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INHIBITORY ACTION OF OXALOACETATE ON SUCCINATE OXIDATION IN RAT-LIVER MITOCHONDRIA AND THE MECHANISM OF ITS REVERSAL

ANNA B. WOJTCZAK

Department of Biochemistry, Nencki Institute of Experimental Biology, Warsaw 22 (Poland)

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SUMMARY

1. Oxidation of succinate in rat-liver mitochondria was studied in the presence of added oxaloacetate, an uncoupler of oxidative phosphorylation and rotenone.

2. At certain concentrations of oxaloacetate, succinate and mitochondria a spontaneous reactivation of succinate oxidation, previously inhibited by oxaloacetate, can be observed. This reactivation is completely abolished by either arsenate, arsenite or lewisite or by the absence of phosphate, and is potentiated by oligomycin. The spontaneous reactivation is accompanied by an increase in the intramitochondrial content of ATP generated by substrate-level phosphorylation.

3. ATP added externally reactivates the oxidation of succinate substantially only in the presence of either carnitine or, to a smaller degree, arsenate.

4. The reactivation produced by ATP *plus* carnitine is potentiated by the addition of small amounts of palmitate. The addition of pyruvate has a small effect and the addition of pyruvate *plus* arsenate almost none.

5. Freshly isolated rat-liver mitochondria contain 15–30 mμmoles free fatty acids per mg protein. This amount is increased during incubation in the presence of KCN, but during aerobic incubation with succinate fatty acids are oxidized, even if 2,4-dinitrophenol is present.

6. Upon addition of oxaloacetate the oxidation of intramitochondrial nicotinamide nucleotides is observed. The steady-state redox level depends on the concentration of added oxaloacetate and on the presence of oxidizable NAD-linked substrates. In the presence of oxaloacetate, intramitochondrial $NAD(P)^+$ is most effectively reduced by isocitrate; ATP *plus* carnitine having a small effect only.

7. The best protection of succinate oxidation against the inhibition by oxaloacetate is provided by ATP *plus* carnitine, by ATP *plus* carnitine *plus* palmitate, or by palmitoyl-carnitine.

8. It is concluded that the oxidation of fatty acids is the most effective factor removing oxaloacetate from the site of succinate dehydrogenase in liver mitochondria. Its effect is due to (i) generation of NADH which can reduce oxaloacetate to malate,

Abbreviations: CCCP, carbonyl-cyanide *m*-chlorophenylhydrazone; FCCP, carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone; EGTA, ethyleneglycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetate.

and (ii) provision of acetyl-CoA which can condense with oxaloacetate to form citrate. A smaller effectiveness of other reactions removing oxaloacetate from mitochondria is discussed.

INTRODUCTION

Oxaloacetate is a potent inhibitor of succinate dehydrogenase (EC 1.3.99.1)[1,2]. However, many studies on the effect of oxaloacetate on succinate oxidation in intact mitochondria revealed the complexity of this inhibition. SCHOLLMEYER AND KLINGENBERG[3] and KUNZ[4] have shown that succinate may be oxidized by intact mitochondria under specific conditions even in the presence of relatively high concentrations of oxaloacetate in the medium. On the other hand, ÅKERBLOM *et al.*[5] have observed a strong inhibition of succinate oxidation even in cases when the concentration of oxaloacetate was extremely low. These differences may be attributed on the one side to different permeability of mitochondria towards oxaloacetate, and on the other side to the removal of oxaloacetate by various metabolic processes occurring in mitochondria. They are: (1) transamination with glutamate; (2) reduction to malate; (3) decarboxylation to pyruvate; (4) decarboxylation to phosphoenolpyruvate; (5) condensation with acetyl-CoA to citrate or to malonyl-CoA and pyruvate. However, under specific conditions only some of these mechanisms can be effective. It is known that succinate oxidation in phosphorylating mitochondria is less sensitive to oxaloacetate than under uncoupled conditions[6,7]. This is an indication that some of the processes removing oxaloacetate are energy-dependent.

The present paper describes the effect of added oxaloacetate on the oxidation of succinate in rat-liver mitochondria. The endogenous formation of oxaloacetate is prevented by rotenone, and the main source of energy, *i.e.* respiratory chain-linked oxidative phosphorylation, is blocked by an uncoupler. It is shown that under these conditions succinate oxidation can be protected against oxaloacetate inhibition by ATP formed in substrate-level phosphorylation or externally added. This ATP (or GTP) is needed for the activation of endogenous fatty acids whose oxidation provides reducing equivalents and acetyl-CoA, both effective in removing oxaloacetate from mitochondria. It is thus suggested that in rat-liver mitochondria the main factors controlling the level of oxaloacetate are the reduction to malate and the condensation to citrate.

