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A PALMITYL-CoA SYNTHETASE STIMULATING FACTOR OF PARTICLE-FREE SUPERNATANTS

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SUMMARY

1. A factor in the particle-free supernatant of tissue homogenates has been shown to stimulate palmityl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3) in particulate subcellular fractions. The factor is a heat-labile, $(NH_4)_2SO_4$-precipitable macromolecule, probably a protein.

2. By repeated fractionation with $(NH_4)_2SO_4$, the factor has been obtained almost free from contaminating palmityl-CoA synthetase in the supernatant.

3. The factor has been found in several organs of the rat, and in livers from animals of different species. The factor from different organs and species stimulated the palmityl-CoA synthetase in all organs tested.

4. It is shown that the effect of the stimulating factor obtained in the supernatant is to increase the formation of palmityl-CoA by the subcellular particulate fractions.

5. In the presence of the supernatant factor, maximum palmityl-CoA synthetase activity is obtained when ATP and Mg^{2+} are added in approximately equimolar amounts. Excess ATP or excess Mg^{2+} inhibits the stimulating effect of the supernatant factor. In the absence of the factor, excess ATP or excess Mg^{2+} has no inhibiting effect on the synthetase activity. The supernatant factor does not change the requirements for CoA.

6. Preincubation of microsomes with supernatant in the presence of Mg^{2+} and ATP activates the microsomal palmityl-CoA synthetase.

7. The possibility that the stimulating factor may be a palmityl-CoA synthetase kinase, is discussed.

INTRODUCTION

Recently we have shown that the particle-free supernatant of rat liver contains a factor of probable protein nature which stimulates the activity of long-chain acyl-CoA synthetase (acid:CoA ligase (AMP), EC 6.2.1.3; palmityl-CoA synthetase)

in isolated mitochondria and microsomes from rat liver[1]. We have also shown that the palmityl-CoA synthetase in rat liver is localized both in microsomal membranes and in the outer membrane of the mitochondria[1,2]. A soluble cytoplasmic factor may therefore stimulate both the mitochondrial and the microsomal synthetase *in vivo*.

In the present paper we report studies on the supernatant factor which show that the maximum stimulation of the palmityl-CoA synthetase depends on the concentration of ATP and Mg^{2+} in the medium, and that the factor is present in several organs from animals of different species.

MATERIALS AND METHODS

Reagents

CoA was purchased from C. F. Boehringer und Soehne, Mannheim, Germany; ATP and crystalline bovine serum albumin from Sigma Chemical Company, St. Louis, Mo. U.S.A. and DL-carnitine from Fluka A.G., Buchs, Switzerland.

DL-[*Me*-^{3}H]Carnitine with specific activity 75 μC/μmole was prepared as previously described by BREMER AND NORUM[3]. The radioactive carnitine was diluted with DL-carnitine to a specific activity 0.6 μC/μmole, or approx. 130 000 counts/min per μmole when counted in a Tri-Carb scintillation spectrometer (500 D).

Palmitate–albumin solutions were prepared as previously described with 6–7 μmoles palmitate/μmole albumin[1].

Carnitine palmityltransferase (EC class 2.3.1) was prepared from calf-liver mitochondria according to NORUM[4]. The enzyme prepared in this way was free from palmityl-CoA synthetase and palmityl-CoA hydrolase (EC 3.1.2.2). The final enzyme extract contained approx. 2 mg protein and 0.4 units of the enzyme per ml.

Animals

Female rats of the Wistar strain weighing 150–200 g were used for most experiments. For one experiment mice and guinea pigs were also used. All animals had free access to food and water until they were killed by a blow on the neck and bled from the jugular veins. The livers and other organs were immediately taken out and cooled on ice.

Preparation of subcellular fractions

Particulate fractions were prepared from rat liver by homogenization and differential centrifugation according to DE DUVE *et al.*[5] as modified by NORUM AND BREMER[6]. The nuclear fraction was discarded since the palmityl-CoA synthetase activity observed in this fraction is due to microsomal and mitochondrial contamination[1]. The L-fraction or 'light mitochondrial fraction' was spun down with the M-fraction or 'heavy mitochondrial fraction' since the lysosomes are free from palmityl-CoA synthetase[1]. Thus two particulate fractions were obtained, namely mitochondria and microsomes.

For some experiments a combined mitochondria *plus* microsomes fraction was spun down at 100 000 $\times$ *g* for 45 min. This fraction is referred to as the 'particulate fraction' in the text. The experiments with other organs than rat liver were carried out with particulate fractions obtained by centrifugation of whole homogenate at 100 000 $\times$ *g* for 45 min.

