Studies on Palmitoyl-Coenzyme A Synthetase

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Acyl-CoA synthetases (EC 6.2.1.x) catalyse the reaction:

$$R\cdot CO_2H + ATP + CoA\cdot SH \rightarrow R\cdot CO\cdot S\cdot CoA + AMP + PP_i$$

(Jones, Black, Flynn & Lipmann, 1953; Mahler, Wakil & Bock, 1953; Kornberg & Pricer, 1953). Long-chain fatty acyl-CoA synthetase (EC 6.2.1.3) has been demonstrated in guinea-pig-liver and rat-liver microsomes by Kornberg & Pricer (1953). Several reaction mechanisms have been suggested, mainly on the basis of work with acetyl-CoA synthetase (EC 6.2.1.1) and medium-length fatty acyl-CoA synthetase (EC 6.2.1.2) (Green, 1952; Beinert *et al.* 1953; Jones, Lipmann, Hiltz & Lynen, 1953; Berg, 1956; Jencks & Lipmann, 1957). The most widely accepted is that suggested by Berg (1956), which includes the following steps:

$$R\cdot CO_2H + ATP \rightleftharpoons R\cdot CO\cdot AMP + PP_i \quad (1)$$

$$R\cdot CO\cdot AMP + CoA\cdot SH \rightleftharpoons R\cdot CO\cdot S\cdot CoA + AMP \quad (2)$$

and was based on the following findings: (*a*) the formation of ATP from pyrophosphate and synthetic acetyl-AMP (i.e. reversal of reaction 1) in the absence of CoA; (*b*) the formation of palmitoyl-CoA from palmitoyl-AMP and CoA (i.e. reaction 2); (*c*) the formation of acetylhydroxamate from ATP, acetate and hydroxylamine (2·5 M) in the absence of CoA, where hydroxylamine seemed to replace CoA; (*d*) the exchange reaction between ^{32}P-labelled PP_i and ATP was dependent on the presence of acetate (reaction 1); (*e*) the exchange between AMP and ATP was dependent on acetate and CoA; (*f*) acetate and acetyl-CoA exchange required PP_i and AMP.

On the other hand, attempts to demonstrate acetyl-AMP formation were unsuccessful; neither could an incorporation of ^{32}P from ^{32}P-labelled ATP into acetyl-AMP be shown. The inability to prove the formation of acetyl-AMP prompted the assumption of a number of reaction schemes in which this substance is not formed at all (Cornforth, 1959; Boyer, 1960) or formed transiently as an enzyme–AMP–acyl-CoA–Mg^{2+} ion complex (Ingraham & Green, 1958).

Evidence for the formation of enzyme-bound acetyl-AMP has recently been obtained by Webster & Campagnari (1962) and Webster (1963). With substrate amounts of enzyme, 1 mol. of acetyl-AMP was formed/mol. of enzyme. The enzyme–acetyl-AMP reacted with CoA to form acetyl-CoA.

In the present paper, several new facts are added on the behaviour of the long-chain fatty acid-CoA synthetase which may be of significance for the understanding of the mechanism of the enzyme reaction.

MATERIALS AND METHODS

Materials. ATP (disodium salt) (Sigma Chemical Co.), hydroxylamine hydrochloride (Hopkin and Williams Ltd.), cysteine hydrochloride (Sigma Chemical Co.) and bovine albumin (Pentex) were neutralized with KOH to pH 7·0 before use. Triton X-100 was a product of City Chemical Corp., N.Y., U.S.A. Calcium phosphate gel (aged) was obtained from Nutritional Biochemicals Corp. Sephadex G-50 was a product of Pharmacia, Uppsala, Sweden. CoA (70% pure) was obtained from Sigma Chemical Co. Hexokinase (EC 2.7.1.1), glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and NADP were obtained from Sigma Chemical Co. or Boehringer und Soehne G.m.b.H.

