SYNTHESIS AND PROPERTIES OF PALMITYL ADENYLATE, PALMITYL COENZYME A, AND PALMITYL GLUTATHIONE*

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For certain studies on the formation of complex lipids, it was found necessary to prepare thiol esters of coenzyme A and long-chain fatty acids. A convenient method for the chemical synthesis of palmityl coenzyme A is presented here, and the preparation of palmityl adenylate and palmityl glutathione are also described. These are modifications of the methods of SIMON AND SHEMIN[1], and of AVISON[2]. Transfer reactions between phosphoryl and sulfhydryl derivatives of short-chain fatty acids have been shown to occur[3–5], and recently, acyl adenylates have been suggested as intermediates in the formation of acyl coenzyme A compounds[6–9]. We have investigated some of these reactions for the long-chain activated fatty acids, and in addition have noted the activity of hydrolytic enzymes. These observations are reported in this paper.

EXPERIMENTAL

Methods

Phosphoryl and thiol derivatives of palmitic acid were estimated with hydroxylamine according to KORNBERG AND PRICER[10]. Free sulfhydryl groups were assayed using the nitroprusside reaction[11]. Adenosine triphosphate was determined spectrophotometrically by reduction of triphosphopyridine nucleotide with glucose, hexokinase and glucose-6-phosphate dehydrogenase[12]. Protein in a clear extract of tissue or an acetone powder extract was measured according to KALCKAR[13]. Adenylic acid was estimated according to LIEBECQ *et al*[14].

Materials

Coenzyme A and adenosine-5-phosphate were obtained from the Pabst Laboratories, Milwaukee, and ^{14}C-glutathione was obtained from the Schwarz Laboratories, Mount Vernon, New York. Palmitic anhydride was prepared from sodium palmitate and palmityl chloride[15], or from palmitic acid and acetic anhydride[16], and was recrystallized from low boiling petroleum ether. Solvents were freed of peroxides immediately before use in the preparation of the thiol esters, or very low yields were obtained. Tetrahydrofuran was purified by adding a small amount of lithium aluminum hydride and distilling after 5 to 10 min. Diethyl ether was washed with ferrous sulfate, water, and distilled.

Preparation of palmityl adenylate

To 20 mg of adenylic acid dissolved in 3.5 ml of a freshly prepared 40% aqueous pyridine solution containing 0.1% of lithium hydroxide was added 60 mg of palmitic anhydride in 7 ml of tetrahydrofuran. The solutions were mixed, allowed to stand at 30° for 10 min, and palmityl adenylate was precipitated by the addition of 50 ml of cold acetone. After 10 min at −10°, the mixture was centrifuged in the cold and the supernatant solution was discarded. The solid was

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suspended in 5 ml of cold water, *N* HCl was added dropwise to a pH of about 2, and 15 ml of ether were added. The phases were mixed thoroughly for a few seconds. Palmityl adenylate, which remained insoluble, was collected by centrifugation in the cold, and the aqueous and ether solutions were carefully removed. The washing was repeated once more.

Preparation of palmityl coenzyme A

50 mg palmitic anhydride in 20 ml of tetrahydrofuran were added to 25 mg coenzyme A in 8.6 ml water and 5.5 ml 0.5 *M* potassium bicarbonate. The mixture was held at 35° for 20 min, cooled in ice, and acidified to a pH of about 2 with *N* HCl. Tetrahydrofuran was removed by concentration in vacuo under nitrogen at a bath temperature of 35° or less, and the excess palmitic anhydride and palmitic acid were removed by extracting 3 times with equal volumes of ether. The white insoluble material in the aqueous phase was collected by centrifugation, and washed once with a few ml of cold water.

Palmityl coenzyme A could also be synthesized from palmitic anhydride and coenzyme A in tetrahydrofuran and aqueous pyridine containing lithium hydroxide as described above for palmityl adenylate.

Preparation of palmityl glutathione

This compound was prepared in an identical manner as palmityl coenzyme A.

