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A Method for the Chemical Synthesis of ^{14}C-Labeled Fatty Acyl Coenzyme A's of High Specific Activity

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A simple and reliable method, based on that described by W. Seubert (1960, *Biochem Prep.* 7, 80–83), has been developed for the chemical synthesis of radioactive acyl coenzyme A's. 1-^{14}C-labeled fatty acids (palmitic, oleic, and linoleic) are converted to their acyl chlorides with oxalyl chloride. The [1-^{14}C]acyl chlorides are then condensed with a two- to three-fold molar excess of coenzyme A in a bicarbonate-buffered tetrahydrofuran solution to form the corresponding [1-^{14}C]acyl coenzyme A's. The overall yields are near 75%, and the purities are greater than 90% based on spectral, chromatographic, and enzymatic properties.

During the course of studying a very low-activity enzyme system in brain which utilizes [^{14}C]acyl CoA as substrate, it became necessary to synthesize this labeled compound at a very high specific activity. A number of chemical and enzymatic methods have been described for the preparation of acyl CoA (1–9). However, none of the procedures was suitable for our purpose. Most of the chemical methods require an excess of the fatty acid derivative to esterify limiting amounts of CoASH, with an unacceptably low conversion of the costly radioactive fatty acid to product. In the enzymatic methods, utilizing acyl-CoA synthetase, the yields are low (<50%) and the product is probably contaminated by other lipids (1,2) unless lengthy procedures are first employed to purify the enzyme (3). We have successfully used the procedure originally described by Seubert (4,5) to prepare non-radioactive acyl CoA, but when the procedure was scaled down for the preparation of radioactive acyl CoA and excess acyl chloride was not used, yields became low and erratic.

Some modifications were therefore necessary to prepare radioactive acyl CoA. We have examined the conditions for the synthesis of acyl CoA in detail with the objective of obtaining a high rate of conversion of radioactive fatty acids to acyl CoA's. By the use of an excess of CoA and by the inclusion of a bicarbonate buffer for pH control, we have developed a method for the preparation of high specific activity [1-^{14}C]acyl CoA's, both saturated and unsaturated, with reproducible yields. The reaction conditions and detailed methods are described here.

MATERIALS AND METHODS

CoASH (85% pure), BHT,[1] DTNB, 1-acyl-*sn*-glycero-3-phosphorylcholine (lysolecithin), palmitoyl chloride, palmitic acid, and oxalyl chloride were obtained from Sigma Chemical Company (St. Louis, Mo.). 1-^{14}C-labeled fatty acids, approximately 50 mCi/mmol, were from New

[1] Abbreviations used: BHT, butylated hydroxytoluene; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

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England Nuclear (Boston, Mass.). Acetone, tetrahydrofuran, and benzene were products of Mallinckrodt (St. Louis, Mo.). Anhydrous ether was supplied by Drake Bros. (Menomonee Falls, Wisc.) and pyridine, by J. T. Baker Chemical Company (Phillipsburg, N. J.). E. Merck's silica gel 60 thin-layer chromatography plates were from Brinkmann Instruments, Des Plaines, Illinois.

Dry benzene was prepared by distillation from CaH_2 and stored in a desiccator containing anhydrous $CaSO_4$ for up to 3 weeks. Tetrahydrofuran was freshly distilled before use to remove peroxides, taking care not to distill it completely to dryness. Acetone and pyridine were dried with anhydrous Na_2SO_4 and CaH_2, respectively.

Fatty acyl chlorides were prepared by reacting fatty acid with oxalyl chloride (10). One to five micromoles of ^{14}C-labeled fatty acids (0.1–50 mCi/mmol), along with an equimolar amount of BHT in the case of an unsaturated fatty acid, were added to a 15-ml Corex tube (No. 8441, Corning Glass Works) and dried three times with dry benzene under a gentle stream of N_2. Dry benzene, 0.8 ml, and 0.4 ml oxalyl chloride were then added, the tube was flushed with nitrogen, stoppered, and incubated for approximately 1 h at 37°C. Solvents were evaporated by a stream of nitrogen, and the oily residue was dried three times with N_2 and aliquots of dry benzene.

