Millipore filter assay for long-chain fatty acid:CoASH ligase activity using ^{3}H-labeled coenzyme A

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Summary A novel radiochemical assay for long-chain fatty acid:CoASH ligase activity (AMP) (EC 6.2.1.3) has been developed based on the conversion of [^{3}H]CoASH to long-chain fatty acyl CoA. Fatty acyl [^{3}H]CoA was quantitatively retained on Millipore filters upon filtration of the acidified reaction mixture under conditions where the [^{3}H]CoASH was not retained. The assay was developed using microsomes derived from isolated fat cells as the source of fatty acid:CoASH ligase activity. The assay performed at 25°C for 10 min was linear with added microsomal protein up to 7 μg. The assay was linear with time up to 24 min when 1 μg of protein was employed. Fatty acid:CoASH ligase activity was strongly dependent on ATP and magnesium, was stimulated by Triton WR-1339, and was two- to fivefold dependent on added fatty acid. The filter assay is easier than existing assays based on incorporation of labeled fatty acid and is equally sensitive.

Supplementary key words isolated fat cells · coenzyme A · palmitoyl CoA · fatty acid · microsomes · lipid synthesis · Triton WR-1339 · fatty acid contamination of microsomes · radiochemical assay

Long-chain fatty acid:CoASH ligase (AMP)[2] (EC 6.2.1.3) activity is found in all mammalian cell types and is apparently ubiquitous in nature. This activity catalyzes the reaction shown in Eq. 1:

$$R{-}\overset{\overset{O}{\|}}{C}{-}OH + ATP + CoASH \xrightarrow{Mg^{2+}} R{-}\overset{\overset{O}{\|}}{C}{-}S{-}CoA + AMP + PP_i \quad \text{Eq. 1}$$

This process is often referred to as "fatty acid activation" because it is a prerequisite for further metabolism of the fatty acid. Ligase activity has been most extensively investigated from liver (1–10), where the activity is largely associated with the microsomal fraction (4, 11). A few reports on the activity from adipose tissue (12, 13), heart (14), and brain (14, 15), and a survey of the activity in kidney, adrenal, testis, and skeletal muscle (14), have appeared. Since the original description of the activity by Kornberg and Pricer in 1953 (1), fatty acid:CoASH ligase has been assayed by a variety of methods. These include a hydroxamate method (1), disappearance of CoASH (3), conversion of labeled fatty acids to fatty acyl CoA (6, 7, 9, 13), and a coupled assay employing fatty acyl CoA carnitine transacylase (4, 15). The hydroxamate assay method requires milligram quantities of protein and millimolar levels of fatty acid. Recent investigations have monitored the conversion of labeled fatty acids to fatty acyl CoA utilizing microgram quantities of protein and micromolar concentrations of fatty acid. However, this method is quite time-consuming because each assay involves three or more liquid–liquid extractions (6, 7, 9, 13, 16).

In this report, we describe a novel radiochemical assay for fatty acid:CoASH ligase activity that is based on the conversion of [^{3}H]CoASH to fatty acyl CoA.

EXPERIMENTAL PROCEDURES

Materials

ATP and CoASH were obtained from P-L Biochemicals, Milwaukee, Wis. Dithiothreitol and bovine serum al-

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[2] This activity has been referred to as palmitoyl CoA synthetase and as long-chain acyl CoA synthetase.

bumin (essentially fatty acid-free) were obtained from Sigma Chemical Co., St. Louis, Mo. Palmitic acid was a product of the Hormel Institute, Austin, Minn. Bovine serum albumin (fraction V) was purchased from Armour Pharmaceutical Co., Omaha, Neb. Millipore filters, 25 mm, type HAWP, 0.45 μm (white), were purchased from Millipore Corp., Bedford, Mass. Triton WR-1339 was obtained from Ruger Chemical Co., Irvington, N.J. Crude collagenase (type I) from *Clostridium histolyticum* was the product of Worthington Biochemical Corp., Freehold, N.J. [^{3}H]Palmitic acid, [^{3}H]CoASH, [^{14}C]palmitoyl CoA, and Aquasol were purchased from New England Nuclear, Boston, Mass.

