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Oxidation of Long Chain Fatty Acids by Rat Liver Mitochondria*

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SUMMARY

Long chain fatty acids are oxidized by rat liver mitochondria provided that endogenous adenosine triphosphate is not depleted.

If endogenous ATP is depleted, *e.g.* by 2,4-dinitrophenol, external ATP as well as carnitine is required for fatty acid oxidation. Atractyloside does not block this reaction in media containing orthophosphate buffer. Oligomycin can substitute for carnitine, but the oligomycin-dependent route is inhibited by atractyloside. These experimental situations localize two ATP-dependent fatty acid-activating systems, one of which is linked to carnitine.

The dinitrophenol-insensitive, GTP-specific fatty acyl coenzyme A synthetase is active only in the absence of orthophosphate. Inhibition of oxidation of fatty acids by atractylate in phosphate-free media is not relieved with oligomycin or carnitine.

The study of fatty acid oxidation, which culminated in the isolation of discrete enzyme systems from mitochondria over a decade ago (1–5), has centered in recent years on the mode of transport of fatty acids into the mitochondrial structure. Fritz (6) and Bremer (7) showed that carnitine could facilitate the oxidation of long chain fatty acids and fatty acyl coenzyme A by mitochondria. The subsequent discovery of palmityl-CoA-carnitine transferase (8) (Equation 1) provided the basis for the hypothesis that acyl transport "into" the mitochondria is accomplished in the form of acylcarnitine.

$$\text{Palmityl—S—CoA} + \text{carnitine} \rightleftharpoons \text{palmitylcarnitine} + \text{CoĀ-SH} \quad (1)$$

Acyl-CoA, the substrate for fatty acid oxidation, is regenerated from acylcarnitine inside the mitochondrion by the same enzyme, and carnitine is set free to recycle. Liver mitochondria appear to be an exception to the general rule, in that long chain fatty acids can be oxidized in the absence of added carnitine under certain conditions (9–12).

As a corollary of the carnitine-linked transport mechanism, the primary activation of fatty acids is pictured as taking place prior to acyl transport, *i.e.* "external" to, or at the surface of, the mitochondrion. Other activating enzymes within the mitochondrion are not categorically excluded. Adenosine triphosphate-dependent fatty acyl synthetase activity (Equation 2) has been demonstrated in both the microsomal (13, 14) and mitochondrial fractions (15, 16) of cells.

$$\text{ATP} + \text{RCOOH} + \text{CoĀ-SH} \rightleftharpoons \text{RCO—S—CoA} + \text{AMP} + \text{PP}_i \quad (2)$$

A guanosine triphosphate-specific activation system has recently been found in bovine liver mitochondria (17) (Equation 3).

$$\text{GTP} + \text{RCOOH} + \text{CoĀ-SH} \rightleftharpoons \text{RCO—S—CoA} + \text{GDP} + \text{P}_i \quad (3)$$

A description of the purification and properties of a GTP-specific long chain acyl-CoA synthetase from rat liver mitochondria will be published soon (18).

The present studies (9, 10) were undertaken to evaluate the requirements for long chain fatty acid oxidation by isolated liver mitochondria, particularly with reference to the mechanisms of fatty acid activation and transport.

EXPERIMENTAL PROCEDURE

Mitochondria—Fresh mitochondria were obtained from livers of Wistar strain albino rats of either sex essentially by the method of Schneider and Hogeboom (19). Mitochondrial pellets were washed twice with 0.25 M sucrose to remove the "fluffy layer." They were finally resuspended in sucrose to approximately 30 mg of protein per ml and stored on ice during the experimental period of 2 hours. Preparations of mitochondria giving acceptor control ratios (20) of less than 5 with α-ketoglutarate as substrate were discarded.

Incubation—The routine incubation mixture included the medium of Chance and Williams (21) containing potassium phosphate buffer (see legend to Fig. 1) in a final volume of 2 ml. Oxygen uptake was measured (22) in the Gilson Oxygraph apparatus by a fixed oxygen electrode (Yellow Springs Instrument Company) immersed in a small beaker containing the incubation

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mixture. Constant stirring was maintained magnetically. Additions to the system were made by microsyringe. Potassium palmitate or oleate was prepared before each experiment as a fine emulsion in water at 60°, adjusted to pH 8 with KOH. Solutions of 20 mM were stable if kept at 55°. Acetoacetate was determined by the method of Barkulis and Lehninger (23), and protein, by the biuret method (24). The content of ATP in mitochondria was measured enzymatically with hexokinase and glucose 6-phosphate dehydrogenase according to Steiner and Williams (25). GTP was also measured by this procedure. Endogenous pyridine nucleotide reduction and oxidation were followed fluorometrically in an Eppendorff apparatus as described by Garland, Shepherd, and Yates (26).

Mitochondrial Swelling—Swelling and shrinking of mitochondria were followed by gravimetric measurement (27, 28). The incubation mixture was diluted 10-fold with 0.25 M sucrose at 0° and centrifuged in tared polypropylene Servall centrifuge tubes for 5 min at 20,000 × g in the cold. The tubes were drained carefully, wiped inside with absorbent paper, capped with Parafilm, inverted, warmed rapidly to room temperature, wiped, and capped again. The tubes were then weighed (mitochondrial wet weight), dried to constant weight overnight at 105°, and weighed again for the mitochondrial dry weight. The dry weight of mitochondrial pellets isolated from liver with 0.25 M sucrose was 27 to 30% of the wet weight. The results shown in Figs. 8 to 12 are expressed in terms of micromoles of water per 100 mg of the wet weight value of fresh mitochondria.

Materials—All reagents were reagent grade. Palmitylcarnitine was prepared as described by Bremer (7). Palmitic and oleic acids were purchased from Merck; yeast hexokinase, from Sigma; coenzyme A, from Nutritional Biochemicals; and glucose 6-phosphate dehydrogenase, from Boehringer. Oligomycin was a gift from Dr. A. L. Lehninger, The Johns Hopkins University School of Medicine; and atractyloside (29), from Dr. A Bruni, University of Padua.

