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MECHANISM OF INHIBITION BY UNCOUPLERS OF SUCCINATE OXIDATION IN ISOLATED MITOCHONDRIA

S. PAPA, N. E. LOFRUMENTO, G. PARADIES AND E. QUAGLIARIELLO

Department of Biochemistry, University of Bari, Bari (Italy)

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SUMMARY

1. The inhibition by uncouplers of succinate oxidation in isolated rabbit-kidney mitochondria has been studied.

2. In the absence of rotenone, the oxidation of 10 mM succinate was inhibited by uncouplers. This inhibition could be prevented by adding glutamate, palmitoylcarnitine or ATP. Activation by ATP occurred in the presence of oligomycin but not of atractyloside.

3. In the presence of rotenone, the uncoupler dicoumarol inhibited the oxidation of succinate when the substrate concentration was 1 mM but not when it was 10 mM. ATP had no effect on the inhibited respiration. Removal of dicoumarol with albumin under conditions in which the mitochondria could subsequently become energized led to a restoration of succinate oxidation.

4. In the absence of rotenone and presence of uncoupler, the activation of respiration by ATP was accompanied by a decline in the level of oxaloacetate and by the formation of phosphoenolpyruvate.

5. In the absence of rotenone and presence of uncoupler, there was an inverse correlation between the rate of respiration and the level of oxaloacetate. The activation of respiration by ATP or palmitoylcarnitine was accompanied by a decline in the level of oxaloacetate.

6. The addition of phosphoenolpyruvate, CO_2 and ADP to mitochondria in the presence of uncoupler and rotenone led to an inhibition of succinate oxidation, presumably due to oxaloacetate formation.

7. It is concluded that the inhibition by uncouplers of succinate oxidation in the absence of rotenone and at relatively high succinate concentrations is due to the formation of oxaloacetate.

INTRODUCTION

It has been known for many years that uncouplers of oxidative phosphorylation, in concentrations which with various substrates elicit respiratory rates equal to or exceeding that of the active coupled state, inhibit the oxidation of succinate by

isolated mitochondria[1–4]. Although this inhibition has been the subject of intensive investigation, its mechanism is still a matter of debate. In the early studies it had already been observed that the inhibition could be abolished by preventing oxaloacetate formation with Amytal[1,2] or by removing oxaloacetate with glutamate[5]. On the basis of these and related findings it was suggested[3–5] that the inhibition of succinate oxidation by uncouplers is due to an increase in the steady-state concentration of oxaloacetate, which inhibits succinate dehydrogenase (EC 1.3.99.1) competitively[6,7]. AZZONE *et al.*[8] and SCHOLLMEYER AND KLINGENBERG[9] threw doubts on this mechanism because of their failure to show a relationship between the rate of succinate oxidation and the level of oxaloacetate.

The inhibition of succinate oxidation by uncouplers can also be prevented or reversed by ATP[1,3]. Various mechanisms have been proposed for this effect of ATP[1,3,5,6,10–12].

More recently WENNER[13] observed that with low concentrations of succinate, uncouplers, in moderate concentrations, inhibit respiration even when rotenone is present. The inhibition has been demonstrated to be competitive with respect to succinate[14,15]. It has also been shown that mitochondria accumulate succinate from the suspending medium and that this process is inhibited by uncouplers[16,17]. It has therefore been proposed that the inhibition by uncouplers of succinate oxidation in the presence of rotenone is due to inhibition of the uptake of succinate[12,15–17]. This mechanism, rather than oxaloacetate inhibition, has also been proposed to explain the inhibition in the absence of rotenone[12].

In this paper an investigation of the inhibition by uncouplers of succinate oxidation in isolated rabbit-kidney mitochondria is presented. The results obtained are consistent with the hypothesis that the inhibition by uncouplers of succinate oxidation, in the presence of rotenone, is due to inhibition of the uptake of succinate by mitochondria. On the other hand, direct evidence is provided that the inhibition, in the absence of inhibitors of the oxidation of NADH *via* the respiratory chain, and at relatively high concentrations of succinate (10 mM), is caused by accumulation of oxaloacetate. It is also shown that in the presence of uncouplers ATP activates succinate oxidation by promoting removal of oxaloacetate. Preliminary accounts of this investigation have been presented[18,19]. Results consistent with these views have also been obtained in the course of studies of the inhibition by uncouplers of succinate oxidation in mitochondria isolated from other tissues[20–23].