Preparation of microsomes. Rat livers were homogenized in 4 vol. of 0·25 M-sucrose in a Potter–Elvehjem-type homogenizer. After centrifugation for 15 min. at 15000g the supernatant was centrifuged in a Spinco centrifuge at 100000g for 30 min. The precipitate obtained was resuspended in 2 ml. of water/g. of liver used, freeze-dried and stored at −15°.

Solubilization and purification of the enzyme. All manipulations were carried out at 4°. Freeze-dried microsomes (150–250 mg.) were suspended in 10 ml. of 0·05 M-tris–HCl buffer, pH 8·0, and treated ultrasonically for 5 min. at 10 kcyc./sec. in a Raytheon oscillator and centrifuged for 30 min. at 100000g. The precipitate was treated with 10 ml. of aq. 0·4–0·5% Triton X-100 solution and recentrifuged as above. The supernatant was mixed with 1·5 mg. of calcium phosphate gel/mg. of protein. The mixture was centrifuged after 10 min. and the precipitate suspended in 10 ml. of 0·1 M-phosphate buffer, pH 5·9. After standing for 10 min. the mixture was centrifuged and the precipitate again suspended in 10 ml. of 0·1 M-phosphate buffer, pH 7·6. The supernatant obtained this time served as the 'purified enzyme'. Ammonium sulphate fractionation did not yield any further purification.

Hydroxamate formation. This was followed by the method of Lipmann & Tuttle (1944), i.e. by trapping the activated acyl compound (i.e. palmitoyl-CoA) with hydroxylamine (0·2 M). The reaction mixture (final vol. 1 ml.) contained 50 μmoles of tris–HCl buffer, pH 7·4, 4 μmoles of $MgCl_2$, 5 μmoles of NaF, 10 μmoles of ATP, 0·5 mg. of CoA, 200 μmoles of hydroxylamine, 22·5 μmoles of cysteine, enzyme as indicated and 2 μmoles of potassium palmitate, added in the order given. After incubation for 1 hr. at 38°,

0·1 ml. of 60% (w/v) $HClO_4$ and 1 ml. of water were added. After centrifugation the precipitate was extracted with 2·5 ml. of 96% (v/v) ethanol, 0·3 ml. of 10% (w/v) $FeCl_3$ in 0·1 N-HCl added and the extinction at 540 mμ read in a Zeiss spectrophotometer. Under these conditions 1 μmole of hydroxamate formed gave an extinction of 0·425. The activity was proportional to the enzyme concentration up to the formation of about 0·25 μmole of hydroxamate (Fig. 1).

Adenosine triphosphate formation from palmitoyladenylate and pyrophosphate. The reversal of the part reaction (1):

$$\text{ATP} + \text{palmitate} \rightleftharpoons \text{palmitoyl-AMP} + PP_i$$

was measured by introducing hexokinase and glucose into the reaction mixture. The glucose phosphate thus formed was then estimated by following the production of NADPH with glucose 6-phosphate dehydrogenase and NADP by measuring the extinction at 340 mμ in a Zeiss spectrophotometer. It was necessary to carry out these coupled reactions in two steps, since palmitate interfered with the spectrophotometric measurement. The first step was terminated by bringing the reaction mixture to pH 5 with acetic acid, boiling the mixture shortly and removing the insoluble constituents by centrifugation at 4°. On the other hand, hexokinase and glucose had to be present in the initial reaction mixture, as otherwise the reaction was not linear and gave lower yields. This was due presumably to the presence of an adenosine triphosphatase and to the reversal of the reaction. The test system (final vol. 1 ml.) contained: 50 μmoles of tris–HCl buffer, pH 7·4, 4 μmoles of $MgCl_2$, 5 μmoles of NaF, 10 mg. of serum albumin, 15 mg. of glucose, 0·25 unit of hexokinase, 2 μmoles of sodium pyrophosphate, enzyme as indicated and 0·5 μmole of palmitoyl-AMP, added in the order given. After incubation at 30° the solution was acidified with 0·05 ml. of 8 M-sodium acetate buffer, pH 5·0, and boiled for 15 sec., cooled and centrifuged for 30 min. at 3000*g*. The supernatant was neutralized with 0·05 ml. of 12 N-KOH, and 0·5 ml. of 0·2 M-tris–HCl buffer, pH 7·4, was added to make 1·6 ml. Then 0·4 ml. of this solution was introduced into a 1 ml. cuvette (1 cm. light-path), 0·05 μmole of NADP, 4 μmoles of $MgCl_2$ and 0·25 unit of glucose 6-phosphate dehydrogenase were added (final vol. 1 ml., with water) and the extinction at 340 mμ was measured in a Zeiss spectrophotometer. Under these conditions 0·1 μmole of NADPH formed gave an extinction of 0·610. With the conditions stated, the formation of ATP was nearly linear with the enzyme concentration up to 1 mg. of microsomes in the reaction mixture (Fig. 2).