RESULTS

As a base-line for this investigation, the behavior of palmitylcarnitine as substrate was examined. Freshly prepared liver mitochondria were incubated at 25° for periods of less than 10 min in a medium containing potassium phosphate buffer (pH 7.0) and magnesium ion (21) (Fig. 1). The mitochondrial preparation used (19) routinely provided high accepter control with α-ketoglutarate or succinate as substrate, and similarly showed good respiratory control and high ADP:O ratios with palmitylcarnitine (Fig. 1) (9, 10, 12, 30). With the latter substrate, oxygen uptake (measured during brief incubations with the Clark oxygen electrode) was stoichiometric with added ADP in the ratio of 2.5 moles of ADP per atom of oxygen. 2,4-Dinitrophenol typically released the inhibition of respiration brought about by oligomycin (31) (Fig. 1). Atractyloside, like oligomycin, blocked ADP-dependent oxygen uptake (29).

Under the conditions of these experiments, palmitylcarnitine oxidation in liver mitochondria was accompanied by formation of acetoacetate in the presence of ADP or dinitrophenol (Table I). Malonate brought acetoacetate generation to its theoretical limit in terms of the ratio of moles of oxygen uptake per mole of acetoacetate produced (calculated value, 1.75 moles of O_2 per mole of acetoacetate).

Free palmitate or oleate was oxidized by mitochondria in this system without benefit of any other additions to the buffered medium (Fig. 2, *lowest curve*). The rate of oxygen uptake was linear provided that the concentration of the fatty acids did not exceed limits characteristic for each fatty acid. With 10 mg of mitochondrial protein per 2 ml of incubation mixture, the value

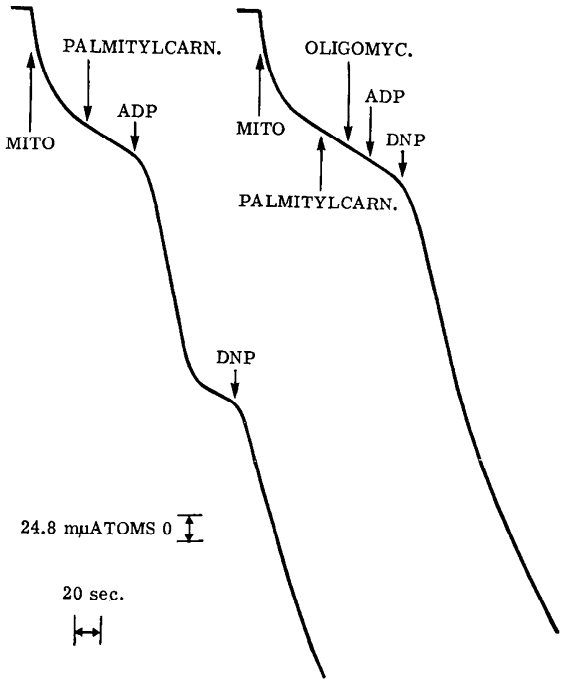

FIG. 1. Oxidation of palmitylcarnitine measured with the oxygen electrode. The incubation system contained 16 mM phosphate buffer (pH 7.4), 12 mM NaF, 26 mM NaCl, 58 mM KCl, and 6 mM $MgCl_2$ (21). At the points indicated by *arrows*, 10 mg of mitochondrial protein (*MITO*), 4 μmoles of palmitylcarnitine, 0.6 μmole of ADP, 0.1 μmole of dinitrophenol (*DNP*), and 5 μg of oligomycin were added. Total volume was 2.0 ml; temperature, 25°.

TABLE I

Oxidation of palmitylcarnitine and formation of acetoacetate in rat liver mitochondria

Experimental conditions were the same as those in Fig. 1. Where indicated, palmitylcarnitine, 4 μmoles; ADP, 2 μmoles; potassium malonate, 2.5 μmoles; and dinitrophenol, 0.1 μmole, were added. The amount of mitochondrial protein was 10 mg. The respiration, measured with the oxygen electrode, was stopped with 1 ml of 12% trichloracetic acid. Acetoacetate was determined by the method of Barkulis and Lehninger (23).

Additions	Oxidation of palmitylcarnitine (A)[a]	Formation of acetoacetate (B)	A:B
	mμmoles/min		
None	25	1.5	
Palmitylcarnitine	27	1.3	
Palmitylcarnitine, ADP	115	48.0	2.40
Palmitylcarnitine, ADP, malonate	123	69.0	1.78
Palmitylcarnitine, dinitrophenol	104	43.1	2.36
Palmitylcarnitine, dinitrophenol, malonate	98	54.0	1.82

[a] O_2 consumed

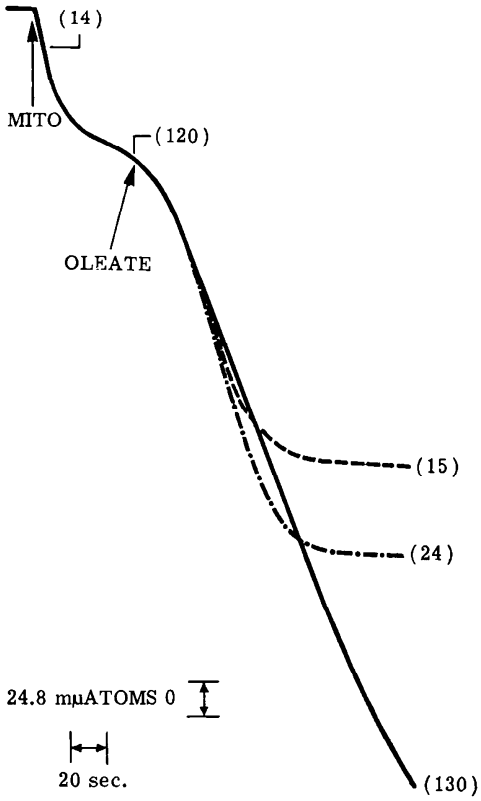

FIG. 2. Endogenous ATP content of mitochondria during oleate oxidation. Experimental conditions were the same as in Fig. 1. Respiration was stopped at the points indicated by addition of 0.1 ml of 70% perchloric acid to 2 ml of incubation mixture. The ATP content of the neutralized perchlorate supernatant was measured enzymatically with hexokinase and glucose 6-phosphate dehydrogenase (25) and is given, in *parentheses*, as millimicromoles per 10 mg of mitochondrial protein. Three oleate levels were used: 0.4 μmole (——), 0.8 μmole (·—·—), and 1.0 μmole (– – –).

for palmitate was 1.0 mM, and for oleate, 0.2 mM.[1] During linear oxygen uptake, 1.0 mM ADP or 1.0 mM ATP and carnitine, separately or in combination, did not stimulate the linear rate of oxygen consumption significantly.