MATERIALS AND METHODS

Isolation of mitochondria. Mitochondria were prepared from rabbit kidney homogenized in 0.25 M sucrose. The homogenate was centrifuged for 5 min at 600 $\times$ *g*, and the mitochondria were sedimented from the supernatant at 4800 $\times$ *g* (10 min), washed once in the sucrose solution and centrifuged for 10 min at 11000 $\times$ *g*. The pellet was suspended in sucrose.

Oxygen determination. O_2 consumption was determined either manometrically or polarographically with an oxygen polarograph (Gilson Medical Electronics, Model K). The final vol. was 1 ml for the manometric measurements, and the temperature was 30°. The reaction was stopped with 0.1 ml 30% $HClO_4$. In the polarographic assays the final vol. was 1.5 ml and the temperature 25° or 30°.

Protein determination. Protein was determined by the method of CLELAND AND SLATER[24] and by a micro-Kjeldahl procedure.

Other assays. Malate[25], pyruvate[26], phosphoenolpyruvate[27] and aspartate[28] were determined enzymically on the neutralized $HClO_4$ extracts with an Aminco–Chance double-beam spectrophotometer. Oxaloacetate was also determined enzymically, after first being converted into aspartate[18]. In separate controls it was found that during this conversion, only a small amount of aspartate was formed as a result of oxidation of malate in the presence of glutamate, rotenone, antimycin and KCN. The values for Δ-oxaloacetate were corrected for this.

Materials. Malate dehydrogenase (EC 1.1.1.37), lactate dehydrogenase (EC 1.1.1.27), pyruvate kinase (EC 2.7.1.40) and aspartate aminotransferase (EC 2.6.1.1) were obtained from Boehringer und Soehne; yeast hexokinase (EC 2.7.1.1) (Type III), oligomycin and antimycin A from Sigma Chem. Co.; dicoumarol from Calbiochem. Rotenone was a gift from Dr. J. M. Tager, sodium atractyloside from Prof. T. Ajello and Prof. A. Quilico, and palmitoylcarnitine and caprinoylcarnitine from Dr. J. Bremer.

RESULTS

Evidence for two types of inhibition by uncouplers of succinate oxidation

Preincubation of rabbit-kidney mitochondria with 20 μM dicoumarol, in the presence of arsenite to abolish substrate-level phosphorylation and to prevent further oxidation of pyruvate deriving from succinate[21–23] (Expt. 1, Table I), caused a strong inhibition of the oxidation of 10 mM succinate. The inhibition could be completely prevented by rotenone or by glutamate. The inhibition could also be largely prevented by oligomycin, probably due to prevention of the uncoupler-induced hydrolysis of endogenous ATP. Indeed, addition of ATP completely abolished the inhibition of respiration. When mitochondria were preincubated with 100 μM dinitrophenol *plus* 3 mM arsenate (Expt. 2, Table I), an inhibition of respiration resulted, which increased with time. The inhibition was reversed by ATP, even in the presence of oligomycin, but not in the presence of atractyloside[29].

In the presence of rotenone (Expt. 3, Table I) dicoumarol caused a slight stimulation of the oxidation at 10 mM succinate and a marked inhibition at 1 mM succinate. In contrast to what was observed in the absence of rotenone, ATP had no effect on the inhibited respiration. However, the inhibition could be released by adding serum albumin, which binds dicoumarol and restores the coupled state[15,30].

Expt. 4 of Table I shows that the inhibition of the oxidation of 10 mM succinate caused by dicoumarol in the absence of rotenone was almost completely released by albumin. However when oligomycin and rotenone were added after the 3-min preincubation with dicoumarol, they did not abolish the inhibition of succinate oxidation by dicoumarol, but completely prevented the restoration of O_2 uptake by albumin.

