THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 242, No. 9, Issue of May 10, pp. 2111–2115, 1967
Printed in U.S.A.

A Guanosine Triphosphate-dependent Acyl Coenzyme A Synthetase from Rat Liver Mitochondria*

(Received for publication, December 2, 1966)

LAURO GALZIGNA, CARLO R. ROSSI, LODOVICO SARTORELLI, AND DAVID M. GIBSON

From the Department of Biochemistry, Indiana University School of Medicine, Indianapolis, Indiana 46207, and the Institutes of Biological and Organic Chemistry, University of Padua, Padua, Italy

SUMMARY

A guanosine triphosphate-specific acyl coenzyme A synthetase has been purified from extracts of sonically disrupted rat liver mitochondria. This enzyme, which is free of adenosine triphosphate-dependent activating systems and from succinyl-CoA synthetase activity, catalyzes the formation of CoA esters of both long and short chain fatty acids. GTP-specific fatty acid activation is inhibited by orthophosphate and fluoride.

A guanosine triphosphate-dependent fatty acyl coenzyme A synthetase (Equation 1) was purified by Rossi and Gibson (1) from buffered extracts of bovine liver mitochondria acetone powder.

$$GTP + RCOOH + Co\bar{A}—SH \rightleftarrows RCO\text{-}S\text{-}CoA + GDP + P_i \quad (1)$$

Soluble fractions were prepared which were free of adenosine triphosphate-linked fatty acyl-CoA synthetase (Equation 2) and succinyl-CoA synthetase (Equation 3).

$$ATP + RCOOH + Co\bar{A}—SH \rightleftarrows RCO\text{-}S\text{-}CoA + AMP + PP_i \quad (2)$$

$$GTP + \text{succinate} + Co\bar{A}—SH \rightleftarrows \text{succinyl-}S\text{-CoA} + GDP + P_i \quad (3)$$

Fatty acids with a chain length of less than 12 carbon atoms were activated by the enzyme preparation derived from acetone powder. Inorganic phosphate and GDP were required in the reverse reaction with butyryl-CoA as substrate. Van den Bergh (2) suggested that the GTP-dependent synthetase was operative in the oxidation of palmitate by dinitrophenol-treated liver mitochondria incubated in phosphate-free media. Addition of orthophosphate greatly inhibited oxygen uptake. In this situation it appeared that fatty acid activation was linked to the "substrate-phosphorylation" step (succinyl-CoA synthetase) coupled to α-ketoglutarate oxidation (3, 4) (see "Discussion"). Yates, Sheperd, and Garland (5) have also provided evidence for the existence of ATP- and GTP-specific acyl-CoA synthetases in intact mitochondria.

A new procedure for the isolation of a GTP acyl-CoA synthetase has been developed starting with extracts of sonically disrupted rat liver mitochondria (6). This enzyme, which was purified in the absence of organic solvents, efficiently catalyzed both short and long chain fatty acid activation (7). The isolation procedure and characterization of the enzyme are presented in this paper.

* This investigation has been supported by Grants TW 00104, HE 04219-07, HE 06308-05, and K3GM 18413 from the United States Public Health Service, by Grant 62G139 from the American Heart Association, and by Grant P178 from the American Cancer Society.

