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Formation of Palmityl-[3′-^{32}P]Coenzyme A from [γ-^{32}P]ATP in Mitochondrial Extracts of Guinea Pig Liver[1]

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Investigations of the incorporation of ^{32}P into acyl-coenzyme A (CoA) in incubation mixtures containing a soluble protein preparation derived from mitochondria, [γ-^{32}P]ATP, and palmityl-CoA have led to the discovery of an enzymatic activity which catalyzes the exchange of palmityl groups between molecules of CoA: CoA* + palmityl-CoA ↔ palmityl-CoA* + CoA. The preparation also contains dephospho-CoA kinase and palmityl-CoA thiolester hydrolase activities. The initial detection of the exchange reaction resulted from the formation of [3′-^{32}P]CoA via the dephospho-CoA kinase reaction with exogenous [γ-^{32}P]ATP. The described preparation of palmityl-[3′-^{32}P]CoA and palmityl-[^{35}S]CoA facilitated demonstration of the reversibility of the reaction and ruled out the possibility that the exchange of fragments of the CoA molecule mediated the observed incorporation. The reversible palmityl group exchange does not appear to be catalyzed by a previously described enzyme. None of the possible acyl group acceptors considered in these studies participated in the reaction as efficiently as CoA itself. The possibility is discussed that the exchange reaction may explain reports of an unknown lipid formed by an oligomycin-sensitive mitochondrial ATPase preparation.

Studies of the incorporation of radioactivity from [γ-^{32}P]ATP into acidic phospholipids in subcellular preparations have proved useful in understanding their metabolism. The present communication deals with the identification of a radioactive substance discovered in acidic phospholipid extracts of incubation mixtures containing [γ-^{32}P]ATP and a soluble protein preparation from guinea pig liver mitochondria. The substance did not share chromatographic properties with any of the acidic phospholipids known to accumulate radioactivity under similar conditions, such as phosphatidic acid (2), phosphatidylinositol phosphate (3, 4), or acyl dihydroxyacetone phosphate (5, 6). Its accumulation was dependent upon the presence of a lipid cofactor derived from whole liver or a particulate fraction of liver mitochondria. Both the lipid cofactor and the radioactive product itself were identified as long-chain acyl-CoA.[3]

MATERIALS AND METHODS

Preparation of [γ-^{32}P]ATP. [γ-^{32}P]ATP was prepared by a modification of the method of Glynn and Chappell (7). After incubation of the essential enzymes, cofactors, and 10 mCi of carrier-free $H_3{}^{32}PO_4$ in a total volume of 5.0 ml, the entire mixture was applied to a column of Dowex 1-8X and washed with water, 0.02 M NH_4Cl in 0.02 M HCl, followed by an additional 40 ml of water. The [γ-^{32}P]ATP was eluted from the column with 0.25 M HCl, titrated to pH 6.5–7.0, and stored at −20°C. The specific activity of the resultant [γ-^{32}P]ATP ranged from 4–7 × 10^8

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[3] Abbreviations used: CoA, coenzyme A; PhIP, phosphatidylinositol phosphate; $PhIP_2$, phosphatidylinositol diphosphate; tlc, thin-layer chromatography; 3′,5′-ADP, adenosine 3′,5′ diphosphate, DHAP, dihydroxyacetone phosphate.

cpm/μmol; the final ATP concentration ranged from 0.75 to 1.0 mM, with a radiochemical purity of 95 to 98%.

Preparation of mitochondrial supernatant fraction. Weighed, blotted portions of chilled guinea pig liver were minced into 9 vol of ice-chilled 0.25 M sucrose containing 2 mM Na_2EDTA and homogenized (8). All subsequent steps were performed at 0–4°C, unless stated otherwise. After centrifugation at 800*g* for 10 min, the resulting supernatant fraction was recentrifuged at 8700*g* for 10 min. The washed mitochondrial pellet was then suspended in 2.0 ml of water/g of liver and frozen in a dry ice–acetone bath. After thawing in a 30°C water bath, the suspension of lysed mitochondria was quickly chilled to 2°C and centrifuged at 100,000*g* for 60 min. The supernatant fraction was used as a source of enzyme activity. The preparation was either used directly or dialyzed against 4 liters of 10 mM Tris–HCl (pH 7.4) for 6 h and 4 liters of fresh buffer for an additional 10 h. After dialysis, the preparation was lyophilized in 5- or 10-ml portions and stored for up to 1 month at −20°C.

The yellowish beige, fluffy powder dissolved quickly in water or buffer to yield a clear yellow solution containing 3 to 4 mg of protein/ml. The protein yield from 1.0 g of liver varied from 8 to 9 mg and solutions had a 260/280-nm absorbance ratio of 0.76.

Preparation of lipid cofactor. The pellet derived from centrifugation of the lysed liver mitochondria was heated to 100°C in a water bath for 15 min, homogenized in 1.0 ml of 0.25 M sucrose/g of liver, and used directly (heated mitochondrial residue) or processed further. A lipid extract of the heated mitochondrial residue was prepared by suspending 0.4 ml of the heated residue in 0.8 ml of water, followed by 4.5 ml of chloroform/methanol (1:2, v/v), 1.5 ml of chloroform, and 1.5 ml of 2.0 M KCl. The mixture was stirred well and centrifuged at 1000*g* for 10 min at room temperature, and the lower layer was dried under N_2 and then sonicated into 0.4 ml of water for use in enzyme incubations or dissolved in chloroform for fractionation on Unisil columns.

A crude lipid extract from frozen guinea pig liver was prepared by treatment of 6 ml of a 20% (w/v) homogenate of liver in water with 22.5 ml of chloroform/methanol (1:2, v/v), 7.5 ml of chloroform, and 7.5 ml of 2.0 M KCl. After dispersion and centrifugation at 1000*g* for 5 min, the lower phase was transferred to a tared tube and dried under N_2. The weighed lipid was dissolved in chloroform to a final concentration of 25 mg/ml and stored at −20°C.

For fractionation of lipid cofactor, the lipid extract of the heated mitochondrial residue from 2 g of liver was dissolved in 2.0 ml of chloroform and applied to a 0.5cm i.d. column containing 0.5 g of chloroform-washed Unisil. Solvent sequences A and B (Table II) were used for fractionation and 2.5 ml of each fraction was dried under N_2 and sonicated in 0.4 ml of water for use in enzyme incubations. Acidic phospholipid extracts were prepared by a modification (9) of the method of Bligh and Dyer (5, 10). The lower phase will be referred to as the acidic phospholipid extract.