Relationship between the rate of respiration and the level of oxaloacetate during succinate oxidation, and the effect of ATP and palmitoylcarnitine

In the experiment of Fig. 1 mitochondria were preincubated with dinitrophenol *plus* arsenite. Succinate or succinate *plus* ATP was then added and the time

TABLE I

EFFECT OF UNCOUPLERS ON SUCCINATE OXIDATION BY RABBIT-KIDNEY MITOCHONDRIA IN THE ABSENCE AND PRESENCE OF ROTENONE

Mitochondria were preincubated 3 min with: 130 mM KCl, 20 mM Tris–HCl, 10 mM potassium phosphate, 1 mM arsenite (Expts. 1 and 4); 130 mM KCl, 20 mM Tris–HCl, 3 mM arsenate (Expt. 2); 250 mM sucrose, 20 mM Tris–HCl, 5 mM KCl, 10 mM potassium phosphate, 2 mM ADP (Expt. 3). Where indicated the following additional components were present during the preincubation, 20 μM dicoumarol, 1 μg rotenone, 15 μg oligomycin, 0.1 mM 2,4-dinitrophenol. Additions: 10 mM glutamate, 2 mM ADP, 2 mM ATP, 15 μg oligomycin, 0.3 mM atractyloside, 1 μg rotenone, 8 mg bovine serum albumin. Mitochondrial protein: 3.9 mg (Expt. 1); 4.0 mg (Expt. 2); 2.0 mg (Expt. 3); 2.4 mg (Expt. 4). Final vol., 1.5 ml; final pH, 7.4. Temp., 25° (Expts. 1, 3, 4) and 30° (Expt. 2). O_2 uptake was measured polarographically. The respiratory rate is given as natoms oxygen/min. When the rate of respiration declined with time both the initial and the final rate (between brackets) are given.

Expt. No.	*Preincubation with*	*I Addition*	*Respiratory rate*	*II Addition*	*Respiratory rate*
1	—	Succinate 10 mM	330	ADP	470
	Dicoumarol	Succinate 10 mM	180	—	
	Dicoumarol, rotenone	Succinate 10 mM	440	—	
	Dicoumarol	Glutamate	80	Succinate 10 mM	580
	Dicoumarol, oligomycin	Succinate 10 mM	360	—	
	Dicoumarol	Succinate 10 mM + ATP	460	—	
2	2,4-Dinitrophenol	Succinate 4 mM	90 (40)	ATP	275
	2,4-Dinitrophenol	ATP + oligomycin	0	Succinate 4 mM	350
	2,4-Dinitrophenol	ATP + oligomycin + atractyloside	0	Succinate 4 mM	115 (35)
3	Rotenone	Succinate 10 mM	210	—	
	Rotenone, dicoumarol	Succinate 10 mM	250	—	
	Rotenone	Succinate 1 mM	120	—	
	Rotenone, dicoumarol	Succinate 1 mM	70 (30)	—	
	Rotenone, dicoumarol	Succinate 1 mM + bovine serum albumin	150	—	
4	—	Succinate 10 mM	200	—	
	Dicoumarol	Succinate 10 mM	100	—	
	Dicoumarol	Succinate 10 mM + bovine serum albumin	180	—	
	—	Rotenone + oligomycin	0	Succinate 10 mM	220
	Dicoumarol	Rotenone + oligomycin	0	Succinate 10 mM	120
	Dicoumarol	Rotenone + oligomycin	0	Succinate 10 mM + bovine serum albumin	110

course of respiration and of the level of oxaloacetate was followed. In the presence of succinate alone, there occurred a progressive accumulation of oxaloacetate and decline in the rate of respiration. ATP stimulated the respiration and prevented to a large extent the accumulation of oxaloacetate. When the rate of respiration was plotted against the concentration of oxaloacetate (Fig. 1), an inverse relationship was found between the amount of oxaloacetate that accumulated and the rate of respiration. Furthermore, the rate of respiration was higher in the presence of ATP only when the concentration of oxaloacetate was lower than that found in its absence.