As indicated in Fig. 2, when the quantity of oleate in the incubation system was greater than 0.4 μmole/10 mg of mitochondrial protein, oxygen uptake quickly came to a halt. The failure of oxygen utilization was coincident with depletion of endogenous mitochondrial ATP. During the first minute of incubation of mitochondria (in the absence of added substrate), synthesis of endogenous ATP takes place, presumably coupled to the oxidation of endogenous substrates or reduced nucleotides. The elevated level of ATP remained for at least 10 min during continued incubation in the absence of substrate. The quantity of endogenous ATP was also maintained during incubation with oleate, if less than 0.4 μmole was added per 10 mg of mitochondrial protein. With higher levels of oleate, the ATP content became relatively much lower, coincident with the failure of oxygen consumption. Since sustained fatty acid oxidation by isolated mitochondria depended upon adequate endogenous energy stores, this high energy phosphate pool must have been accessible for fatty acid activation *in situ*.

[1] Similar optimal values were also observed by Van den Bergh (11).

After respiration had ceased in the systems containing higher oleate and palmitate concentrations, the addition of ATP was necessary to restore the rate of oxygen uptake to normal. However, carnitine was now also required in order to effect this restoration (Fig. 3). Carnitine, which was not needed for the oxidation of oleate (at concentrations less than 0.4 μmole/10 mg of mitochondrial protein), was necessary whenever external ATP was added. CoA could not substitute for carnitine in this situation. Acetoacetate was generated stoichiometrically at low concentrations of oleate and palmitate during oxygen uptake (Table II). With the higher levels of fatty acids, proportionately less acetoacetate was formed before cessation of respiration.

Similar patterns of change were also obtained by other means. Oxygen uptake in the presence of low concentrations of free long chain fatty acids came to a halt if dinitrophenol, malonate, or the "hexokinase trap" was added to the incubation system (see Figs. 4, 5 and 6, respectively). Fatty acid oxidation resumed only when both ATP and carnitine were added. Here, again, CoA did not replace carnitine. Although the mechanisms of action of the three agents cited are different, the common result appeared to be the depletion of endogenous ATP in the mitochondria (see

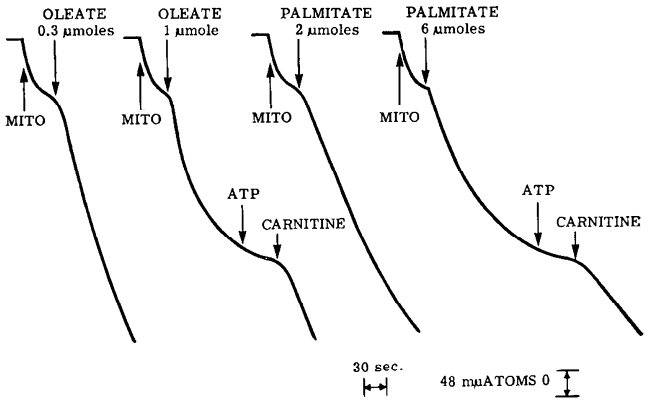

FIG. 3. Requirement for ATP and carnitine at high concentrations of oleate and palmitate. Experimental conditions were the same as in Fig. 1. The amount of mitochondrial protein (*MITO*) was 10.5 mg. At the points indicated by the *arrows*, 2 μmoles of ATP or 2 μmoles of carnitine were added.

TABLE II

Oxidation of palmitate and oleate and production of acetoacetate in rat liver mitochondria

The conditions were the same as those in Fig. 2. No ATP or carnitine was added.

Additions	Amount	Oxidation (A)[a]	Formation of acetoacetate (B)	A:B
	μmoles	*mμmoles/min*		
None		25	1.5	
Palmitate	2	110	60.0	1.83
	4	95	30.1	3.15
	6	131	29.0	4.40
	8	110	23.2	4.75
Oleate	0.3	155	63.0	2.40
	0.5	180	72.4	2.49
	1.0	90	11.0	8.20

[a] O_2 consumed.

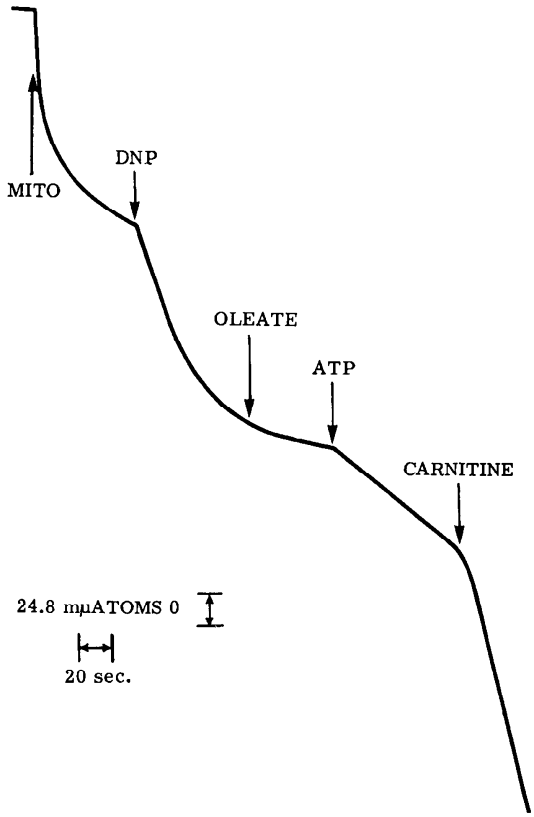

FIG. 4. Requirement for ATP and carnitine in the presence of dinitrophenol (*DNP*). Experimental conditions were the same as in Fig. 1. At the points indicated by *arrows*, 9.5 mg of mitochondrial protein (*MITO*), 0.1 μmole of dinitrophenol, 0.5 μmole of oleate, 2 μmoles of ATP, or 2 μmoles of carnitine were added.

Fig. 2 and Table III). The need for external ATP was clear, but the emergent requirement for carnitine was less obvious.

It was observed that ATP, by itself, at a concentration of 2 mM, could diminish the ordinarily linear rate of oxidation of 0.2 mM oleate (Fig. 7). Supplementation with carnitine restored the initial rate of oxygen uptake. This result suggested that external ATP determined, in part, the requirement for carnitine in the oxidation of fatty acids by liver mitochondria. Since mechanochemical effects of ATP on mitochondria have been described (32), an attempt was made to correlate the physical state of mitochondria with their capacity for oxidizing fatty acids.