Particle-free supernatants were obtained from organs homogenized in 5 vol. of 0.25 M sucrose by centrifugation at 100 000 × *g* for 45 min. The supernatants were decanted and stored at −10° until used.

Enzyme assays

The mitochondrial and microsomal fractions were tested for mutual contamination by the assay of marker enzymes which were carnitine palmityltransferase for the mitochondria[6], and glucose-6-phosphatase (EC 3.1.3.8) for the microsomes[7]. Palmityl-CoA synthetase was assayed as previously described by measuring the formation of L-palmityl-[*Me*-^{3}H]carnitine from palmitate, labelled carnitine and ATP in the presence of catalytic amounts of CoA and excess amounts of carnitine palmityltransferase[1]. It should be noted that L-[*Me*-^{3}H]carnitine was added in such amounts that no significant dilution of the radioactive carnitine by the endogenous, unlabelled carnitine occurred.

The protein content of the different fractions was assayed by a biuret reaction.

Table I shows that the microsomes were contaminated with mitochondria to a slight degree, whereas the mitochondria were relatively more contaminated with microsomes. From these results it can be calculated that about one-third of the synthetase activity of the mitochondria was of microsomal origin while only 3% of the microsomal activity was of mitochondrial origin.

TABLE I

THE DISTRIBUTION OF PALMITYL-CoA SYNTHETASE IN SUBCELLULAR FRACTIONS OF RAT LIVER IN RELATION TO MARKER ENZYMES FOR MICROSOMES AND MITOCHONDRIA

E, cytoplasmic extract; M, heavy mitochondrial fraction; L, light mitochondrial fraction; P, microsomal fraction; and S, final supernatant. Palmityl-CoA synthetase was assayed with the addition of: 0.1 μmole of CoA; 5 μmoles of GSH; 5 μmoles of L-[*Me*-^{3}H]carnitine; 2.5 μmoles of ATP; 2.5 μmoles of $MgCl_2$; 1.0 μmole of palmitate–albumin; 100 μmoles of Tris buffer (pH 7.6) and carnitine palmityltransferase in an amount of about 0.04 unit (0.2 mg protein). Total vol. 1.0 ml. Incubation temp. was 30°. Incubation time 10 min. The reaction was stopped by the addition of 0.1 ml conc. HCl and 1.9 ml water, and the L-palmityl-[*Me*-^{3}H]carnitine formed was extracted with 1.0 ml of *n*-butanol.

Enzyme	*Percentage values*				*Per cent recovery*
	E	*M + L*	*P*	*S*	
Carnitine palmityl transferase	100	84	6	6	96
Glucose-6-phosphatase	100	20	78	8	106
Palmityl-CoA synthetase	100	40	57	6	103

RESULTS

Partial isolation of the supernatant stimulating factor

The final, particle-free supernatant or the 'S-fraction' always stimulated the palmityl-CoA synthetase in isolated particulate fractions. The stimulating activity was quantitatively recovered with the protein fraction after gel filtration of supernatant on Sephadex G-25 and from the precipitate after saturation of the supernatant with $(NH_4)_2SO_4$. Furthermore, the stimulating effect was completely lost

when the supernatant was heated at 60° for 10 min. These findings strongly suggested that the stimulating factor is a protein.

The final supernatant always contained some palmityl-CoA synthetase as seen from Table I. Assays of the synthetase activity in particulate fractions in the presence of supernatant therefore made corrections of the stimulating effect necessary. Attempts were made to obtain the stimulating factor free from palmityl-CoA synthetase. Fig. 1 shows that a partial separation was obtained by repeated $(NH_4)_2SO_4$ fractionation. By the assay of the stimulating activity of different $(NH_4)_2SO_4$ fractions from their ability to stimulate the palmityl-CoA synthetase of isolated microsomes, it was found that the fraction precipitated between 40 and 60% contained the main part of the stimulating factor and a small amount of the contaminating synthetase. This fraction was resuspended in 0.25 M sucrose and refractionated with $(NH_4)_2SO_4$. Fig. 1 shows that 0.4 mg protein of the precipitate from the second