[^{3}H]Palmitoyl CoA was synthesized from [^{3}H]palmitic acid via the *N*-hydroxysuccinimide ester according to the procedure of Al-Arif and Blecher (17).

All other reagents were the highest grade commercially available.

Preparation of microsomes

Isolated fat cells were prepared from the parametrial fat of 200–250-g Charles River CD female rats essentially according to the method of Rodbell (18) as described by Czech and Lynn (19). The microsomal membrane fraction was obtained after the isolated cells were collected by centrifugation and broken in a Teflon–glass homogenizer containing 0.25 M sucrose with 1 mM EDTA and 5 mM Tris, pH 7.4, by 10 rapid up-and-down strokes, essentially according to Jarett (20). The microsomal pellet was suspended in 50 mM potassium phosphate, pH 7.0. Protein was determined by a microbiuret procedure (21) using bovine serum albumin as a standard.

Assay of fatty acid:CoASH ligase

Assays were run at 25°C. The standard assay mixture contained 380 mM Tris–HCl, pH 7.4, 8 mM $MgCl_2$, 10 mM ATP, 0.5 mM dithiothreitol, 50 μM [^{3}H]CoASH (10 μCi/μmole), 30 μM palmitic acid, and 1 mg/ml Triton WR-1339, in a final volume of 200 μl. The concentrations of ATP, CoASH, and palmitic acid were found to be saturating under the conditions used. The reaction was started by the addition of microsomal protein and was stopped by adding 20 μl of 1 mg/ml bovine serum albumin (essentially fatty acid-free) and 2.0 ml of ice-cold 0.3 M trichloroacetic acid. The tubes were held in ice for 5 min, then the contents of each tube was poured over a 25-mm, 0.45 μm, HAWP Millipore filter and filtered on a Millipore filter box. The tubes were washed three times with 2.0 ml of cold trichloroacetic acid, and these washings were in turn poured over the filter. The filters were dissolved in 8 ml of Bray's solution (22) and counted in an Intertechnique (Dover, N.J.) scintillation counter. Tritium was counted at 30% efficiency.

Identification of ^{3}H-labeled material retained on filter

The ^{3}H-labeled material retained on the Millipore filter was characterized by ascending paper chromatography. Several assays were performed using 3 μg of microsomal protein in the standard assay mixture for 10 min. The acidified reaction mixtures were processed as usual. One filter was counted directly. The others were extracted twice with 3 ml of $CHCl_3$–MeOH 2:1 by shaking for a few minutes at room temperature. The liquid phase was transferred to a tube and concentrated to dryness under nitrogen. A portion of this material was applied directly to Whatman no. 3 paper. Another portion was treated with 1.0 M neutral hydroxylamine for 30 min at room temperature prior to being spotted on the paper. Authentic [^{3}H]CoASH and [^{3}H]palmitoyl CoA were also spotted. The chromatogram was developed in 1-butanol–acetic acid–water 5:2:3. The material was also characterized using isobutyric acid–ammonium hydroxide–water 57.7:3.8:38 (v/v/v). The chromatograms were dried, and the distribution of label was determined by cutting each lane into sections. Each section was added to a scintillation vial, and 0.8 ml of water and 8 ml of Aquasol were added.

RESULTS

Rationale

An assay for fatty acid:CoASH ligase activity (see Eq. 1) based on conversion of [^{3}H]CoASH to fatty acyl [^{3}H]CoA should be possible provided a rapid, simple separation of [^{3}H]CoASH and the labeled fatty acyl CoA's could be achieved.