In the experiments cited in Figs. 8 to 12, swelling and shrinking of liver mitochondria were measured gravimetrically (27, 28) following incubations under a variety of conditions. In the *upper tracing* of Fig. 8, oxygen uptake with 0.4 mM oleate is followed. As shown previously (Fig. 2), with this concentration of oleate oxygen uptake quickly returns to the base-line rate, and resumption of oxidation is obtained only after ATP and carnitine are both added. In the *lower part* of Fig. 8, the water content of the mitochondria increased (swelling) during incubation (32). Following the introduction of ATP, the water content rapidly decreased, indicating mitochondrial shrinkage back to the initial state (32). Carnitine, itself, appeared to have no influence on the shrinking-swelling process (see also Figs. 9 and 10).

Swelling was also obtained in the systems containing malonate (Fig. 9) or dinitrophenol (Fig. 10) (28). ATP induced shrinkage had but little effect on oleate oxidation until carnitine was added.

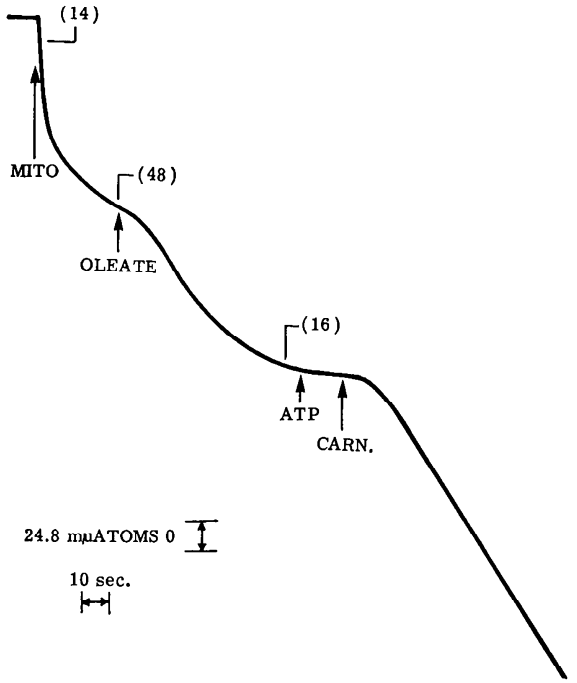

FIG. 5. Requirement for ATP and carnitine in the presence of malonate. The incubation system was similar to that of Fig. 1, but 2.5 μmoles of malonate and 11 mg of mitochondrial protein (*MITO*) were added. ATP content of mitochondria was determined at the points in time indicated. At the *arrows*, 0.3 μmole of oleate, 4 μmoles of ATP, or 2 μmoles of carnitine were added.

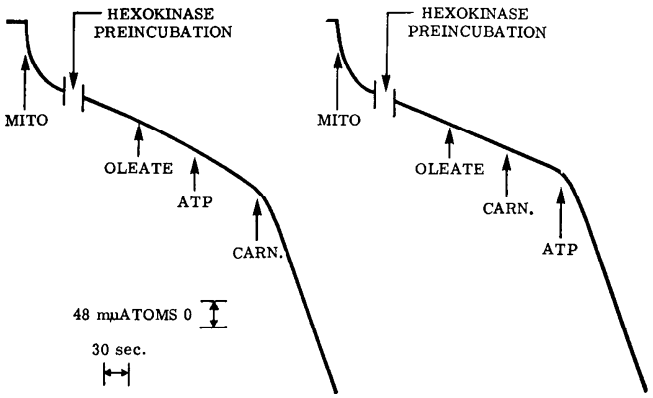

FIG. 6. Requirement for ATP and carnitine after preincubation with hexokinase. The test system was the same as in Fig. 1 and contained, in addition, 5 μg of oligomycin, 150 μg of hexokinase, 10 mM glucose, and 10 mg of mitochondrial protein (*MITO*). The preincubation of mitochondria with hexokinase lasted 6 min, before the further addition of 0.3 μmole of oleate, 4 μmoles of ATP, and 2 μmoles of carnitine. (Oligomycin enhanced the efficiency of the hexokinase-induced ATP drain, probably by preventing rephosphorylation of ADP.)

Mitochondria which were "aged" in 0.25 M sucrose for 24 hours at 0° did not oxidize oleate unless ATP was added (Fig. 11). Again, the requirement for ATP was the consequence of depleted endogenous ATP stores (Table III). In contrast to fresh mitochondria, no carnitine was necessary when external ATP was added. Examination of the water content of these mitochondria (*lower part* of Fig. 11) indicated that swelling took place during incubation with oleate. Addition of ATP in this instance, how-

TABLE III

Endogenous ATP levels in mitochondria

Mitochondria obtained from systems incubated with oleate plus dinitrophenol (Fig. 4), with oleate plus hexokinase (Fig. 6), and aged mitochondria incubated with oleate (Fig. 11) were analyzed for endogenous ATP.

Preparation	ATP level	Oleate oxidation rate
	mμmoles/10 mg protein	*mμmoles/min/10 mg protein*
Fresh mitochondria....	42.5	120
After dinitrophenol....	0.9	7
After hexokinase.....	0	10
Aged 24 hours.....	0.6	12

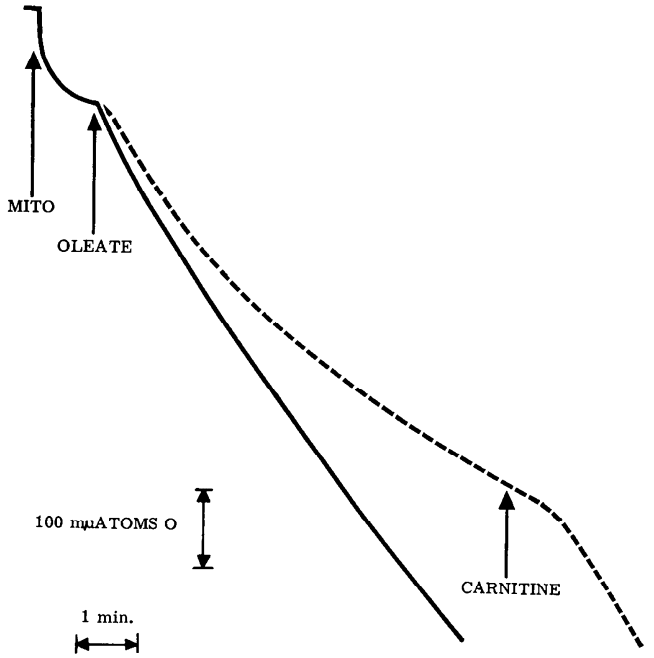

FIG. 7. Oxidation of oleate in the presence of ATP. In the experiment represented by the *upper curve* (- - -), 4.0 μmoles of ATP were present in the incubation mixture, and 2 μmoles of carnitine were added later (*arrow*). In the second experiment (——) no ATP was added. Otherwise, the incubation system was the same as in Fig. 1 (9 mg of mitochondrial protein (*MITO*), 0.4 μmole of oleate).

ever, caused only a small, transitory contraction. These findings suggest that carnitine was not necessary for fatty acid oxidation by mitochondria which remained in a relatively swollen state (see below).

