

Fat Metabolism in Higher Plants. The Determination of Acyl-Acyl Carrier Protein and Acyl Coenzyme A in a Complex Lipid Mixture^{1,2}

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A method has been developed that permits the quantitative analysis of [¹⁴C]acyl-acyl carrier proteins and [¹⁴C]acyl CoAs from a typical reaction mixture. The method is based on (a) the initial extraction of free fatty acids and the less polar lipids into petroleum ether from aqueous isopropanol; (b) the precipitation of [¹⁴C]acyl-acyl carrier proteins in the presence of ammonium sulfate and chloroform-methanol; and (c) the final separation of acyl CoAs from the more polar lipids by selective adsorption on neutral alumina gel. All fractions can then be analyzed for the composition of complex lipids and ¹⁴C-labeled fatty acids by the usual methods.

Acyl thioesters have been determined in biological samples by converting these compounds to hydroxamate derivatives and then measuring the product colorimetrically as the ferric-hydroxamate complex (1). This procedure, however, has several disadvantages; that is, it is insensitive at the nanomole range and acyl oxygen esters can also interfere. The method of Barron and Mooney (2) appeared to overcome these limitations. The method was based on the selective reduction of acyl thioesters to the corresponding alcohols in the presence of acyl oxygen esters, using NaBH₄ and tetrahydrofuran. The acyl alcohols were readily analysed by gas chromatography. However, Nichols and Stafford (3) have recently demonstrated that the reactivity of acyl oxygen esters toward NaBH₄ was dependent on the polarity of the particular lipid as well as the concentration of tetrahydrofuran employed in the reaction. Thus, although triacylglycerols and free fatty acids were stable under the

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conditions of the Barron and Mooney method (2), the acyl oxygen esters of more polar lipids such as phospholipids and monogalactosyl diglycerides were in part reduced to the corresponding alcohols. Recently, a new method has been reported which determines enzymatically the total concentration of long chain acyl-CoA (4). Since this method, however, is not suitable for the analysis of the fatty acid composition of acyl CoAs, it cannot be employed in those studies involving modifications of the acyl chains nor in those where labeled fatty acids are used in the picomole range.

In this publication, a method is described that permits the quantitative analysis of acyl-acyl carrier proteins (acyl ACPs) and acyl CoAs in biological samples. The sensitivity of the procedure is limited only by the gas chromatography and counting equipment available.

MATERIALS AND METHODS

Materials. Trifluoroacetic anhydride, defatted bovine albumin, and the CdCl_2 complex of *sn*-glycerol 3-phosphorylcholine were from Sigma Chemical Company; neutral alumina AG 7 (100–200 mesh) was from Bio Rad Laboratories; Triton X-100, 2,5-diphenyloxazole (PPO), and dimethyl PPO were from Packard Instrument Company, Inc. and [^3H]acetic acid, [$1\text{-}^{14}\text{C}$]myristyl CoA, [$1\text{-}^{14}\text{C}$]palmitic acid, and [$1\text{-}^{14}\text{C}$]oleic acid were purchased from New England Nuclear. All other materials were of reagent grade and obtained from commercial sources.

Preparation of labeled substrates. Mixed potato [^3H]lipids were prepared from [^3H]acetate by the procedure of Galliard (5); [$\text{O-}^{14}\text{C}$]stearyl ACP⁴ was prepared from [$1\text{-}^{14}\text{C}$]acetate and *Escherichia coli* ACP by the procedure of Jaworski and Stumpf (6), and 1,2-di[$1\text{-}^{14}\text{C}$]oleyl lecithin was prepared from [$1\text{-}^{14}\text{C}$]oleate and the CdCl_2 complex of *sn*-glycerol 3-phosphorylcholine in the presence of trifluoroacetic anhydride by the procedure of Pugh and Kates (7).

Gas chromatography. The methyl esters were chromatographed on a Varian Aerograph model 920 gas chromatograph equipped with a thermal conductivity detector and connected to a Nuclear Chicago Biospan 4998 proportional tube radioactivity detector. The gas phase was helium maintained at a flow rate of 60 ml/min. The glc was equipped with a 5-ft stainless steel column (0.25 in. o.d.) packed with 10% EGG-S-X on 100–200 mesh Gas Chrom P operated at 150°C.

Liquid scintillation counting. The samples to be counted were dissolved in 1.0 ml H_2O to which was added 10.0 ml toluene–Triton X-100 (2:1, v/v) containing 3.5 g PPO and 0.1 g dimethyl PPO per liter. Radioactivity was monitored in a Beckman LS-230 liquid scintillation system.