Separation of [^{14}C]palmitoyl CoA and [^{3}H]CoASH

Because long-chain fatty acyl CoA's are known to be acid precipitable (17, 23), we tested whether [^{14}C]palmitoyl CoA could be quantitatively retained on a Millipore filter upon acidification of a solution containing buffer, magnesium, ATP, fatty acid, and CoASH (all the necessary components of a fatty acid:CoASH ligase assay mixture). As shown in **Table 1,** more than 99% of the [^{14}C]palmitoyl CoA was retained by the filter provided that 20 μg of bovine serum albumin was added prior to acidification with trichloroacetic acid. Under these conditions, less than 0.3% of the labeled [^{3}H]CoASH was retained on the filter. Hence, a rapid separation of labeled fatty acyl CoA and CoASH was achieved by Millipore filtration.

Because this separation would form the basis of the proposed fatty acid:CoASH ligase assay, we proceeded to investigate several factors that might influence the retention of either [^{14}C]palmitoyl CoA or [^{3}H]CoASH on the filter. These data are also presented in Table 1. Carrier palmitoyl CoA was not necessary for the quantitative recovery of

labeled palmitoyl CoA provided albumin was added. It was noted that 90% of the labeled palmitoyl CoA was retained when the solution was not acidified and the filter was washed with water. Because this assay should be useful for characterizing fatty acid:CoASH ligase activity under a variety of conditions, under which the ionic strength might change, we investigated whether KCl added to final concentrations of 0.33, 0.66, and 1.1 M affected the retention of palmitoyl CoA on the filter. No differences were noted. Further, the quantity of [^{3}H]CoASH retained on the filter did not vary under any conditions tested.

Assay of fatty acid:CoASH ligase

Preliminary experiments demonstrated that [^{3}H]CoASH was converted to material retained on a Millipore filter upon filtration of an acidified reaction mixture. (Evidence will be presented later that demonstrates that the material is fatty acyl CoA.) This conversion was dependent on microsomal protein, magnesium, fatty acid, and ATP (see **Table 2**). The quantity of [^{3}H]CoASH retained was linear with the amount of added microsomal protein up to 7 μg during a 10-min assay period (see **Fig. 1**). The retention of [^{3}H]CoASH was linear with time for 24 min when 1 μg of protein was employed (**Fig. 2**). No lag phase was observed. The assay was linear with time for at least 10 min at all microsomal protein concentrations tested (1–5 μg).

Requirements for assay of fatty acid:CoASH ligase

The dependence of the fatty acid:CoASH ligase assay was investigated under conditions where the assay was demonstrated to be linear with time and amount of protein. The results, shown in Table 2, using 2 μg of protein for 10 min, demonstrated that the assay is totally dependent on added microsomal protein. No activity was observed using boiled microsomal protein or if the reaction mixture was acidified prior to addition of microsomal protein. Further, the reaction was strongly dependent on ATP and Mg^{2+}. The effect of dithiothreitol was small, but it was routinely included to ensure reduction of the labeled CoASH. The reaction was only 2.4-fold dependent on added palmitic acid (Table 2). The dependence on added palmitic acid varied from two- to fivefold in different microsomal preparations. This suggested that different microsomal preparations contained different amounts of free fatty acid. The reaction was quite dependent on addition of the fatty acid in Triton WR-1339. The reaction rate independent of added fatty acid was also stimulated by addition of Triton WR-1339.

Characterization of [^{3}H]CoASH reaction product

The data presented in Table 1 demonstrated that palmitoyl CoA was quantitatively retained on Millipore filters and that CoASH was not. Therefore, the [^{3}H]CoASH was presumed to be retained on the Millipore filter because it had been converted to a long-chain fatty acyl CoA derivative during the incubation period. Evidence supporting this presumption was obtained by characterizing the material retained on the filter produced under conditions in which

TABLE 1. Separation of palmitoyl CoA and CoASH from an assay mixture by Millipore filtration