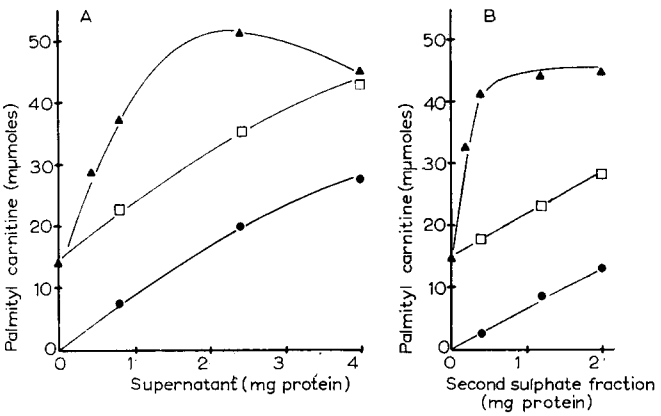

Fig. 1. Effect of partial isolation of the palmityl-CoA synthetase stimulating factor in particle-free supernatant of rat liver. A. The effect of addition of native supernatant to microsomes obtained from 12.5 mg of rat liver. B. The effect of addition of the second 40–60% $(NH_4)_2SO_4$ fraction added to the same amount of isolated rat-liver microsomes as in A. Other additions as stated in Table I. ●——●, activity of contaminating palmityl-CoA synthetase in the supernatant added; ▲——▲, activity of palmityl-CoA synthetase after addition of supernatant; □——□, the palmityl-CoA synthetase activity expected from a constant amount of microsomes and increasing amounts of supernatant.

40–60% saturation gave maximal stimulation. This amount contained almost no synthetase. Of the original supernatant about 1.5 mg protein was required to obtain maximal stimulation, and then a substantial amount of synthetase was introduced.

The stimulating effect of the supernatant factor on isolated subcellular fractions of rat liver

Fig. 2 shows the effect of the addition of supernatant on the formation of L-palmityl-[*Me*-^{3}H]carnitine by various amounts of isolated microsomes and mitochondria from rat liver. The synthetase activity increased both in microsomes and mitochondria to about the same extent. A moderate inhibition of the synthetase activity was observed with larger amounts of supernatant. The maximum stimulating effect occurred with about the same amount of supernatant protein both with different amounts of mitochondria and different amounts of microsomes.

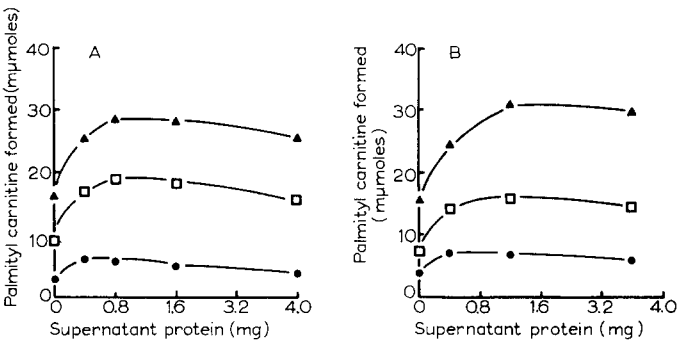

Fig. 2. The effect of increasing amounts of partially isolated supernatant synthetase-stimulating factor on the formation of L-palmityl-[*Me*-^{3}H]carnitine by isolated subcellular particles from rat liver. All values were corrected for the contaminating palmityl-CoA synthetase in the added supernatant. Other additions as stated in Table I. ●—·—●, Mitochondria from 2.5 mg rat liver (A), or microsomes from 1.7 mg rat liver (B); □—–—□, mitochondria from 7.5 mg rat liver (A), or microsomes from 4 mg rat liver (B); ▲—–—▲, mitochondria from 15 mg rat liver (A) or microsomes from 8.5 mg rat liver (B).

The stimulating effect on the formation of L-palmityl-[*Me*-^{3}H]carnitine is not a result of stimulation of the carnitine palmityltransferase as seen from Table II. Assayed with the isotope-exchange method[4], or by the formation of L-palmityl-[*Me*-^{3}H]carnitine from labelled carnitine and palmityl-CoA[8], this enzyme was insensitive to the addition of the supernatant factor. Table II also shows that the addition of carnitine palmityltransferase was necessary for the maximum formation of L-palmityl-[*Me*-^{3}H]carnitine, especially in the presence of the supernatant factor. This

TABLE II

EFFECT OF THE SUPERNATANT FACTOR ON THE CARNITINE PALMITYLTRANSFERASE, AND ON THE PALMITYL-CoA SYNTHETASE

Expt. 1. Carnitine palmityltransferase assayed by the isotope-exchange method with the addition of: 0.12 μmole of CoA; 0.25 μmole of L-[*Me*-^{3}H]carnitine; 0.5 μmole of DL-palmitylcarnitine; 5 μmoles of GSH and 100 μmoles of Tris buffer (pH 7.6). Total vol. 1.0 ml. Incubation time 10 min. Incubation temp. 30°. Expt. 2. Carnitine palmityltransferase assayed by the formation of L-palmityl-[*Me*-^{3}H]carnitine from 0.5 μmole of palmityl-CoA and 2.5 μmoles of L-[*Me*-^{3}H]-carnitine. Other additions as in Expt. 1, except for DL-palmitylcarnitine which was omitted. Expt. 3. Palmityl-CoA synthetase assayed in the absence and presence of the supernatant factor and carnitine palmityltransferase. Other additions as stated in Table I.