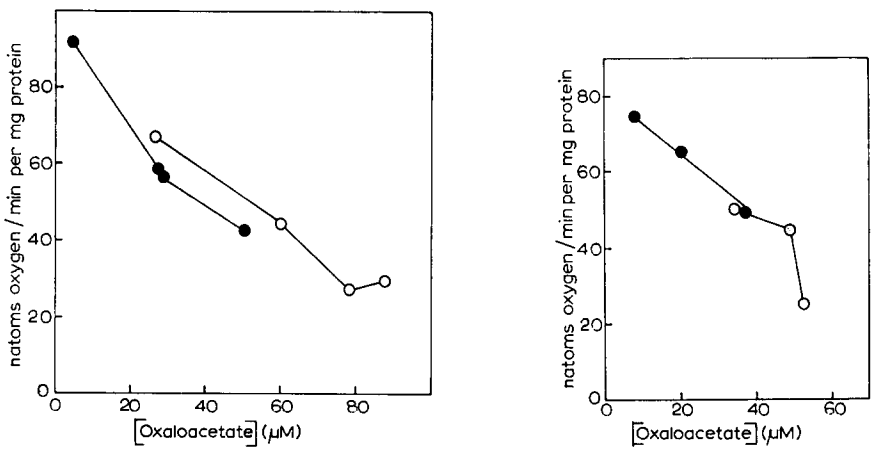


Fig. 1. Rate of succinate-supported respiration in rabbit-kidney mitochondria as a function of the concentration of oxaloacetate that accumulated during the incubation: effect of ATP. Mitochondria were preincubated in Warburg vessels for 6 min with 130 mM KCl, 20 mM Tris, 1 mM arsenite and 0.1 mM dinitrophenol before adding 10 mM succinate (○—○) or succinate *plus* 2 mM ATP *plus* 15 μg oligomycin (●—●). Final pH 7.4. Temp. 30°. The reaction was stopped in separate flasks after 2, 10, 14, 18 min by adding $HClO_4$. Oxaloacetate was determined on the neutralized extracts. The respiratory rate was measured at the same time at which parallel samples were stopped for analysis of oxaloacetate.

Fig. 2. Rate of succinate-supported respiration in rabbit-kidney mitochondria as a function of the concentration of oxaloacetate that accumulated during the incubation: effect of palmitoylcarnitine. Mitochondria were preincubated 6 min in the medium of Fig. 1, adding 10 mM succinate (○—○), or succinate *plus* 0.15 mM palmitoylcarnitine (●—●). Assays of oxaloacetate were carried out after 10, 20 and 26 min. 150 μM palmitoylcarnitine *plus* 5 mM malate gave a very small rate of O_2 uptake which was subtracted from that observed in the presence of 10 mM succinate *plus* 150 μM palmitoylcarnitine. The corrected values were used for the plot.

An analogous experiment was carried out with palmitoylcarnitine instead of ATP. Palmitoylcarnitine can be oxidized after conversion by palmitoyl-CoA: carnitine acyltransferase[31] into palmitoyl-CoA. This gives rise to acetyl-CoA, which can remove oxaloacetate by the citrate synthase (EC 4.1.3.7) reaction. Palmitoylcarnitine gave a marked stimulation of the succinate-supported respiration and a considerable decrease in the steady-state concentration of oxaloacetate. Fig. 2 shows that the activation of succinate oxidation brought about by palmitoylcarnitine is correlated with its ability to lower the level of oxaloacetate (*cf.* Fig. 1). Similar results were obtained with caprinoylcarnitine.

Role of phosphopyruvate carboxylase in the control of succinate oxidation in rabbit-kidney mitochondria

Rabbit-kidney mitochondria have a high phosphopyruvate carboxylase (GTP:oxaloacetate carboxy-lyase (transphosphorylating), EC 4.1.1.32) activity[32]. The possibility has therefore been examined that added ATP, after transphosphorylation with endogenous GDP (Reaction 1), removed oxaloacetate by Reaction 2.

$$ATP + GDP \rightleftharpoons ADP + GTP \quad (1)$$

$$GTP + \text{oxaloacetate} \rightleftharpoons GDP + \text{phosphoenolpyruvate} + CO_2 \quad (2)$$

Table II shows the effect of ATP on O_2 uptake, and the formation of malate, oxaloacetate, phosphoenolpyruvate and pyruvate during succinate oxidation by rabbit-kidney mitochondria incubated with dinitrophenol *plus* arsenite. In the absence of ATP respiration proceeded at a low rate, and a significant accumulation of oxaloacetate occurred. Some pyruvate also accumulated, but very little phosphoenolpyruvate. ATP markedly activated O_2 uptake and malate formation, but the extent of activation decreased with time. The activation of respiration by ATP was accompanied by a decrease in the level of oxaloacetate and a considerable formation of phosphoenolpyruvate.