EXPERIMENTAL PROCEDURE

Methods

Enzyme Isolation—Mitochondria were separated from rat liver homogenates (in 0.25 M sucrose) by the method of Schneider and Hogeboom (8) as described by Rossi *et al.* (9). Freshly prepared mitochondria from male rats of the Holtzman strain displayed good respiratory control (Gilson oxygraph equipped with a Clark electrode) when incubated with α-ketoglutarate and other substrates in buffered media (9, 10). The packed, washed mitochondrial pellet from 30 g of rat liver (800 mg of protein) was resuspended in 15 ml of 0.5% aqueous Triton X-100 and sonically disrupted (20 kilocycles, Biosonik apparatus) for 5 min at a temperature between 0° and 5°. The suspension was centrifuged at 80,000 × *g* for 15 min. The supernatant, containing 520 mg of protein, was designated Fraction 1 (see Table I). This solution was adjusted (with stirring at 0°) to pH 3.4 with 0.1 M HCl, and then readjusted to pH 7.0 without delay. The formed precipitate was removed by centrifugation at 80,000 × *g* for 15 min and discarded. To the supernatant (73 mg of protein, Fraction 2) was added aged calcium phosphate gel (2 mg per mg of protein). Unadsorbed protein was recovered by centrifugation (70,000 × *g*, for 10 min); 33 mg of protein remained in the supernatant (Fraction 3A). By adding ammonium sulfate to a concentration of 30% saturation, 15 mg of inactive protein were precipitated. The supernatant obtained after centrifugation was passed through a Sephadex G-25 column (1 × 20 cm) equilibrated with 0.5 M KCl. This process removed both ammonium sulfate and Triton X-100. (The biuret-reacting effluent was designated Fraction 3B.) Finally this fraction was run through a Dowex 50-W column (H^+ form, 1 × 20 cm). The adsorbed protein was eluted with 0.2 M Tris-HCl buffer (pH 8.0). The second component, emerging from the column between 24 and

40 ml of the recovered effluent (Fraction 4; 9 mg of protein), possessed a high specific activity with GTP and oleate as substrates (see Table I).

Enzyme Assays—Three assays for GTP-specific acyl-CoA synthetase activity were routinely employed: acyl hydroxamate formation (11), sulfhydryl disappearance (12), and inorganic orthophosphate release (13, 14). The procedures were employed interchangeably since a 1:1:1 stoichiometry was observed among these components at all stages of purification (1). The assay incubation mixtures for acyl hydroxamate formation and for orthophosphate release were essentially the same: hydroxylamine, pH 7.4, 280 μmoles (or Tris-HCl, pH 7.4, 100 μmoles, in the orthophosphate assay); $MgCl_2$, 7.5 μmoles; glutathione, 16 μmoles; fatty acid (potassium salt), 3 μmoles; albumin, 6 mg; coenzyme A, 1 μmole; GTP (or ATP), 1 μmole; and mitochondrial protein, 0.5 to 1 mg, in a final volume of 0.5 ml. Incubation was carried out for 30 min at 38°.

The incubation mixture for the nitroprusside assay was: Tris-HCl (pH 7.4), 100 μmoles; $MgCl_2$, 2.5 μmoles; KBH_4, 1 μmole; fatty acid (potassium salt), 1 μmole; coenzyme A, 0.4 μmole; GTP (or ATP), 1 μmole; and mitochondrial protein, 0.03 to 0.1 mg, in a final volume of 0.2 ml. The duration of incubation was 10 or 15 min at 38° under nitrogen.

Protein concentration was determined by the biuret reaction (15) or by measuring the absorbance at 280 mμ (serum albumin standard).

Reagents—Nucleotide reagents were obtained commercially and were checked for purity with the DEAE-cellulose thin layer chromatography (16). CoA was completely reduced before use with sodium amalgam (17). Palmityl-CoA was prepared from palmitic acid anhydride by the method of Goldman and Vagelos (18).

RESULTS

The product of the isolation procedure, designated Fraction 4 in Table I, possessed a specific enzyme activity (GTP-oleate)

TABLE I

Purification of GTP acyl-CoA synthetase

Fractions were derived from rat liver mitochondria according to the scheme outlined under "Methods." Specific enzyme activities are expressed in terms of micromoles of —SH disappearance per hour per mg of protein. Identical values were observed for orthophosphate release (in GTP-succinate and GTP-oleate assays). Details of these enzyme assay systems are presented under "Methods." In the nitroprusside assay (—SH disappearance) 0.05 to 0.10 mg of enzyme was incubated in a final volume of 0.2 ml for 15 min at 38° under nitrogen. In the orthophosphate assay, 0.5 to 1.0 mg of enzyme was incubated in a final volume 0.5 ml for 30 min at 38°.