METHODS

Rat-liver mitochondria were isolated according to HOGEBOOM[8] in 0.25 M sucrose + 1 mM Tris–HCl (pH 7.4). The isolation medium for mitochondria used in swelling–contraction experiments also contained 0.1 mM EDTA.

O_2 uptake was measured with Clark type oxygen electrode. The simultaneous recording of light scattering and O_2 uptake was accomplished by a device described by CHAPPELL AND CROFTS[9].

ATP was determined enzymatically with hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49)[10]. Fluorescence was measured in Eppendorf fluorimeter.

Free fatty acids were determined by microtitration according to DOLE AND MEINERTZ[11]. The extraction and separation of free fatty acids were as follows. Incubation samples were inactivated by immersing for 2–3 min in a water bath at 100° and were centrifuged. The precipitated protein was extracted several times with a mixture of ethyl ether and methanol (2:1, v/v) at 30°. Combined extracts were evaporated under N_2 and the residue was dissolved in petroleum ether. The ether solution was then shaken with equal volume of cold alkaline ethanol (0.06 M NaOH in 50% ethanol); the ether layer was separated and extracted twice with new portions of alkaline ethanol, and finally discarded (it contained esterified fatty acids, mainly phospholipids). All extractions with alkaline ethanol were carried out at 0° to prevent the hydrolysis of phospholipids. Combined ethanol layers were acidified with H_2SO_4 to pH 3–4 and extracted several times with petroleum ether. Combined petroleum ether extracts were washed with water and used for the titrimetric estimation of fatty acids.

Protein was determined by the biuret method[12] after solubilizing mitochondria with deoxycholate.

Oxaloacetic acid and ATP were obtained from both Sigma (St. Louis, Mo., U.S.A.) and Boehringer (Mannheim, Germany) and DL-carnitine–HCl from Light (Colnbrook, Great Britain), hexokinase and glucose-6-phosphate dehydrogenase were from Boehringer. Sodium succinate and other substrates were of analytical grade. DL-Palmitoyl-carnitine was kindly offered by Dr. P. B. GARLAND and carbonyl-cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) and carbonyl-cynanide *m*-chlorophenylhydrazone (CCCP) by Dr. P. G. HEYTLER of E.I. Du Pont de Nemours and Co. Inc. (Wilmington, Del., U.S.A.).

RESULTS

Permeability of mitochondria to oxaloacetate

CHAPPELL AND HAARHOFF[13] have shown that various anions differ in their ability to penetrate the mitochondrial membranes. In this respect the anions can be divided into four groups: (1) freely penetrating; (2) penetrating in the presence of P_i; (3) penetrating in the presence of P_i *plus* L-malate; and (4) non-penetrants. The question thus arose as to the penetrating ability of oxaloacetate.

An experiment carried out in a system described by CHAPPELL AND HAARHOFF[13] demonstrated that mitochondria suspended in 0.1 M ammonium oxaloacetate did not swell until 2 mM ammonium phosphate was added (Fig. 1). This indicates that oxaloacetate belongs to the group of anions, such as succinate, D- and L-malate and malonate, whose penetration through mitochondrial membranes is phosphate-dependent.

Effect of oxaloacetate at various energetic states of mitochondria

As shown in Fig. 2, the effect of oxaloacetate on succinate oxidation strongly depends on the energetic state of mitochondria. With 3 mM oxaloacetate no inhibition occurs in States 4 and 3, but the inhibition sets in very quickly when a low energy state is induced by either 2,4-dinitrophenol or gramicidin *plus* K^+. In Trace A the addition of KCl in the presence of gramicidin induces both an increase of O_2 uptake and the swelling of mitochondria. The later effect is a manifestation of an increased transport of ions, *i.e.* of K^+ and, most likely, of both succinate and oxaloacetate. The inhibition

of respiration which appears subsequently might be a result of either an increased penetration of oxaloacetate, or the lowering of the energy state of mitochondria. Traces C and D point to the later possibility.