Isolation of stearyl ACP. To simulate a typical reaction mixture, [$\text{O-}^{14}\text{C}$]

⁴ [$\text{O-}^{14}\text{C}$]Stearyl ACP was a gift from Dr. Ward E. Shine.

^{14}C]Stearyl ACP (25,940 cpm), [^{14}C]myristyl CoA (27,071 cpm), and 500- μg of spinach leaf lipids were dissolved in 1 ml isopropanol and 1 ml of 0.05 M potassium phosphate, pH 7.2 containing 10 μmoles MgCl_2 and 2 mg bovine albumin in a 16×125 mm screw-cap culture tube. The solution was acidified with 25 μl of glacial acetic acid and extracted three times with 2 ml of petroleum ether saturated with 50% aqueous isopropanol to remove the free fatty acids and less polar lipids. Two drops (50 μl) of saturated $(\text{NH}_4)_2\text{SO}_4$ solution were mixed with the washed aqueous phase and then 8 ml of chloroform-methanol (1:2, v/v) were added slowly to the vortexing solution. The mixture was set aside for 20 min at room temperature and then centrifuged for 5 min in a clinical centrifuge to pellet the salt-protein precipitate. The pellet and tube were rinsed with 5 ml of chloroform-methanol (1:2, v/v), the suspension was then centrifuged and the supernatant fractions which contained acyl CoAs were combined.

To analyse the acyl ACP fraction, the pellet was partially dissolved in 1 ml of 0.05 M potassium phosphate, pH 7.2, 0.5 ml of isopropanol and completely dissolved by acidifying with 25 μl of glacial acetic acid. After adding 0.5 ml 40% KOH and 500 μg of carrier palmitic acid, the solution was saponified by heating for 30 min at 80°C . The solution was cooled and then acidified with 1.0 ml of 6 N HCl and the free fatty acids were extracted into petroleum ether. The petroleum ether extract was evaporated to dryness in a stream of nitrogen and the radioactivity determined. Alternatively the fatty acids were methylated by incubation for 5 min at 80°C with 2 ml of 4% methanolic HCl, extracted into petroleum ether after dilution with water, and analysed by glc.

Isolation of acyl CoAs. The acyl CoAs as well as complex lipids were in the combined supernatant fractions. The combined fractions were poured over a column (0.8 \times 30 cm) containing 0.75 g neutral alumina AG 7. The tube which had contained the fractions and column were rinsed with 5 ml chloroform-methanol (1:2, v/v) and then the gel was eluted with 20 ml chloroform-methanol (1:2, v/v). All the eluates were collected for further analysis as oxygen ester complex lipids. Acyl CoAs were retained on the gel. The column was next rinsed with 10 ml diethyl ether, flushed with nitrogen and the dry gel was collected and placed in a screw-cap tube. Three ml of 25% KOH in methanol-water (1:1) and 500 μg of carrier palmitic acid were added and the tube heated at 80°C for 30 min. Then 2 ml of 1% Triton X-100 in isopropanol-water (1:1) and 3 ml of 6 N HCl were added and the free fatty acids were extracted into petroleum ether. The petroleum ether extract was evaporated to dryness and the radioactivity determined or the fatty acids were methylated and analysed by glc. Data obtained from this fraction represented the composition of the acyl CoAs of the reaction mixture.

To recover the intact acyl CoA, the gel was washed with 20 ml of 0.1

M potassium phosphate, pH 7.2–methanol (1:1). The eluate was acidified with 50 μ l of glacial acetic acid (pH \sim 5) and the methanol evaporated under vacuum. An aliquot of this solution was reduced with NaBH_4 (2) and the resulting fatty alcohol and any possible free fatty acids were extracted into petroleum ether. The fatty acid (as methyl ester) and the fatty alcohol (as TMS derivative) were analysed by glc. Since no methyl esters of fatty acids were present, there was no hydrolysis during the procedure. Furthermore, these results proved that all oxygen ester lipids had been separated from acyl CoAs by the chloroform–methanol elution of the alumina gel. The remaining acyl CoAs were then extracted into butanol by washing the aqueous solution three times with 2 ml of water saturated butanol. This procedure can be readily adapted for the isolation and purification of acyl CoAs.