TABLE II

EFFECT OF ATP ON OXYGEN UPTAKE AND THE FORMATION OF MALATE, OXALOACETATE, PHOSPHOENOLPYRUVATE AND PYRUVATE DURING SUCCINATE OXIDATION BY UNCOUPLED RABBIT-KIDNEY MITOCHONDRIA

Mitochondria (5 mg protein) were preincubated for 6 min with 130 mM KCl, 25 mM Tris, 1 mM arsenite and 0.1 mM dinitrophenol before adding 10 mM succinate, or succinate *plus* 2 mM ATP *plus* 15 μg oligomycin. Final pH, 7.4. Final vol., 1 ml. Temp., 30°.

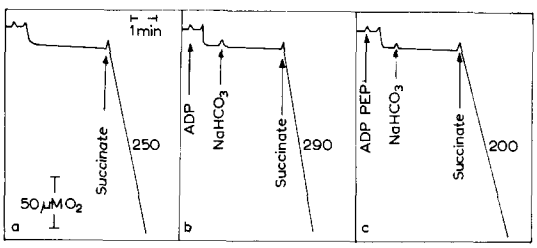
Additions	*Time (min)*	*ΔO (μatoms)*	*ΔMalate (μmoles)*	*ΔOxaloacetate (nmoles)*	*ΔPhosphoenolpyruvate (nmoles)*	*ΔPyruvate (nmoles)*
Succinate	2	0.45	0.167	9.7	14	36
	22	4.30	1.845	31.9	0	90
Succinate + ATP + oligomycin	2	0.90	0.399	1.8	68	45
	22	6.45	2.100	10.5	661	208

In another experiment it was found that when mitochondria were incubated for 4 min with succinate in the presence of dicoumarol and arsenite, 5–10 nmoles oxaloacetate had accumulated. On the addition of ATP *plus* rotenone, respiration was activated and 1 min later, the oxaloacetate level was zero, and a corresponding amount of phosphoenolpyruvate was found. Rotenone on its own had no effect, either on respiration or on the oxaloacetate level.

The experiments described show that ATP activates succinate oxidation by converting oxaloacetate into phosphoenolpyruvate. The system can also work in the reverse direction. The polarographic recordings of Fig. 3 show that the addition of phosphoenolpyruvate in the presence of $NaHCO_3$ and ADP brought about an inhibition of succinate oxidation, which in various experiments, however, did not exceed

30%. It is possible that mitochondria are not freely permeable to phosphoenolpyruvate.

That added phosphoenolpyruvate generates oxaloacetate is shown in Fig. 4. In this experiment, nicotinamide nucleotides were allowed to become maximally reduced by the addition of succinate and oligomycin. Rotenone was then added, followed by ADP and $NaHCO_3$. On addition of phosphoenolpyruvate oxidation of nicotinamide nucleotides occurred.



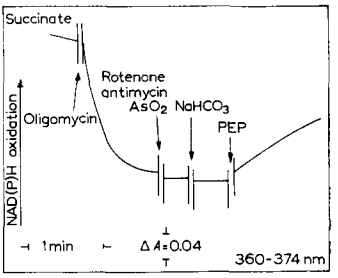


Fig. 3. Effect of oxaloacetate generated from phosphoenolpyruvate on succinate oxidation in rabbit-kidney mitochondria in the presence of rotenone. Mitochondria (4.5 mg protein) were incubated with 130 mM KCl, 20 mM Tris, 0.1 mM dinitrophenol and 2 μg rotenone. Final concentration of additions, 10 mM succinate, 5 mM ADP, 10 mM $NaHCO_3$ and 5 mM phosphoenolpyruvate (PEP). Temp., 30°. Final vol., 1.5 ml. Final pH, 7.4.

