EFFECT OF FATTY ACIDS ON PYRUVATE CARBOXYLATION IN RAT LIVER MITOCHONDRIA

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1. Introduction

Free fatty acids have long been regarded as uncouplers of oxidative phosphorylation [1–5]. On the other hand, Wojtczak et al. [6, 7] demonstrated that long chain fatty acids inhibit the translocation of adenine nucleotides through mitochondrial membranes. Recently, Pande and Blanchaer [8], Shug et al. [9, 10] and Vaartjes et al. [11, 12] have shown that CoA esters of fatty acids are much stronger inhibitors of the translocation than free acids.

Carboxylation of pyruvate to oxaloacetate occurs in mitochondria. It requires ATP as energy donor and is inhibited by ADP [13]. It has been observed that low concentrations of octanoate [14] and oleate [15] increase the rate of pyruvate carboxylation under conditions of state 3 respiration of mitochondria. This effect has been interpreted [14] as being due to the inhibition of adenine nucleotide translocation by fatty acids and an increased ATP/ADP ratio inside mitochondria. Recently, Stucki et al. [15] have shown that oleate and octanoate act by different mechanisms; octanoate diminishes the pool size of exchangeable adenine nucleotides while oleate inhibits adenine nucleotide translocase. A similar explanation of the stimulation of carboxylation by medium chain and long chain fatty acids was presented by ourselves [16] in a preliminary form.

In the present investigation a large spectrum of fatty acids was examined and it was shown that short and medium chain acids (up to 14 carbon atoms) differ in their mode of action on pyruvate carboxylation from long chain acids (16 carbon atoms and more). Evidence is also presented that long chain acids are effective as CoA esters whereas octanoate is active as free acid. Furthermore, it is shown that the effect of fatty acids, or their CoA esters, is additive to that of acetyl-CoA which is formed during the oxidation of fatty acids.

2. Materials and methods

Mitochondria were obtained from livers of albino rats by the conventional procedure [17]. The translocation of [^{14}C]ATP was measured by the inhibitor-stop method as described previously [6] except that the temperature was 25°. Pyruvate carboxylation was measured in the following incubation medium: 125 mM KCl, 5 mM triethanolamine-HCl buffer and 5 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 10 mM sodium pyruvate, 10 mM $KHCO_3$, 10 mM glucose, 1 mM ADP, 10 units/ml hexokinase, and mitochondria containing about 10 mg protein in a total volume of 2.0 ml. Incubation time was 10 min if not indicated otherwise, temperature 36°. Samples were incubated in Warburg or Erlenmeyer flasks under gentle shaking. Incubation was stopped by perchloric acid and the deproteinated samples were neutralized by $KHCO_3$. The amount of pyruvate carboxylated was determined by either the formation of malate and citrate or the incorporation of $^{14}CO_2$. In the latter case the medium contained 1 μCi [^{14}C]$KHCO_3$. Malate and citrate were

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measured spectrophotometrically by enzymatic assays [18, 19]. According to Walter and Stucki [14], the sum of these two citric acid cycle intermediates formed corresponds to more than 90% of total pyruvate carboxylated. Acetoacetate was determined by the method of Walker [20]. Protein was measured by the biuret procedure [21].

Malate dehydrogenase was purified according to Dupourgue and Kun [22] omitting the final column chromatography. Citrate lyase, CoA and sodium pyruvate were obtained from Boehringer. L-carnitine derivatives of acetate, octanoate, palmitate and oleate were kindly provided by Dr. J. Bremer and Dr. B.O. Christophersen (Institute of Clinical Biochemistry, University of Oslo). Atractyloside was from Calbiochem, free fatty acids from British Drug Houses, $[^{14}C]Na_2CO_3$ from the Institute of Nuclear Research (Świerk, Poland), and [8-^{14}C]ATP from Radiochemical Centre (Amersham, England). Other chemicals were of highest purity commercially available. Carbonyl-cyanide-*m*-chlorophenylhydrazone was a gift of Dr. P.G. Heytler of Du Pont de Nemours (Wilmington, Del., USA).

3. Results and discussion

The effect of oleate on pyruvate carboxylation is shown in table 1. It is evident that the carboxylation is lower in state 3 than in state 4 and is increased by 50 nmoles oleate/mg mitochondrial protein almost to the level of state 4. A similar effect is produced by atractyloside, while uncouplers and higher concentrations of oleate are strongly inhibitory. The stimulatory effect of low concentration of oleate, fully compatible with recent observations of Stucki et al. [15], clearly indicates that at that concentration oleate does not uncouple oxidative phosphorylation, since the carboxylation is an energy-requiring process. Therefore, the inhibition of ATP–P_i exchange and lowering of P/O ratio which we observed previously [7] were due to the inhibition of the translocation rather than to uncoupling when low concentrations of oleate were present.