RESULTS AND DISCUSSION

Separation of acyl ACP from reaction mixture. Acyl ACP was isolated from a typical reaction mixture by coprecipitation of the proteins and ammonium sulfate in a solvent system containing chloroform and methanol. The flocculation time of the proteins was critical (Fig. 1). Thus, if flocculation was rapid (as in the presence of high salt) or if the solution was centrifuged within a few minutes of adding the chloroform–methanol, then 15–30% of the acyl ACP fraction remained in the supernatant (Fig. 1). However, by allowing sufficient time for protein aggregation (20 min), essentially complete precipitation of [^{14}C]acyl ACP occurred. In the absence of albumin, the very small mass of acyl ACP present did not precipitate on addition of chloroform–methanol. A cleaner separation of acyl ACP from the other lipids was observed when a small amount of ammonium sulfate was mixed with the protein solution prior to precipitation (Fig. 1).

In the step preceding protein precipitation, free fatty acids were extracted into petroleum ether after acidification of the reaction mixture. If the solution was acidified with 1 drop of 6 N HCl to about pH 1 rather than with glacial acetic acid to a pH of 3–4, the recovery of [$\text{O-}^{14}\text{C}$]stearyl ACP in the pellet following precipitation was only about 60–70%. In the presence of low salt, at a pH of 3–4, and with sufficient time allowed for flocculation, only 1–2% of acyl ACP remained in the supernatant following addition of chloroform–methanol. Using this procedure, [$\text{O-}^{14}\text{C}$]stearyl ACP was recovered essentially free of contamination with [$1\text{-}^{14}\text{C}$]myristyl CoA (Table 1).

Some polar lipids were also extracted with petroleum ether together with free fatty acids and neutral lipids. Thin-layer chromatography of the two phases obtained after the extraction of spinach lipids showed that the petroleum ether extract contained all of the fatty acids and neutral

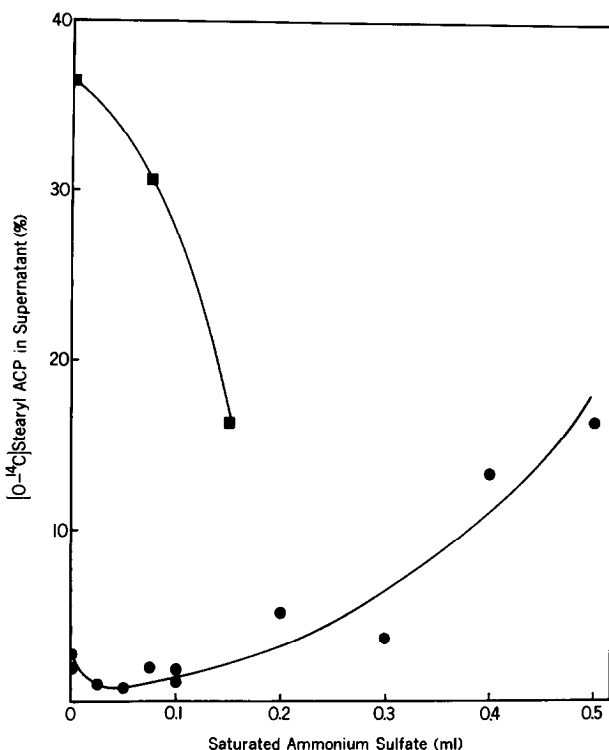


FIG. 1. The effect of ammonium sulfate and flocculation time on the precipitation of [$O\text{-}^{14}\text{C}$]stearyl ACP. [$O\text{-}^{14}\text{C}$]stearyl ACP (11,000 cpm) was dissolved in 1 ml of 0.05 M potassium phosphate, pH 7.2 containing 10 μmoles MgCl_2 and 2 mg bovine albumin. The designated volume of a saturated solution of ammonium sulfate was added followed by 25 μl glacial acetic acid and 1 ml isopropanol. The proteins and salt were coprecipitated by addition of 8 ml chloroform-methanol (1:2, v/v) to the vortexing solution. The tubes were set aside for the indicated time and then centrifuges in a clinical centrifuge for 5 min at top speed. A sample (5 ml) of the supernatant fraction was withdrawn, evaporated to dryness with a stream of nitrogen and counted. Flocculation time: (■) 2 min; (●) 20 min.

lipids and a representative fraction of the polar lipids. The aqueous phase contained the remainder. The precise proportion of polar lipids that were distributed between the two phases cannot be established because the type of lipids that are present will depend on the nature of the reaction mixtures. Quantitative data were obtained with mixed potato [^3H]lipids and with [$1\text{-}^{14}\text{C}$]dioleoyl phosphatidyl choline and are presented in Table 2.