Fig. 4. Spectrophotometric recording of oxidation of intramitochondrial nicotinamide nucleotides on the addition of phosphoenolpyruvate and $NaHCO_3$ to rabbit-kidney mitochondria. Mitochondria (5.7 mg protein) were incubated with 130 mM KCl, 20 mM Tris, 5 mM P_i, 5 mM $MgCl_2$, 2 mM ADP and 5 mM succinate. Other additions, 15 μg oligomycin, 2 μg rotenone, 0.5 μg antimycin, 1 mM arsenite, 10 mM $NaHCO_3$ and 5 mM phosphoenolpyruvate (PEP). Final pH, 7.4. Final vol., 3 ml.

Relationship between substrate-level phosphorylation and succinate oxidation

It has been observed that in order to obtain maximal inhibition of succinate oxidation not only the respiratory-chain phosphorylation, but also the substrate-level

TABLE III

EFFECT OF SUBSTRATE-LEVEL PHOSPHORYLATION ON SUCCINATE OXIDATION BY RABBIT-KIDNEY MITOCHONDRIA

Mitochondria (4 mg protein) were preincubated for 4 min with 130 mM KCl, 20 mM Tris and 0.1 mM dinitrophenol. Then 1 mM arsenite was added, followed 1 min later by 10 mM succinate. Where indicated the following additional components were present during the preincubation: 5 mM P_i, 15 μg oligomycin; 5 mM α-oxoglutarate, 5 mM ADP, 30 mM glucose and 28 I.U. yeast hexokinase. Temp., 30°. Final vol., 1.5 ml. O_2 uptake was measured polarographically. Since the rate of respiration declined with time, both the initial and the final rate (between brackets) are given. The respiratory rate is given as natoms oxygen/min.

Preincubation with	*I Addition*	*Respiratory rate*	*II Addition*	*Respiratory rate*
—	AsO_2	0	Succinate	70 (35)
P_i, oligomycin	AsO_2	0	Succinate	100 (60)
P_i, oligomycin, α-oxoglutarate	AsO_2	0	Succinate	130 (100)
P_i, oligomycin, α-oxoglutarate, ADP, glucose, hexokinase	AsO_2	0	Succinate	80 (35)

phosphorylation must be uncoupled[1,20]. The data of Table III show that when substrate-linked phosphorylation of endogenous nucleotide diphosphates was favoured by adding inorganic phosphate, α-oxoglutarate and oligomycin to the medium during the preincubation of mitochondria with dinitrophenol, the inhibition of succinate oxidation was largely prevented. However, when ADP, hexokinase and glucose were also added to the system to trap the energy-rich phosphate bond, this activating effect was suppressed.

DISCUSSION

The results presented allow one to distinguish two types of inhibition by uncouplers of succinate oxidation in isolated mitochondria. The inhibition observed in the presence of rotenone can be due to inhibition of the entry of succinate into the mitochondria[12,15–17,33]. It was, indeed, abolished by increasing the concentration of added succinate, or by removing the uncoupler with albumin. However, the possibility that dicoumarol also competes with succinate for its binding site on succinate dehydrogenase[14] can not be dismissed on the basis of the data available. The inhibition of succinate oxidation, caused by uncouplers in the absence of inhibitors of NADH oxidation *via* the respiratory chain, has the following features that distinguish it from the former type: (i) at 10 mM succinate the respiration was still inhibited by the uncouplers. In contrast, in the presence of rotenone there was no inhibition by uncouplers at this succinate concentration. (ii) ATP abolished the inhibition of respiration. In contrast, it had no effect on the inhibition the uncouplers caused in the presence of rotenone. (iii) The beneficial effect on respiration of removal of the uncouplers with albumin was prevented by adding, after the preincubation with the uncoupler, rotenone *plus* oligomycin. Thus, in this case the effect of albumin is not due to the removal of the uncoupler *per se*, but probably depends upon the resynthesis of endogenous ATP and/or restoration of the succinate-driven reduction of NAD^+ (ref. 34), reactions that remove oxaloacetate.