Incubation Mixture	Radiolabel Added	Precipitation and Wash	% Radioactivity Retained on Filter
Complete system	[^{14}C]Palmitoyl CoA	TCA	99.7
	[^{3}H]CoASH		0.3
Complete system	[^{14}C]Palmitoyl CoA	Water	89.6
	[^{3}H]CoASH		0.3
Complete system minus bovine serum albumin	[^{14}C]Palmitoyl CoA	TCA	92.3
	[^{3}H]CoASH		0.3
Complete system minus bovine serum albumin plus 1 mM palmitoyl CoA	[^{14}C]Palmitoyl CoA	TCA	92.8
	[^{3}H]CoASH		0.3
Complete system plus 0.33 M KCl	[^{14}C]Palmitoyl CoA	TCA	101.2
	[^{3}H]CoASH		0.3
Complete system plus 0.66 M KCl	[^{14}C]Palmitoyl CoA	TCA	102.2
	[^{3}H]CoASH		0.3
Complete system plus 1.1 M KCl	[^{14}C]Palmitoyl CoA	TCA	99.9
	[^{3}H]CoASH		0.3

[^{14}C]Palmitoyl CoA (77,200 dpm) or [^{3}H]CoASH (224,200 dpm) was added to the standard assay mixture minus microsomal protein. Then bovine serum albumin and trichloroacetic acid (TCA) were added, and the contents were filtered. See Experimental Procedures for details. The filters were counted and the percentage of radioactivity retained was determined.

TABLE 2. Dependencies of the assay

Assay Conditions	Conversion to Fatty Acyl [^{3}H]CoA
	nmoles/min/mg
Complete system[a]	44.5[b]
Minus microsomes[a]	0
Boiled microsomes	0
Time 0, control	0
Minus ATP	0.3
Minus palmitic acid	18.0
Minus Mg^{2+}	3.2
Minus Mg^{2+} plus 1 mM EDTA	0
Minus dithiothreitol	38.0
Minus Triton WR-1339 minus palmitic acid	3.9
Minus Triton WR-1339	8.1

Assays were run for 10 min using the standard assay conditions described under Experimental Procedures. Reactions employed 2 μg of microsomal protein.

[a] In the complete system, 6077 cpm was observed with a background (minus microsomes) of 150 cpm.

[b] Specific activities have ranged from 20 to 120 nmoles/min/mg in different microsomal preparations. A value of 45 nmoles/min/mg was typical.

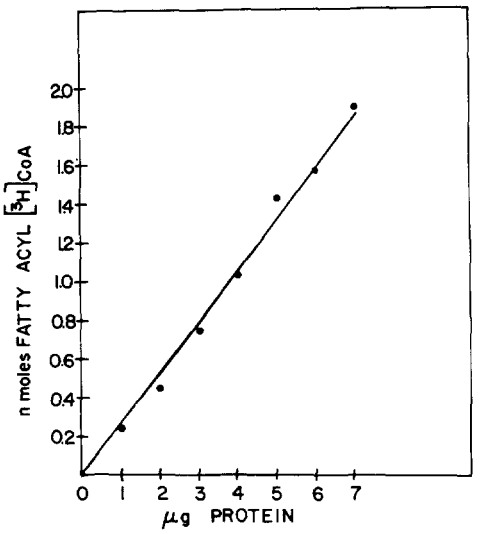

Fig. 1. Linearity of Millipore filter assay with amount of protein. Fatty acid:CoASH ligase activity was assayed at various microsomal protein levels for 10 min as described under Experimental Procedures.

the reaction was linear with time and protein and with the dependencies as detailed in Table 2. Over 75% of the tritiated material was extracted from the filters and chromatographed on Whatman no. 3 paper in two solvent systems (**Figs.** 3 and 4). Over 96% of the label migrated similarly to authentic [^{3}H]palmitoyl CoA, and no peak of label migrated like [^{3}H]CoASH (Figs. 3*B* and 4*B*). The labeled material was treated with neutral hydroxylamine to form a hydroxamate derivative of the fatty acid and thereby release the labeled CoASH. When this material was chromatographed, over 93% of the label chromatographed similarly to [^{3}H]CoASH (Figs. 3*D* and 4*D*). Hence, all of the labeled material appeared to be long-chain fatty acyl CoA. When the labeled reaction product formed in the absence of