The experimental conditions of the present investigation slightly differ from those of Stucki et al. [15]. These authors included ATP in their incubation system and state 3 was obtained by addition of creatine

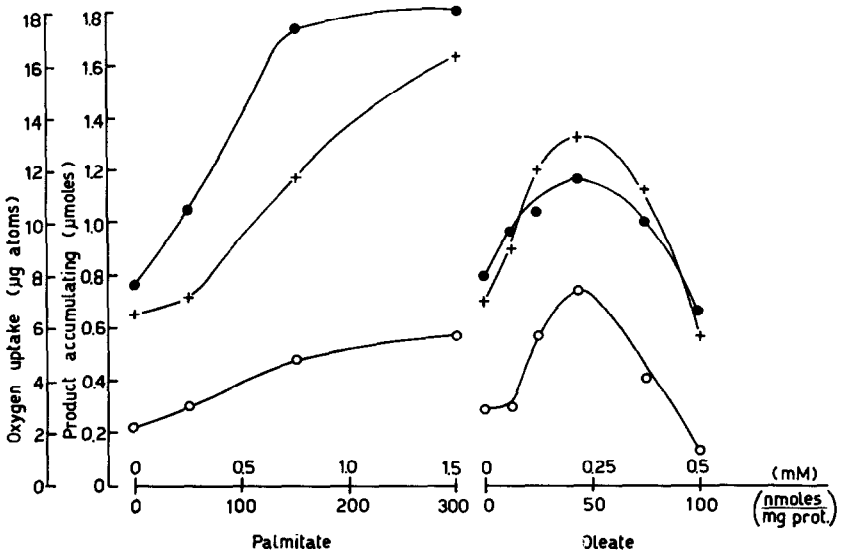

Fig. 1. Effect of palmitate and oleate on the formation of malate and acetoacetate and the uptake of oxygen by mitochondria incubated with pyruvate in state 3. The data are expressed per sample (10 mg mitochondrial protein) per 15 min. (○—○—○) Malate formed; (●—●—●) acetoacetate formed; (+—+—+) O_2 uptake.

Table 1
Effect of oleate, an uncoupler of oxidative phosphorylation (carbonyl-cyanide-*m*-chlorophenylhydrazone, CCCP) and atractyloside on the carboxylation of pyruvate, oxygen uptake and accumulation of acetoacetate.

Composition of the medium	Malate	Citrate	Malate + citrate (μmoles)	Acetoacetate	O_2 uptake (μg atoms)
ADP and hexokinase omitted (state 4)	0.56	1.80	2.36	0.31	7.3
Complete (state 3)	0.28	0.65	0.93	0.61	9.0
+ Oleate 25 nmoles/mg protein	0.28	1.49	1.77	0.84	11.1
+ Oleate 50 nmoles/mg protein	0.68	1.62	2.30	0.90	14.6
+ Oleate 150 nmoles/mg protein	0.14	0.00	0.14	0.58	8.6
+ CCCP 2.5 μM	0.05	0.26	0.31	1.74	10.6
+ Atractyloside 25 μM	0.57	2.20	2.77	0.39	6.9

The data are expressed per sample (10.5 mg mitochondrial protein) per 15 min. The formation of citrate plus malate is a measure of pyruvate carboxylated. Composition of the complete medium is described under Methods.

and creatine kinase. In our system no ATP was added and state 3 conditions were produced by the glucose–hexokinase trap. Nevertheless, the effect of oleate was similar in both systems with the exception that in our experiments there was a sharp optimum concentration of about 50 nmoles/mg protein (fig. 1) and a decrease of carboxylation rate at higher concentrations of oleate, whereas in the system of Stucki et al. [15] 100 nmoles oleate/mg protein was still stimulatory. Fig. 1 also shows that the optimum concentration for palmitate is much higher and corresponds to about 300 nmoles/mg protein or higher. The decrease of

Table 2
Effect of fatty acids of various chain length on pyruvate carboxylation in states 3 and 4.

Fatty acid added (shorthand designation)	Concentration (mM)	Carboxylation (relative values) State 3	State 4
None		100	100
C_2	25	180	108
C_4	10	208	23
C_6	5	195	21
C_8	2.5	214	3
C_{10}	1.0	212	3
C_{12}	0.25	205	0
C_{14}	0.25	225	30
C_{16}	1.0	158	122
C_{18}	1.0	128	99
$C_{18:1}$	0.25	172	128
Atractyloside	0.025	318	146

The carboxylation was measured as the sum of malate and citrate formed and expressed in percentage of the control (without added fatty acid) in the respective state. The absolute amounts were 1.16 μmoles and 3.64 μmoles in states 3 and 4, respectively (per sample). The concentrations of fatty acids used were found to give maximum stimulation in state 3.