Expt. No.	*Transferase preparation (mg protein)*	*Supernatant preparation (mg protein)*	*Palmityl-CoA synthetase (mg protein)*	*Counts/min in butanol extract*
1		0.4		250
	0.05			2 700
	0.05	0.4		2 900
2		0.2		150
	0.02			5 600
	0.02	0.2		5 900
3			0.5	3 000
	0.20		0.5	6 900
		0.4	0.5	3 900
	0.20	0.4	0.5	18 200

finding indicates that the stimulating effect of the supernatant factor is due to an increased formation of palmityl-CoA, and that the formation of L-palmityl-[*Me*-^{3}H]carnitine is the rate-limiting step when no extra carnitine palmityltransferase is added.

A further confirmation for this conclusion was obtained by the following experiment: When microsomes were incubated with palmitate, CoA and ATP, the addition of the supernatant factor significantly increased the disappearance of CoA assayed by amperometric titration with $AgNO_3$.

The butanol phase from one of the tubes where the synthetase was maximally stimulated by the addition of the supernatant factor, was chromatographed in a two-dimensional thin-layer chromatographic system as described by WITTELS AND BRESSLER[9]. Only one radioactive spot with an R_F value corresponding to palmitylcarnitine was found. It is therefore concluded that the increased radioactivity in the butanol extract when isolated subcellular fractions are stimulated by the supernatant factor really represents acyl-carnitines, and not other carnitine derivatives.

TABLE III

THE EFFECT OF PARTICLE-FREE SUPERNATANTS FROM LIVER, HEART AND KIDNEY ON PALMITYL-CoA SYNTHETASE IN PARTICULATE FRACTIONS OF THESE ORGANS IN THE RAT

The particulate fraction of liver was prepared from 5 mg of tissue. All other fractions were prepared from 15 mg of the respective tissues. Palmityl-CoA synthetase activity was assayed as in Table I.

Particulate fraction	*Supernatant*	L-*Palmityl-[Me-*^{3}*H]carnitine (mμmoles)*				*Per cent stimulation**
		Particulate fraction (I)	*Supernatant (II)*	*Combined fractions (I + II)*		
				Expected (I + II)	*Found III*	
Liver	Liver	12.4	2.7	15.1	45.0	250
Liver	Kidney	12.4	2.2	14.6	41.6	210
Liver	Heart	12.4	4.1	16.5	39.1	180
Kidney	Liver	7.0	2.7	9.7	14.7	70
Kidney	Kidney	7.0	2.2	9.2	12.7	40
Kidney	Heart	7.0	4.1	11.1	13.1	30
Heart	Liver	3.4	2.7	6.1	14.4	320
Heart	Kidney	3.4	2.2	5.6	12.2	190
Heart	Heart	3.4	4.1	7.5	11.3	110

* $\left(\frac{\text{III} - \text{II}}{\text{I}} - 1\right) \times 100$.

The occurrence of the supernatant factor in different organs of the rat, and in livers of different species

Table III shows that the supernatants from liver as well as from heart and kidney of the rat stimulated the palmityl-CoA synthetase in the particulate fractions from all these organs. The liver apparently contains the highest concentration both of palmityl-CoA synthetase and of supernatant factor. However, the absolute degree

TABLE IV

EFFECT OF PARTICLE-FREE SUPERNATANT OF LIVERS FROM DIFFERENT SPECIES ON THE PALMITYL-CoA SYNTHETASE IN PARTICULATE FRACTIONS OF LIVERS

The rat-liver particulate fraction consisted of microsomes from 12 mg of tissue, the other particulate fractions were obtained by centrifugation of 15 mg of whole homogenates. The rat-liver supernatant was a partially isolated supernatant factor and contained 1.2 mg protein. Other supernatants were obtained from 15 mg of tissue. Palmityl-CoA synthetase was assayed as stated in Table I.