Fraction	Total protein	Specific enzyme activity			Yield of GTP oleate
		GTP-succinate	ATP-oleate	GTP-oleate	
	mg				%
Sonically disrupted mitochondria	800	0.75	0.83	0.20	100
Fraction 1	520	0.80	0.40	0.30	98
Fraction 2	73	1.60	0	2.14	97
Fraction 3A	33	0	0	2.50	52
Fraction 4	9	0	0	6.05	35

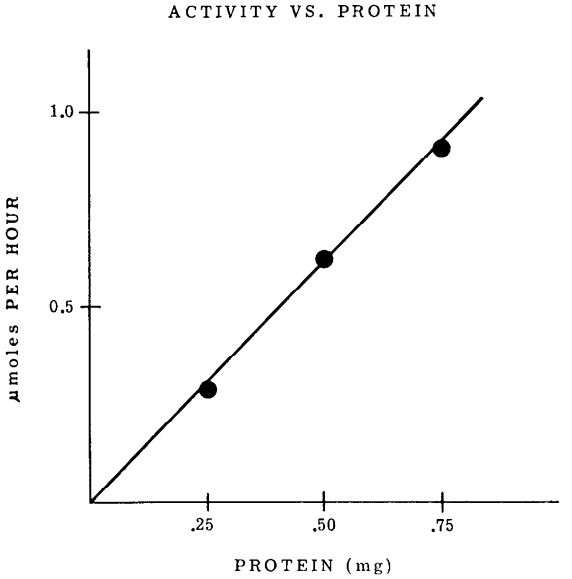

FIG. 1. GTP-acyl-CoA synthetase activity, in terms of micromoles of acyl hydroxamate formed per hour (ordinate), is plotted against milligrams of protein (Fraction 3A) added to the incubation mixture (*abscissa*). In a total volume of 0.5 ml, oleate concentration was 5 mM, GTP was 2 mM, and CoA—SH 2 mM (see "Methods"). Similar results were obtained by following orthophosphate release and —SH disappearance.

30-fold greater than the initial suspension of sonically disrupted liver mitochondria. ATP-dependent long chain fatty acyl-CoA synthetase (19, 20) and succinyl-CoA synthetase (3, 21) activities were absent in the purified fraction. The former enzyme was labile in the acid pH step (22), and the latter enzyme was adsorbed on calcium phosphate gel (1). Pyrophosphatase, palmityl-CoA deacylase, ATPase, and GTPase activities could not be detected in the blank reactions for the synthetase assays of Fraction 4. Carnitine acyl-CoA transferase assays (23) likewise were negative. At each stage of the purification, GTP-acyl-CoA synthetase activity was linear with protein concentration (Fig. 1).

Enzyme purification was also followed by acrylamide gel zone electrophoresis (24, 25). Fraction 4 displayed only one band. The molecular weight of Fraction 4 was estimated by the gel filtration analysis (Sephadex G-75) of Whitaker (26). Highly purified samples of pepsin, serum albumin, myoglobin, and cytochrome *c* were used for calibration of the Sephadex column (Table II). A molecular weight of $2.0 \pm 0.3 \times 10^4$ was calculated for the GTP synthetase protein from the observed retention volume-void volume ratio (V/V_0). The ultraviolet absorption spectrum of the protein eluted from Sephadex G-75 column showed a $A_{280}:A_{260}$ ratio of 1.63.

In the analytical ultracentrifuge (Spinco, model E) 80% of the protein migrated as a single peak. A sedimentation coefficient of 1.0×10^{-13} was calculated from the sedimentation velocity of the major component (protein concentration was 7.5 mg per ml in 0.1 M Tris-HCl buffer, pH 7.4). The observed sedimentation coefficient is consistent with a molecular weight of the magnitude determined by Sephadex chromatography.

Stoichiometry of Reactants—Palmitate, oleate, octanoate, and butyrate were active as substrates in this system. The apparent K_m values are presented in Table III. Similar to succinic thiokinase (1, 21), ITP could replace GTP, but CTP and UTP were inactive. With the purified enzyme a one to one correspondence

TABLE II

Molecular weight determination by gel filtration

Molecular weight of Fraction 4 was estimated by the procedure of Whitaker (26): Sephadex G-75 column (67 × 1 cm); eluting buffer, sodium acetate, 0.1 M (pH 6.0), sodium chloride, 0.4 M; flow rate, 0.33 ml per min; temperature, 25°. The void volume (V_0), determined with Blue Dextran (Pharmacia, Uppsala; molecular weight, 2×10^6), was 15.0 ml. Horse heart cytochrome *c* (type II), crystalline bovine serum albumin, and pepsin (crystallized twice), were obtained from Sigma. Crystalline sperm whale myoglobin (27) was the gift of Professor F. R. N. Gurd.