The time course of succinate oxidation in the presence of oxaloacetate: inhibition and reactivation

When various amounts of oxaloacetate were added to the system containing mitochondria (6–9 mg protein), succinate (5 mM), 2,4-dinitrophenol and rotenone (in the final volume of 3.0 ml), it could be observed that 1–2 mM oxaloacetate had little or no effect on O_2 uptake, while 3–4 mM oxaloacetate produced a substantial but transient inhibition followed by a spontaneous reactivation up to 50% of the initial activity. When the amount of oxaloacetate was further increased the extent of this reactivation gradually decreased and finally disappeared. A typical picture of

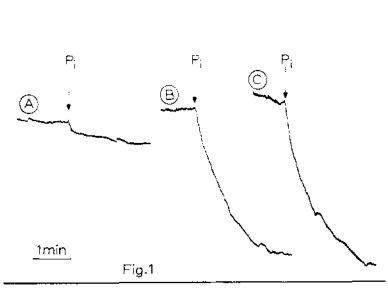

Fig. 1

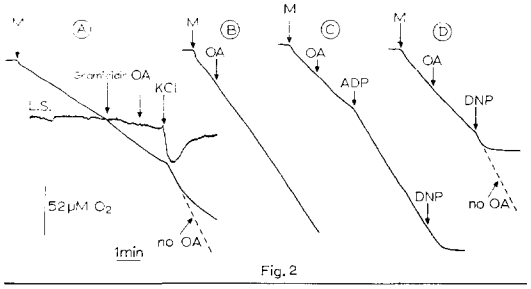

Fig. 2

Fig. 1. Swelling of mitochondria in solutions of NH_4Cl, ammonium oxaloacetate and ammonium succinate. Incubation media: 100 mM solutions of (A) NH_4Cl, (B) ammonium oxaloacetate, and (C) ammonium succinate. In each case 5 mM Tris–HCl (pH 7.4), 0.1 mM ethyleneglycol bis-(β-aminoethyl ether)*N,N'*-tetraacetate (EGTA) and 1 μM rotenone were present. 2 mM ammonium phosphate (P_i) was added as indicated. Mitochondria, 2.5 mg protein/ml. Temp. 30°. Downward deflection of light scattering indicates swelling.

Fig. 2. Effect of oxaloacetate on succinate oxidation at various energetic states of mitochondria. Incubation medium: 200 mM sucrose, 10 mM Tris–HCl (pH 7.4), 2 mM Tris phosphate, 5 mM Tris succinate and 1 μM rotenone; mitochondrial protein, 1.3 mg/ml (in Trace A) and 2.0 mg/ml (in Traces B, C and D). Temp. 26°. Additions: mitochondria (M), 0.2 μg/ml gramicidin, 0.5 mM (Trace A) and 3.3 mM (Traces B, C and D) oxaloacetate (OA), 9 mM KCl, 1.7 mM ADP, 0.2 mM 2,4-dinitrophenol (DNP) (final concentrations). Downward deflection of light scattering (L.S., Trace A) indicates mitochondrial swelling.

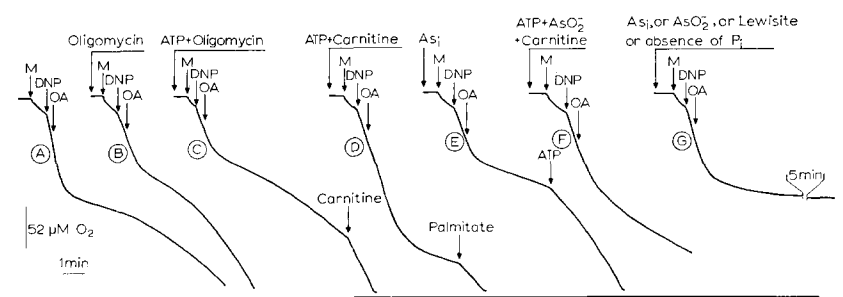

Fig. 3. Succinate oxidation in the presence of oxaloacetate under various conditions. Incubation medium: 125 mM KCl, 10 mM Tris–HCl, 2 mM P_i, 5 mM sodium succinate, 1 μM rotenone and 2.0 mg mitochondrial protein/ml, pH 7.4; temp. 26°. Additions: mitochondria (M), 0.1 mM 2,4-dinitrophenol (DNP), 3.3 mM oxaloacetate (OA), 2 μg/ml oligomycin, 2 mM ATP, 1 mM DL-carnitine, 30 μM palmitate, 2 mM arsenate (As_i), 2 mM sodium arsenite (AsO_2^-), 2 μg/ml lewisite.