Tissue preparations

Homogenates of rat brain and rat liver were prepared at 0° with 0.25 *M* sucrose using a Potter-Elvehjem apparatus. The supernatant fraction of brain homogenate was obtained by centrifugation at 23,000 *g* for 30 min[17]. The guinea-pig-liver residue fraction was the material which sedimented at high speed and which was then lyophilized according to the directions of KORNBERG AND PRICER[10]. Acetone powders were stirred for 1 h at 0° with 0.002 *M* tris buffer, pH 7.4, and the extracts were clarified by centrifugation and used as indicated. When the residue fraction was extracted, it was treated in a similar manner.

RESULTS

Properties of palmityl compounds

Yield and purity. Yields were determined by measurement of the quantity of hydroxamic acid formed at 37° with hydroxylamine for 15 min, and averaged about 50 % based on adenylic acid or coenzyme A. The yield of palmityl glutathione was less, usually about 35 %. By comparison of dry weight to hydroxamic acid formed, the purity of palmityl adenylate and palmityl coenzyme A was found to be 80 to 90 % in various preparations. No free sulfhydryl group could be detected in palmityl coenzyme A.

The hydroxamic acid produced from preparations of palmityl glutathione was much less than expected. A preparation which gave a 50 % yield of hydroxamic acid by incubation at 37° with hydroxylamine was incubated with the same quantity of hydroxylamine at 100° for 5 min. As shown in Table I, incubation at the higher

TABLE I

VARIATION WITH TEMPERATURE OF HYDROXAMIC ACID FORMED FROM PALMITYL ADENYLATE AND PALMITYL GLUTATHIONE

Palmityl derivative	*Hydroxamic acid*	
	37°	*100°*
	μmole	*μmole*
Palmityl adenylate	0.68	0.65
Palmityl glutathione	0.45	0.84

Reaction mixtures contained the palmityl compound and 1200 μmoles hydroxylamine, pH 6.7, in 1.2 ml volume. Incubation at 37° was for 15 min and at 100° for 5 min.

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temperature gave an amount of hydroxamic acid almost twice that obtained at 37°. Longer incubation at 37° of palmityl glutathione and hydroxylamine did not increase the yield. With palmityl adenylate, on the other hand, the same amount of hydroxamic acid was formed at the two temperatures. Since it has been shown that acetamide[18] and acetyl glycine[19] react quantitatively with hydroxylamine only at 100°, it may be assumed that the palmityl glutathione used in the experiments shown in Table I was a mixture of 54% of palmityl-S-glutathione and 46% of palmityl-N-glutathione.

Optical properties. A solution of palmityl adenylate at pH 7 had an absorption spectrum similar to adenylic acid with a maximum at 260 mμ. The ratio at this pH of 280:260 mμ was 0.25, and of 250:260 was 0.76. Palmityl glutathione showed an absorption peak at 229 mμ, which is in a range characteristic of several S-acyl compounds[20,21,1]. A maximum for palmityl coenzyme A was exhibited only at 259 mμ.

Stability of palmityl adenylate, palmityl coenzyme A, and palmityl-S-glutathione. The stability of these compounds was tested under a variety of conditions of temperature and pH, using as the assay the amount of hydroxamic acid formed on incubation with hydroxylamine at 37°. They are insoluble in acid and stable to acid. After 15-min exposure to 0.1 *N* HCl at 30°, less than 10% of hydrolysis occurred. At 0° under these conditions, no hydrolysis could be detected.

In contrast, these derivatives of palmitic acid are labile to alkali. After 2 min at 37° in 0.1 *N* NaOH, palmityl adenylate was completely split. At pH values closer to neutrality, the three compounds are far more resistant to hydrolysis, but palmityl adenylate is the least stable. In 0.1 *M* Tris buffer, pH 8.8, after 15 min at 30°, the extent of hydrolysis of palmityl adenylate was 25%, and that of palmityl coenzyme A and palmityl glutathione less than 10%. At a pH generally used in metabolic studies, 7.4, obtained with 0.1 *M* Tris buffer, suspension for 30 min at 37° caused less than 10% of hydrolysis of any of the three compounds. Neutral solutions, which are really turbid suspensions, unless very dilute, have been stored at — 10° for several months without detectable alteration. Palmityl adenylate appears to be more soluble than the other two compounds at pH 7.4.