Degradation of phospholipid. Lipid extracts were subjected to alkaline methanolysis (11, 12) by the procedure of Hajra *et al.* (5). A mixture containing equal volumes of sample and alkaline phosphatase solution [EC 3.1.3.1, 350 units/mg from calf intestine, 15 μg/ml in 0.32 M ethanolamine-HCl (pH 10.1) containing 10 mM magnesium acetate] was incubated for 10 min at 37°C. Another sample was treated with 7 vol of 0.1 N HCl at 100°C for 10 min. The reaction mixtures were dried under N_2 and the residues were dissolved in water. The enzymatic, alkaline, and acid digests were submitted to formic acid high-voltage electrophoresis.

Preparation of palmityl-[3′-^{32}P]CoA. The incubation mixture was composed of mitochondrial supernatant fraction (15 mg of protein), 88 mM Tris–HCl (pH 7.4), 8.8 mM $MgCl_2$, 8.8 mM NaF, 2 mM dithiothreitol, 88 μM [γ-^{32}P]ATP (1×10^9 cpm), and 90 μM dephospho-CoA in a final volume of 20.3 ml. The mixture was incubated at 37°C for 45 min before the addition of 0.7 ml of 1.75 mM palmityl-CoA (with swirling) and incubation was extended for 20 min. Three-tenths milliliter of 12 M perchloric acid, 7 ml of 0.17 M perchloric acid, and 14 ml of "washed" Hyflo-SuperCel suspension in 0.17 M perchloric acid were added (with swirling) and the mixture was allowed to stand at 0–4°C for 60 min.

With slight modifications, the method of Galliard and Stumpf (13) was used for isolation of labeled palmityl-CoA. The acidified incubation mixture was agitated to suspend the SuperCel, and then was applied to a column (4°C) of washed SuperCel (3.2 × 0.5 cm) in a glass column fitted with a sintered-glass disk and eluate flow was maintained by N_2 pressurization. After loading the sample, the column was washed with 150 ml of 0.17 M perchloric acid, followed by 150 ml of 80% (v/v) ethanol at a flow rate of 1.5–2.0 ml/min. The column was then warmed and maintained at 40°C, and palmityl-CoA was eluted with 90 ml of isopropanol:pyridine:water (1:1:1, v/v/v) at 40°C at a flow rate no greater than 0.5 ml/min. The use of colorless pyridine is imperative; if impure pyridine is used, the final palmityl-CoA solution will contain a yellow, insoluble flocculent material which binds palmityl-CoA quite strongly. The eluate from the column was stored at −20°C at this stage of purification.

The radiochemical amount and purity of palmityl-[3′-^{32}P]CoA recovered from the incubation mixture were determined by measurement of the percentage of the total radioactivity of a nitrogen-dried sample of the column eluate, dissolved in bovine serum albumin (4 mg/ml), that adhered to the origin

after electrophoresis. Carrier palmityl-CoA was added to the column eluate to yield an initial specific activity of 5×10^6 cpm/μmol. The solution was then concentrated on a rotary evaporator at 40°C, mixed with 5 vol of water, and lyophilized. The resultant fluffy powder was then dissolved in an amount of water to yield a final palmityl-CoA concentration of 300 μM and stored at −20°C.

If the purify of the preparation did not exceed 90%, the palmityl-CoA was purified by precipitation with 0.17 M perchloric acid, followed by a cold acetone wash. Spectral analyses indicated that concentrations of from 200 to 400 μM were obtained routinely. The variability probably reflects the presence of varying amounts of endogenous long-chain acyl-CoA in the mitochondrial supernatant preparation. The radiochemical purity of these preparations averaged over 90% (range, 75–95%). [γ-^{32}P]ATP was the principal contaminant, with lesser contributions by $^{32}P_i$ and [3′-^{32}P]CoA.

Preparation of palmityl-[^{35}S]CoA. The method of Sen and Leopold (14) was modified for the preparation of [^{35}S]CoA. One gram of lyophilized yeast cells from a culture containing 5 mCi of $Na_2{}^{35}SO_4$ was added to 5 ml of boiling water and heated for 5 min at 100°C. Small chips of ice were added to cool the suspension, which was then centrifuged at 10,000*g* for 10 min at 4°C. The supernatant solution was concentrated to 0.5 ml under N_2, followed by the addition of 4 mg of dithiothreitol and 5 μl of 1 M Tris–HCl (pH 8.0). The mixture was agitated and allowed to stand at 4°C for 16 h under N_2; final pH of the solution was 7.5. A suitable aliquot of the reduced ^{35}S extract was submitted to high-voltage electrophoresis in oxalate buffer with 0.15 μmol of CoA carrier. After localization by ammonium molybdate spray reagent, the CoA region was assayed for ^{35}S content.

Preparation of palmityl-[^{35}S]CoA from the yeast cell extract was accomplished using a "thiokinase" incubation mixture (15): 3.4 mM Na_2ATP, 0.34 mM CoA, 10 mM cysteine-HCl, 16.7 mM NaF, 5 mM $MgCl_2$, 0.67 mM ammonium palmitate, 33.3 mM potassium phosphate (pH 7.4), guinea pig liver microsomes (2.0 mg of protein), 0.2 ml of ^{35}S-labeled yeast extract, and water in a final volume of 12.0 ml. The mixture was incubated at 37°C for 60 min and the reaction was terminated by the addition of 15 ml of cold 0.14 M perchloric acid. The suspension was centrifuged at 12,000*g* for 5 min at 4°C. The precipitate was washed twice with 0.14 M perchloric acid and twice with 80% (v/v) ethanol by resuspension and centrifugation. The pellet was then extracted with 2 ml of isopropanol:pyridine:water (1:1:1, v/v/v) for 5 min at 37°C and recentrifuged, and the supernatant fraction was removed and stored at −20°C. Aliquots of the extract derived from the thiokinase incubation mixture were submitted to silica gel H-oxalate thin-layer chromatography, along with a palmityl-CoA standard, using solvent system 2; the palmityl-CoA area was assayed for ^{35}S content. The extract was dried under N_2 and redissolved in 0.2 ml of water for use in enzyme incubations. Approximately 4% of the total radioactivity was recovered in the extract, of which 0.6% was recovered as CoA, even with the use of the dithiothreitol reduction step to avoid a loss of CoA due to its reaction with glutathione (16). Use of a CoA-containing extract in the thiokinase incubation led to the recovery of 0.7% of the radioactivity as palmityl-[^{35}S]CoA, which was 70% radiochemically pure.

Other methods. Protein concentrations were determined by the biuret method of Gornall *et al.* (17). Turbidity interference was overcome by the use of KCN (18).

Paper chromatographic separations were performed by the method of Bressler and Wakil (19), using isobutyric acid/ammonia/water (66:1:33, v/v/v), pH 3.7). Descending chromatography was performed on Whatman 3MM paper for 22 h at 22°C (solvent front migration, 34 cm).

Silica gel oxalate plates (20) 0.5 mm thick were used with two solvent systems: (1) chloroform/methanol/water (45:45:15, v/v/v) (21) and (2) chloroform/methanol/water/ammonia (90:90:22:8, v/v/v/v) (20). Plates developed with solvent system 1 were washed by chloroform migration before use and activated at 100°C for 60 min. Plates used with solvent system 2 were activated, but were not washed.