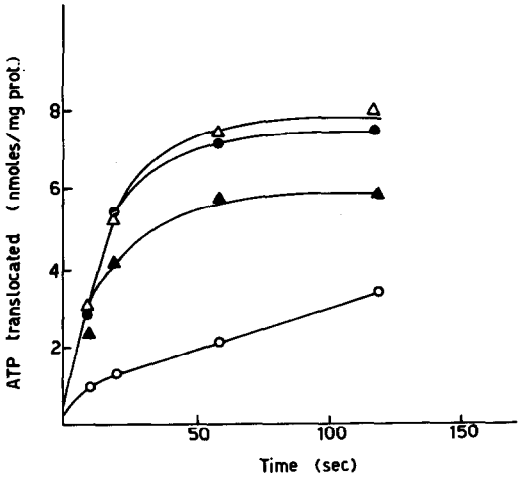

Fig. 2. Effect of oleate, octanoate and α-ketoglutarate on [^{14}C]ATP uptake by mitochondria. The incubation medium contained 120 mM KCl, 20 mM Tris-HCl pH 7.4, 1 mM $MgCl_2$, 25 mM sucrose, 60 μM [^{14}C]ATP, and 10 mg mitochondrial protein in the total volume of 2.2 ml. (△—△—△) No additions; (▲—▲—▲) 30 nmoles octanoate/mg protein; (●—●—●) octanoate (same amount) and 5 mM α-ketoglutarate; (○—○—○) 30 nmoles oleate/mg protein.

Table 3
Effect of oleate, CoA and 2,4-dinitrophenol (DNP) on ATP translocation.

Additions	ATP translocated (nmoles/mg protein)	(% of the control)
None (control)	5.2	100
DNP 0.1 mM	5.8	111
CoA 0.3 mM	4.8	92
Oleate 30 nmoles/mg protein	3.1	92
Oleate + CoA	0.8	15
Oleate + DNP	5.5	106

Conditions as in fig. 3; incubation time 40 sec.

pyruvate carboxylation at higher concentrations of oleate may be due to the true uncoupling and is also paralleled by a decrease in fatty acid activation, as reflected in fig. 1 by a decrease of oxygen uptake and acetoacetate production (cf. also [23]). In the experiment illustrated in fig. 1 only malate was determined as the carboxylation product since it has been shown in table 1 (and also in table 4) that changes in malate formation can reflect the rate of carboxylation.

Using a large spectrum of fatty acids, it was shown (table 2) that all of them were activatory in state 3.

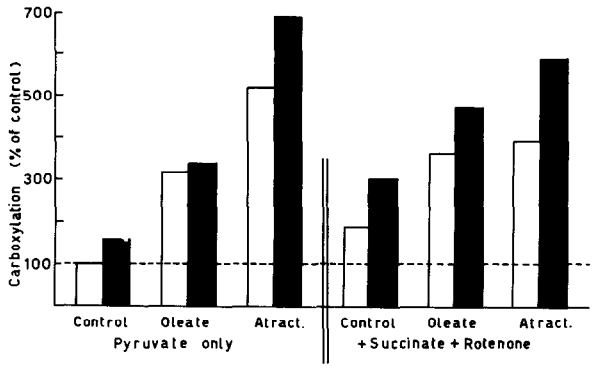

Fig. 3. Effect of acetyl-carnitine on pyruvate carboxylation in state 3. The carboxylation was measured by $^{14}CO_2$ incorporation. The right part of the figure represents samples to which 5 μM rotenone and 5 mM succinate were added. The data are expressed in percentage of the control without rotenone and succinate. Concentrations: oleate 0.15 mM (30 nmoles/mg protein), atractyloside 25 μM. White bars, no acetyl-carnitine; black bars, with 5 mM acetyl-carnitine.

Contrary to this, in state 4 short and medium chain acids (C_4–C_{12}) were strongly inhibitory. Only long chain acids, and also atractyloside, appeared to be slightly activatory or had no effect. It is also noteworthy that the concentrations giving maximum stimulation in state 3 decreased, for saturated fatty acids, with increasing chain length up to C_{12} and C_{14} and then increased. The lowest optimum concentration was also found for the unsaturated $C_{18:1}$, i.e. oleate. The dependence on the chain length and unsaturation is the same as that of mitochondrial swelling [24] and probably reflects a partial damage of mitochondrial integrity and/or of their coupling system by higher concentrations of the acids.

The opposite effects of short and medium chain fatty acids in states 3 and 4 are most likely connected with the fact that these acids are activated inside mitochondria [25] resulting in the accumulation of AMP and strongly competing for ATP with the carboxylation system. In state 4 this results in an inhibition of the carboxylation. Contrary to this, in state 3, due to a diminished ATP plus ADP pool in the presence of fatty acids, a high rate of oxidative phosphorylation maintains a higher ration of ATP/ADP than in the absence of fatty acids [15], thus resulting in increasing the carboxylation rate. On the other hand, the action of long chain fatty acids, which are activated mostly outside, is due to their effect on the translocase of adenine nucleotides, thus also increasing the ATP/ADP ratio inside mitochondria.