TABLE II

ENZYMIC HYDROLYSIS OF PALMITYL ADENYLATE, PALMITYL COENZYME A, AND PALMITYL GLUTATHIONE

Enzymic extract	*Hydrolysis of*		
	palmitate adenylate	*palmityl coenzyme A*	*palmityl glutathione*
	μmoles	*μmole*	*μmole*
None	0.18	0.13	0.10
Rat-liver acetone powder (6.5 mg protein)	1.73	—	—
Rat-brain acetone powder (6.7 mg protein)	2.10	0.84	0.95
Guinea-pig-liver residue fraction (6.5 mg protein)	2.18	0.60	0.35

Each tube contained 2.9 μmoles of substrate, 40 μmoles Tris buffer, pH 7.4, and extract as indicated in a final volume of 1.6 ml. Enzymic extracts were treated at 0° for 15 min with 0.5 vol. of Dowex-1 chloride and then for 15 min with 10 mg of Norit per ml before centrifugation. After incubation for 30 min at 37°, the reaction was stopped with perchloric acid and the precipitate was assayed at 37° with hydroxylamine.

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During alkaline hydrolysis, palmityl adenylate released an amount of adenylic acid equivalent to the decrease in hydroxamic acid (Table III).

Enzymic hydrolysis of palmityl adenylate, palmityl coenzyme A, and palmityl glutathione. The presence of hydrolytic enzymes for these compounds in extracts of rat-liver and rat-brain acetone powders and guinea-pig residue fraction was tested. Since transferases may have been present, the extracts were treated with Dowex-1 chloride and with Norit in order to remove nucleotides and minimize or prevent transfer activities. Considerable hydrolytic activity was noted (Table II), which was much greater for palmityl adenylate than for the thiol esters. It is apparent from this result that palmityl coenzyme A could not have been an intermediate in the splitting of palmityl adenylate, even if traces of coenzyme A remained in the extracts. No requirement for magnesium ions was found. In an experiment which is not shown here, addition of 2 μ-moles of magnesium chloride per ml caused a decrease of 25 % in hydrolytic activity.

Enzymic hydrolysis of palmityl coenzyme A and palmityl glutathione was accompanied by an appearance of free sulfhydryl groups which corresponded closely to the decrease in activated palmitate measured by the reaction with hydroxylamine at 37° (Table III).

TABLE III

HYDROLYSIS OF PALMITYL COMPOUNDS WITH RELEASE OF ADENYLIC ACID, COENZYME A, AND GLUTATHIONE

Palmityl compound	*Initial*	*Disappearance*	*Appearance of AMP, CoASH and GSH*
	μmoles	*μmole*	*μmole*
Palmityl adenylate	1.86	0.54	0.46
Palmityl coenzyme A	0.61	0.39	0.41
Palmityl glutathione	2.52	0.84	0.89

Palmityl adenylate was incubated with 40 μmoles of Tris buffer, pH 8.8, at 50° for 5 min. Palmityl coenzyme A and palmityl glutathione were incubated with an extract of rat-liver acetone powder containing 2.57 mg protein, treated as described in Table II, 40 μmoles Tris buffer, pH 7.4, at 37° for 30 min. The final volume was 1 ml. For sulfhydryl assay, the reaction was stopped with metaphosphoric acid. For hydroxamic acid assay, perchloric acid was used.

Transfer reactions

Formation of palmityl coenzyme A and ATP from palmityl adenylate. Palmityl transfer from palmityl adenylate to coenzyme A was measured using the disappearance of free sulfhydryl groups as the assay procedure[11]. The results obtained with a number of tissues and extracts are shown in Table IV. Since hydrolases for palmityl coenzyme A are present, the values are minimum values. The guinea-pig-liver residue fraction was found to be active not only for the activation of palmitate with ATP and coenzyme A as shown by KORNBERG AND PRICER[10], but active also in the formation of palmityl coenzyme A from palmityl adenylate and coenzyme A. The rate of the latter reaction was twice as great as the rate of the activation reaction from palmitate, ATP, and coenzyme A, in contrast with the results obtained by WHITEHOUSE *et al.* with octanoate[22]. This may, however, be due to the lower solubility of palmitate compared to palmityl adenylate. When ATP was replaced by AMP, palmitate was not activated. Extraction of the guinea-pig residue fraction followed by dialysis of