Commercial 250-μm silica gel plates were activated and developed with an additional solvent system composed of chloroform/methanol/glacial acetic acid (90:2:8, v/v/v) (22). Solvent system 2 was used with unactivated commercial plates as well.

For high-voltage electrophoresis (Gilson Medical Electronics Model D electrophorator), 8% (v/v) formic acid was used for pH 1.9 and 0.06 M sodium oxalate for pH 1.5 (23).

Radioactive substances were usually localized by radioautography. Phosphate-containing substances on thin-layer plates were visualized by the Dittmer and Lester spray reagent (24). Paper-bound phosphates were localized by a modification of the method of Hanes and Isherwood (25), in which the reagent was composed of 10 ml of 10% (w/v) ammonium molybdate tetrahydrate, 5 ml of 2 M HCl, 10 ml of 12 M perchloric acid, and 175 ml of water. Papers were sprayed and allowed to dry partially before color development under ultraviolet lamps. The presence of sulfhydryl groups was determined with a nitroprusside spray reagent (26).

Materials. Phosphatidylinositol phosphate and diphosphate were prepared according to the methods of Hendrickson and Ballou (27). CoA and its derivatives were obtained from P-L Biochemicals, Inc., Milwaukee, Wisconsin. Alkaline phosphatase, oligomycin, carnitine, and D-pantetheine were products of the Sigma Chemical Company, St. Louis,

Missouri. Glyceraldehyde-3-phosphate dehydrogenase, 3-phosphoglycerate kinase, and hexokinase were obtained from Boehringer Mannheim Corporation, New York, New York. Hyflo-SuperCel was a product of Johns-Manville, Celite Division, New York, New York and was purified before use. Silica gel H and precoated silica gel plates were products of Brinkmann Instruments, Westbury, New York. Unisil was a product of Clarkson Chemical Company, Williamsport, Pennsylvania. Bovine serum albumin was obtained from Miles Research Laboratories, Pentex Division, Kankakee, Illinois. Palmitic acid was a product of the Hormel Institute, Austin, Minnesota. *sn*-Glycerol 3-phosphate was obtained from Calbiochem, La Jolla, California. All radioactive compounds were obtained from New England Nuclear, Boston, Massachusetts. Male, albino guinea pigs (250–300 g) were obtained from Camm Research Institute, Inc., Wayne, New Jersey. Sphingosine, psychosine, and cholesterol were gifts of Dr. N. S. Radin. Dihydroxyacetone phosphate and its derivatives, as well as 1-acyl-3-glycerol phosphate, were gifts of Dr. A. K. Hajra. A pure culture of *Saccharomyces cerevisiae* was a gift of Dr. E. Juni. An oligomycin-sensitive ATPase (EC 3.6.1.4) preparation from mitochondria was a gift of Dr. Efraim Racker.

RESULTS

Formation of Labeled Lipid from [γ-^{32}P]ATP

The incorporation of radioactivity from [γ-^{32}P]ATP into an acidic phospholipid extract of incubation mixtures was dependent upon the presence of the soluble protein preparation and a heat-stable cofactor, both obtained from liver mitochondria. Table I summarizes typical results from initial tests of the incorporation system. The total mitochondrial supernatant or the heated mitochondrial residue alone was ineffective in labeling the acidic phospholipid extract. The effectiveness of the soluble protein preparation in labeling the acidic phospholipid extract was completely destroyed by heat treatment, and most of the cofactor activity of the heated mitochondrial residue could be recovered in a lipid extract.

Incubation of the protein preparation and lipid cofactor with [γ-^{32}P]ATP appeared to promote the labeling of a single unknown substance, designated lipid X. Lipid X was highly polar and contained at least one equivalent of phosphate. It did not share chromatographic properties with phospholipids known to accumulate radioactivity from [γ-^{32}P]ATP under similar conditions (5, 6). Phospholipid extracts from incubation mixtures utilizing both the heated mitochondrial residue and its lipid extract as cofactor preparations were applied to silica gel H–oxalate plates along with purified preparations of phosphatidylinositol phosphate (PhIP) and phosphatidylinositol diphosphate ($PhIP_2$) from beef brain. Whether the heated mitochondrial

TABLE I

REQUIREMENTS FOR FORMATION OF LABELED LIPID FROM [γ-^{32}P]ATP[a]

Incubation	Lipid-soluble radioactivity (^{32}P cpm) Experiment			
	1	2	3	4
Mitochondrial supernatant	900	1,200	–	–
Heated mitochondrial residue	3,900	300	300	300
Heated mitochondrial supernatant[b] and heated mitochondrial residue	–	–	–	300
Mitochondrial supernatant and heated mitochondrial residue	16,100	17,300	19,800	25,500
Mitochondrial supernatant and lipid extract of heated mitochondrial residue	–	–	–	19,800

[a] Incubations were for 10 min in air with agitation at 37°C and contained 0.1 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 0.01 M NaF, 0.1 mM [γ-^{32}P]ATP (5×10^7 cpm), 1.5 mg of protein, and 0.4 ml of cofactor preparations in a final volume of 1.2 ml.

[b] Heated for 10 min at 100°C and homogenized before use.

residue or its lipid extract was used as a source of cofactor, most of the ^{32}P-labeled lipid from either type of incubation mixture migrated with an R_f of 0.49, while the inositides migrated with R_fs of 0.55 (PhIP) and 0.31 ($PhIP_2$) (28).

Lipid Cofactor Requirement

Several experiments were performed to characterize the lipid cofactor requirement. The rate of labeling of lipid X in the presence of the lipid extract of the heated mitochondrial residue was linear for 10 min and continued for up to 30 min at a lower rate. The incorporation system was stimulated by additions of the lipid extract of up to 0.4 ml.

The efficacy of fractions of lipid extracts of liver derived from Unisil columns in stimulating the labeling of the acidic phospholipid extract was tested several times (Table II). The lipid extract of the heated mitochondrial residue was less effective than the residue itself, as is seen in Table I. The most effective fraction from solvent sequence A was II, which stimulated the labeling of the acidic phospholipid extract nearly as effectively as the unfractionated material. More extensive fractionation of the cofactor preparation by solvent sequence B indicated that the active component trailed from the column in all of the fractions, none of which was nearly as effective as the lipid extract of the heated mitochondrial residue itself. It was apparent that the lipid extract of whole liver was active as a stimulatory agent in the system and could be used to replace the heated mitochondrial residue or its lipid extract. None of the fractions of the lipid extract of whole liver was as effective as the unfractionated material in acting as the source of the lipid cofactor necessary for the labeling of the acidic phospholipid extract (28).