Radioactive acyl chloride was quantitated by reacting it with methanol in pyridine and measuring the methyl ester formed. Direct measurement of acyl chloride itself was not possible due to its instability during handling and during thin-layer chromatography. Aliquots of a solution containing acyl chloride (approximately 0.1–0.4 μmol) were mixed with 2 ml of a 1/1 mixture of methanol and dry pyridine. After a 2-h incubation at 37°C, solvents were evaporated with nitrogen. When the original acyl chloride solution had contained $NaHCO_3$ (used for pH control; see later sections), the residue after drying was extracted by the Bligh and Dyer method under acidic conditions (11) to allow recovery of free fatty acid. In either case, the final residue was separated by thin-layer chromatography using petroleum ether/diethyl ether/glacial acetic acid (50/50/2) as developing solvent. After localization by radioautography, the methyl ester at R_f 0.82 and fatty acid at R_f 0.48 were scraped off and counted by liquid scintillation (12). Under these conditions, maximum yields of methyl ester from acyl chloride ranged from 90 to 95%, while less than 1% of free fatty acid could be converted to the ester. The conversion of acyl chloride to its methyl ester was not affected by the presence of small amounts (10–50 μl) of tetrahydrofuran/150 mM aqueous $NaHCO_3$, pH 8.8 (2.2/1.0), in the esterification solution.

The optimum conditions for the preparation of [1-^{14}C]palmitoyl CoA, as determined in this communication (see Results) are as follows. [1-^{14}C]Palmitic acid (5.0 μmol) was converted to its acyl chloride as described above. To the dry acyl chloride, 1.28 ml of a solution (adjusted to pH 8.8 with NaOH) containing 15 μmol CoASH in tetrahydrofuran/150 mM $NaHCO_3$ (2.2/1.0) was added all at once and mixed well. The tube was flushed with nitrogen, stoppered, and incubated at 37°C for 30 min. The reaction was terminated by the addition of 20 μl 10% (v/v) $HClO_4$, to give a pH of about 4, and solvents were then evaporated off by a stream of N_2.

The acyl CoA was then purified basically as described by Seubert (4), with slight modification. To the dried residue in the Corex tube, 2.3 ml of aqueous 1.3% $HClO_4$ was added. The tube was chilled on ice and centrifuged at 20,000g for 15 min, and the supernatant was removed. In like manner, the pellet was washed successively with the following solvents: 2.3 ml of 1.3% $HClO_4$, 4 ml of dry acetone[2] and twice with 4 ml of

[2] The commercial acetone contained variable amounts of water, which interfered with the pelleting of the acyl CoA. The problem was aggravated by humid weather, making the drying procedure mandatory.

dry ether. The last traces of ether had to be removed from the fluffy residue by incubating the tube in a shaking water bath heated to 37°C. The final product was taken up in 10 mM phosphate buffer, pH 6.0, and stored under a N_2 atmosphere at −20°C. The overall yield was approximately 75%. (see Table 1).

[1-^{14}C]Oleoyl CoA and [1-^{14}C]linoleoyl CoA were prepared in a similar manner. An equimolar amount of BHT was added to the fatty acid before reacting with oxalyl chloride, as described above. A 1.0-μmol amount of unsaturated fatty acid was generally used rather than the 5 μmol used for palmitoyl CoA synthesis, and all reagent amounts used in the condensation reaction were reduced accordingly. The purified unsaturated [^{14}C]acyl CoA's were taken up in a 10 mM phosphate buffer, pH 6.0, containing BHT (0.1 mol/mol acyl CoA) and stored under a N_2 atmosphere at −20°C. Yields for both were about 75%.

The purities of the [1-^{14}C]acyl CoA's were determined by their spectral properties, by thin-layer chromatography, and by their reactivities in two enzymatic systems. The extinction coefficient at 260 nm was taken as 15.4 mM^{-1} cm^{-1}, and the thioester bond was measured at 232 nm; A_{232}/A_{260} = 0.56 for palmitoyl CoA[3] (13,7). The radiochemical purity was determined by thin-layer chromatography using butanol/water/acetic acid (50/30/20) as solvent (14), with recoveries of applied radioactivity > 90%. After localization by radioautography, the radioactive spots were scraped off and counted by liquid scintillation (12). The activity of these acyl CoA's toward two different enzymes, the acyl-CoA hydrolase (palmitoyl-CoA hydrolase EC 3.1.2.2) and the lysolecithin acyltransferase (acyl-CoA:1-acylglycero-3-phosphocholine *O*-acyltransferase, EC 2.3.1.23) was also checked (15). Soluble protein ($3 \times 10^6 g_{min}$ supernatant) from a 12-day rat brain was used for the acyl-CoA hydrolase assay (16). Guinea pig liver microsomes ($0.2–3 \times 10^6$ g_{min} residue) were used for the acyltransferase assay, because of the relatively high ratio of acyltransferase to hydrolase activity found in this tissue (15). In both cases, the reaction was allowed to go to completion, and the released CoASH was measured at 412 nm, after its reaction with DTNB (ϵ_{412} = 13.6 mM^{-1} cm^{-1}) (15).