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TABLE IV

FORMATION OF PALMITYL COENZYME A FROM PALMITYL ADENYLATE

Enzyme	*Substrate*	*Incubation time*	*Coenzyme A*	
			Initial	*Disappearance*
		min.	*μmole*	*μmole*
None	Palmityl adenylate	30	0.175	0.005
Guinea-pig-liver residue fraction (2 mg)	Palmityl adenylate	3	0.160	0.045
Guinea-pig-liver residue fraction (2 mg)	Palmityl adenylate	10	0.160	0.070
Guinea-pig-liver residue fraction (2 mg)	Palmityl adenylate	30	0.160	0.105
Guinea-pig-liver residue fraction (2 mg)	Palmitate + ATP	10	0.185	0.033
Guinea-pig-liver residue fraction (2 mg)	Palmitate + AMP	10	0.191	0.006
Guinea-pig-liver residue fraction (9 mg)	Palmityl adenylate	15	0.232	0.195
Dialyzed extract of guinea-pig-liver residue fraction (9 mg)	Palmityl adenylate	15	0.197	0.019
10% rat-liver homogenate (1 ml)	Palmityl adenylate	20	0.360	0.135
10% rat-brain homogenate (1 ml)	Palmityl adenylate	20	0.212	0.012
Supernatant of 10% rat-brain homogenate (1.4 ml)	Palmityl adenylate	20	0.212	0.077
Rat-liver acetone-powder extract (1.1 mg protein)	Palmityl adenylate	30	0.175	0.022
Rat-brain acetone-powder extract (4.7 mg protein)	Palmityl adenylate	30	0.195	0.000

Each tube contained 40 μmoles Tris buffer, pH 7.6, 4 μmoles $MgCl_2$, 4 μmoles of substrate, and coenzyme A and tissue preparations as indicated in a final volume of 2 ml. The reaction was stopped with metaphosphoric acid. Corrections were made for enzymic oxidation of coenzyme A in absence of substrate.

the extract against 0.002 *M* tris buffer, pH 7.6, for 16 h at 0° caused almost complete loss of transferase activity.

Activity was also observed in rat liver and in rat brain. Formation of palmityl coenzyme A from palmityl adenylate and coenzyme A was detected in a whole-liver homogenate, but not in a whole-brain homogenate. The supernatant fraction obtained by centrifugation of brain homogenate at 23,000 *g*, however, contained considerable activity. This situation in rat brain is identical to that noted for the activation of palmitic acid by ATP and coenzyme A[23], and may be due to the presence in whole rat-brain homogenate of acceptors for the generated palmityl coenzyme A, with subsequent release of the coenzyme. Acetone-powder extracts of rat liver and rat brain had little or no activity.

The formation of ATP from palmityl adenylate and pyrophosphate could also be demonstrated. With an extract of guinea-pig residue fraction, a small but significant increase in ATP was found when palmityl adenylate was added to the pyrophosphate-containing medium (Table V).

Conversion of palmityl adenylate and palmityl coenzyme A to palmityl glutathione. The transfer of palmityl from adenylate and coenzyme A was tested conveniently with ^{14}C-glutathione by determining the amount of radioactivity in the perchloric acid-insoluble material obtained after incubation. In Table VI, it is shown that a low but measurable quantity of palmityl glutathione was formed from either palmityl

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TABLE V

FORMATION OF ATP FROM PALMITYL ADENYLATE AND PYROPHOSPHATE

Enzyme	*Palmityl adenylate added*	*ATP Formed*
	μmole	*μmole*
+	0.00	0.003
+	0.20	0.017
—	0.20	0.000

Each tube contained 40 μmoles Tris buffer, pH 7.6, 4 μmoles $MgCl_2$, 100μmoles NaF, 4 μmoles sodium pyrophosphate, palmityl adenylate and an extract of guinea pig-residue fraction containing 0.5 mg protein as indicated in a final volume of 2 ml. The extract was previously treated at 0° for 30 min with 0.5 volume of Dowex-1 chloride. Incubation was carried out at 30° for 30 min. The reaction was stopped with acid, and an aliquot of the supernatant obtained by centrifugation was neutralized and assayed for ATP according to KORNBERG[12].