Particulate fraction	*Supernatant*	L-*Palmityl-[Me-*^{3}*H]carnitine (mμmoles)*				*Per cent stimulation**
		Particulate fraction (I)	*Supernatant (II)*	*Combined fractions (I + II)*		
				Expected (I + II)	*Found III*	
Rat	Rat	23.1	0.2	23.3	32.3	40
Rat	Guinea pig	23.1	6.2	29.3	51.5	95
Rat	Mouse	23.1	5.4	28.5	56.9	120
Guinea pig	Rat	16.9	0.2	17.1	28.5	70
Guinea pig	Guinea pig	16.9	6.2	23.1	53.8	180
Guinea pig	Mouse	16.9	5.4	22.3	56.0	200
Mouse	Rat	13.8	0.2	14.0	20.0	45
Mouse	Guinea pig	13.8	6.2	20.0	38.4	130
Mouse	Mouse	13.8	5.4	19.2	42.3	165

* $\left(\frac{III - II}{I} - 1\right) \times 100$.

of stimulation cannot be taken as a direct measure of the content of the supernatant factor in the different organs because the stimulation does not increase proportionally with the concentration of the factor. The table also shows that the maximum palmityl-CoA synthetase activity *in vitro* was almost 10 times higher in liver than in the other organs.

Table IV shows that particle-free supernatant from both guinea-pig and mouse liver stimulated the palmityl-CoA synthetase in isolated microsomes from rat liver as well as in the particulate fractions from guinea-pig and mouse liver. The partial isolated supernatant factor from rat liver, almost free from contaminating palmityl-CoA synthetase, also stimulated the synthetase activity in particulate fractions from livers of guinea pig and mouse.

Effect of the supernatant factor on the requirements for the co-factors for palmityl-CoA synthetase activity

Fig. 3 shows that the addition of the supernatant factor to the incubation medium did not change the requirements for CoA for maximum formation of L-palmityl-[*Me-*^{3}H]carnitine. A high concentration of CoA gave a moderate inhibition of the synthetase activity both in the absence and presence of the supernatant factor. The figure also shows that the inhibition was essentially the same with both mitochondria and microsomes.

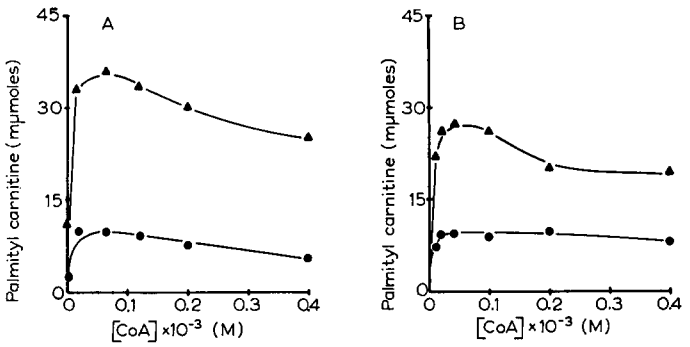

Fig. 3. The effect of CoA on the palmityl-CoA synthetase activity in microsomes from 2.5 mg rat liver (A), and in mitochondria from 7.5 mg rat liver (B), in the absence and presence of a constant amount of partially isolated supernatant factor. ●——●, no supernatant added; ▲——▲, with partially isolated supernatant factor (1.2 mg protein). Other additions as stated in Table I.

Accurate determination of the K_m for CoA was impossible since the particulate fractions contained some CoA, but it can be roughly estimated to be less than 10^{-5} M both in the absence and presence of the supernatant factor.

Fig. 4 shows that the addition of the supernatant to isolated microsomes markedly changed the effects of ATP and Mg^{2+}. In the absence of the supernatant factor the synthetase activity increased with increasing concentrations of ATP to approx. $2 \cdot 10^{-3}$ M, and remained unchanged when the ATP concentration was further increased. From Fig. 4A it is seen that the K_m for ATP is about $5 \cdot 10^{-4}$ M when the palmityl-CoA synthetase is assayed in microsomes in the absence of the