Van den Bergh (11, 33) observed that ATP could not induce oleate oxidation by mitochondria treated with dinitrophenol unless oligomycin was also added to the system. In other words, oligomycin and carnitine, superficially, have the same effect on oxygen uptake. The contribution of oligomycin was interpreted by Van den Bergh (11, 33) in the framework of its inhibitory

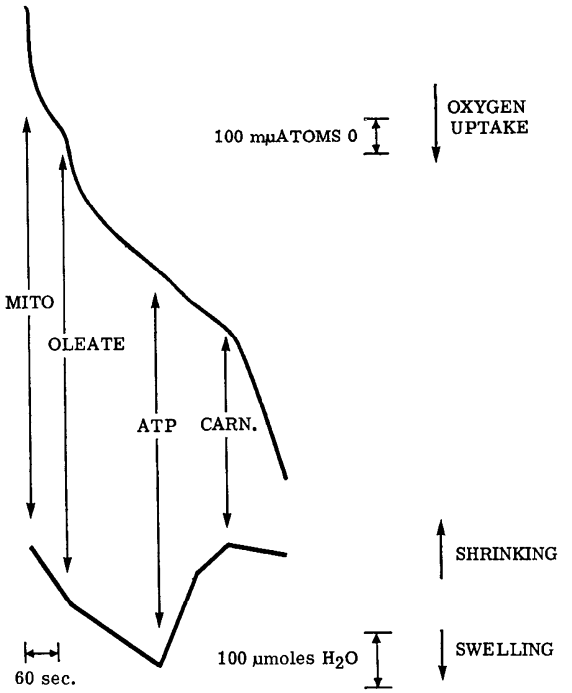

FIG. 8. Oxidation of oleate and mitochondrial swelling. The *upper curve* is the Oxygraph tracing during oleate oxidation as in Fig. 2. The *lower curve* is the water content of mitochondria (taken from a parallel series incubated in the Oxygraph vessel under the same conditions) in terms of micromoles of water per 100 mg of freshly isolated mitochondria. At the points indicated by *arrows*, 9 mg of mitochondrial protein (*MITO*), 0.8 μmole of oleate, 2 μmoles of ATP, or 2 μmoles of carnitine were added.

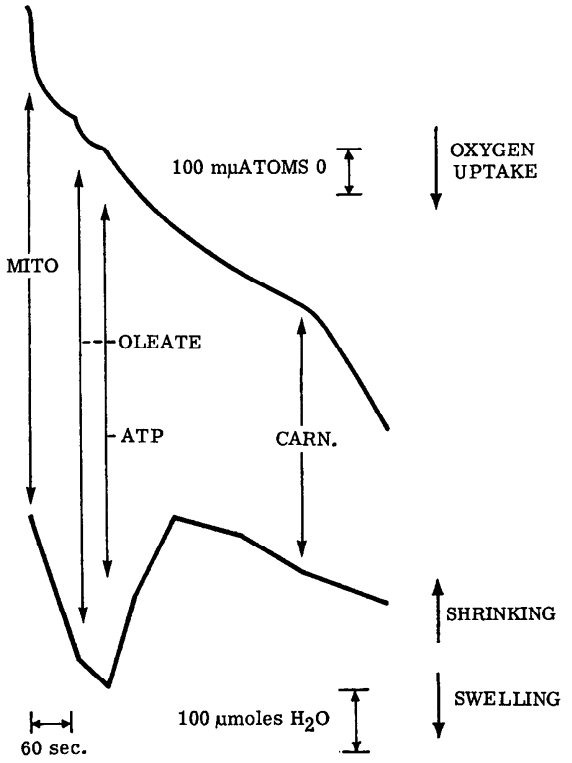

FIG. 9. Oxidation of oleate and mitochondrial swelling in the presence of malonate. Experimental conditions were the same as in Fig. 8. At the points indicated by *arrows*, 10 mg of mitochondrial protein (*MITO*), 0.3 μmole of oleate, 2 μmoles of ATP, or 2 μmoles of carnitine were added. Malonate (2.5 μmoles) was present in the medium from the beginning.

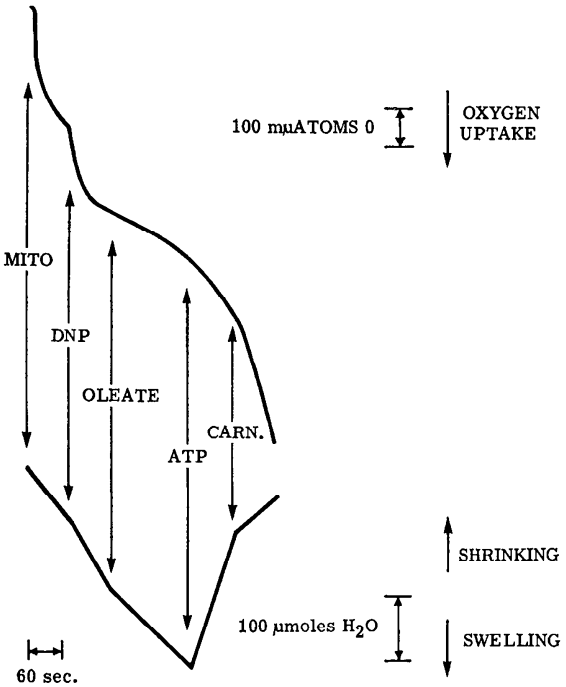

FIG. 10. Oxidation of oleate and mitochondrial swelling in the presence of dinitrophenol (*DNP*). Experimental conditions were the same as in Fig. 8. At the points indicated by *arrows*, 10.5 mg of mitochondrial protein (*MITO*), 0.1 μmole of dinitrophenol, 0.4 μmole of oleate, 2 μmoles of ATP, or 2 μmoles of carnitine were added.