RESULTS

Purification procedure. When the purification steps described were tested by the two methods of assay, it turned out that the specific activity of the enzyme increased 5–6-fold as assayed by hydroxamate formation. However, there was no purification, or even loss of activity/mg. of protein, when ATP formation served as index. ATP formation in the Triton X-100 extract could not be determined, owing to interference of the Triton with the assay (Table 1).

Dependence on coenzyme A. Contrary to the ATP-forming reaction, in which no CoA had to be added, the hydroxamate formation was dependent on the presence of CoA. In its absence, very little hydroxamate was formed with the microsomes and still less with the purified enzyme. This was not markedly changed by the omission of the sodium fluoride from the mixture and by the addition of pyrophosphatase. Pyrophosphate formation paralleled that of hydroxamate and was also dependent on CoA (Table 2). With the concentra-

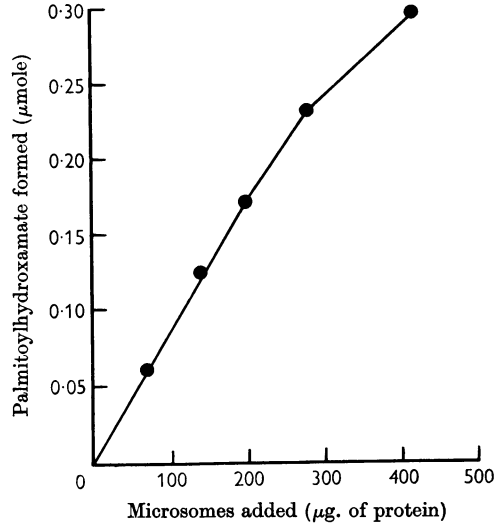

Fig. 1. Hydroxamate formation from palmitic acid, ATP and CoA. Details are given in the Materials and Methods section.

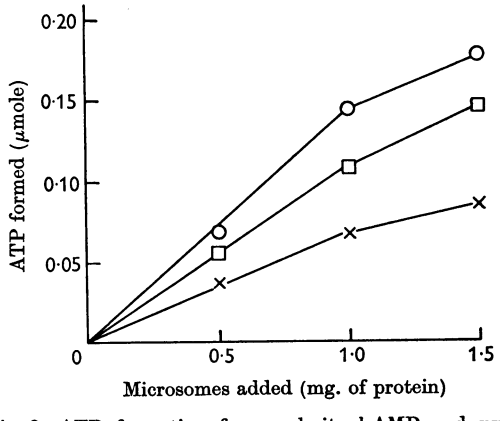

Fig. 2. ATP formation from palmitoyl-AMP and pyrophosphate after incubation for: ×, 10 min.; □, 15 min.; ○, 20 min. Details are given in the Materials and Methods section.

tion of enzyme used, even increasing the hydroxylamine concentration to 2·2M did not yield hydroxamate in the absence of CoA.