TABLE II

ACTIVITY OF UNISIL COLUMN FRACTIONS OF PREPARATIONS OF LIPID COFACTOR[a]

Fractionation	Lipid-soluble radioactivity (^{32}P cpm)
Heated mitochondrial residue	5,100
Lipid extract of heated mitochondrial residue	2,700
Column fractions from:	
Solvent sequence A[b]	
I Chloroform	700
II Chloroform/methanol, 1:1	2,500
III Methanol	900
Solvent sequence B[b]	
I Chloroform	100
II Chloroform/methanol, 9:1	620
III Chloroform/methanol, 7:3	840
IV Chloroform/methanol, 1:1	370

[a] Incubation conditions were as for Table I. The [γ-^{32}P]ATP specific activity was 10^8 cpm/μmol.

[b] Solvent composition is v/v.

The ^{32}P-labeled lipid formed in incubation mixtures using either fraction II of solvent sequence A (A-II) from Unisil columns or the lipid extract of whole liver as sources of the lipid cofactor comigrated with lipid X on silica gel H–oxalate plates developed with solvent system 2. As a result of these studies, fraction A-II of the lipid extract of the heated mitochondrial residue was used as the source of the lipid cofactor in most of the remaining preliminary studies.

The addition of EDTA or $CaCl_2$ to the standard incubation medium appeared to slightly inhibit the labeling of lipid X (28). The omission of NaF had a pronounced inhibitory effect, while the omission of $MgCl_2$ from the incubation mixtures abolished all labeling of lipid. The replacement of $MgCl_2$ by $CaCl_2$ lowered the labeling of lipid below the enzyme control level.

Using fraction A-II from Unisil columns as the source of the lipid cofactor, the rate of labeling of lipid X was proportional to the concentration of protein present in the incubation mixtures between 0.33 and 1.25 mg/ml (Fig. 1). Figure 2 represents the effects of two concentrations of fraction A-II on the time course of labeling of lipid X. The rate of labeling of lipid X appeared to be a linear function of the concentration of lipid cofactor over the range considered in this study. This observation, together with the finding that the extent of labeling of lipid X at equilibrium was also proportional to the amount of lipid cofactor present, suggested that the lipid cofactor participated stoichiometrically in the labeling of lipid X and that the concentrations of

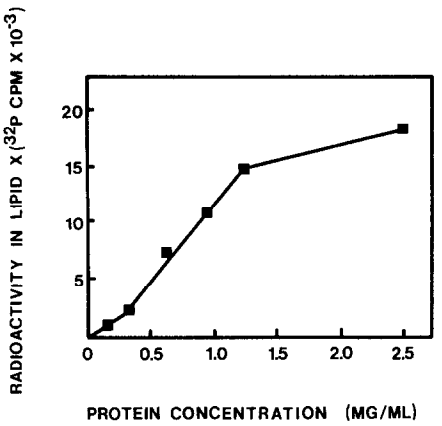

FIG. 1. Dependence of lipid X labeling on protein concentration. Incubations were for 5 min at 37°C, and 0.4 ml of Unisil fraction II of solvent sequence A (A-II) was used as a source of lipid cofactor. Incubation conditions were those of Table I.

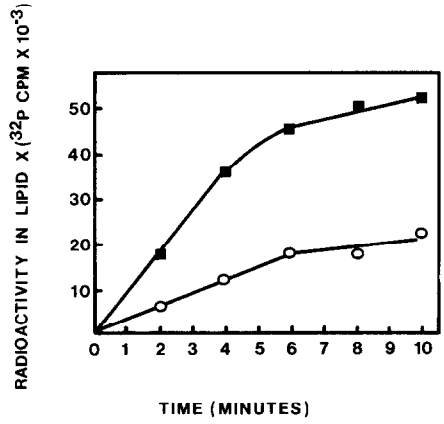

FIG. 2. Dependence of reaction rate and equilibrium on lipid cofactor concentration. Incubation mixtures contained 1.5 mg of mitochondrial supernatant protein and the equivalent of either 0.5 ml (open circles) or 1.5 ml (closed squares) of Unisil fraction II of solvent sequence A as a source of lipid cofactor. Incubation conditions were those of Table I.

cofactor used in these experiments were below the K_m for its participation in the reaction system.

Replacement of Lipid Cofactor by Palmityl-CoA

The naturally occurring ionic detergents, palmityl-CoA and palmityl dihydroxyacetone phosphate, as well as a synthetic, nonionic detergent, Triton X-100, were tested for their ability to influence the labeling of lipid X. The effects of such agents on the degree of labeling of lipid X were observed in incubation mixtures with and without the use of a lipid extract of whole liver as a source of lipid cofactor (Table III). There was an apparent replacement by palmityl-CoA of the requirement for a lipid cofactor. The incorporation of ^{32}P into lipid X was significantly greater when the lipid extract of whole liver was omitted from incubation mixtures which included palmityl-CoA. The other detergents appeared to inhibit the labeling of lipid X. The inclusion of CoA markedly decreased the accumulation of ^{32}P in lipid X. Substances labeled in incubation mixtures containing the lipid cofactor isolated from natural sources and synthetic palmityl-CoA were submitted to thin-layer chromatography on silica gel H–oxalate plates (20). The labeled material from both types of incubation mixture comigrated with lipid X. Several additional characterizations of lipid X were performed. The combined acidic phospholipid extracts from four incubation mixtures were fractionated on a Unisil column using solvent sequence A (as in Table II). The bulk of the ^{32}P-labeled material eluted from the column was found in the second fraction (A-II). Approximately one-third could not be eluted from the column with the use of the three solvents of sequence A. Unexpect-

TABLE III

EFFECTS OF DETERGENTS AND CoA ON THE LABELING OF LIPID X IN THE PRESENCE AND ABSENCE OF LIPID COFACTOR[a]

Additions	Lipid-soluble radioactivity (^{32}P cpm)	
	Without lipid extract of whole liver	With lipid extract of whole liver
None	–	3,580
Palmityl-DHAP[b] (0.25 μmol)	2,470	3,560
Trition X-100 (0.1 mg)	1,130	4,150
Palmityl-CoA (0.09 μmol)	12,640	10,160
CoA (0.09 μmol)	–	920

[a] Conditions were as for Table I, except that incubations were for 5 min and mixtures contained 1 mg of lipid cofactor preparation.

[b] DHAP is dihydroxyacetone phosphate.

edly, the bulk of lipid X was eluted from Unisil by the same solvent that eluted the most active lipid cofactor preparation derived from the fractionation of the lipid extract of the heated mitochondrial residue.