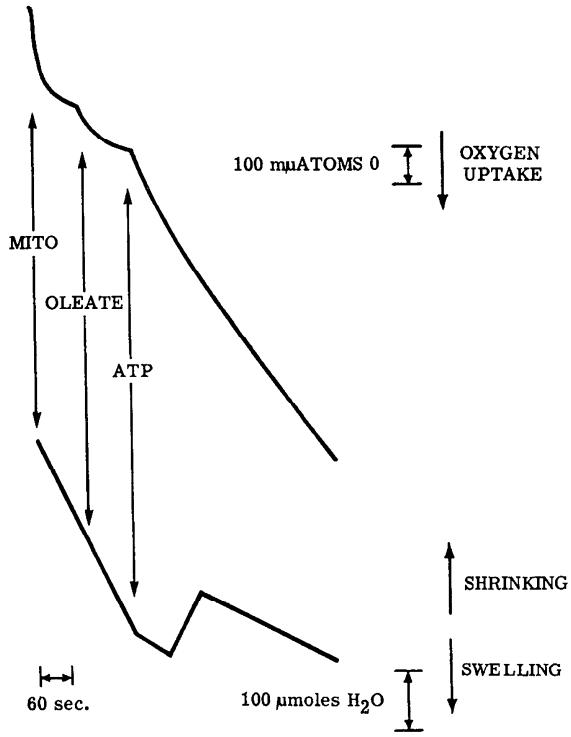

FIG. 11. Oxidation of oleate and mitochondrial swelling in aged mitochondria. Mitochondria were aged in 0.25 M sucrose at 0° for 24 hours. Otherwise the conditions were the same as in Fig. 8. At the points indicated by *arrows*, 9 mg of mitochondrial protein (*MITO*), 0.4 μmole of oleate, or 2 μmoles of ATP were added.

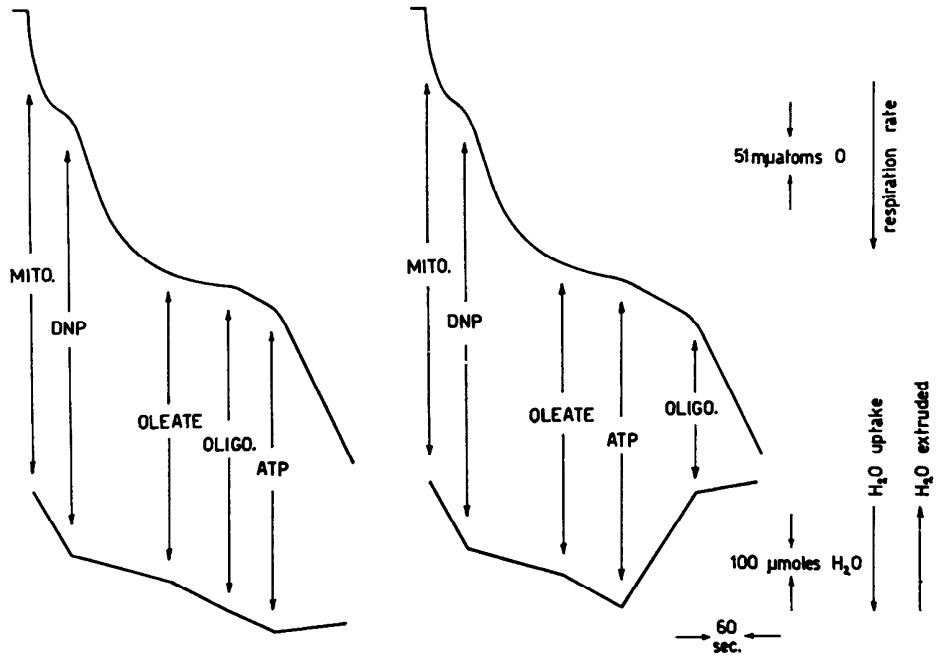

FIG. 12. Requirement for oligomycin in mitochondria treated with dinitrophenol (*DNP*). Experimental conditions were the same as in Figure 8 (16 mM potassium phosphate buffer). At the points indicated by *arrows*, 10 mg of mitochondrial protein (*MITO*), 0.1 μmole of dinitrophenol, 0.2 μmole of oleate, 2 μmoles of ATP, or 10 μg of oligomycin were added.

effect on the dinitrophenol-induced ATPase (31); *i.e.* oligomycin permits external ATP (and free fatty acids) to pass to "internal" activation sites in dinitrophenol-treated mitochondria.

In the present study the effect of oligomycin is not necessarily linked to its known inhibitory action on ATP-induced shrinkage of mitochondria (30, 34–38). A "carnitine-like" action on oxygen uptake was achieved by adding oligomycin to dinitrophenol-treated mitochondria either before or after ATP was introduced into the system (Fig. 12). Oligomycin added before ATP prevented ATP-induced shrinkage (Fig. 12, *left side*). However, when oligomycin was added after ATP, *i.e.* when mitochondria were fully contracted (Fig. 12, *right side*), the oxidation rate was promptly stimulated, even though there was no reversal of contraction.

In the presence of dinitrophenol and oligomycin the rate of

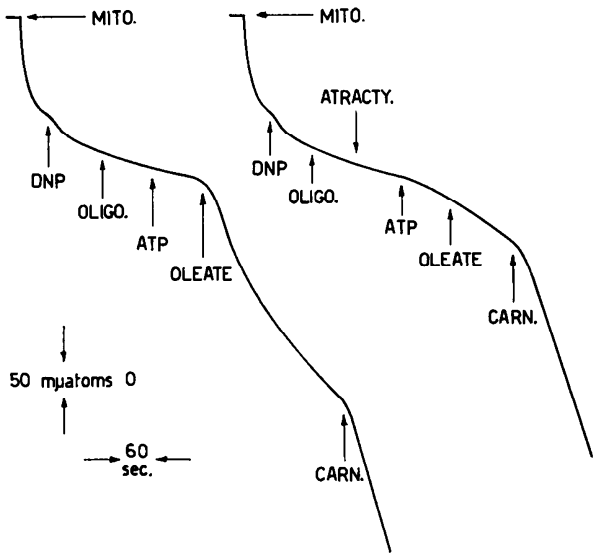

FIG. 13. Effect of atractyloside on oleate oxidation in the presence of dinitrophenol (*DNP*) and oligomycin. The incubation system contained 10 mM malonate; otherwise the conditions were the same as in Fig. 12. Where indicated, 1.0 mM atractyloside was added.