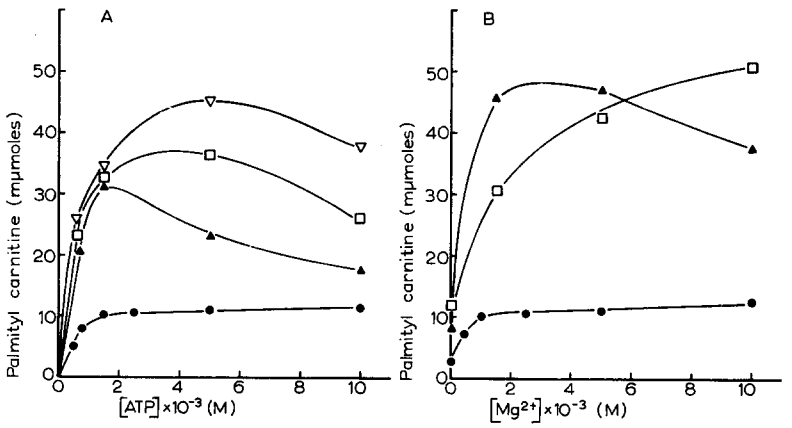

Fig. 4. The effect of Mg^{2+} and ATP on microsomal palmityl-CoA synthetase in the absence and presence of a constant amount of partially isolated supernatant factor. A. The effect of ATP on the formation of L-palmityl-[*Me*-^{3}H]carnitine by microsomes from 10 mg rat liver. ●——●, no supernatant added, $5 \cdot 10^{-3}$ M Mg^{2+}; ▲——▲, with supernatant (1.2 mg protein) and $1.5 \cdot 10^{-3}$ M Mg^{2+}; □——□, with supernatant (1.2 mg protein) and $5 \cdot 10^{-3}$ M Mg^{2+}; ▽——▽, with supernatant (1.2 mg protein) and 10^{-2} M Mg^{2+}. B. The effect of Mg^{2+} on the formation of L-palmityl-[*Me*-^{3}H]carnitine by microsomes from 10 mg rat liver. ●——●, no supernatant added, $1.5 \cdot 10^{-3}$ M ATP; ▲——▲, with supernatant (1.2 mg protein) and $1.5 \cdot 10^{-3}$ M ATP; □——□, with supernatant (1.2 mg protein) and 10^{-2} M ATP. Other additions as stated in Table I.

supernatant factor. Fig. 4B shows that the synthetase activity is also dependent on the presence of Mg^{2+} in the medium. In the absence of the supernatant factor no inhibition by high concentrations of ATP or Mg^{2+} was observed with microsomal palmityl-CoA synthetase.

When supernatant was added to the incubation medium, the synthetase activity in microsomes was stimulated with increasing concentration of ATP up to a maximum, and a further increase in the ATP concentration then resulted in an inhibition of the stimulating effect. Fig. 4A shows that with a low concentration of Mg^{2+} in the medium, the maximum stimulation was reached with a low ATP concentration. With higher concentrations of Mg^{2+} in the medium, the maximum stimulation occurred with higher concentrations of ATP.

Fig. 4B shows that in the presence of the supernatant factor, the effect of Mg^{2+} on the synthetase activity was dependent on the concentration of ATP in the medium. At a high ATP concentration more Mg^{2+} was required to obtain maximum stimulation. The maximum stimulating effect in all experiments was found when ATP and Mg^{2+} were added in approximately equimolar concentrations. Thus in the presence of the supernatant factor it appears that excess of either ATP or Mg^{2+} inhibits the stimulating effect on the palmityl-CoA synthetase, whereas no such effects were observed in the absence of the supernatant.

Fig. 5 shows that in the absence of the supernatant factor, the requirements for ATP and Mg^{2+} in mitochondria were almost identical with those in microsomes. When supernatant factor was added to the incubation medium, the stimulation of the synthetase activity was dependent on the concentrations of ATP and Mg^{2+} in a similar way as in microsomes. However, excess of ATP or Mg^{2+} had a more pronounced inhibiting effect in mitochondria than in microsomes. This may partly be due to activation of mitochondrial ATPase by Mg^{2+}.