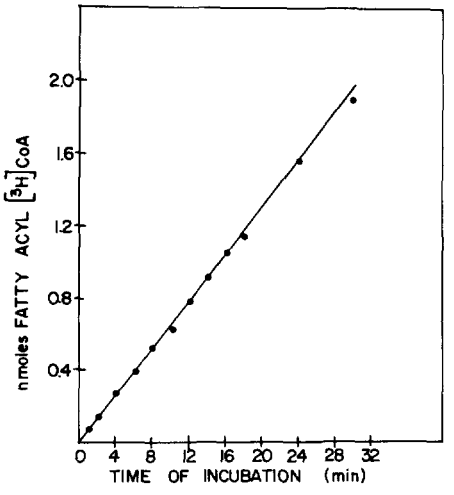

Fig. 2. Linearity of Millipore filter assay with time. Fatty acid:CoASH ligase activity was assayed at 1 μg of microsomal protein for various periods of time using conditions described under Experimental Procedures.

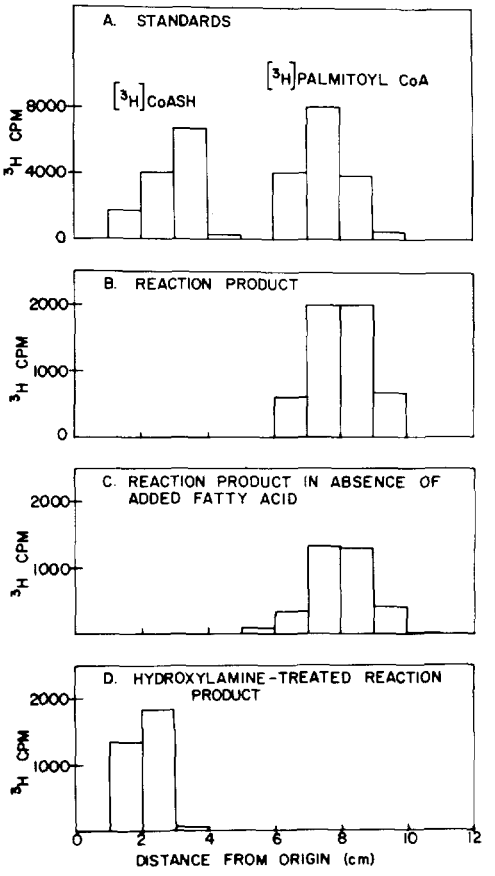

Fig. 3. Characterization of labeled reaction product by paper chromatography. The labeled material retained on Millipore filters was chromatographed on Whatman no. 3 paper using 1-butanol–acetic acid–water 5:2:3 (v/v/v), as described under Experimental Procedures. *A*, authentic [^{3}H]CoASH and [^{3}H]palmitoyl CoA standards run in separate lanes and plotted together for comparison; *B*, ^{3}H-labeled product formed in the presence of 30 μM palmitic acid; *C*, ^{3}H-labeled reaction product formed in the absence of exogenous fatty acid; *D*, ^{3}H-labeled reaction product (formed in the presence of 30 μM palmitic acid) treated with neutral 1 M hydroxylamine. For details, see Experimental Procedures.

added fatty acid was chromatographed in both solvent systems, over 94% of the labeled material migrated similarly to palmitoyl CoA (Figs. 3*C* and 4*C*). Thus, the lack of total dependence on added fatty acid most likely resulted from endogenous fatty acid associated with the microsomes.