the inhibition produced by 3 mM oxaloacetate is shown in Fig. 3, Curve A. The inhibition appears after a short lag period of about 30–60 sec and is usually followed by a spontaneous slow reactivation. This reactivation occurs when appropriate proportions between succinate, oxaloacetate and mitochondria are kept. The spontaneous reactivation is greatly enhanced by oligomycin (Fig. 3, Curve B) and is completely abolished by the addition of either 2 mM arsenite, 2 mM arsenate, or 2 mg/ml lewisite (β-chlorovinylarsenious oxide) (Fig. 3, Curve G). The reactivation is also inhibited when P_i is omitted from the medium. The protective effect of P_i can also be visualized when endogenous P_i is bound as strontium salt within the mitochondria. In this case a complete inhibition of succinate oxidation is obtained with even lower concentrations of oxaloacetate. In all subsequent experiments 2 mM P_i was included into the incubation medium. This concentration was found to be optimal, while higher concentrations of P_i, above 10 mM, potentiated the inhibitory effect of oxaloacetate.

TABLE I

CHANGES OF ATP CONTENT IN RAT-LIVER MITOCHONDRIA DURING SUCCINATE OXIDATION

Incubation medium as in Fig. 3; 3 mM oxaloacetate was added after 1 min of incubation; other additions as indicated; temp. 37°.

Additions	*ATP (mμmoles/mg protein) after*		
	0 min	*1 min*	*5 min*
FCCP (0.5 μM)	4.9	1.28	(+ oxaloacetate) 1.04
FCCP (0.5 μM) + oligomycin (2 μg/ml)			(+ oxaloacetate) 1.51
FCCP (0.5 μM) + arsenite (2 mM)		1.40	(+ oxaloacetate) 0.00
FCCP (0.5 μM)	6.9	1.74	(+ oxaloacetate) 1.62
FCCP (0.5 μM) + oligomycin (2 μg/ml)		2.40	(+ oxaloacetate) 3.20
FCCP (0.5 μM) + oligomycin (2 μg/ml)		2.43	2.68

On the basis of these observations it may be supposed that ATP (or GTP) generated by the substrate-level phosphorylation plays a role in the reactivation of succinate oxidation inhibited by oxaloacetate. In order to check this point, the changes of intramitochondrial ATP were followed during the oxidation of succinate in the presence of an uncoupler (FCCP) and oxaloacetate. It appeared (Table I) that the content of ATP rapidly decreases during the first minute of incubation and decreased only slowly after the addition of oxaloacetate (Expts. 1 and 2, 1st row). In the presence of oligomycin the content of ATP also decreased during the first minute, but was increased when oxaloacetate was added (Expt. 2, 2nd row, compare with 3rd row). In the presence of arsenite all mitochondrial ATP disappeared during a few minutes. This synthesis of ATP was due to substrate-level phosphorylation as indicated by its insensitivity to FCCP and oligomycin and the inhibition by arsenite. These experiments indicate a parallelism between the rate of succinate oxidation in the presence of oxaloacetate and the content of intramitochondrial ATP.

The reactivation by ATP

The effect of added ATP on the inhibition by oxaloacetate of succinate oxidation has often been studied. In uncoupled kidney mitochondria ATP reversed the inhibi-

tion[6], whereas in uncoupled rat-liver mitochondria it had only a small effect[3,14,15]. However, AZZONE AND ERNSTER[7,16] observed that ATP reversed the inhibition in rat-liver mitochondria preincubated in the presence of 2,4-dinitrophenol and arsenate. It was therefore interesting to reinvestigate the effect of ATP in our system.