Protein	Amount added	Molecular weight	V/V_0
	mg		
Cytochrome *c*	10.0	13,000	2.67
Myoglobin	12.0	16,900	2.4
Pepsin	10.0	35,500	2.0
Serum albumin	7.5	70,000	1.6
Fraction 4	7.0	20,000[a]	2.3

[a] Calculated.

TABLE III

Michaelis constants

With the use of Fraction 3B in the orthophosphate release assay, the apparent K_m values were determined from a Lineweaver-Burk (reciprocal) plot (28). The K_m values were calculated from five levels of the variable substrate: 0.2, 0.5, 1.5, 2.0, and 3.0 mM. The invariant pair of substrates was held at 3 mM concentration. The remaining reagents were at the concentration indicated for the assay under "Methods."

Substrate	Michaelis Constants
	moles/liter
Oleate	2.1×10^{-3}
Palmitate	3.1×10^{-3}
Butyrate	2.2×10^{-4}
Octanoate	2.0×10^{-4}
CoA (with butyrate)	3.3×10^{-3}
GTP (with butyrate)	4.3×10^{-3}

was observed for sulfhydryl appearance, acyl hydroxamate formation, thioester bond formation, and orthophosphate release (Table IV). In the reverse reaction both GDP and orthophosphate were required for palmityl-CoA utilization. Acyl hydroxamate disappearance and sulfhydril formation were equivalent (Table V). Also, arsenolysis of palmityl-CoA (in the absence of GDP and orthophosphate) was shown (Table VI).

These results confirm that Equation 1 represents the GTP-specific acyl-CoA synthetase reaction (1).

A broad pH optimum between pH 5 and 8 was observed with the three enzyme assays. From the ratio $k_1:k_2$ of the forward (k_1) and backward (k_2) initial reaction velocities the apparent equilibrium constant was calculated to be 0.6 at 38° and pH 7.4 (Table VII). This value was of the same order as other acyl-CoA synthetase systems (22, 30). A Q_{10} value of 2.3 was obtained up to 55°.

Activators and Inhibitors—Under the conditions of the orthophosphate release assay (see "Methods"), *i.e.* in the presence of Mg^{++} (15 mM), both K^+ and Ca^{++} at 10 mm concentration accelerate the reaction velocity by 50%. Added Na^+ (20 mM), by contrast, is inhibitory (Table VIII). Preincubation with sulfhydryl group inhibitors slowed the reaction velocity as did metal-chelating agents. The most striking effect, however, was elicited with fluoride and added orthophosphate (Tables VIII and IX). Fluoride influenced the GTP-specific acyl-CoA synthetase but not succinyl-CoA synthetase and ATP-dependent acyl-CoA synthetase (Table IX). Fluoride also appeared to block oleate oxidation by intact mitochondria in phosphate-free media (see below, and Fig. 2). Pyrophosphate, when compared at equimolar concentrations with orthophosphate, is not inhibitory in systems containing the purified synthetase. In a separate study (29), atractyloside was found to be inhibitory at 200 μg per mg protein.

TABLE IV

Stoichiometry of GTP acyl-CoA synthetase

Kinase activity was measured with oleate (5 mM) as substrate and Fraction 1. The assay incubation conditions were those described under "Methods." Thioester bond formation was measured at 232 mμ (22, 44). All observed rates were converted to micromoles of change in concentration per hour per mg of enzyme.

	Minus change of —SH	Plus change of		
		Acyl hydroxamate	Acyl-CoA ester	P_i
GTP-synthetase	0.21	0.20	0.19	0.23

TABLE V

Reverse reaction

Two assay systems were employed to measure the reverse reaction. (*a*) Acyl hydroxamate assay: $MgCl_2$, 7.5 μmoles; glutathione, 16 μmoles; palmityl-CoA, 0.5 μmole; GDP, 0.5 μmole; K_2HPO_4, 1.0 μmole; and Fraction 2, 150 μg, in a final volume of 0.5 ml. Following incubation at 38° for 30 min, 280 μmoles of hydroxylamine were added (see "Methods"). (*b*) Sulfhydryl production: Tris-HCl, 100 μmoles; $MgCl_2$, 2.5 μmoles; palmityl-CoA, 1.2 μmoles; GDP, 1 μmole; K_2HPO_4, 1 μmole; and Fraction 2, 100 μg, in a final volume of 0.2 ml. Incubation was carried out for 15 min at 38°. Rate of change is expressed in micromoles per mg protein per hour.

