th>
<th>Lipid Addition (0.2 mM)</th>
<th>Organic Solvent Used in Adding Lipids to Acetone-Ether Powder</th>
<th>20 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>SE Activity</td>
<td>% SE Activity</td>
</tr>
<tr>
<td>1</td>
<td>Control</td>
<td>&lt;5 C Benzene</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1,2-Dipalmitin</td>
<td>&lt;5 C Benzene</td>
<td>134</td>
<td>163</td>
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<tr>
<td>1,3-Dipalmitin</td>
<td>&lt;5 C Benzene</td>
<td>295</td>
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<tr>
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<td>Control</td>
<td>0 C Diethyl Ether</td>
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<td>100</td>
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<tr>
<td>1,2-Dipalmitin</td>
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<td>1,3-Dipalmitin</td>
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<td>Tripalmitin</td>
<td>0 C Diethyl Ether</td>
<td>105</td>
<td>97</td>
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</tr>
<tr>
<td>3</td>
<td>Control</td>
<td>0 C Acetone</td>
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<tr>
<td>Palmitic Acid</td>
<td>0 C Acetone</td>
<td>134</td>
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<td>4</td>
<td>Control</td>
<td>0 C Diethyl Ether</td>
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<td>100</td>
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<td>PC (egg yolk)</td>
<td>0 C Diethyl Ether</td>
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<td>PE (egg yolk)</td>
<td>0 C Diethyl Ether</td>
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</tbody>
</table>
[4-14C]cholesteryl ester biosynthesis and its ability to inhibit the incorporation of [1-14C]palmitate from [1-14C]palmitoyl-CoA into steryl ester suggest that fatty acyl-CoA was not the acyl donor in these assays. The predominant reaction in the palmitoyl-CoA assays was its hydrolysis to palmitic acid and CoA, but palmitic acid was not the acyl donor since [16-14C]palmitic acid was not incorporated into steryl ester. Significant amounts of labeled 1,2-diglycerides, however, were produced, and they may have been the actual acyl donors.

The results suggest that the 1,2-diglyceride generated in the degradation of phosphatidylcholine was the acyl donor involved in spinach leaf steryl ester biosynthesis. The ability of [4-14C]acyl-labeled 1,2-diglyceride and [1-14C]palmitate-labeled dipalmitin to incorporate label into steryl ester as well as the ability of unlabeled 1,2-dipalmitin and 1,2-diolein to stimulate [4-14C]cholesteryl ester biosynthesis verify the 1,2-diglyceride acyl donor assignment.

The theoretical maximum amount of labeled monoglyceride produced by the end of the 60-min [1-14C]palmitate-labeled dipalmitin assay was approximately 9,000 cpm (monoglyceride + free fatty acid in Table II). This is less than the 90,000 cpm of 1-monopalmitin (45,000 cpm for each enantiomer) and about equal to the 10,000 cpm of 2-monopalmitin at the beginning of the [1-14C]palmitate-labeled monopalmitin assay. If either 1- or 2-monopalmitin were the exclusive or preferred acyl donor for steryl ester biosynthesis, then the initial rate of incorporation of label from monopalmitin into cholesteryl palmitate should have been much greater than the rate of incorporation of label from dipalmitin into cholesteryl palmitate. Since this was not the case (Fig. 4), it is most unlikely that 1- and 2-monopalmitin were the exclusive or preferred acyl donors.

Of the various acyl-labeled glycerides tested, [4-14C]acyl-labeled 1,2-diglyceride was the most effective in terms of total radioactivity added in incorporating label into steryl ester. [1-14C]Palmitate-labeled dipalmitin was the next most effective. [1-14C]Palmitate-labeled monopalmitin was the least effective. The heterogeneous fatty acid composition of [4-14C]acyl-labeled 1,2-diglyceride compared to the homogeneous fatty acid composition of [1-14C]palmitate-labeled dipalmitin may have accounted for the preference of [4-14C]acyl-labeled 1,2-diglyceride. The predominance of the 1,3 isomer as well as the presence of 1,2-dipalmitin enantiomers in [1-14C]palmitate-labeled dipalmitin may also have been important factors.