DISCUSSION

The fatty acid:CoASH ligase assay described here represents an important technological advance in the study of long-chain fatty acid:CoASH ligase activity. The assay, like the assay using labeled fatty acids (6, 7), is orders of magnitude more sensitive than the earlier assays based on hydroxamate formation or CoASH disappearance. The assay is very sensitive, allowing accurate assessments of fatty acid:CoASH ligase activity using as little as 1 μg of

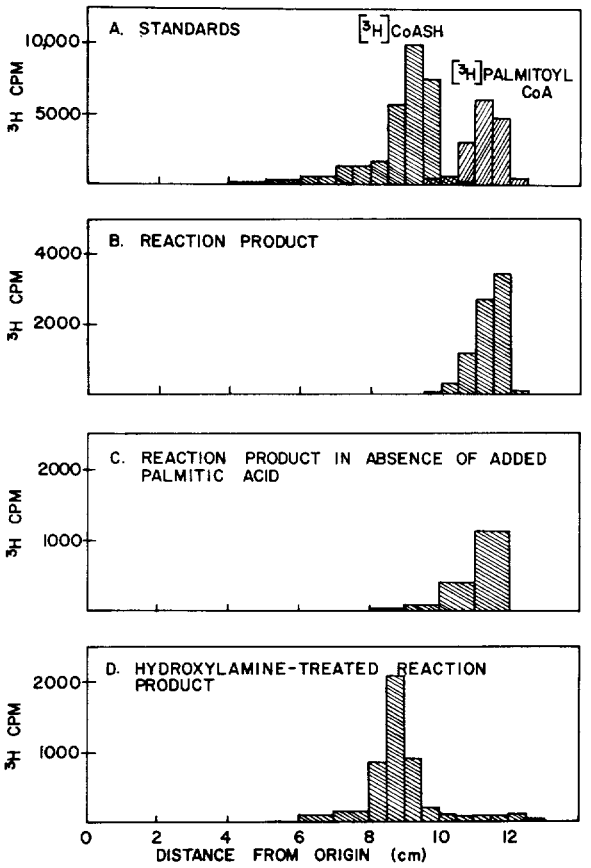

Fig. 4. Characterization of labeled reaction product by paper chromatography in a second solvent system. The labeled material retained on Millipore filters was characterized in a second solvent system, isobutyric acid–ammonium hydroxide–water 57.7:3.8:38 (v/v/v), in a manner analogous to that described in Fig. 3. β-Mercaptoethanol was added during the hydroxylamine treatment to prevent oxidation of the labeled CoASH. Panels are as described in the legend to Fig. 3.

microsomal protein in 10 min. The assay employs low levels of added fatty acid and measures product formation directly. A principal advantage of the filter assay is that it is rapid and avoids the time-consuming liquid–liquid extractions of the assay using labeled fatty acids. Moreover, because product formation is monitored by [^{3}H]CoASH incorporation, comparative studies on the fatty acid specificity of the ligase can now be conveniently performed without the necessity of synthesizing numerous labeled fatty acids.

The filter assay, like the assay employing labeled fatty acids (6, 7), does employ the detergent Triton WR-1339. The presence of detergent at 1 mg/ml makes it unlikely that the fatty acid is present in solution as the monomer, as stated by previous workers (7), even though the fatty acid was added below its critical micellar concentration. The fatty acid undoubtedly is associated with a mixed micelle.

The filter assay permits investigations of ligase activity independent of fatty acid. In fact, our microsomal preparations from isolated rat fat cells appear to be contaminated with added fatty acid. This observation may necessitate a reevaluation of rate and specificity studies in which only the dependence on added labeled fatty acid was monitored (6, 7). Further, because intracellular CoASH pools are known to be low, endogenous CoASH can be swamped by addition of labeled CoASH, thus allowing fatty acid: CoASH ligase activity to be assayed in crude extracts of any cell type. The filter assay has been employed for detecting ligase activity in liver extracts and microsomes, and in crude extracts of isolated fat cells, in which endogenous fatty acid would presumably interfere with a determination of ligase activity using the labeled fatty acid assay.

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