TABLE IV

Oleate oxidation in presence of atractylate

Experimental conditions were similar to those in Figs. 2 to 5: dinitrophenol, 0.1 μmole; ATP, 2 μmoles; and, where indicated, carnitine, 2 μmoles, malonate, 2.5 μmoles, and atractylate, 150 μg. The amount of mitochondrial protein was 9.5 mg.

Additions	Oxidation of oleate	
	−Atractylate	+Atractylate
	mμmoles/min/9.5 mg protein	
Oleate (0.3 μmole)	110	110
Oleate[a] (0.6 μmole) + ATP + carnitine	190	160
Oleate (0.3 μmole) + dinitrophenol[a] + ATP + carnitine	160	160
Oleate (0.3 μmole) + malonate[a] + ATP + carnitine	130	120

[a] The transitory preliminary rise in oxygen uptake induced by 0.6 μmole of oleate, by dinitrophenol, and by 0.3 μmole of oleate in the presence of malonate was subtracted throughout.

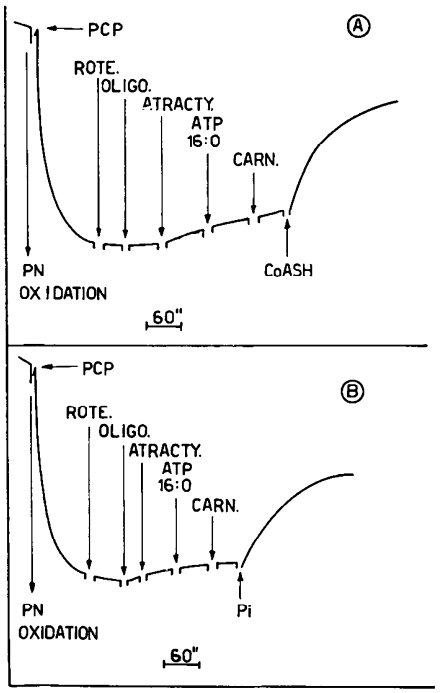

FIG. 14. Effect of orthophosphate on palmitate-dependent reduction of pyridine nucleotides (*PN*) by mitochondria treated with pentachlorophenol (*PCP*). The fluorescence of reduced pyridine nucleotides was measured with the modified Eppendorff photometer (26). The incubation system contained 10 mM Tris-HCl buffer (pH 7.4), 26 mM NaCl, 58 mM KCl, 6 mM $MgCl_2$, 10 mM malonate, and 10 mg of mitochondrial protein (*MITO*). At the points indicated by *arrows*, 0.02 μmole of pentachlorophenol 0.8 μg of rotenone, 10 μg of oligomycin, 2 μmoles of atractyloside, 0.1 μmole of palmitate (*16:0*), 2 μmoles of ATP, or 2 μmoles of carnitine were added. Total volume was 2 ml. Extra additions: *Experiment A*, 0.1 μmole of CoA; *Experiment B*, 1.6 μmoles of orthophosphate. In separate experiments, oleate could substitute for palmitate.

oleate oxidation induced by ATP progressively decreased (Fig. 13, *left side*). The terminal rate could be restored with carnitine. The oligomycin effect was strongly inhibited with atractyloside (Fig. 13, *right side*). Yet carnitine restored oxygen uptake under these conditions. In the presence of phosphate buffer this action of atractyloside can serve to distinguish the "ATP-carnitine" from the "ATP-oligomycin" pathways to fatty acid oxidation by dinitrophenol-treated mitochondria. Atractyloside in a concentration range of 0.1 to 1.0 mM did not inhibit the oxidation of oleate or palmitate, which is initiated by external ATP and carnitine under a variety of other conditions (Table IV).

The action of atractyloside is quite different if dinitrophenol-treated (or pentachlorophenol-treated) liver mitochondria are incubated in the absence of orthophosphate. Chappell and Crofts (30, 39) observed that 1 mM atractyloside completely blocked the reduction of endogenous pyridine nucleotides (followed fluorometrically) of liver mitochondria which were incubated in a phosphate-free medium in the presence of ATP, palmitate, and carnitine. Yates, Shepherd, and Garland (40) later reported that added CoA could restore pyridine nucleotide reduction in this system. The latter experiment was repeated in the present study (Fig. 14, *Curve A*). In the same manner, the addition of orthophosphate to a concentration of 0.8 mM permitted reduction to continue in the presence of atractyloside (Fig. 14, *Curve B*).

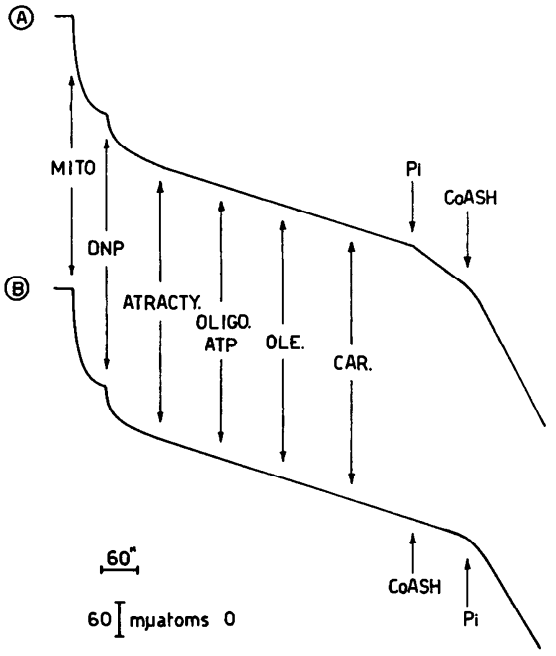

FIG. 15. Effect of phosphate on oleate oxidation. The incubation system was the same as in Fig. 14. At the points indicated by *arrows*, 10 mg of mitochondrial protein (*MITO*), 0.1 μmole of dinitrophenol (*DNP*), 2 μmoles of atractyloside, 10 μg of oligomycin, 2 μmoles of ATP, 0.1 μmole of oleate, 1 μmole of carnitine, 1.6 μmoles of orthophosphate, or 0.1 μmole of CoA were added. Palmitate could substitute for oleate.