System	Minus change in acyl hydroxamate	Plus change in —SH
Complete	1.46	1.68
No phosphate	0.04	0.08
No GDP	0.04	0.06
No enzyme	0.03	0.08

TABLE VI

Arsenolysis of palmityl-CoA

The following incubation mixture was employed for arsenolysis of palmityl-CoA: Tris-HCl, 100 μmoles; $MgCl_2$, 2.5 μmoles; palmityl-CoA, 0.25 μmole; $NaBH_4$, 1 μmole; potassium arsenate, 5 μmoles; and Fraction 4, 160 μg, in a final volume of 0.2 ml. The duration of incubation was 15 min at 38°. The change in concentration of sulfhydryl is expressed as micromoles per mg of protein per hour.

Mixture	Plus change in —SH
Without arsenate	0.008
With arsenate	0.360

Intact Mitochondria—Mitochondria incubated in the absence of added phosphate and in the presence of DNP will efficiently oxidize long chain fatty acids although regeneration of ATP via electron transport appears to be precluded (2). The means by which fatty acids are activated prior to oxidation must be linked in this case to the GTP-specific succinyl-CoA synthetase system which is coupled to α-ketoglutarate dehydrogenase (3). Orthophosphate arising concurrently with fatty acyl-CoA formation

$$\alpha\text{-Ketoglutarate} + NAD^+ + CoA\text{—}SH \rightarrow \text{succinyl-}S\text{-CoA} + NADH + H^+ + CO_2 \quad (4)$$

(Equation 1) could recycle through the succinyl-CoA synthetase step, thus permitting a continuous supply of GTP. As indicated in the present studies, both orthophosphate and fluoride inhibit the purified GTP-specific acyl-CoA synthetase (Tables VIII and IX). Under conditions which are similar to those used by Van den Bergh (2), both fluoride and orthophosphate inhibited uptake of oxygen in the presence of dinitrophenol (Fig. 2). These results again suggest that the dinitrophenol-insensitive activation process is the GTP-specific fatty acyl-CoA synthetase of mitochondria.

TABLE VII

Apparent equilibrium constant

At pH 7.4 and 38°, the specific initial velocities of the forward (k_1) and backward (k_2) reactions were measured. The ratio $k_1:k_2$ provides an estimation of the equilibrium constant under these conditions (28). In the forward reaction, palmitate (2 mM), GTP (2 mM), and CoA—SH (2 mM) were substrates, and the activity was measured by following hydroxamate formation and sulfhydryl disappearance (see "Methods"). In the backward reaction, palmityl-CoA (2 mM), GDP (2 mM), and P_i (2 mM) were the substrates: hydroxamate disappearance (hydroxylamine added following incubation) and sulfhydryl appearance were the methods used for the activity assay. The values for k_1 and k_2 are expressed in micromoles of change in concentration per mg of enzyme protein per hour.

Method of assay	k_1	k_2	k_{eq}
	× 10^{-2}		
Hydroxamate	1.1	1.8	0.61
Nitroprusside	1.6	2.5	0.66

TABLE VIII

Inhibition of GTP acyl-CoA synthetase

Assays were carried out in 0.5 ml of incubation mixture, containing 0.3 mg of Fraction 4. The agents listed inhibited the acyl-CoA synthetase to the extent indicated. *K*-Oleate (5 mM), GTP (2 mM), and CoA—SH (2 mM) were substrates (see "Methods"). Inhibition by fluoride, EDTA, oxalate, Na^+, *p*-chloromercuribenzoate, and iodoacetate was measured by sulfhydryl disappearance and orthophosphate release. The effect of orthophosphate was measured by sulfhydryl disappearance and hydroxamate formation. Control activity: 5.3 μmoles of change in concentration per hour per mg of enzyme protein. In the case of —SH group inhibitors, 3 mg of enzyme protein were incubated with 0.2 mM *p*-chloromercuribenzoate or 2.0 mM iodoacetate in a total volume of 1 ml for 30 min at 25°. The enzyme was then dialyzed against KCl at 0° for 12 hours. An aliquot containing 0.3 mg of enzyme was then used for the activity assay. Control samples were not affected by preincubation or dialysis.