Alkaline methanolysis of lipid X derived from incubation mixtures utilizing either the heated mitochondrial residue or its lipid extract as a source of lipid cofactor indicated that all of the radioactivity was associated with a water-soluble degradation product (28). No ^{32}P was found in the organic phase, whereas the isotope recovery in the aqueous phase varied from 67 to 99%. High-voltage electrophoresis of the water-soluble material in formic acid indicated that the ^{32}P label resided neither in glycerol 3-phosphate nor P_i. The treatment of the water-soluble material with a potent alkaline phosphatase resulted in the liberation of all of the ^{32}P as P_i. However, the ^{32}P of the water-soluble product of alkaline methanolysis of lipid X was relatively resistant to treatment with 0.1 N HCl at 100°C for 10 min. Approximately one-third of the labeled material was converted to a substance migrating slightly faster than the untreated sample (28), but conversion to $^{32}P_i$ was not observed in acid treatments under these conditions.

The aforementioned similarity in the conditions of elution of both lipid X and the lipid cofactor, palmityl-CoA, from Unisil columns led to the final identification of lipid X. Evidence for the identity of lipid X was found with the use of four chromatography systems (Table IV). Lipid X and palmityl-CoA comigrated in each of the chromatographic systems. In addition, acidic phospholipid extracts from incubation mixtures were submitted to alkaline methanolysis along with authentic palmityl-CoA and the water-soluble degradation products subjected to high-voltage electrophoresis in oxalate buffer with CoA, P_i, and palmityl-CoA standards. Phosphate-containing substances were visualized by radioautography and the molybdate spray reagent (Fig. 3). Seven or eight phosphate-containing substances were produced by the alkaline methanolysis of palmityl-CoA, as resolved by the electrophoresis at pH 1.5. All but two migrated faster, and one comigrated with the CoA standard, while two substances migrated slower than CoA. The ^{32}P-labeled degradation product of lipid X from the incubation mix-

TABLE IV

CHROMATOGRAPHIC BEHAVIOR OF LIPID X AND RELATED COMPOUNDS

Compound	R_f in system[a]				
	A	B	C		D
			Single development	Double development	
CoA	0.23	–	0.09	0.14	0.33
Dephospho-CoA	0.35	–	0.07	–	–
Acetyl-CoA	0.25	–	0.11	0.15	0.45
Acetoacetyl-CoA	0.26	–	0.10	0.14	0.38
Palmityl-CoA	0.49	0.27	0.37	0.42	0.66
Lipid X	0.49	0.27	0.37	0.42	0.66
ATP	0.04	–	0.02	–	0.17
PhIP	0.53	–	–	–	–
$PhIP_2$	0.31	–	–	–	–

[a] Systems: A, silica gel H–oxalate plate tlc (20); B, commercial silica gel plate tlc (20); C, silica gel H–oxalate plate tlc (21); D, paper chromatography (19).

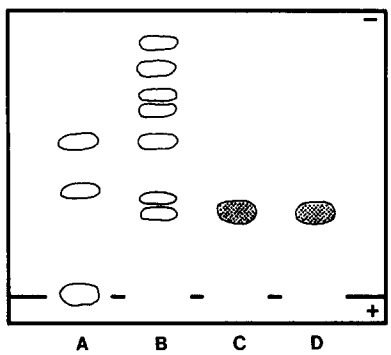

FIG. 3. Oxalate electrophoresis of lipid X degradation products. A contains palmityl-CoA at the point of application, CoA (faster), and P_i (slower) (ammonium molybdate spray reagent). B contains the water-soluble products of the alkaline methanolysis of palmityl-CoA (ammonium molybdate spray reagent). C contains the water-soluble products of the alkaline methanolysis of extracts from incubation mixtures containing lipid extract of whole liver (^{32}P radioautography). D contains the same sample as C, but prepared from incubation mixtures containing palmityl-CoA (^{32}P radioautography). The duration of electrophoresis was 20 min at 4000 V.

tures containing either the lipid cofactor isolated from natural sources or palmityl-CoA co-migrated with the slowest of the phosphate-containing substances produced by the alkaline methanolysis of palmityl-CoA. No appreciable amount of labeled material comigrated with either CoA or P_i standards. The palmityl-CoA standard adhered to the origin. The small amount of endogenous acyl-CoA present precluded identification of the fatty acid group present. Investigation of the effect of fatty acid chain length of added synthetic cofactor was not pursued.

Mechanism of Palmityl-CoA Labeling from [γ-^{32}P]ATP

Table V summarizes the results of two experiments in which a variety of substances was tested for their ability to promote the accumulation of ^{32}P in the lipid extract from incubation mixtures. The combination of dephospho-CoA and palmityl-CoA greatly promoted palmityl-CoA labeling, while the additions of CoA and palmitate or dephospho-CoA and palmitate were less effective. The most potent inhibition was seen when CoA was present in a palmityl-CoA-activated system. Dephospho-CoA or CoA alone did not stimulate incorporation.

TABLE V

EFFECTS OF VARIOUS COMPOUNDS ON LABELING OF PALMITYL-CoA[a]

Incubation conditions	Lipid-soluble radioactivity (^{32}P cpm)
Experiment A	
Mitochondrial supernatant	1,720
Additions:	
Palmityl-CoA (0.09 μmol)	31,060
Palmityl-CoA and CoA (0.09 μmol)	7,410
Palmityl-CoA and dephospho-CoA (0.09 μmol)	42,360
Experiment B	
Mitochondrial supernatant	1,000
Additions:	
Palmityl-CoA (0.09 μmol)	8,380
CoA (0.09 μmol)	1,360
CoA and potassium palmitate (0.1 μmol)	1,000
CoA and palmityl-CoA	2,200
Dephospho-CoA (0.09 μmol)	1,070
Dephospho-CoA and potassium palmitate	1,090
Dephospho-CoA and palmityl-CoA	13,400

[a] Incubation conditions were those of Table III, except that no lipid cofactor preparations were included in the mixture.

Table VI shows the distribution of ^{32}P in various water-soluble substances found in incubation mixtures containing [γ-^{32}P]ATP. The addition of dephospho-CoA stimulated the incorporation of ^{32}P into CoA and a substance with an R_{ATP} of 0.5 (assumed to be ADP or 3′,5′-ADP) by 177 and 30%, respectively. The addition of CoA had little effect on the distribution of ^{32}P among these substances except for a marked stimulation of P_i labeling.

Experiments were performed to determine whether or not ATP was required for synthesis of palmityl-CoA or only for the prelabeling of CoA (Table VII). The additions of dephospho-CoA markedly stimulated the synthesis of CoA and the labeling of palmityl-CoA. The depletion of ATP by more than 99% had a minor effect on the labeling of palmityl-CoA. The greatest decrease in the labeling of palmityl-CoA was produced by the interjection of a heat treatment in the ATP depletion protocol. Even with a high level of labeled CoA present, the treatment of the incubation mixture at 100°C for 2 min abolished label-

TABLE VI

DISTRIBUTION OF ^{32}P IN LABELED COMPOUNDS FROM INCUBATION MIXTURES[a]

Incubation	Radioactivity (cpm × 10^{-3})			
	1.00[b] ATP	0.83 CoA	0.59 P_i	0.49 "ADP"
Mitochondrial supernatant control	2730	354	799	697
Additions:				
Dephospho-CoA (0.1 μmol)	1625	983	839	902
CoA (0.1 μmol)	2163	380	1042	814

[a] Incubation conditions were those of Table III. Reactions were terminated by freezing, and 20-μl aliquots were submitted to oxalate electrophoresis for 20 min at 4000 V. The mean sum of recovered radioactivity in each incubation was (4440 ± 120) × 10^3 cpm (SD).