In a separate experiment, oxygen consumption was measured under similar conditions. (Dinitrophenol was used instead of pentachlorophenol.) Here again, the small concentration of orthophosphate (0.8 mM) revived the atractyloside-inhibited system (Fig. 15). External CoA exerted relatively little influence in the presence of 0.8 mM orthophosphate, and none under the usual Oxygraph conditions of 16 mM phosphate.

In the presence of phosphate fatty acid activation is brought about only by ATP; GTP is not active, *i.e.* under the conditions of Figs. 3 to 6. A GTP-dependent, long chain fatty acid-activating system has been isolated in fractions derived from sonically disrupted rat liver mitochondria (18). This enzyme is probably identical with the GTP-linked acyl kinase previously identified in bovine liver mitochondria (17). It is reasonable to conclude that the fatty acid oxidation observed with dinitrophenol-treated mitochondria in phosphate-free media is linked to the GTP-specific, long chain acyl-CoA synthetase (11, 33). The purified, soluble GTP-activating enzyme is inhibited by both orthophosphate and fluoride (18). The same agents inhibit fatty acid oxidation in dinitrophenol-treated mitochondria in phosphate-free media (18). In recent experiments,[2] atractylate was found to inhibit fatty acid oxidation by dinitrophenol-treated mitochondria in phosphate-free media. Adding ATP, oligomycin, and carnitine could not restore fatty acid oxidation. The addition of phosphate, however, permitted oxidation to ensue (Figs. 14 and 15) via the carnitine-ATP-dependent activation, which is insensitive to atractylate. These findings are consistent with another study in which it was observed that GTP-dependent acyl-CoA synthetase purified from rat liver mitochondria was inhibited by atractylate if assayed in the absence of borohydride (42).

Atractylate appears to block the acyl-CoA synthetase systems that do not require carnitine for linkage with the fatty acid oxidation enzymes (*e.g.* the oligomycin-dependent and the phosphate-free processes). The oxidation of added palmitylcarnitine which is coupled to the phosphorylation of ADP, was also blocked by atractylate (similar to the action of oligomycin in Fig. 1). However, the oxidation of palmitylcarnitine by dinitrophenol-treated mitochondria was not influenced by atractylate.

DISCUSSION

As long as endogenous ATP and GTP (or other high energy phosphate) stores can be maintained, long chain fatty acids can be oxidized by liver mitochondria without the requirement for added carnitine.

When endogenous ATP is depleted (*e.g.* by dinitrophenol, malonate, high concentrations of fatty acid, or preincubation with hexokinase), then both ATP and carnitine must be added to ensure oxidation of fatty acids. This suggests that the fatty acid activation which is manifest under these particular conditions takes place at an "external" site, since the long chain acyl group must be transferred from the acyl-CoA to carnitine as a prerequisite for transport to the "internal" sites of β oxidation. Alternative hypotheses explaining the need for carnitine can be advanced (9, 10): (*a*) endogenous carnitine depletion, (*b*) saturation of catalytic levels of endogenous CoA (as acyl-CoA) in the presence of an excess of ATP (30, 35), or (*c*) inhibition of carnitine-Acyl-CoA transferase. Existing evidence does not support these latter possibilities in the situations described: *viz.* added CoA did not restore oxidation in the presence of ATP, nor were the acyltransferase enzyme and the acyl-CoA synthetase enzyme (assayed in whole liver mitochondria or with partially purified subfractions) inhibited by dinitrophenol or malonate.

ATP-induced contraction of liver mitochondria may be of importance in interfering with the inward transport of long chain acyl-CoA, but not of ATP or of free fatty acids. Oligomycin added after ATP does not promptly relieve mitochondrial contraction (Fig. 12). Nevertheless, oleate oxidation proceeds as soon as oligomycin is added. Carnitine is not necessary for oleate oxidation by aged mitochondria, since they are damaged and do not contract in the presence of ATP. Consequently ATP and oleate, or acyl-CoA, may have access to exposed sites of β oxidation in the aged preparation.

In liver mitochondria treated with dinitrophenol and incubated with ATP and long chain fatty acids, oligomycin can substitute for carnitine in the initiation of fatty acid oxidation (11). The oligomycin-dependent oxidation again indicates that fatty acids can reach "internal" activation sites of liver mitochondria without the necessity of added carnitine. Exogenous ATP can pass into mitochondria or be utilized for fatty acid activation as long as dinitrophenol-induced ATPase is blocked with oligomycin (11, 31). Atractylate inhibits the oligomycin-dependent route in dinitrophenol-treated mitochondria, but this reagent does not influence the carnitine-stimulated process. That is, atractylate may interfere with the passage of ATP to "internal" sites, or with the binding of ATP (29, 39, 41) to the "internal" acyl-CoA synthetase enzymes. Conversely, the "external" activation locus is unaffected by atractylate (*viz.* the site of the carnitine-dependent cycle).

The "carnitine" and "oligomycin" alternative pathways are

[2] Unpublished observations.

evident only in media containing phosphate buffer. In phosphate-free media, fatty acids are efficiently oxidized by dinitrophenol-treated liver mitochondria (11). Since such oxidation is inhibited by malonate or phosphate (11), it is referable to the GTP-dependent acyl-CoA synthetase. The inhibition of purified GTP-dependent acyl-CoA synthetase by phosphate (17, 18) can serve as a diagnostic for the participation of the GTP-specific system in whole mitochondria (18). In the presence of phosphate and malonate, fatty acid oxidation is restored by adding ATP and oligomycin (Fig. 13, *left side*). This activation, which is blocked by atractyloside (Fig. 13, *right side*), probably occurs at "internal" sites, since the introduction of carnitine permits the demonstration of the "external" ATP-carnitine-linked fatty acid activation (Fig. 13, *right side;* Figs. 14 and 15).

Recent evidence of Allmann *et al.* (42) points to the fact that all of the enzymes concerned with fatty acid oxidation in bovine heart mitochondria are located in fractions derived from the outer membrane. The suggestion of these investigators that the inner and outer faces of the external membrane of mitochondria are separated by a permeability barrier seems valid in the present study. It may be visualized that free fatty acids and acylcarnitine, but not acyl-CoA, can pass through this barrier. Similarly, added ATP could be utilized within this barrier provided that oligomycin blocked ATPase activity.

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