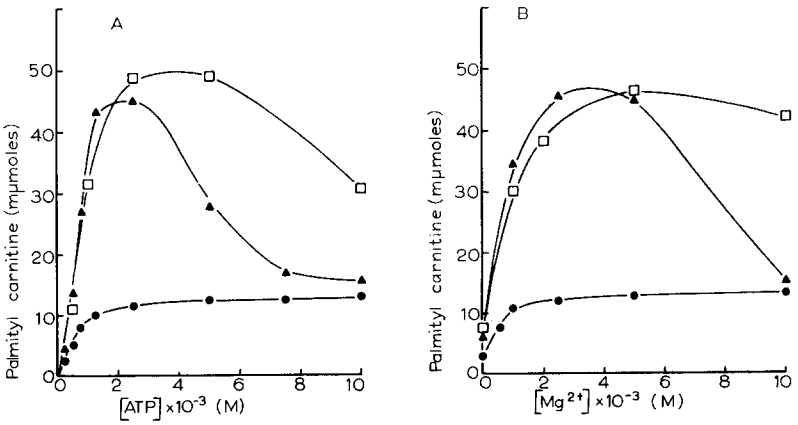

Fig. 5. The effect of Mg^{2+} and ATP on mitochondrial palmityl-CoA synthetase in the absence and presence of a constant amount of partially isolated supernatant factor. A. The effect of ATP on the formation of L-palmityl-[*Me*-^{3}H]carnitine by mitochondria from 15 mg of rat liver. ●——●, no supernatant added, $2.5 \cdot 10^{-3}$ M Mg^{2+}; ▲——▲, with 1.2 mg supernatant protein and $2.5 \cdot 10^{-3}$ M Mg^{2+}; □——□, with 1.2 mg supernatant protein and 10^{-2} M Mg^{2+}. B. the effect of Mg^{2+} on the formation of L-palmityl-[*Me*-^{3}H]carnitine by mitochondria from 15 mg of rat liver. ●——●, no supernatant added, $2.5 \cdot 10^{-3}$ M ATP; ▲——▲, with 1.2 mg supernatant protein and $2.5 \cdot 10^{-3}$ M ATP; □——□, with 1.2 mg supernatant protein and 10^{-2} M ATP.

Effect on microsomal palmityl-CoA synthetase of preincubation with particle-free supernatant

Isolated microsomes were preincubated with supernatant and Mg^{2+} in the presence and absence of ATP. Microsomes were also preincubated with ATP and Mg^{2+} and varying amounts of supernatant. For these experiments, amounts of supernatant giving 50–100% stimulation of the synthetase activity in microsomes were used. At zero time, CoA, labelled carnitine, palmitate, carnitine palmityltransferase and varying amounts of ATP and supernatant were added to give an identical content in all vessels during the incubation period.

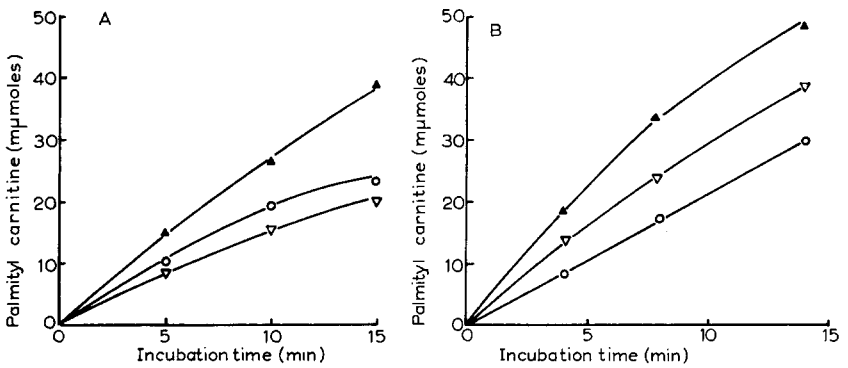

Fig. 6. The effect on palmityl-CoA synthetase of preincubation with supernatant. Microsomes were preincubated for 10 min with $3.3 \cdot 10^{-3}$ M Mg^{2+}, 0.1 M Tris buffer (pH 7.6), $5 \cdot 10^{-3}$ M GSH in the presence and absence of supernatant and ATP. At zero time was added: 0.3 μmole CoA, 15 μmoles L-[*Me*-^{3}H]carnitine, 0.12 unit of carnitine palmityltransferase and supernatant or ATP to give identical content in all vessels during the incubation period. Total vol. 3.0 ml. A. The effect of ATP on preincubation with supernatant. ▽-——▽, no additions of ATP and supernatant; ○—○, with supernatant protein, no ATP added; ▲—▲, with 0.6 mg supernatant protein and $3.3 \cdot 10^{-3}$ M ATP. B. The effect of the amount of supernatant during preincubation. ○—○, without supernatant, $3.3 \cdot 10^{-3}$ M ATP; △—△, with 0.6 mg supernatant protein and $3.3 \cdot 10^{-3}$ M ATP; ▲—▲, with 1.2 mg supernatant protein and $3.3 \cdot 10^{-3}$ M ATP.