The last finding as well as the fact that the inhibition by uncouplers of the oxidation of 10 mM succinate (or higher concentrations) can be prevented by rotenone or Amytal and reversed by glutamate suggest that the inhibition is due, in this case, to accumulation of oxaloacetate. Direct evidence for this is the demonstration that the level of oxaloacetate during the oxidation of succinate in uncoupled mitochondria (in the absence of rotenone) is high enough to account for inhibition of succinate dehydrogenase. Furthermore, an inverse relationship exists between the rate of respiration and the concentration of oxaloacetate that accumulated. The addition of ATP or palmitoylcarnitine to uncoupled mitochondria oxidizing succinate prevented to a large extent the accumulation of oxaloacetate and this was accompanied by a marked stimulation of respiration. However, for equal concentrations of oxaloacetate the rate of respiration in the presence of ATP or of palmitoylcarnitine was practically equal to that in their absence. Thus the reactivation of succinate oxidation by palmitoylcarnitine or ATP is the result of their effect on the concentration of oxaloacetate (contrast refs. 10, 11).

ATP promoted the removal of oxaloacetate by converting it into phosphoenolpyruvate; this was accompanied by activation of succinate oxidation. Further support for a functional relationship between phosphopyruvate carboxylase and succin-

ate dehydrogenase is given by the finding that oxaloacetate generated by added phosphoenolpyruvate caused inhibition of succinate oxidation.

There is a third enzymatic system whose activity is associated with phosphoenolpyruvate carboxylase and succinate dehydrogenase. This is the α-oxoglutarate dehydrogenase complex. GTP generated by substrate-level phosphorylation promotes phosphoenolpyruvate formation, thus preventing oxaloacetate inhibition of succinate dehydrogenase. In fact to get maximal inhibition of succinate oxidation, arsenate or arsenite must be added together with dinitrophenol or dicoumarol to abolish substrate-level phosphorylation. On the other hand, the inhibition of succinate oxidation by dinitrophenol is largely prevented when substrate-level phosphorylation of endogenous nucleoside diphosphates is favoured.

The results presented show that in uncoupled rabbit-kidney mitochondria the phosphopyruvate carboxylase reaction is a very active reaction by which ATP removes oxaloacetate. It should, however, be stressed that in mitochondria with low phosphopyruvate carboxylase activity, like those from rat liver or rat heart, and possibly even in rabbit-kidney mitochondria under certain conditions, ATP could also promote removal of oxaloacetate by activating the oxidation of endogenous fatty acids[35], or by supporting NAD^+ reduction by succinate.

Recent studies[36–38] have shown that succinate dehydrogenase is a site of metabolic control in the tricarboxylic acid cycle. Our data indicate that the activity of the cycle may be controlled at this site by the ratio [succinate]:[oxaloacetate], which in turn is governed by the energy state of mitochondria and the extent of phosphorylation of ADP and GDP.