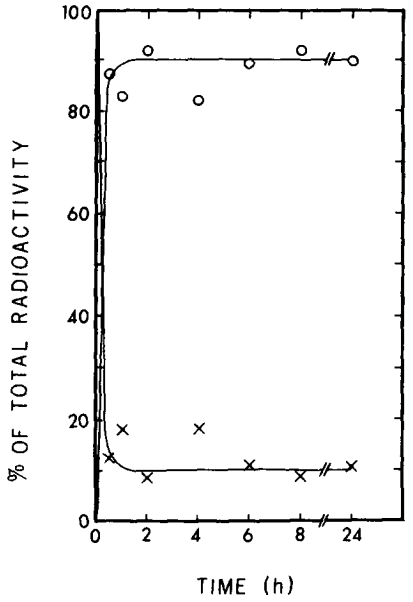

FIG. 1. Time course of formation of acyl chloride. Ten micromoles of [^{14}C]palmitic acid (240 dpm/nmol) was incubated with a mixture of oxalyl chloride (0.4 ml) and dry benzene (0.8 ml) at 37°C. At designated times, 50-μl aliquots were removed, dried under N_2, and reacted with methanol and pyridine for formation of methyl palmitate as described under Materials and Methods. ○, Acyl chloride (as methyl palmitate); ×, free fatty acid. Points represent the averages of duplicate determinations.

RESULTS

Preparation of Acyl Chloride

The reaction of fatty acid with oxalyl chloride at 37°C to form the acyl chloride was found to be largely complete within 30 min (Fig. 1). The maximum yield varied

[3] The inclusion of 0.1 *M* eq BHT in the unsaturated acyl CoA solutions caused a negligible interference with A_{232}/A_{260} measurements as the extinction coefficient of BHT at these two wavelengths was found to be 0.3 and 0.8 mM^{-1} cm^{-1}, respectively.

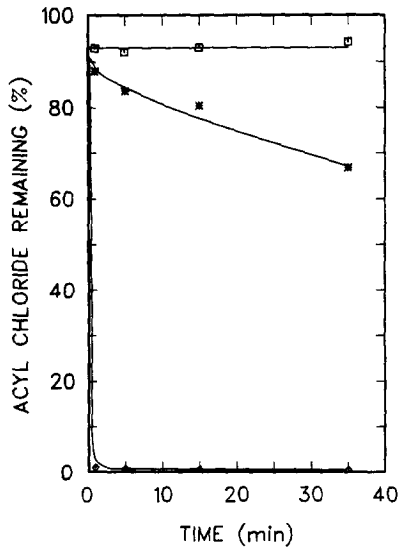

FIG. 2. Stability of acyl chloride in different solvents. [^{14}C]Palmitic acid (400 dpm/nmol) was converted to the acyl chloride as described under Materials and Methods. Designated solvents were added to a portion of dried acyl chloride to give a concentration of 4 μmol/ml, and 25-μl aliquots were removed and added to tubes containing methanol/pyridine at the specified times. Acyl chloride was quantitated as the methyl ester as detailed under Materials and Methods. ⊡, Benzene; ★, tetrahydrofuran; ◈, tetrahydrofuran/150 mM $NaHCO_3$, ph 8.8 (2.2/1.0). Points represent the averages of triplicate determinations.

from 90 to 95%, as measured by its conversion to methyl ester (see Materials and Methods). The only other radioactive product detected was free fatty acid. Although the formation of acyl chloride is described to be quantitative (10), anhydrous methanol was not used in this methylation procedure and a portion of acyl chloride is probably hydrolyzed rather than esterified, accounting for the lower measured yield.

Hydrolysis of Acyl Chloride

The stability of [^{14}C]palmitoyl chloride was tested in various solvent systems relevant to the synthesis of palmitoyl CoA (Fig. 2). Acyl chloride is very stable in dry benzene and can be stored in this solvent for relatively long periods of time. An appreciable rate of hydrolysis was observed in freshly distilled tetrahydrofuran (Fig. 2). It is also seen in Fig. 2 that acyl chloride was hydrolyzed very rapidly when incubated under the conditions used for condensation of acyl chloride with CoASH, namely, in tetrahydrofuran/150 mM $NaHCO_3$, pH 8.8 (2.2/1.0). Degradation was measured to be nearly 99% within the first minute; however, the actual hydrolysis may not be quite this high due to inaccuracies in the measurement procedure, as mentioned in the preceding paragraph.