Fig. 6 shows that the palmityl-CoA synthetase activity was markedly increased in microsomes preincubated with supernatant in the presence of ATP and Mg^{2+}. Preincubation with supernatant in the absence of ATP had no obvious effect on the synthetase activity (Fig. 6A). Addition of various amounts of supernatant to the medium during preincubation resulted in differences in the palmityl-CoA synthetase activity during the incubation period (Fig. 6B). Preincubation with ATP in the absence of supernatant had no effect on the synthetase activity. Thus both ATP and supernatant had to be present during preincubation to obtain increased synthetase activity during the incubation period. Preincubation in the absence of Mg^{2+} was not possible with the crude preparations used here since some synthetase activity was found in microsomes even when no Mg^{2+} was added to the incubation medium (Fig. 4B).

DISCUSSION

The present results confirm our previous observation of a stimulating effect of the particle-free supernatant on the palmityl-CoA synthetase in isolated mito-

chondria and microsomes of rat liver[1]. They also indirectly support the observation that the mitochondrial palmityl-CoA synthetase is localized in the outer membrane of the mitochondria[2]. This localization is consistent with a stimulating effect of a soluble cytoplasmic factor.

The stimulating factor is found in the supernatants from liver, heart and kidney in the rat, and in livers from mice and guinea pigs. The supernatant factor stimulates the synthetase activity in several organs from the same animal, and also in organs from animals of different species. These findings suggest that the stimulating factor from different organs and different species has an identical effect on the palmityl-CoA synthetase, and that the factor is present in all tissues.

The nature of the stimulating effect on palmityl-CoA synthetase by the supernatant factor is so far unknown. The present findings show that the increased butanol-extractable radioactivity found when supernatant was added to the incubation medium really represented palmityl-carnitine, and that the increased formation of palmityl-carnitine was due to an increased formation of palmityl-CoA. Furthermore, it is likely that the stimulation is due to a macromolecule which is heat labile and precipitable by $(NH_4)_2SO_4$. The factor therefore seems to be a protein, or a factor firmly bound to a protein.

The mechanism of fatty acid activation is relatively well established. The acyl-CoA synthetases are all dependent on ATP (or GTP[10]), Mg^{2+} and CoA[11,12]. BERG[13] has presented experimental evidence for an intermediate acetyl-AMP in the activation of acetate. BAR-TANA AND SHAPIRO[14] found that purified palmityl-CoA synthetase was activated by ATP and CoA. They therefore suggested that, in the formation of palmityl-CoA, an enzyme–CoA is formed initially, followed by the formation of an enzyme–CoA–palmityl-AMP complex. No extra protein was required for the activation in their system. The activation of palmityl-CoA synthetase by the supernatant factor thus seems to be different from the activation observed by BAR-TANA AND SHAPIRO[14]. In our system the preincubation experiments also revealed no requirements for CoA. However, this cannot be completely excluded as the microsomes used most likely contained traces of CoA.

In the absence of the supernatant factor, the palmityl-CoA synthetase activity in subcellular particles is insensitive to variations in the concentrations of ATP and Mg^{2+} between 10^{-3} and 10^{-2} M. This is not the case in the presence of the supernatant factor. The maximum stimulation of the synthetase activity then occurs when ATP and Mg^{2+} are added in approximately equimolar concentrations. Excess of any of these co-factors inhibits the stimulating effect of the supernatant factor. These findings suggest that excess ATP or Mg^{2+} competes with an ATP–Mg^{2+} complex for a common site in the activated system. This site may be different from the activating site on the palmityl-CoA synthetase which is apparently insensitive to excess ATP or Mg^{2+}. A similar inhibiting effect of excess amounts of ATP or Mg^{2+} has previously been demonstrated for phosphorylase *b* kinase (EC 2.7.1.38) by KREBS *et al.*[15,16]. Thus it seems probable that the stimulating factor of the particle-free supernatant may be a new enzyme kinase, and that the stimulating effect on the palmityl-CoA synthetase is to phosphorylate an inactive form of the enzyme, converting it into an active, phosphate-containing form.

Recently SMITH AND HÜBSCHER[17] have reported that a soluble cytoplasmic factor, probably a protein, stimulates glyceride formation in rat-liver mitochondria.

They concluded that this could not be explained by an effect on palmityl-CoA synthetase, as only a small stimulating effect on this enzyme was found. However, they tested the effect of their supernatant factor on the synthetase with ATP and Mg^{2+} concentrations at which we have found the supernatant factor to be nearly inactive. On the other hand, the incorporation of fatty acids into glycerides was assayed with ATP and Mg^{2+} concentrations which permit maximum activation of the palmityl-CoA synthetase by the supernatant factor. It is therefore likely that the effect of the supernatant on glyceride synthesis which they observe is due to activation of the mitochondrial palmityl-CoA synthetase, thus increasing the available palmityl-CoA in the system.

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