Added ATP had a small and variable effect on the inhibition of succinate oxidation by oxaloacetate. In some experiments it potentiated, in others it partly released, the inhibition. However, ATP *plus* carnitine always reversed the inhibition (Fig. 3, Curve C). Carnitine alone, in the absence of ATP, had no effect (not shown). The variable effect of ATP alone probably depended on the content of endogenous fatty acids in mitochondria, as the addition of a small amount of oleate or palmitate together with ATP always produced a substantial activation of the respiration. This activation was potentiated by carnitine to such an extent that no inhibition by oxaloacetate could be observed. This effect of carnitine strongly suggests the participation of fatty acids in the reactivating action of ATP. This is further illustrated by an experiment where ATP and carnitine were added to the incubation medium before oxaloacetate. In this case the inhibitory effect of oxaloacetate was largely delayed (Fig. 3, Curve D). If then a microquantity of palmitate was added, O_2 uptake was stimulated. This stimulation was partly due to the oxidation of palmitate (with oxaloacetate and oxygen as electron acceptors) but, as shown by control experiments without succinate, this amounted to less than 10 % of O_2 uptake observed in Trace D (Fig. 3) after the addition of palmitate. This indicates that a real reactivation of succinate oxidation occurred. The addition of a new portion of ATP was without effect (not shown). This experiment indicates that endogenous fatty acids are the limiting factor in the reactivating effect of ATP. When endogenous fatty acids are exhausted, added fatty acids are equally active.

Oxaloacetate used in these experiments contained 5–10 % pyruvate. It could be therefore supposed that pyruvate is responsible for the removal of oxaloacetate from mitochondria and, consequently, for the spontaneous reactivation of succinate oxidation. BREMER[17] has observed that the oxidation of pyruvate in phosphorylating rat-liver mitochondria is depressed by long-chain acylcarnitines which compete with pyruvate for intramitochondrial CoA. In our hands the utilization of 0.3 mM pyruvate in uncoupled liver mitochondria was depressed by 0.6–2 mM ATP by about 30 %. This observation might explain the inhibitory effect of ATP on the spontaneous reactivation.

The question thus arises whether the oxidation of pyruvate was exclusively responsible for the spontaneous reactivation. That it was not is shown by the effect of arsenate which completely prevented the spontaneous reactivation (Fig. 3, Curves E and G), although was without effect on the oxidation of pyruvate. (Arsenate uncouples substrate-level phosphorylation coupled to the oxidation of α-ketoglutarate[18].)

It can be thus concluded that pyruvate (added together with oxaloacetate) is oxidized *via* the tricarboxylic acid cycle with the generation of ATP due to substrate-level phosphorylation. This ATP (or GTP) then promotes the oxidation of endogenous fatty acids. This process is most probably responsible for the spontaneous reversal of the inhibition of succinate oxidation. The oxidation of pyruvate alone, if not accompanied by substrate-level phosphorylation, is not effective in this respect, as shown by the experiment with added arsenate. When the oxidation of pyruvate was inhibited by arsenite, ATP *plus* carnitine had still an activating, although smaller, effect (Fig. 3, Trace F).

The reactivation by added ATP *plus* arsenate was more efficient than by ATP alone (Fig. 3, compare Traces C and E). This can be explained by assuming that arsenate increases the breakdown of succinyl-CoA liberating CoA which can be utilized in fatty acid oxidation. This is also suggested by experiments in which the oxidation of ^{14}C-labelled oleate was measured in the presence of succinate, ATP, 2,4-dinitrophenol and oligomycin (A. B. WOJTCZAK, unpublished observations). Under these conditions arsenate increased the disappearance of oleate 2-fold while the increase produced by carnitine was 5-fold. The effect of arsenate is thus similar to that of carnitine, although the mechanisms of both effects are different.

Oxidation of endogenous fatty acids

The experiments described in the preceding section clearly show that the effect of ATP on the inhibition of succinate oxidation by oxaloacetate is in rat-liver mitochondria mainly due to the activation of endogenous fatty acids. To further test this point the level of fatty acids was determined in isolated mitochondria under various conditions of incubation. Freshly isolated mitochondria contain from 15 to 30 mμmoles non-esterified fatty acids per mg mitochondria protein. This amount increases by about 50% during the incubation at room temperature, especially if the respiratory chain is inhibited (Table II). In the presence of succinate and 2,4-dinitrophenol this increase is not observed, suggesting that an oxidation of fatty acids occurs. This is completely abolished by arsenate but not by arsenate *plus* ATP. The disappearance of fatty acids is most probably due mainly to their oxidation[19,20]. Under specific conditions, especially in the presence of α-glycerophosphate, fatty acids are also incorporated into mitochondrial phospholipids[21]. This process is, however, much slower than the oxidation of fatty acids and in the present experiments its participation in the removal of endogenous fatty acids can be neglected.