To analyse the acyl ACP fraction, the pellet was first saponified and the resulting fatty acids were extracted and counted, or methylated and chromatographed by glc. Complete saponification was achieved if the pellet was first dissolved by adding phosphate buffer, acetic acid, and isopropanol. Cold carrier palmitic acid and 40% KOH (aqueous) were

TABLE 1
THE FRACTIONATION OF [0-¹⁴C] STEARYL ACP AND [1-¹⁴C] MYRISTYL CoA^a

Fraction	Radioactivity (cpm)	Composition ^b
Supernatant	26,434	14:0 only
Pellet	26,650	93% 18:0; 5% 16:0 and 20:0; <2% 14:0

^a [¹⁴C] Stearyl-ACP (25,940 cpm) and [¹⁴C] myristyl-CoA (27,071 cpm) were dissolved in 1 ml of isopropanol and 1 ml of potassium phosphate, pH 7.2 containing 10 μmoles of MgCl₂ and 2 mg of bovine albumin. The solution was acidified with 25 μl of glacial acetic acid and the proteins were precipitated according to the procedure. The pellet lipid was saponified and the radioactivity of the supernatant and pellet was determined.

^b The fatty acid composition was analysed by glc. Stearyl ACP has initially 5% of 16:0 and 20:0 acyl groups as contaminants.

TABLE 2
THE FRACTIONATION OF LABELED LIPIDS^a

Fraction	[¹⁴ C] myristyl CoA (%)	Potato [³ H] lipids (%)	[¹⁴ C] phosphatidyl choline (%)
Petroleum ether	<0.1	87.6	31.0
Alumina gel	99.5	1.3	1.4
Column eluate	0.5	11.1	67.6

^a [1-¹⁴C] Myristyl CoA (45,100 cpm), mixed [0-³H] lipids from potato (50,500 cpm), or [1-¹⁴C] dioleoyl phosphatidyl choline (16,700 cpm) were dissolved in 1 ml of 0.05 M potassium phosphate, pH 7.2 containing 10 μmoles MgCl₂ and 2 mg bovine albumin. The samples were fractionated according to the procedure and the radioactivity was determined.

TABLE 3
EXTRACTION OF FREE FATTY ACID WITH PETROLEUM ETHER^a

Petroleum ether extract	Palmitic acid recovered	
	(cpm)	(%)
I	201,204	88.2
II	21,663	9.5
III	3301	1.4
IV	761	0.3
Unextracted	1278	0.6

^a [1-¹⁴C] Palmitic acid (228,207 cpm) was dissolved in 1 ml 0.05 M potassium phosphate, pH 7.2 containing 10 μmoles MgCl₂ and 2 mg bovine serum albumin. One milliliter isopropanol was added and the solution was acidified with 25 μl glacial acetic acid. The solution was washed four times with 2.0 ml of 50% isopropanol-saturated petroleum ether (40°-60°bp) and the radioactivity was determined.

then added and the mixture was saponified. Because of the very low mass of ^{14}C -labeled fatty acid which was derived from the $[^{14}\text{C}]$ acyl ACP, carrier fatty acid was added to improve recoveries. Also, some loss of counts were observed if the pellet was saponified with 25% KOH in 50% aqueous methanol rather than with 40% aqueous KOH.

Isolation of acyl CoAs. The remaining supernatant fraction contained the acyl CoA and small amounts of phospholipids and galactolipids. The free fatty acids had already been removed prior to the acyl ACP precipitation step, by extracting the acidified 50% aqueous isopropanol solution of the reaction mixture with petroleum ether that was preequilibrated with the lower phase solvents. Using this system, over 99% of the acid was removed by three petroleum ether extractions (Table 3). Under these conditions, less than 0.1% of $[1\text{-}^{14}\text{C}]$ myristyl CoA was extracted (Table 2). It is essential that this solvent system be employed since $[^{14}\text{C}]$ myristyl CoA can be extracted by petroleum ether from water or 50% aqueous methanol and also from 50% aqueous isopropanol when mixed lipids were present.