Inhibitor	Inhibition
	%
Fluoride (2 mM)	100
Orthophosphate (5 mM)	70
EDTA (10 mM)	25
Oxalate (10 mM)	20
Na^+ (20 mM)	40
p-Chloromercuribenzoate (preincubation, 0.2 mM)	45
Iodoacetate (preincubation, 2 mM)	85

TABLE IX

Stoichiometry of acyl-CoA synthetase enzymes in presence and absence of fluoride

Kinase activity was measured with oleate (5 mM) or succinate (5 mM) as substrate and Fraction 1. The assay incubation conditions were those described under "Methods." Fluoride was present at 5 mM concentration in the systems indicated. All observed rates were converted to micromoles of change in concentration per hour per mg of enzyme.

Conditions	Acyl-CoA (GTP) synthetase		Succinyl-CoA synthetase		Acyl-CoA (ATP) synthetase	
	Alone	Plus fluoride	Alone	Plus fluoride	Alone	Plus fluoride
Minus change in —SH	0.21	0.03	0.78	0.76	0.22	0.22
Plus change in acylhydroxamate	0.20	0	0.80	0.78	0.25	0.25
Plus change in P_i	0.23	0	1.08	0.95		

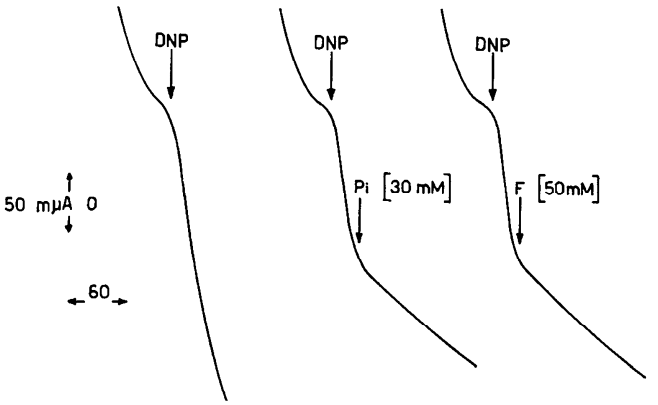

FIG. 2. Fluoride and orthophosphate inhibition of dinitrophenol-uncoupled rat liver mitochondria was studied in the Gilson oxygraph (9, 10). Oxygen uptake is represented in the *ordinate*. The initial incubation system contained, in a volume of 2.0 ml, the following components at the indicated concentration: KCl, 18 mM; $MgCl_2$, 6 mM; EDTA, 2.4 mM; Tris-HCl buffer (pH 7.5), 60 mM; potassium oleate, 0.1 mM; and 10 mg of mitochondrial protein. Where indicated by the *arrow*, 0.2 μmole of dinitrophenol (*DNP*) was added to separate incubation mixtures. In the *middle curve* potassium orthophosphate (P_i) buffer (pH 7.5) was added to bring the system to a concentration of 30 mM. In the *right curve*, KF was added (final concentration, 50 mM).

DISCUSSION

The GTP-specific acyl-CoA synthetase from bovine liver mitochondria, originally described in 1962 (1, 31), was active only with fatty acids of chain length less than 12 carbon atoms. The enzyme from rat liver mitochondria described in the present paper is active with both long and short chain fatty acids. The difference seems to lie in the fact that the latter enzyme was prepared

in the absence of organic solvents. In a separate study (7), evidence has been presented that protein-bound lecithin is essential for maximal activity, and that endogenous lecithin may ensure reactivity with long chain fatty acids. This role of lecithin is in keeping with the observation that the GTP-specific synthetase is bound to mitochondrial membranes (29, 32). The enzyme has been shown in fractions of heart mitochondria (29), but not in brain mitochondria (33).