TABLE II

THE CONTENT OF FREE FATTY ACIDS IN RAT-LIVER MITOCHONDRIA UNDER VARIOUS CONDITIONS

Incubation medium as in Fig. 3; mitochondrial protein 30.6 mg; final vol. 2.0 ml; temp. 20°. Additions where indicated: 5 mM succinate, 0.1 mM 2,4-dinitrophenol, 2 mM KCN, 2 mM ATP, 2 mM arsenate. Incubation was carried out for 30 min under constant shaking. Fatty acids were extracted and determined as described under METHODS. Determinations of free fatty acids were done by Miss E. ŁAGWINSKA.

Additions	*Free fatty acids* (μ*moles*)
None, sample not incubated	0.92
None (incubated)	1.31
KCN	1.47
Succinate + 2,4-dinitrophenol	0.90
Succinate + 2,4-dinitrophenol + ATP	0.72
Succinate + 2,4-dinitrophenol + arsenate	1.82
Succinate + 2,4-dinitrophenol + ATP + arsenate	0.66

In the presence of rotenone fatty acids can be oxidized if oxaloacetate is also present. In this case oxaloacetate is the electron acceptor. This is illustrated by Table III which shows that in the presence of oxaloacetate the oxidation of oleate, as measured by the formation of acid-soluble oxidation products, is independent of the

presence of rotenone. However, the production of acetoacetate is much greater in the presence of rotenone than in its absence. This fact provides a further evidence that in the presence of rotenone oxaloacetate is an electron acceptor and is therefore less accessible for citrate synthesis (*cf.* ref. 23).

Thus, the oxidation of endogenous or added fatty acids provides both the reducing equivalents, which can reduce oxaloacetate to malate, and acetyl-CoA which can condense with oxaloacetate to form citrate. The problem thus arises which of these two processes plays an essential part in removing oxaloacetate from the site of succinate dehydrogenase in liver mitochondria. To elucidate this question the effect of oxaloacetate on the oxidoreduction state of intramitochondrial nicotinamide nucleotides was studied.

TABLE III

OXIDATION OF [1-^{14}C]OLEATE IN RAT-LIVER MITOCHONDRIA

Without rotenone: 125 mM KCl, 2 mM phosphate buffer and 10 mM Tris–HCl buffer (pH 7.4), 0.1 mM 2,4-dinitrophenol, 1.3 mM oxaloacetate, 10 μM [1-^{14}C]oleate (400 000 counts/min ^{14}C), 5.25 mg mitochondrial protein; final vol. 3.0 ml; temp. 26°; incubation time 5 min. *With rotenone*: The same conditions as in above except that 5 mM succinate and 1 μM rotenone were added and the concentration of oxaloacetate was increased to 3.3 mM. The samples were deproteinized with $HClO_4$ and centrifuged, then $KClO_4$ was precipitated and removed. A sample of the supernatant was treated with aniline citrate[22] and $^{14}CO_2$ evolved was absorbed in NaOH. Counts corresponding to acetoacetate are the counts of this CO_2 multiplied by the factor of 2.

Additions	*Oxidation products soluble in $HClO_4$ (counts/min)*			
	Without rotenone		*With rotenone*	
	Total	*Acetoacetate*	*Total*	*Acetoacetate*
None	43000	1900	33350	10800
ATP (2 mM)	41500	2300	47000	11200
ATP (2 mM) + carnitine (1 mM)	168300	30000	250000	72000

Redox changes in nicotinamide nucleotides

Fig. 4A shows that externally added oxaloacetate can oxidize intramitochondrial nicotinamide nucleotides and that the state of oxidation depends on the concentration of oxaloacetate. The oxidation is very rapid, contrary to the effect of oxaloacetate on succinate oxidation where a lag is observed. At a constant level of oxaloacetate a steady state is obtained, resulting from the oxidation of the nucleotides by oxaloacetate from one side and their reduction by NAD-linked substrates from the other side. The level of NAD^+ reduction at the steady state depends on the oxidizable substrate. As can be seen in Fig. 4B isocitrate is more effective in preventing the oxidation of nicotinamide nucleotides than is β-hydroxybutyrate. ATP alone is without effect but together with carnitine it increases the level of reduction; this effect is, however, small as compared to that of isocitrate (Fig. 4C). A steady state of the oxidoreduction of mitochondrial nicotinamide nucleotides in the presence of oxaloacetate can also be maintained by endogenous substrates. This is, however, possible only in the coupled state (Fig. 4D). The addition of an uncoupler results in an oxidation of the nucleotides, the rate of this oxidation being further strongly increased by arsenite. This experiment supports the view that the oxidation of endogenous substrates requires energy and that substrate-level phosphorylation (blocked by arsenite) may be one of the energy sources.