Activation and inactivation of the adenosine triphosphate-forming system. The discrepancy between the two methods of assay may have been due to activation or inactivation of the enzyme by constituents of the reaction mixture, present in one assay method but absent in the other. ATP, CoA and fatty acid were present only in the hydroxamate-formation assay mixture. The enzyme was therefore preincubated with combinations of these factors and the activity measured by the ATP-forming assay after the removal of added ATP by filtering the mixture through Sephadex G-50 (Gelotte, 1960). Preincubation of the microsomes with ATP and CoA caused a 50 % increase in the activity as measured by ATP formation. ATP alone was inactive, and CoA alone was inhibitory. The activation was greater with the purified enzyme (250 %) than with the microsomes. Palmitate alone was without effect, and its addition to CoA and ATP almost abolished the activation (Table 3). Incubation of the enzyme with ATP and palmitate, on the other hand, caused considerable inactivation. The inactivation was still more marked with palmitoyl-AMP, the product in the enzyme-catalysed reaction; CoA prevented this inactivation. The effect of preincubation with various CoA and ATP concentrations is presented in Figs. 3 and 4. CoA alone inhibited the reaction (Fig. 4 and Table 3). This was due to the fact that filtration through Sephadex gel did not remove the CoA from the preincubation mixture and its presence decreased the yield in the ATP-forming assay system, presumably owing to competition with pyrophosphate for the palmitoyl-AMP. ATP alone (Fig. 3) had a small activity with microsomes

Table 1. *Solubilization and purification of rat-liver microsomal palmitoyl-coenzyme A synthetase*

The purification procedure is described in the Materials and Methods section. The hydroxamate-formation and ATP-formation methods of assay are also described in the Materials and Methods section; in the former the activity units are expressed as μmoles of hydroxamate formed/hr. from palmitic acid, ATP and CoA, and in the latter as μmoles of ATP formed/10 min. from palmitoyl-AMP and pyrophosphate.

		Total activity (units)		Sp. activity (units/mg. of protein)	
Enzyme	Yield (mg. of protein)	Hydroxamate-formation method	ATP-formation method	Hydroxamate-formation method	ATP-formation method
Freeze-dried microsomes	15·35	15·8	7·4	1·03	0·48
Ppt. after ultrasonic treatment	9·4	15·0	3·75	1·60	0·40
Soln. in Triton X-100	9·1	15·0	—	1·65	—
Eluate from calcium phosphate gel	0·91	4·65	0·135	5·1	0·15

Table 2. *Dependence on coenzyme A of the palmitoylhydroxamate- and pyrophosphate-forming palmitoyl-coenzyme A-synthetase system*

Hydroxamate formation was assayed as described in the Materials and Methods section. For pyrophosphate formation, the $HClO_4$ supernatant obtained from the same reaction mixture was shaken with 150 mg. of acid-washed Norit A for 10 min., water was added to 5 ml. and the mixture centrifuged. Pyrophosphate was determined in 1 ml. samples as the inorganic phosphate liberated by 10 min. hydrolysis with N-HCl, according to the method of Fiske & Subbarow (1925).

	Formation of product (μmole)	
Enzyme	Hydroxamate	Pyrophosphate
Freeze-dried microsomes (225 μg.)		
Full system	0·25	0·26
CoA omitted	0·06	0·065
Purified enzyme (70 μg.)		
Full system	0·33	0·32
CoA omitted	0·02	0·0
Purified enzyme (140 μg.)		
Full system	0·63	0·72
CoA omitted	0·04	0·0
Freeze-dried microsomes (200 μg.)		
Full system with 0·28M-NH_2·OH	0·23	—
CoA omitted	0·02	—
Full system with 2·2M-NH_2·OH	0·21	—
CoA omitted	0·01	

Table 3. *Effects of preincubation with adenosine triphosphate, coenzyme A and palmitate on the adenosine triphosphate-forming activity of palmitoyl-coenzyme A synthetase*

Preincubation was carried out with 15 mg. of freeze-dried microsomes (5·25 mg. of protein) or with 1·33 mg. of purified enzyme, 22 μmoles of cysteine, 5 μmoles of NaF, 4 μmoles of $MgCl_2$, and ATP and CoA as indicated (final vol. 1·6 ml.) at 30° for 15 min. under nitrogen. The reaction mixture was then put on a column (12 cm. × 0·7 cm.) of Sephadex G-50 and eluted with 0·05 M-NaCl at room temperature. The enzyme was eluted with the front of eluent and could be identified by its turbidity or by its protein content. It was free of ATP but contained CoA. ATP formation with the eluted enzyme was measured as described in the Materials and Methods section (10 min. incubation).