As indicated previously (1), the GTP-acyl-CoA synthetase behaves formally as the succinyl-CoA synthetase system (3, 21), and not as the ATP-linked fatty acid-activating enzymes (20, 22). No acyl hydroxamate is formed in the absence of CoA (1); GDP and P_i are required in the reverse reaction; and arsenolysis of palmityl CoA is readily shown. Many of the kinetic constants are similar to those of the succinic kinases (30). The possibility of a phosphorylated enzyme intermediate emerges, as a consequence of the recent findings with purified succinyl-CoA synthetase (30, 34, 35).

Although orthophosphate would be expected to inhibit both acyl-CoA and succinyl-CoA formation, the effect of fluoride appears to be specific for the GTP-acyl-CoA synthetase. In phosphate-free media, fluoride is probably diagnostic for this reaction in intact mitochondria. The lack of a fluoride effect on succinyl-CoA synthetase has been observed previously (36). It should be noted that fluoride can depress ATP-dependent oxidation of fatty acids in intact mitochondria (37), probably through its action on pyrophosphatase (38, 39).

The metabolic significance of the GTP fatty acid-activating enzyme has not been determined. The enzyme may be essential for "intramitochondrial" fatty acyl-CoA formation in liver (9). However, this is difficult to assess since the system is inhibited by orthophosphate. If uncoupling of cytochrome-linked phosphorylation is of physiological importance, the GTP enzyme stands ready for direct coupling with succinic thiokinase.

In other situations, the synthetase could operate in the reverse direction, perhaps as a control link, *e.g.* by reducing the concentration of long chain acyl-CoA which is known to inhibit many reactions (40), including the formation of citrate (41), or by liberating CoA—SH from long chain acyl-CoA, thereby augmenting the intramitochondrial CoA—SH pool (42, 43).

The relative concentrations of ATP- and GTP-specific acyl-CoA synthetases may be compared in the mitochondrial Fraction 1 (Table I). The existence of three sites for fatty acid activation by liver mitochondria was described in a preceding paper (9). Two of these sites were apparently ATP-dependent, while the third was attributed to the GTP-requiring system. (The latter was studied in phosphate-free media; Fig. 2.) Regardless of the conditions used for showing either ATP- or GTP-linked routes for fatty acid oxidation, oleate was oxidized by liver mitochondria at approximately the same specific rate (9).

Acknowledgments—The authors gratefully acknowledge the skillful technical assistance of Suzanne Baugh. They also thank Dr. G. T. Gifford for the acrylamide electrophoresis.