The ability of 1,2-dipalmitin and the inability of 1-monopalmitin and tripalmitin to stimulate steryl ester biosynthesis suggest an acylglycerol specificity (Table III). Since 1,2-dipalmitin stimulated steryl ester biosynthesis more effectively than did 1,3-dipalmitin, the enzyme exhibited a positional specificity. The ability of 1,2-diolein to stimulate steryl ester biosynthesis more effectively than 1,2-dipalmitin could mean that the enzyme exhibited a fatty acid specificity, but it could also mean that 1,2-diolein was more suitably dispersed in the aqueous solution and, therefore, was more available for enzymatic attack.

Since PC and PE were rapidly catabolized to 1,2-diglyceride by spinach leaf acetone powder preparations (Fig. 1), the PE (egg yolk) and PE (egg yolk) should also have been rapidly catabolized to 1,2-diglyceride (Table III). The greater stimulation with the egg yolk phopholipids could be explained if the heterogeneous fatty acid composition of their 1,2-diglyceride catabolites more nearly approximated the fatty acid specificity requirement of the enzyme than did 1,2-dipalmitin or 1,2-diolein. A second possibility is that the enzyme, like many membrane-bound enzymes (4), had a phospholipid cofactor requirement. A third possibility is that the generated diglyceride was the preferred isomer.

Figure 5 summarizes the various enzyme activities encountered in spinach leaf acetone powder preparations during this investigation. The appearance of [4-14C]acyl-labeled phosphatidic acid from [4-14C]acyl-labeled phosphatidylcholine suggests that phospholipase D was present. The disappearance of label from phosphatidic acid and the appearance of label in 1,2-diglyceride indicate the presence of phosphatidic acid phosphatase. Cheniae (3) has previously shown that spinach leaf microsomes synthesize phosphatidic acid, monoglyceride, diglyceride, and triglyceride when incubated with labeled sn-glycerol-3-P. He has also shown that phosphatidic acid in the precursor for these glycerides. This investigation has extended this earlier work by studying the metabolism of diglyceride and monoglyceride individually in spinach leaf acetone powder preparations and showing that their fatty acids are incorporated into several different metabolic pathways.

One involved the transfer of an acyl moiety from 1,2-diglyceride to a free sterol. Although this is a new steryl ester biosynthetic pathway, its universal occurrence in plants requires further testing.

**Table IV.** Effect of 1,2-Diolein and 1,2-Dipalmitin on the Fatty Acid Composition of [14C]Cholesteryl Esters

<table>
<thead>
<tr>
<th>Reaction Mixtures</th>
<th>Control</th>
<th>1,2-Dipalmitin</th>
<th>1,2-Diolein</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) (14C)Cholesteryl and unlabeled trioleate were added to the acetone powder.*</td>
<td>0.70%</td>
<td>0.70%</td>
<td>0.70%</td>
</tr>
<tr>
<td>(b) Rennin was added by free-feeding. Incubation time was 60 min.</td>
<td>0.45%</td>
<td>0.45%</td>
<td>0.45%</td>
</tr>
<tr>
<td>Samples were counted in a Nuclear-Chicago 720 series liquid scintillation system for 10 min and quenching was corrected by the channel ratio method.</td>
<td>0.45%</td>
<td>0.45%</td>
<td>0.45%</td>
</tr>
</tbody>
</table>

No. of Double Bonds in Acyl Mole of ST

<table>
<thead>
<tr>
<th>SE dpm</th>
<th>% Recovery</th>
<th>SE dpm</th>
<th>% Recovery</th>
<th>SE dpm</th>
<th>% Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>00</td>
<td>1,280</td>
<td>83</td>
<td>1,320</td>
<td>84</td>
<td>2,840</td>
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<tr>
<td>01</td>
<td>163</td>
<td>6</td>
<td>220</td>
<td>6</td>
<td>1,500</td>
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<tr>
<td>02</td>
<td>169</td>
<td>6</td>
<td>212</td>
<td>7</td>
<td>261</td>
</tr>
<tr>
<td>03</td>
<td>125</td>
<td>3</td>
<td>124</td>
<td>3</td>
<td>104</td>
</tr>
<tr>
<td>Total</td>
<td>2,717</td>
<td>1,957</td>
<td>4,605</td>
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</tr>
</tbody>
</table>

LITERATURE CITED


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ACYL DONOR IN STERYL ESTER BIOSYNTHESIS


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