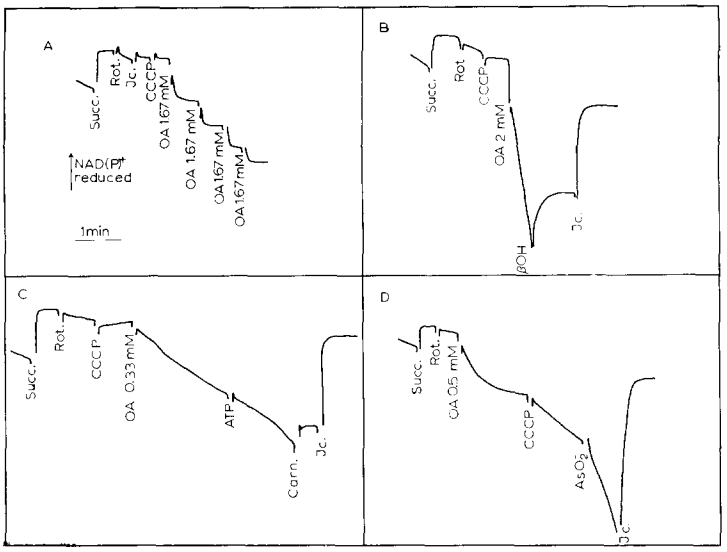

Fig. 4. Redox changes of intramitochondrial $NAD(P)^+$ in the presence of oxaloacetate under various conditions. Incubation medium: 120 mM KCl, 20 mM Tris–HCl (pH 7.4), 2 mM P_i, 2 mM $MgCl_2$ and 2.0 mg mitochondrial protein/ml. Temp. 23°. Additions: 3.3 mM succinate (Succ.), 4 mM isocitrate (Ic.), 2 mM β-hydroxybutyrate (βOH), 1.3 mM DL-carnitine (Carn.), 1.5 mM ATP, 1 μM rotenone (Rot.), 1 μM CCCP, 2 mM arsenite (AsO_2^-) and oxaloacetate (OA) as indicated.

The reactivation by respiratory substrates

The effectiveness of various substances in preventing the inhibition of succinate oxidation by oxaloacetate or in reversing this inhibition, is shown in Table IV. It can be seen that ATP alone (*plus* oligomycin to inhibit the ATPase) has no effect. However, ATP *plus* carnitine, or ATP *plus* carnitine *plus* palmitate, or palmitoyl-carnitine can reverse the inhibition. A similar effect is exerted by isocitrate but is partially abolished if either arsenate or arsenite (not shown) are present. β-Hydroxybutyrate is less effective than isocitrate. Pyruvate can effectively prevent the inhibition if present from the beginning of the incubation, but is not able to release the inhibition if it has already occurred. This is in contrast with palmitoyl-carnitine or palmitate *plus* carnitine *plus* ATP which are effective both when present from the beginning of the incubation and added after the inhibition.

The comparison of Table IV and Fig. 4 reveals that there is no close parallelism between the ability of an oxidizable substrate to maintain a high level of reduced nicotinamide nucleotides in the presence of oxaloacetate and the ability to prevent or reverse the inhibitory effect of oxaloacetate on succinate oxidation. Isocitrate is a potent reducing agent for mitochondrial NAD^+ *plus* $NADP^+$ (Figs. 4B and C), and is effective in preventing or reversing the inhibition of succinate oxidation produced by oxaloacetate (Table IV). On the other hand, ATP *plus* carnitine produce only a slight reduction of mitochondrial nicotinamide nucleotides (Fig. 4C), but are as effective in preventing the inhibition as is isocitrate (Table IV).

It is to be noted that in all cases when a spontaneous or induced reactivation of succinate oxidation occurred, no substantial decrease in oxaloacetate concentration in the incubation medium could be observed.

TABLE IV

EFFECT OF ATP AND OXIDIZABLE SUBSTRATES ON THE RATE OF SUCCINATE OXIDATION IN THE PRESENCE OF OXALOACETATE

Incubation medium as in Fig. 3; 3 mM oxaloacetate, 0.1 mM 2,4-dinitrophenol; temp. 26°.