Enzyme preparation	Additions for preincubation	Sp. activity of ATP formation (unit/mg. of protein)
Freeze-dried microsomes	None	0·11
	CoA (0·75 mg.)	0·06
	ATP (10 μmoles)	0·12
	CoA (0·75 mg.) + ATP (10 μmoles)	0·16
	Palmitate (2 μmoles)	0·11
	Palmitate (2 μmoles) + ATP (10 μmoles)	0·07
	Palmitate (2 μmoles) + ATP (10 μmoles) + CoA (0·75 mg.)	0·12
	Palmitoyl-AMP (3 μmoles)	0·0
	Palmitoyl-AMP (3 μmoles) + CoA (0·75 mg.)	0·11
Purified enzyme	None	0·12
	ATP (10 μmoles) + CoA (0·1 mg.)	0·41

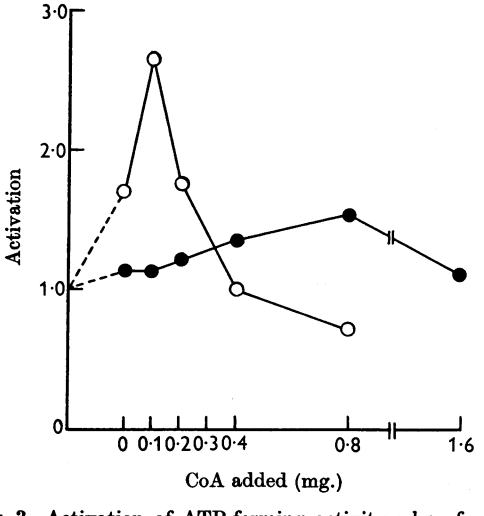

Fig. 3. Activation of ATP-forming activity when freeze-dried microsomes (●) and purified enzyme (○) were preincubated with various CoA concentrations. 'Activation' is the specific activity obtained after preincubation with CoA and ATP related to that without these compounds. Preincubation and assay of activity were as given in Table 3 and the Materials and Methods section, with 10 μmoles of ATP.

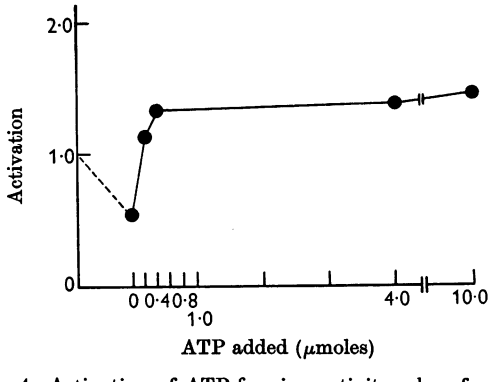

Fig. 4. Activation of ATP-forming activity when freeze-dried microsomes were preincubated with various ATP concentrations. 'Activation' is the specific activity obtained after preincubation with CoA and ATP related to that without these compounds. Preincubation and assay of activity were as given in Table 3 and the Materials and Methods section, with 0·75 mg. of CoA.

and a considerable one with the purified enzyme. This may have been due to some CoA present in the preparations and to the much lower CoA concentration required by the purified enzyme. Optimum combinations of both ATP and CoA gave considerable activation, which was much higher with the purified enzyme. Owing to the presence of CoA in the assay system, the activations found were lower than would be the case if this factor could have been removed. Filtration through Norit charcoal caused loss of most of the enzyme protein and could not be employed.

Reactivation of 'inactivated enzyme'. After inactivation by preincubation with ATP and palmitate or with palmitoyl-AMP, activity was restored by a second preincubation with ATP and CoA (Table 4).