That octanoate does not inhibit adenine nucleotide translocase but changes the proportion of mitochondrial nucleotides is evident from fig. 2. It shows that the initial rate of the translocation, as measured after 10 sec of the incubation, is only slightly affected by octanoate whereas it is strongly decreased by oleate. The effect of octanoate is, on the other hand, manifested after 60 sec when an isotopic equilibrium, is attained. This can be interpreted by assuming that the exchangeable pool of mitochondrial adenine nucleotides is diminished. This interpretation is compatible with the observation that the effect of octanoate can be completely abolished by α-ketoglutarate, known to convert mitochondrial AMP to ADP [26]. α-Ketoglutarate had no effect on the rate of ATP translocation in the presence of oleate (not shown). These results confirm the observation [11] that octanoate and its CoA ester have no effect on

Table 4
Comparison of the effects of fatty acids and their carnitine esters ± CoA on pyruvate carboxylation.

Additions (nmoles/mg protein)	Without CoA				With CoA 0.3 mM			
	Malate (μmoles)	Citrate (μmoles)	Malate + citrate (μmoles)	(%)	Malate (μmoles)	Citrate (μmoles)	Malate + citrate (μmoles)	(%)
Expt. 1								
None	0.26	0.77	1.03	100*	–	–		
Octanoate (500)	0.82	1.09	1.91	186	0.88	0.84	1.72	167
Octanoyl-carnitine (160)	0.52	0.78	1.30	126	0.55	0.78	1.33	129
Expt. 2								
None	0.16	0.71	0.87	100*	0.26	0.57	0.83	96
Palmitate (50)	0.26	0.91	1.17	136	1.29	0.91	2.20	253
Oleate (50)	0.54	1.23	1.77	203	1.38	1.13	2.51	290
Palmitoyl-carnitine (5)	0.23	0.71	0.94	108	0.32	0.82	1.14	131
Palmitoyl-carnitine (25)	0.26	0.92	1.18	136	1.30	1.05	2.35	270
Oleoyl-carnitine (5)	0.19	0.71	0.90	103	0.37	1.16	1.53	176
Oleoyl-carnitine (25)	0.32	0.83	1.15	132	1.26	1.22	2.48	285

* The carboxylation in the absence of fatty acids or their derivatives and CoA was taken as 100. The data are expressed per sample (10.0 mg protein) per 10 min.

adenine nucleotide translocation, but show that this medium chain fatty acid decreases the sum of ATP and ADP, apparently due to the intramitochondrial formation of octanoyl-CoA [25].

Table 3 shows that the effect of oleate on the translocation is strongly potentiated by CoA and is completely abolished by dinitrophenol. Thus, it seems likely that free oleate does not inhibit the translocation and its effect is only due to some formation of oleoyl-CoA.

In agreement with the concept of different mechanisms of action on the carboxylation of medium and long chain fatty acids are the results of experiments with the carnitine esters of fatty acids. They show (table 4) that, in contrast to free octanoate, octanoyl-carnitine either with or without CoA has very little effect on the carboxylation while oleoyl and palmitoyl-carnitines are activatory, especially in the presence of CoA.

The stimulatory effect of fatty acids on gluconeogenesis has been primarily ascribed to the activation of pyruvate carboxylase by the increased level of acetyl-CoA [27]. Nevertheless, from the present investigation as well as those by other authors [12, 14, 15] it seems likely that the inhibition of the translocation of adenine nucleotides may be even more important. In order to differentiate between thse two possible effects acetyl-carnitine was added together with oleate. In this experiment the carboxylation was determined by measuring the incorporation of $^{14}CO_2$ into acid soluble compounds. This is a less precise measure of pyruvate carboxylation than by a direct determination of the carboxylation products, but for the purpose of this experiment this was of a secondary importance. It was shown (fig. 3) that the presence of acetyl-carnitine had no additional stimulatory effect under conditions when oleate was oxidized (left side of the figure). When rotenone was added to inhibit fatty acid oxidation, oleate (right side of the figure) and other long chain fatty acids (not shown) retained their stimulatory effect. In this case, however, acetyl-carnitine further potentiated this effect. Acetyl-carnitine was always stimulatory in the presence of atractyloside and in the controls, indicating that under these conditions pyruvate carboxylase was not maximally saturated with acetyl-CoA.

These results show that under conditions when oleate and other long chain fatty acids are oxidized their effect on carboxylation is dual: i) by the inhibition of the adenine nucleotide translocation and ii) by increasing intramitochondrial acetyl-CoA.

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