Condensation of Acyl Chloride with CoASH

When the same tetrahydrofuran/$NaHCO_3$ solution, but containing CoASH, was added to the acyl chloride it was found that esterification was more rapid than the hydrolysis, as 70% of acyl chloride was converted to

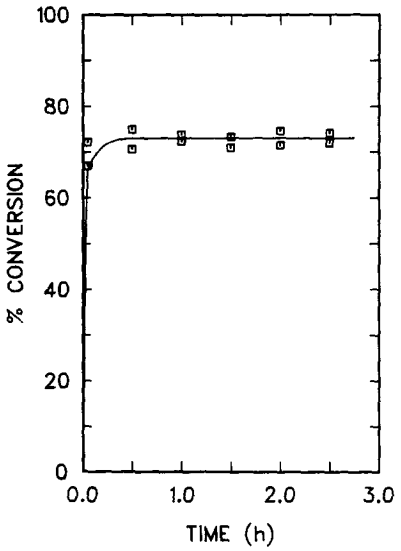

FIG. 3. Time course of condensation of acyl chloride with CoASH. Six hundred and forty microliters of tetrahydrofuran/150 mM $NaHCO_3$, pH 8.8 (2.2/1.0) containing 5.0 μmol CoASH was added to 2.5 μmol palmitoyl chloride and incubated at 37°C. The reaction was terminated at designated times with $HClO_4$. The palmitoyl CoA was purified by precipitation from acid and organic solvents, and then quantitated by its absorbance at 260 nm. The ordinate represents the percentage conversion of the acyl chloride to acyl CoA. (See text.)

acyl CoA within 3 min (Fig. 3). Incubation for longer times gave only a small increase in the yield of acyl CoA.

Increasing the amount of CoASH in the reaction mixture led to increased conversion of acyl chloride to acyl CoA (Fig. 4). Optimal yields were obtained when the concentration of CoASH was at least twice that of acyl chloride and a further increase of CoASH concentration gave only a small increase in the amount of acyl CoA formed (Fig. 4).

Purity of Acyl CoA's

Table 1 summarizes optical, radiochemical, and enzymatic determinations of the purity of [1-^{14}C]acyl CoA's. Typical values are shown for one set of acyl CoA's. Similar results were obtained with different preparations of palmitoyl and linoleoyl CoA. By thin-layer chromatography of these compounds, most (90–96%) of the radioactivity migrated with standard palmitoyl CoA (R_f 0.46) with three minor bands (total of 4% of the radioactivity, R_f's 0.50, 0.55, 0.61) above the main band. With unsaturated acyl CoA's, another minor band (2–6% of the radioactivity) was present below the acyl CoA band at R_f 0.42. This band, probably an oxidation product, could have formed during the handling, as no effort was made to exclude oxygen during the procedure. Free fatty acid (R_f 0.86) was undetectable in all the fatty acyl CoA's. All acyl CoA's were found to be active in the two enzymatic determinations employed (Table 1).

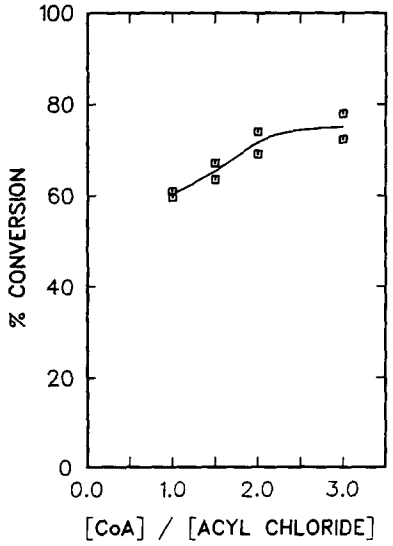

FIG. 4. Optimum concentration of CoASH for the formation of acyl CoA. The reaction conditions are as stated in the legend to Fig. 3, except that variable amounts of CoASH were used as designated here, and the reaction time was 2 h.