Table 4. *Reactivation of inactivated freeze-dried microsomes*

Details are as in Table 3 and in the Materials and Methods section. Expts. A and B were carried out with different preparations of freeze-dried microsomes.

	Treatment of enzyme system	Sp. activity of ATP formation (unit/mg. of protein)
Expt. A	(*a*) None	0·17
	(*b*) Preincubated with ATP (10 μmoles) + palmitate (2 μmoles)	0·12
	(*c*) As A (*b*) with additional preincubation with ATP (10 μmoles) + CoA (0·75 mg.)	0·18
Expt. B	(*a*) None	0·11
	(*b*) Preincubated with palmitoyl-AMP (3 μmoles)	0·0
	(*c*) As B (*b*) with additional preincubation with ATP (10 μmoles) + CoA (0·8 mg.)	0·18

DISCUSSION

The dependence of hydroxamate formation on the presence of CoA, shown in the present work as well as by other authors (Kornberg & Pricer, 1953), is difficult to reconcile with part reaction (1), as formulated by Berg (1956), namely:

$$\text{Palmitate} + \text{ATP} \rightleftharpoons \text{palmitoyl-AMP} + \text{PP}_i$$

since palmitoyl-AMP reacts rapidly with hydroxylamine at neutral pH.

If palmitoyl-AMP is formed in an enzyme-bound form in analogy to the enzyme–acetyl-AMP found by Webster (1963), one has to assume that it is protected, not only from the palmitoyl-AMP hydrolase present in the preparation, but also from the interaction with hydroxylamine.

An alternative explanation for low yields of hydroxamate when low concentrations of hydroxylamine (0·2–0·4 M) are used was suggested by Jencks (1958), who demonstrated the formation of labile *O*-acylhydroxamates that are hydrolysed by water and thus do not yield the coloured iron complex. However, this explanation does not hold in the present case, since pyrophosphate formation was also negligible in the absence of CoA. An alternative possibility would be that this part reaction too is CoA-dependent, as already postulated by Ingraham & Green (1958).

The supposition that CoA takes part in the catalysis of reaction (1) met initially with the difficulty that the reverse of the reaction, i.e. ATP formation from palmitoyl-AMP and pyrophosphate, proceeded in the absence of added CoA. This difficulty could be resolved by the finding that preincubation of the microsomes with CoA and ATP caused considerable activation of the ATP-forming capacity. CoA is thus involved in the reaction in both directions. It was assumed that the activity exhibited by the microsomes before preincubation with ATP and CoA was due to the presence of some enzyme-bound CoA (enzyme–CoA), whereas the preincubation converted that part of the enzyme that was devoid of CoA into the active enzyme–CoA form. In corroboration of this assumption is also the finding that, on solubilization of the microsomal enzyme and its purification, the specific activity as measured by the hydroxamate-forming assay increased 5–6-fold, whereas that measured by ATP formation even decreased. The main difference between these two assay systems is the presence of ATP and CoA in the first and their absence in the second. Therefore, if during the purification procedure some of the enzyme is converted into the inactive CoA-free form, it will be reactivated by ATP and CoA present in the hydroxamate-forming assay system, but not in the ATP-forming one. This interpretation is substantiated by the finding that the activation of the purified enzyme by preincubation with ATP and CoA is much more extensive than that of the microsomes and could account for most of the activity lost during the purification process.

Activation of mitochondrial octanoyl-CoA synthetase by incubation with malate has been reported by Harel, Mendoza & LeBreton (1961). It has to be determined whether this activating system is related to the one described in the present paper.