[b] R_{ATP} of marker compound.

TABLE VII

EFFECT OF ATP DEPLETION ON DISTRIBUTION ON ^{32}P IN LABELED COMPOUNDS FROM INCUBATION MIXTURES[a]

Tube number	Radioactivity (cpm × 10^{-3})						
	1.00[b] ATP	0.88 CoA	0.82 Glucose 6-phosphate	0.55 P_i	0.49 "ADP"	0.0 Acyl-CoA	Sum
1	11.85	0.16	0.0	0.50	0.37	0.027	12.90
2	8.90	0.83	0.0	0.41	1.20	0.690	12.03
3	0.06	1.30	9.9	0.35	0.27	0.610	12.49
4	0.10	1.20	9.1	0.32	0.47	0.020	11.20

[a] Incubation mixtures containing 0.1 M Tris–HCl (pH 7.4), 0.01 M $MgCl_2$, 0.01 M NaF, 0.1 mM [γ-^{32}P]ATP (10^6 cpm), and 0.75 mg of protein in a final volume of 0.6 ml were pre-incubated for 2 min at 37°C. Tube 1 received 20 μl of water and tubes 2–4 received 0.045 μmol of dephospho-CoA in 10 μl of water. Tube 2 received 10 μl of water and tubes 3–4 received 2 μg of hexokinase (EC 2.7.1.1, 140 units/mg from yeast) and 1 μmol of D-glucose in 10 μl of water. In all cases, incubation time was extended for 3 min to provide for the synthesis of radioactive CoA from exogenous precursor (tubes 2–4) and the depletion of the ATP pool by the enzymatic trap (tubes 3–4). Tubes 1–3 received 0.09 μmol of palmityl-CoA in 10 μl of water and incubation time was extended for 5 min to provide for the synthesis of radioactive acyl-CoA by the exchange reaction. Tube 4 was heated for 2 min at 100°C before the addition of palmityl-CoA and extension of the incubation time as for tubes 1–3. Reactions were terminated by freezing, and 10-μl portions were submitted to oxalate electrophoresis for 45 min at 4000 V.

[b] R_{ATP} of marker compound.

ing of palmityl-CoA stimulated by palmityl-CoA addition.

The nature of the material with an R_{ATP} of 0.5 is not certain. Both ADP and 3′,5′-ADP comigrate in this region on oxalate high-voltage electrophoresis. The addition of 0.1 μmol of AMP to a normal incubation mixture resulted in a sixfold increase in the labeling of the unknown substance in 8 min of incubation at 37°C.

Reaction Reversibility

The reversibility of the reaction responsible for the labeling of palmityl-CoA was demonstrated by the use of palmityl-[3′-^{32}P]CoA as a substrate (Table VIII). CoA greatly stimulated the release of labeled CoA from the substrate. An increase in the amount of ^{32}P found in CoA occurred in concert with a decrease in the amount of ^{32}P found in palmityl-CoA. The amount of radioactivity in CoA in the substrate blank indicated a 1% contamination of the substrate by [3′-^{32}P]CoA. Incubations containing the two nucleotides, AMP and 3′,5′-ADP, showed no stimulation of [3′-^{32}P]CoA release by these compounds. CoA itself was the only substrate capable of stimulating the release of [3′-^{32}P]CoA from the substrate.

Table IX summarizes the results of a typical experiment comparing the reactivities of both palmityl-[3′-^{32}P]CoA and palmityl-[^{35}S]CoA in the incubation system; both served as substrates in the reaction. The observed difference in the efficiency of reaction with the two substrates may be the result of a number of factors, but the most significant aspect of the experiment was the qualitative effect of CoA addition: stimulation of the release of radioactive CoA.

No substance capable of stimulating the release of CoA from palmityl-[3′-^{32}P]CoA was more effective than CoA in these studies. The summary of the data derived from a number of experiments (Table X) indicates that no substance among those tested was more than 25% as effective as CoA in serving as a cosubstrate in the enzyme-catalyzed reaction.

Oligomycin-Sensitive ATPase Studies

The data indicate that the oligomycin-sensitive ATPase preparation was capable of promoting labeling of palmityl-CoA. The most heavily labeled substance found in acidic phospholipid extracts of ATPase incubation mixtures containing oligomycin comigrated with palmityl-CoA in the three tlc systems employed in these studies. The presence of five or six other substances was detected with the use of the Dittmer and Lester spray reagent and was

to be expected in light of the observation that the ATPase preparation contains 30% phospholipid (29). None of these substances was labeled by ^{32}P and all of them migrated with R_f's greater than that of palmityl-CoA. The labeling of palmityl-CoA was stimulated approximately sixfold by the addition of oligomycin, while ATPase activity was inhibited by 70% under the same conditions (Fig. 4).

TABLE VIII

REVERSIBILITY OF EXCHANGE REACTION[a]

Incubation	^{32}P cpm recovered in compound		
	Palmityl-CoA	CoA[b]	Sum
Substrate blank	114,200	1,100	115,300
Substrate + mitochondrial supernatant	102,700	9,170	111,800
Additions:			
CoA (0.025 μmol)	90,900	20,000	110,000
3′,5′-ADP (0.2 μmol)	101,400	10,700	112,100
CoA + 3′,5′-ADP	87,400	23,400	110,800
AMP (0.2 μmol)	101,400	10,400	111,800
CoA + AMP	88,800	21,300	110,100

[a] Incubation mixtures contained 0.1 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 0.01 M NaF, 0.7 mg of dialyzed protein, and 0.02–0.04 μmol of palmityl-[3′-^{32}P]CoA (10^5 cpm) in a final volume of 0.35 ml. A substrate blank incubation contained bovine serum albumin in place of the mitochondrial supernatant protein. Incubations were for 5 min at 37°C, and reactions were terminated by freezing. Thirty-microliter portions were submitted to oxalate electrophoresis for 45 min at 4000 V with 0.15 μmol of CoA carrier.

[b] The mean radioactivity in CoA in incubation mixtures from which CoA was absent was 10,000 cpm. The mean radioactivity in CoA in incubation mixtures in which CoA was present was 21,600 cpm.