TABLE VI

TRANSFER OF PALMITYL FROM PALMITYL ADENYLATE AND PALMITYL COENZYME A TO PALMITYL GLUTATHIONE

Palmityl derivative	*Radioactivity in acid-insoluble material*	
	with enzyme	*no enzyme*
	counts/min	*counts/min*
Palmityl adenylate, 1.85 μmoles	254	0
Palmityl coenzyme A, 0.9 μmole	204	2

Each tube contained 40 μmoles Tris buffer, pH 7.4, 2 μmoles $MgCl_2$, 0.17 μmoles ^{14}C-glutathione (8,200 counts/min total radioactivity), palmityl derivative, and 2 mg of guinea-pig-liver residue fraction as indicated in a final volume of 1.05 ml. After incubation for 15 min at 37°, palmityl derivatives were precipitated and washed 3 times with 3.5% perchloric acid. The radioactivity at zero time was 56 counts/min and has been subtracted from the values shown.

adenylate or palmityl coenzyme A. It may be calculated that this amount, which was approximately the same whether the adenylate or coenzyme A derivative was used, was only one-twentieth as much as the amount of palmityl coenzyme A formed from palmityl adenylate and coenzyme A under the same conditions of incubation. Since traces of coenzyme A might have been present in the enzyme mixture, transfer to glutathione in this experiment may have proceeded from palmityl adenylate to glutathione via coenzyme A. No attempts were made to determine whether the product was the thiol ester or the amide, because very small amounts of palmityl glutathione were synthesized in the enzymic reactions.

Several attempts were made to determine whether palmityl glutathione and coenzyme A could react to give palmityl coenzyme A and glutathione, but no evidence for such a reaction in the tissues tested was obtained.

DISCUSSION

A procedure for the preparation, not only of palmityl coenzyme A, but of palmityl adenylate has been developed for several reasons. A source of palmityl coenzyme A which might be enzymically generated would allow the use of smaller quantities of the less readily obtainable coenzyme A. Data presented here indicate that transferase activity is present in guinea-pig liver, rat liver, and rat brain. The usefulness of palmityl adenylate as a source of palmityl coenzyme A is limited, however, because these

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tissues contain enzymes which are highly active in splitting palmityl adenylate. Separation of the transferase and hydrolase activities will be necessary before palmityl adenylate can serve adequately for this purpose. It is of interest to note that the transferase and hydrolase enzymes are apparently different proteins because hydrolase activity is present in extracts of acetone powder preparations and in extracts of guinea-pig-liver residue fraction, but transferase activity is almost completely lost after these treatments.

A second reason for the preparation of palmityl adenylate was to test whether reactions which have been shown to occur with short-chain compounds may be demonstrated for the long-chain substances. As would be expected, similar reactions take place. Not only does palmityl transfer from adenylate to coenzyme A occur, but a formation of ATP from palmityl adenylate and pyrophosphate can be detected. The rate of palmityl transfer from adenylate to coenzyme A was rapid, but transfer to glutathione was far slower. This is of interest in view of the possible physiological role of the transfer reaction.

The identity in metabolic behavior of the long-chain to the short-chain fatty acid derivatives is further emphasized by the finding that a relatively active hydrolytic activity is present in several tissues for palmityl-S-glutathione. This extends the demonstration of KIELLEY AND BRADLEY[24] concerning thiolesterase activity of mouse liver for acetyl and butyryl glutathione.

SUMMARY

Procedures for the preparation of palmityl adenylate, palmityl coenzyme A, and palmityl glutathione have been described. The stability of these compounds under different pH conditions has been investigated. Hydrolytic activities for each of the palmityl derivatives were demonstrated in several tissue extracts. Palmityl transferase activities from palmityl adenylate to coenzyme A and to glutathione were shown, and ATP formation by guinea-pig liver was detected from palmityl adenylate and pyrophosphate.

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