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REFERENCES

1 G. F. AZZONE AND L. ERNSTER, *J. Biol. Chem.*, 236 (1961) 1518.
2 P. BORST AND E. C. SLATER, *Biochim. Biophys. Acta*, 8 (1961) 362.
3 J. B. CHAPPELL, in T. W. GOODWIN AND O. LINDBERG, *Biological Structure and Function*, Vol. 2, Academic Press, New York, 1961, p. 71.
4 E. A. JONES AND H. GUTFREUND, *Biochem. J.*, 87 (1963) 639.
5 E. C. SLATER AND W. C. HULSMANN, *Proc. Natl. Acad. Sci. U.S.*, 47 (1961) 1109.
6 A. B. PARDEE AND V. R. POTTER, *J. Biol. Chem.*, 176 (1948) 1085.
7 N. B. DAS, *Biochem. J.*, 31 (1937) 1124.
8 G. F. AZZONE, L. ERNSTER AND M. KLINGENBERG, *Nature*, 188 (1960) 552.
9 P. SCHOLLMEYER AND M. KLINGENBERG, *Biochem. Biophys. Res. Commun.*, 4 (1961) 43.
10 C. GREGOLIN AND P. SCALELLA, *Biochim. Biophys. Acta*, 99 (1965) 185.
11 A. AKERBLOM, L. ERNSTER, O. LINDBERG AND E. C. WEINBACH, *Proc. 6th Intern. Congr. Biochem., New York, 1964*, Vol. 10, Academic Press, New York, 1964, p. 773.
12 E. J. HARRIS, M. P. HOFER AND B. C. PRESSMAN, *Biochemistry*, 6 (1967) 1348.
13 C. E. WENNER, *Federation Proc.*, 24 (1965) 544.
14 D. F. WILSON AND R. D. MERZ, *Arch. Biochem. Biophys.*, 119 (1967) 470.
15 K. VAN DAM, *Biochim. Biophys. Acta*, 131 (1967) 407.
16 E. J. HARRIS, K. VAN DAM AND B. C. PRESSMAN, *Nature*, 213 (1967) 1126.
17 E. QUAGLIARIELLO AND F. PALMIERI, *European J. Biochem.*, 4 (1968) 20.
18 S. PAPA, N. E. LOFRUMENTO AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 110 (1965) 442.

19 S. PAPA, N. E. LOFRUMENTO, G. PARADIES AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 153 (1968) 306.
20 S. PAPA, N. E. LOFRUMENTO, A. G. SECCHI AND E. QUAGLIARIELLO, *Biochim. Biophys. Acta*, 131 (1967) 288.
21 E. QUAGLIARIELLO, S. PAPA, N. E. LOFRUMENTO AND G. PARADIES, *Abstr. 7th. Intern. Congr. Biochem., Tokyo, 1967*, p. 869.
22 S. PAPA, N. E. LOFRUMENTO, G. DE GREGORIO, G. PARADIES AND E. QUAGLIARIELLO, *Boll. Soc. Ital. Biol. Sper.*, 43 (1967) 1233.
23 A. B. OESTREICHER, *Abstr. 5th Meeting Federation European Biochem. Soc., Prague 1968*, p. 50.
24 K. W. CLELAND AND E. C. SLATER, *Biochem. J.*, 53 (1953) 547.
25 H. J. HOHORST, in H. U. BERGMEYER, *Methods in Enzymatic Analysis*, Academic Press, New York, 1963, p. 328.
26 TH. BUCHER, R. CZOK, W. LAMPRECHT AND E. LATZKO, in H. U. BERGMEYER, *Methods in Enzymatic Analysis*, Academic Press, New York, 1963, p. 253.
27 R. CZOK AND L. ECKERT, in H. U. BERGMEYER, *Methods in Enzymatic Analysis*, Academic Press, New York, 1963, p. 224.
28 G. PFLEIDERER, in H. U. BERGMEYER, *Methods in Enzymatic Analysis*, Academic Press, New York, 1963, p. 381.
29 J. B. CHAPPELL AND A. R. CROFTS, *Biochem. J.*, 95 (1965) 707.
30 E. C. WEINBACH AND J. GARBUS, *J. Biol. Chem.*, 241 (1966) 3708.
31 J. BREMER, *J. Biol. Chem.*, 238 (1963) 2774.
32 R. BANDURSKI AND F. LIPMANN, *J. Biol. Chem.*, 219 (1956) 741.
33 K. VAN DAM AND E. C. SLATER, *Proc. Natl. Acad. Sci. U.S.*, 58 (1967) 2015.
34 B. CHANCE AND B. HAGIHARA, *J. Biol. Chem.*, 237 (1962) 3540.
35 A. B. WOJTCZAK AND J. B. CHAPPELL, *Abstr. 3rd Meeting Federation European Biochem. Socs.*, Warsaw, 1966, Academic Press and Polish Scientific Publishers, London and Warsaw, 1966, p. 141.
36 G. SCHAFER, P. BALDE AND M. LAMPRECHT, *Nature*, 214 (1967) 20.
37 R. W. VON KORFF, *Nature*, 214 (1967) 23.
38 G. R. WILLIAMS, *Can. J. Biochem.*, 43 (1965) 603.