TABLE IX

COMPARISON OF PALMITYL-[^{35}S]CoA AND PALMITYL-[3′-^{32}P]CoA AS SUBSTRATES FOR EXCHANGE[a]

Incubation	cpm in CoA	
	^{35}S	^{32}P
Substrate blank	200	1,310
Mitochondrial supernatant	1,060	6,120
Mitochondrial supernatant + CoA (0.025 μmol)	2,500	21,130

[a] Incubation conditions were as in Table VIII, except that the palmityl-[^{35}S]CoA preparation (1.5×10^5 cpm) and 0.05 μmol of palmityl-CoA replaced the palmityl-[3′-^{32}P]CoA in the ^{35}S incubation mixtures.

TABLE X

ACTIVITY OF POSSIBLE ACYL GROUP ACCEPTORS

Substance	Percentage of CoA reactivity (equimolar basis)
CoA	100.0
Dephospho-CoA	12.0
Acetyl-CoA	1.7
D-Pantetheine	0.0
Cholesterol	26.0
1-Acyl-3-glycerol phosphate	19.4
(±)-Carnitine	10.7
3-Glycerol phosphate	8.7
Dihydroxyacetone phosphate	7.0
Sphingosine	7.2
Psychosine	1.4

The ATPase preparation was also capable of promoting the labeling of a substance which comigrated with CoA on oxalate electrophoresis (Table XI). The addition of oligomycin greatly enhanced the labeling of this substance as well as of the origin, where palmityl-CoA would adhere in the presence of protein. However, the addition of dephospho-CoA did not greatly enhance the labeling of the CoA area or the origin. Addition of both dephospho-CoA and palmityl-CoA did enhance the labeling of the origin.

DISCUSSION

The results of this study indicate that a soluble protein preparation derived from mitochondria is capable of catalyzing the incorporation of radioactivity from [γ-^{32}P]ATP into a unique lipid in the presence of a lipid cofactor. Both the final product and the lipid cofactor have been identified as long-chain acyl-CoA. The soluble protein preparation is also capable of catalyzing the phosphorylation of dephospho-CoA. The formation of radioactive palmityl-CoA apparently involves the initial production of radioactive CoA and the subsequent exchange of palmityl groups between molecules of CoA. The rapid exchange of palmityl groups appears to be catalyzed by a novel enzyme and does not require ATP.

The characterizations of lipid X derived

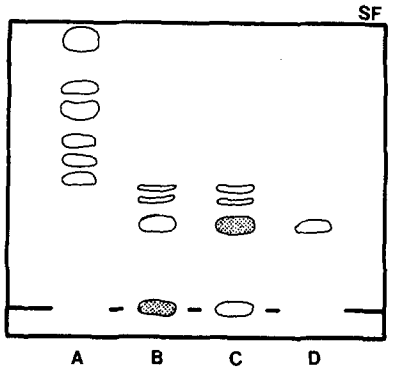

FIG. 4. Silica gel tlc of acidic phospholipid extracts of ATPase incubation mixtures. Incubation mixtures contained 1.8 mg of ATPase, 0.17 mM [γ-^{32}P]ATP, 0.08 M Tris-HCl (pH 7.2), 1.7 mM $MgCl_2$, 0.05 mM dephospho-CoA, and 0.075 mM palmityl-CoA in a final volume of 1.2 ml. For tubes containing oligomycin (40 μg), the inhibitor was transferred to tubes in diethyl ether, which was evaporated under N_2. All components were combined and sonicated prior to addition of the ATPase and its substrate. The reaction was initiated by the addition of ATP after a preincubation period of 2 min at 30°C. Incubations were of 10-min duration at 30°C, and acidic phospholipid extracts were prepared as described in Materials and Methods. The extracts from duplicate incubation mixtures were combined and dried under a stream of N_2. The film was dissolved in 30 μl of chloroform/methanol (2:1, v/v). A contains the acidic phospholipid extracts of the oligomycin-sensitive ATPase preparation alone (Dittmer and Lester spray reagent). B contains the acidic phospholipid extract of an incubation mixture lacking oligomycin (^{32}P radioautography). C contains an acidic phospholipid extract of an incubation mixture containing oligomycin (^{32}P radioautography). D contains a sample of pure palmityl-CoA (Dittmer and Lester spray reagent). Samples were run on a commercial plate using solvent system 2.

from the degradation studies are consistent with its identification as an acyl-CoA, as is the observed comigration with palmityl-CoA in four different chromatographic systems. The alkaline methanolysis of palmityl-CoA yields a variety of phosphate-containing substances including CoA, which is generated very rapidly because of the lability of the thiolester linkage. The further degradation of CoA by alkali results in the production of several fragments, including AMP, 3′,5′-ADP, pantetheine-3′,4′-cyclic hydrogen phosphate, pantothenate 4′-phosphate, and pantothenate-3′,4′-cyclic hydrogen phosphate (30). The lack of ^{32}P in phosphate-containing substances comigrating with CoA indicated that no intact CoA survived the alkaline methanolysis of lipid X. It also suggests that a phosphate-containing substance seen following the alkaline degradation of authentic palmityl-CoA and comigrating with CoA is not CoA.

Acid hydrolysis of the water-soluble degradation products of lipid X increased the rate of migration of the labeled substance produced by the alkaline methanolysis treatment. Such behavior would be expected from the glycosidic cleavage of either of two possible nucleotide degradation products, AMP and 3′,5′-ADP. A similar change in the negativity of the two possible cyclic phosphodiester degradation products would be observed if a lowering of phosphate pK_a accompanied the cleavage to phosphomonoesters by acid treatment. The total conversion of the radioactivity in the water-soluble degradation product to $^{32}P_i$ by the intestinal phosphatase militates against the presence of ^{32}P in a phosphodiester linkage. On high-voltage electrophoresis in oxalate buffer, AMP mi-

TABLE XI

OLIGOMYCIN-SENSITIVE ATPase STUDY OF CoA LABELING[a]

Incubations	Co-migrating radioactivity (^{32}P cpm)	
	CoA	Palmityl-CoA
Control	9,800	900
Additions:		
Oligomycin (40 μg)	36,700	6,400
Oligomycin + dephospho-CoA (0.14 μmol)	40,800	7,300
Oligomycin + dephospho-CoA + palmityl-CoA (0.175 μmol)	33,200	8,300

[a] Incubations contained 1.8 mg of ATPase, 0.04 M Tris-HCl (pH 7.2), 3.3 mM $MgCl_2$, 0.4 mM [γ-^{32}P]ATP (5×10^5 cpm), and 0.3 mM dithiothreitol in a final volume of 0.6 ml. Mixtures were prepared and treated as indicated in Fig. 4, and reactions were terminated by freezing. Twenty-microliter portions were submitted to oxalate electrophoresis with 0.12 μmol of CoA carrier at 4000 V for 45 min. Products were located by radioautography and the ammonium molybdate spray reagent.

grated toward the cathode slightly, while 3′,5′-ADP comigrated with the ^{32}P-labeled degradation product of lipid X.