The supernatant, free of FFA and acyl ACP but containing acyl CoA and complex lipids, was passed through the neutral alumina column in the presence of chloroform-methanol (1:2, v/v). Polar lipids were readily removed from the alumina gel by washing with chloroform-methanol (1:2, v/v). Thus under the conditions used, $[1\text{-}^{14}\text{C}]$ dioleoyl phosphatidyl choline and $[^3\text{H}]$ lipids from potato were almost quantitatively eluted from neutral alumina, but $[1\text{-}^{14}\text{C}]$ myristyl CoA remained on the gel (Tables 2 and 4). This gel has a very high affinity for acyl CoA, and did not release myristyl CoA even in the presence of 10% methanolic

TABLE 4
THE FRACTIONATION OF $[1\text{-}^{14}\text{C}]$ MYRISTYL CoA AND MIXED $[0\text{-}^3\text{H}]$ LIPIDS^a

Fraction	^3H -channel (cpm)	^{14}C -channel (cpm)	$^3\text{H} + ^{14}\text{C}$ -channel (cpm)
$[1\text{-}^{14}\text{C}]$ myristyl CoA	8679	16,886	26,118
$[0\text{-}^3\text{H}]$ lipids	52,477	677	53,295
Pellet	270	352	632
Petroleum ether	49,823	773	50,727
Alumina gel	8557	15,348	25,432
Column eluate	3038	308	2338

^a $[1\text{-}^{14}\text{C}]$ Myristyl CoA (26,118 cpm) and mixed $[0\text{-}^3\text{H}]$ lipids from potato (53,295 cpm) were counted in both ^3H and ^{14}C channels. Then the compounds were dissolved together in 1 ml of isopropanol and 1 ml of 0.05 M potassium phosphate, pH 7.2, containing 10 μ moles of MgCl_2 and 2 mg bovine albumin. The solution was acidified with 25 μ l of glacial acetic acid, the protein precipitated and the supernatant fractionated according to the previously described procedure.

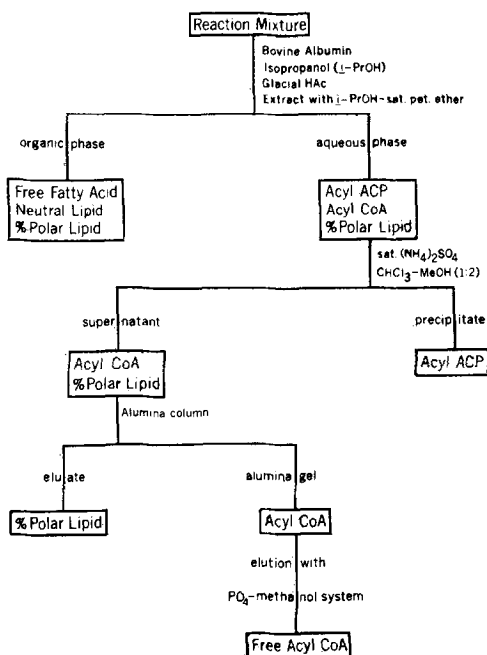


FIG. 2. Flow diagram summarizing the analytic procedures for the separation of acyl ACPs, acyl CoAs, and complex lipids in a reaction mixture.

pyridine or 2 N HCl-methanol (1:1, v/v). However, over 90% of adsorbed acyl CoA can be eluted by washing the gel with an eluting solution of 0.1 M potassium phosphate at pH 7.2 and methanol (1:1).

Labeled acyl CoA, bound to the alumina gel, may be analyzed by two procedures: (a) saponification of the acyl CoA on the gel and determination of the radioactivity of the free fatty acids released or by glc of the corresponding methyl esters. Complete saponification was achieved if the gel was heated with 25% KOH in aqueous methanol and extraction was improved when carrier palmitic acid and Triton X-100 were present; (b) by recovery of acyl CoA from gel by ionic displacement with 0.1 M potassium phosphate, pH 7.2-methanol (1:1) in a yield of 90%. After the methanol was evaporated under vacuum, the acyl CoA was extracted into butanol in a yield of 95%. The acyl CoAs can then be further analyzed by the Barron and Mooney procedure (2) or by interesterification to form methyl esters of the acyl components.

In summary, this procedure allows for the quantitative determination of acyl ACPs, acyl CoAs, triacylglycerols, free fatty acids, galactolipids, and phospholipids in a reaction mixture. Figure 2 presents a flow sheet for the various separating steps. This method avoids the problems encountered when either the hydroxylamine method or the Barron and

Mooney method was employed in the analysis of complex mixtures. It can be used also as a preparative method to isolate and purify acyl ACPs and acyl CoAs.

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