These considerations lead to the following formulation of the first steps in synthetase reaction:

$$\text{Enzyme} + \text{CoA} \xrightarrow{\text{ATP}} \text{enzyme–CoA} \quad (3)$$

$$\text{Enzyme–CoA} + \text{ATP} + \text{palmitate} \rightarrow \text{enzyme–CoA–palmitoyl-AMP} + \text{PP} \quad (4)$$

$$\text{Enzyme–CoA–palmitoyl-AMP} \rightarrow \text{enzyme–CoA–palmitate} + \text{AMP} \quad (5)$$

The set of reactions postulated yields enzyme-bound palmitoyl-CoA. For the reaction to continue catalytically, one has to postulate a mechanism for the recovery of active enzyme, i.e. enzyme–CoA, at the end of the reaction steps. The reactivation of the enzyme by reaction (3) cannot be assumed as obligatory, as in this case two ATP

molecules would be required for each palmitoyl-CoA molecule formed, which is contrary to the 1:1 stoicheiometry of the reaction found by Kornberg & Pricer (1953). A possibility to be considered would be the reaction:

$$\text{Enzyme–CoA–palmitate} + \text{CoA} \rightleftharpoons \text{enzyme–CoA} + \text{palmitoyl-CoA} \quad (6)$$

i.e. the exchange of palmitoyl-CoA for CoA on the enzyme.

In the absence of free CoA this reaction would be replaced by relatively slow hydrolysis, with formation of inactive enzyme:

$$\text{Enzyme–CoA–palmitate} + H_2O \rightarrow \text{enzyme} + \text{palmitoyl-CoA} \quad (6a)$$

Only in the presence of added CoA will the enzyme be reconverted into its active form (reaction 6) and be able to act catalytically. The inactivation of the enzyme, when incubated with ATP and palmitate, or with palmitoyl-AMP in the absence of CoA, could be demonstrated by reisolating the so incubated enzyme by filtration through Sephadex gel, which removes the ATP used, and testing it by the ATP-forming assay. In this assay no ATP or CoA is added and it can therefore serve as an assay system for the active enzyme–CoA. Palmitoyl-AMP was the most efficient inactivator. ATP or palmitate, when added separately, had no effect. The formation of palmitoyl-AMP was thus necessary for the inactivation, as postulated in the reaction scheme presented. The addition of CoA prevented the inactivation.

The inability to obtain accumulation of palmitoyl-AMP is explained by the fact that in the absence of CoA the enzyme becomes inactive, whereas in the presence of CoA the reaction proceeds to the formation of palmitoyl-CoA.

This picture is also in agreement with the findings of Webster (1963) that maximally 1 mol. of acyl-AMP is formed/mol. of enzyme. This maximum was attained in very short incubation times (0·2–0·5 min.) and fits with the formulation of reaction (4). Their yields thereafter show a gradual decline, as would be expected according to reactions (5) and (6*a*). By this time reaction (4) does not continue to pile up new product, since the enzyme was inactivated. The assay system used by Webster (1963) would not reveal this inactivation, as it depended on the acetate-dependent disappearance of CoA, i.e. had all the ingredients for reactivation.

The necessity for a certain concentration of free CoA to keep the thiokinase active may be an important regulatory factor in palmitate metabolism. Thiokinase will not consume all the CoA present and will leave an adequate concentration of free CoA for the succeeding thiolase reactions. Only when the activated fatty acids have been degraded to acetyl-CoA and these are metabolized by the tricarboxylic acid cycle, with the liberation of CoA, will the thiokinase proceed to activate additional molecules of fatty acid.

SUMMARY

1. Palmitoyl-CoA synthetase of rat-liver microsomes was solubilized and purified.

2. According to a hydroxamate-forming assay, a 5–6-fold purification was obtained. By an assay based on ATP formation from palmitoyl-AMP and pyrophosphate, no purification or even a fall in activity was found.

3. Hydroxamate formation was dependent on the presence of CoA.

4. The ATP-forming activity by microsomes was activated by preincubation with ATP and CoA by about 50 %. The purified enzyme increased its activity 3–4-fold on preincubation.

5. Incubation with ATP and palmitate, or with palmitoyl-AMP, caused a fall in ATP-forming capacity. This fall was prevented by the presence of CoA. The inactivated enzyme could be reactivated by incubation with ATP and CoA.

6. The results are discussed in the light of the assumption of two forms of the enzyme, an active CoA-containing one and an inactive one.

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