The direct stimulation of the incorporation system by palmityl-CoA and the properties of the added lipid cofactor are consistent with its identification as an acyl-CoA. Its presence in the heated mitochondrial residue and lipid extracts of whole liver is also consistent with the known occurrence of acyl-CoA in mammalian systems (31).

The strength of binding of palmityl-CoA to protein has been documented by Ayling *et al.* (32). After reaction of labeled palmityl-CoA with a sample of purified yeast fatty acid synthetase, 30 extractions with butanol:ethanol:0.03 M aqueous HCl (1:1:1, v/v/v) were required for the quantitative removal of excess palmityl-CoA. The extraction of the lipid cofactor from tissue preparations by the methods used in these studies would undoubtedly yield variable quantities of palmityl-CoA and other long-chain acyl-CoA compounds. The observed minor incorporation of ^{32}P into lipid extracts of incubation mixtures containing the protein preparation alone was probably due to the presence of small amounts of acyl-CoA in the preparation. The behavior of the lipid cofactor on Unisil columns is also consistent with its identification as an acyl-CoA. Long-chain acyl-CoAs bind strongly to silica gel and the requirement for large volumes of elution solvents is not unusual (33).

The labeling of CoA by [γ-^{32}P]ATP could occur by either of two known mechanisms – through the activity of pantothenate kinase or dephospho-CoA kinase. The former would require the presence of all of the enzymes and substrates required for the biosynthesis of CoA. It would not be surprising to find them present in the mitochondria as well as in the cytoplasm, since mitochondrial membranes are impermeable to CoA and acyl-CoA compounds (34). One could, in fact, anticipate such labeled CoA would be labeled in two positions – at the phosphoryl group of 4′-phosphopantetheine and at the 3′ position of 3′,5′-ADP. Such *doubly* labeled CoA should, however, yield more than one labeled degradation product upon alkaline methanolysis. The reported result, then, suggests that dephospho-CoA kinase is more likely responsible for the observed CoA labeling. Further support is derived from the observations of increased CoA labeling upon addition of dephospho-CoA. The marked inhibitory effect of CoA addition could then be mediated by a direct lowering of the specific activity of CoA or by the inhibition of dephospho-CoA kinase activity. Presumably, a stimulation would have been observed had a CoA phosphomonoesterase activity been present. The susceptibility of the ^{32}P in the water-soluble products of alkaline methanolysis to alkaline phosphatase attack also supports the conclusion that the label resides in a phosphomonoester linkage.

Dephospho-CoA is apparently an obligatory precursor of [3′-^{32}P]CoA production from [γ-^{32}P]ATP. The production of palmityl-[3′-^{32}P]CoA from [3′-^{32}P]CoA and palmityl-CoA involves the exchange of a labeled moiety of CoA with an identical moiety of unlabeled palmityl-CoA. The concentrations of the reactants and products are the same at equilibrium, and the reaction involves no free energy change. The lack of any stimulatory effect of the addition of dephospho-CoA and palmitate or CoA and palmitate is evidence that a thiokinase is not involved in the incorporation system. A depletion study indicated that there is no ATP requirement in the formation of palmityl-CoA from prelabeled CoA. Given the location of ^{32}P at the 3′-adenylate position, its exchange into palmityl-CoA seems most likely to occur at the thiolester or at the pyrophosphate linkage. Enzymatic cleavage of the amide linkages of CoA has not been observed, whereas dephospho-CoA pyrophosphorylase (35) and various palmityltransferase activities have been described in the literature.

Further studies required an assay for the exchange reaction which was independent of the participation of dephospho-CoA kinase. Preparation of prelabeled CoA was not simple. Kurooka *et al.* have described the synthesis of [3′-^{32}P]CoA from dephospho-CoA and *p*-nitrophenyl-[^{32}P]phosphate by cell suspensions of *Proteus mirabilis*. The final product was obtained through purification by ion-ex-

change chromatography (36). Trams *et al.* (37) have described the synthesis of [3′-^{32}P]CoA from dephospho-CoA and [γ-^{32}P]ATP by crude preparations of dephospho-CoA kinase. The final product was purified by preparative cellulose thin-layer chromatography, because of the difficulty of resolving ATP and CoA by ion-exchange column chromatography. Neither of these procedures, nor isolation of labeled CoA from our incubations, could provide adequate quantities of pure [3′-^{32}P]CoA for use as a substrate in these studies.

The preparation of palmityl-[3′-^{32}P]CoA and palmityl-[^{35}S]CoA did make it possible to establish both the reversibility of the reaction and its mechanism. The release of CoA in control incubations where CoA was absent was undoubtedly due to the activity of palmityl-CoA thiolester hydrolase (38). Experiments involving palmityl-[3′-^{32}P]CoA and 3′,5′-ADP did not establish the reaction mechanism. If the exchange involved the pyrophosphate bond of CoA, the addition of 3′,5′-ADP to incubation mixtures would result in the apparent inhibition of the reaction, assuming that any 3′,5′-ADP involved in the exchange had access to the soluble pool of unlabeled 3′,5′-ADP. The use of palmityl-[^{35}S]CoA established the acyl transfer reaction by excluding the possible exchange of fragments of the CoA molecule. This conclusion raised the possibility that the activity responsible for the catalysis of palmityl transfer between CoA molecules was due to one of a number of previously described enzymes and reflected the absence of a more suitable substrate for the acceptance of the acyl group:

palmityl-S-CoA* + HX →
palmityl-X + *CoA-SH
palmityl-S-CoA* + CoA-SH →
palmityl-S-CoA + *CoA-SH

Reactivity could not be demonstrated with a number of putative acceptors such as glycerol 3-phosphate, sphingosine, etc. The lack of reactivity with dephospho-CoA indicates a high degree of specificity for CoA as a substrate. Dithiothreitol stimulates the release of radioactive CoA in the exchange assay, but interpretation is complicated by the fact that a nonenzymatic exchange of acyl groups can occur between the sulfhydryl reagent and CoA (28, 39). No other acyl group acceptor considered in these studies participated in the reaction as effectively as CoA itself.

Studies of the ability of the oligomycin-sensitive ATPase preparation to promote the labeling of palmityl-CoA were prompted by the report of Hill *et al.* (40) on the labeling of an unknown phospholipid under similar conditions. A consideration of its chemical characteristics, in light of the present work, suggested that the lipid might be a long-chain acyl-CoA. The requirement for oligomycin in the labeling of the lipid and the substance comigrating with CoA probably reflects its inhibition of ATPase activity. The presence of dephospho-CoA kinase in the preparation is not firmly established because of the diminutive increase in the labeling of material comigrating with CoA upon the addition of dephospho-CoA. The presence of dephospho-CoA in the ATPase preparation after dialysis and passage over Sephadex columns is not unlikely since tight binding of CoA (41) and dephospho-CoA (42) to protein has been observed.

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