TABLE 1

CHARACTERIZATION OF PURIFIED [^{14}C]ACYL CoA[a]

Measurement	Acyl CoA 16:0	18:1	18:2
A_{260}[b]	100	100	100
Radioactivity[b,c]	110	102	100
Acyl-CoA hydrolase[b]	92	87	87
Lysolecithin acyltransferase[b]	95	100	92
Free -SH[b]	0	0	0
A_{232}/A_{260}	0.52	0.52	0.56
Radiochemical purity[d]	96	94	90
Yield[e]	79	74	75

[a] Individual [1-^{14}C]acyl CoA's (approximately 50 mCi/mmol) were synthesized according to the method described in the text. Numbers represent the averages of 2 to 10 determinations of each parameter on each preparation.

[b] The concentrations of acyl CoA solutions determined by these measurements are expressed as a percentage of that determined by its absorbance at 260 nm (ϵ_{260} = 15.4 mM^{-1} cm^{-1}).

[c] Determined by the specific activity of the precursor ^{14}C-labeled fatty acid.

[d] Determined by thin-layer chromatography as a percentage of the recovered radioactivity in the acyl CoA spot.

[e] Determined by the percentage recovery of precursor fatty acid radioactivity in the purified product.

DISCUSSION

Three general methods for the chemical synthesis of fatty acyl CoA are in common use, differing mainly in the particular ac-

tivated fatty acid derivative used. These derivatives are (a) the *N*-hydroxysuccinimide ester (6), (b) the fatty acid anhydride (7) or the mixed acid anhydride (8,9), and (c) the acyl chloride (4,5). These methods were developed for the synthesis of nonradioactive acyl CoA and, consequently, were optimized to conserve the more expensive CoASH by using an excess of the fatty acid derivative, up to 70-fold. Modifications of these procedures have been used by some workers for the synthesis of radioactive acyl CoA, but are either lengthy (17) or give a low yield (18). In the development of the procedure described here, we found the acyl chloride method to be the simplest, because this compound can be synthesized easily in high yield without the necessity of a lengthy isolation and purification procedure. In adapting Seubert's method for its condensation with CoASH (4,5), we encountered difficulties with pH regulation and with the hydrolysis of acyl chloride.

The control of pH is important during the condensation of acyl chloride with CoASH since HCl produced during the nonbuffered reaction causes a rapid drop in pH and in the reaction rate. The pH needs to be maintained at a high enough level for the condensation to occur, but not so high as to hydrolyze the thioester bond of the newly formed acyl CoA. In experiments that were buffered in the range 9.0 to 9.2, sporadically low yields were observed, probably due to this product hydrolysis. Buffering at a slightly lower pH, 8.8, with sodium bicarbonate was found to give the best results.

Another major problem encountered was that of hydrolysis of acyl chloride during its handling. Early in this study, tetrahydrofuran was used to transfer the freshly synthesized acyl chloride to another tube containing CoASH (5), and variable yields between 20 and 40% were observed. Subsequently, it was found that acyl chloride is hydrolyzed at an appreciable rate when in solution in tetrahydrofuran (Fig. 2). Although this was not a problem in the original procedure where acyl chloride was in excess, in our method where acyl chloride is the limiting reagent, hydrolysis was reflected directly in a reduction of the yield of acyl CoA. By reversing the addition, i.e., by adding the CoASH solution directly to the dried acyl chloride, the yield was improved to a stable 75%.

By various criteria, it was shown that the acyl CoA's synthesized here were all greater than 90% pure (Table 1). Enzymatic tests of purity were deemed necessary as these compounds were synthesized to be used in biological systems, and, in addition, an earlier study (19) reported that a batch of linoleoyl CoA that was optically pure was only 25% reactive in an enzymatic system. The [^{14}C]-linoleoyl CoA synthesized in this study was found to be about 90% enzymatically reactive (Table 1) and remained so for at least 2 weeks after it was made. However, after prolonged storage (3–4 months, in 10 mM phosphate buffer, pH 6.0, with 0.1% BHT under N_2 at −20°C) two of the batches were found to be only partially reactive toward acyltransferase. The optical properties were also found to have changed; the A_{232}/A_{260} ratio had increased from initial values of 0.56 to about 0.9. This was probably due to oxidation or peroxidation of double bonds, which possibly could have been prevented by the use of more antioxidant during storage. Batches of [^{14}C]palmitoyl and [1-^{14}C]-oleoyl CoA, which had also been stored for 3 months, both retained spectral properties and enzymatic reactivities identical to that originally measured and reported in Table 1.

Though this procedure was worked out for the synthesis of [^{14}C]acyl CoA's we have also used it on two occasions to prepare nonradioactive acyl CoA using a small excess of palmitoyl chloride over CoASH, with good yields.

ACKNOWLEDGMENTS

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