REFERENCES

1. Rossi, C. R., and Gibson, D. M., *J. Biol. Chem.*, **239**, 1694 (1964).
2. Van den Bergh, S. G., *Biochim. Biophys. Acta*, **98**, 442 (1965).
3. Sanadi, D. R., Gibson, D. M., Ayengar, P., and Jacob, M., *J. Biol. Chem.*, **218**, 505 (1955).
4. Lehninger, A. L., *J. Biol. Chem.*, **161**, 437 (1945).
5. Yates, D., Shepherd, D., and Garland, P., *Nature (London)*, **209**, 1213 (1966).
6. Galzigna, L., Rossi, C. R., Sartorelli, L., and Gibson, D. M., *Fed. Proc.*, **25**, 339 (1966).
7. Sartorelli, L., Galzigna, L., Rossi, C. R., and Gibson, D. M., *Biochem. Biophys. Res. Commun.*, **26**, 90 (1967).
8. Schneider, W. C., and Hogeboom, G. H., *J. Biol. Chem.*, **183**, 123 (1950).
9. Rossi, C. R., Galzigna, L., Alexandre, A. and Gibson, D. M., *J. Biol. Chem.*, **242**, 2102 (1967).
10. Rossi, C. R., Galzigna, L., and Gibson, D. M., in E. Quagliariello, E. C. Slater, S. Papa, and J. M. Tager (Editors), *Regulation of metabolic processes in mitochondria*, American Elsevier Publishing Company, New York, 1966, p. 143.
11. Lipmann, F., and Tuttle, L. C., *Biochim. Biophys. Acta*, **4**, 301 (1950).
12. Grunert, R. R., and Phillips, P. H., *Arch. Biochem.*, **30**, 217 (1951).
13. Berenblum, I., and Chain, E., *Biochem. J.*, **32**, 295 (1938).
14. Martin, J. B., and Doty, D. M., *Anal. Chem.*, **21**, 965 (1949).
15. Gornall, A. G., Bardawill, C. J., and David, M. M., *J. Biol. Chem.*, **177**, 751 (1949).
16. Stickland, R. G., *Anal. Biochem.*, **10**, 108 (1965).
17. Beinert, H., Von Korff, R. W., Green, D. E., Buyske, D. A., Handschumacher, R. E., Higgins, H., and Strong, F. M., *J. Biol. Chem.*, **200**, 385 (1953).
18. Goldman, P., and Vagelos, P. R., *J. Biol. Chem.*, **236**, 2620 (1961).
19 Kornberg, A., and Pricer, W. E., Jr., *J. Biol. Chem.*, **204**, 329 (1953).
20. Bar-Tana, J., and Shapiro, B., *Biochem. J.*, **93**, 533 (1964).
21. Mazumder, R., Sanadi, D. R., and Rodwell, V. W., *J. Biol. Chem.*, **235**, 2546 (1960).
22. Mahler, H. R., Wakil, S. J., and Bock, R. M., *J. Biol. Chem.*, **204**, 453 (1953).
23. Bremer, J., *J. Biol. Chem.*, **237**, 2228 (1962).
24. Ornstein, L., *Ann. N. Y. Acad. Sci.*, **121**, 321 (1964).
25. Gifford, G. T., and Yuknis, L., *J. Chromatogr.*, **20**, 150 (1965).
26. Whitaker, J. R., *Anal. Chem.*, **35**, 1950 (1963).
27. Hardman, K. D., Eylar, E. H., Ray, D. K., Banaszak, L. J., and Gurd, F. R. N., *J. Biol. Chem.*, **241**, 432 (1966).
28. Dixon, M., and Webb, E. C., *Enzymes*, Academic Press, New York, 1958, p. 63.
29. Allmann, D. W., Galzigna, L., McCaman, R., and Green, D. E., *Arch. Biochem. Biophys.*, in press.
30. Hager, L. P., in P. D. Boyer, H. Lardy, and K. Myrbäck (Editors), *The enzymes, Vol. 6*, Academic Press, New York, 1962, p. 387.
31. Rossi, C. R., and Gibson, D. M., *Abstracts of the American Chemical Society Meeting, Atlantic City, September 1962*, p. 32C.
32. Lands, W. E. M., *Annu. Rev. Biochem.*, **34**, 313 (1965).
33. Beattie, D. S., and Basford, R. E., *J. Biol. Chem.*, **241**, 1412 (1966).
34. Mitchell, R. A., Butler, L. G., and Boyer, P. D., *Biochem. Biophys. Res. Commun.*, **16**, 545 (1964).
35. Cha, S., Chung-Ja, M. C., and Parks, R. E., Jr., *J. Biol. Chem.*, **240**, PC3700 (1965).
36. Kaufman, S., Gilvarg, C., Cori, O., and Ochoa, S., *J. Biol. Chem.*, **203**, 869 (1953).
37. Kennedy, E. P., and Lehninger, A. L., in W. D. McElroy and B. Glass (Editors), *Phosphorus metabolism, Vol. II*, The Johns Hopkins Press, Baltimore, 1952, p. 253.
38. Seal, U. S., and Binkley, F., *J. Biol. Chem.*, **228**, 193 (1957).
39. Rafter, G. W., *J. Biol. Chem.*, **230**, 643 (1958).
40. Taketa, K., and Pagell, B. M., *J. Biol. Chem.*, **241**, 720 (1966).
41. Wieland, O., and Weiss, L., *Biochem. Biophys. Res. Commun.*, **13**, 26 (1963).
42. Chappell, J. B., and Crofts, A. R., *Biochem. J.*, **95**, 707 (1965).
43. Klingenberg, M., and Pfaff, E., in E. Quagliariello, E. C. Slater, S. Papa, and J. M. Tager (Editors), *Regulation of metabolic processes in mitochondria*, American Elsevier Publishing Company, New York, 1966, p. 180.
44. Jaenicke, L., and Lynen, F., in P. D. Boyer, H. Lardy, and K. Myrbäck (Editors), *The enzymes, Vol. 3*, Academic Press, New York, 1960, p. 27.