Expt. No.	*Protein (mg)*	*Initial additions*	*Rate of O_2 uptake (mμatoms/min)*					
			Initial	*Time (min) after the addition of oxaloacetate*				
				1	*2*	*3*	*Subsequent additions*	*4*
1	7.2	None	680	340	102	102	None	136
		ATP (2 mM) + oligomycin (2 μg/ml)	680	255	85	85	Carnitine (2 mM)	458
		ATP (2 mM) + oligomycin (2 μg/ml)	680	255	85	119	Isocitrate (1 mM)	442
		Arsenate (2 mM)	764	340	0	0	Isocitrate (1 mM)	85
		Arsenate (2 mM) + isocitrate (1 mM)	764	425	306	204	None	170
		Isocitrate (1 mM)	595	595	595	*		
2	8.5	None	595	476	85	51	None	68
		None	595	476	85	51	Pyruvate (4 mM)	51
		Pyruvate (4 mM)	595	595	272	*		
		Pyruvate (4 mM) + arsenate (3 mM)	595	425	136	51	None	0
3	6.7	None	630	460	51	85	None	100
		ATP (0.6 mM) + carnitine (1 mM)	595	425	255	85	Palmitate (33 μM)	170
		None	630	460	51	68	ATP (0.6 mM) + carnitine (1 mM)	170
		None	630	460	51	85	Palmitoyl-carnitine (30 μM)	136
		β-Hydroxybutyrate (5 mM)	595	425	170	85	None	85

* O_2 exhausted.

DISCUSSION

The controlling effect of oxaloacetate in succinate oxidation seems now to be well established, but the control of oxaloacetate level in mitochondria presents several intriguing points. One of them is the permeability of mitochondria to exogenous oxaloacetate. The present investigation suggests that mitochondria are permeable to oxaloacetate. This is indicated by (i) an immediate swelling of mitochondria suspended in 100 mM ammonium oxaloacetate, and (ii) a very rapid oxidation of intramitochondrial NAD(P)H by added oxaloacetate (1–3 mM). At lower concentrations of oxaloacetate (below 1 mM) its penetration is probably facilitated by an energy-dependent cation transport, as recently shown by ROBINSON AND CHAPPELL[24] and HASLAM AND KREBS[25].

Another problem represents the energy dependence of succinate oxidation. It is well known that this oxidation in uncoupled mitochondria undergoes a spontaneous inhibition after a short period of initial high rate[4,6,7,15]. AZZONE AND ERNSTER[7] have suggested that the succinate oxidase system is in some way energy-controlled. However, SLATER AND HÜLSMANN[26] and CHAPPELL[6] postulated that oxaloacetate formed endogenously was responsible for the inhibition. It has been also shown[3,6,14] that succinate oxidation is more susceptible to added oxaloacetate in the uncoupled state than in phosphorylating mitochondria. It has been also observed that addition of ATP reverses the inhibition. The present investigation supports the view expressed earlier by SLATER AND HÜLSMANN[26] that the oxidation of endogenous fatty acids promotes the removal of oxaloacetate from mitochondria and that ATP, either formed in mitochondria or added, is necessary for the activation of these acids.

There are two mechanisms by which the oxidation of fatty acids can remove oxaloacetate. The first of them is the reduction of oxaloacetate to malate by NADH formed during the oxidation of fatty acids. This mechanism is especially important in the presence of rotenone or amytal, *i.e.* when the reoxidation of NADH but the respiratory chain is blocked. In fact, in this case oxaloacetate is the electron acceptor for the oxidation of fatty acids (Table III). That this pathway is effective in removing oxaloacetate from mitochondria is also indicated by the fact that other NAD-linked substrates, especially isocitrate, can also protect against oxaloacetate inhibition. Compatible with this are observations that amytal or rotenone effectively protect against the inhibitory effect of added oxaloacetate[15]. ROBERTON[27] showed that in a submitochondrial particle system oxaloacetate which had been formed during succinate oxidation could be reduced by NADH generated by the reversal of electron transport. This may be in fact the way in which succinate oxidation is controlled in coupled mitochondria. However, in systems containing an uncoupler and/or rotenone, like in the present investigation, this mechanism does not occur. In this case NAD^+ is reduced only by endogenous or added NAD-linked substrates. The effectiveness of various substrates depends on several factors, as the redox potential, activities of particular dehydrogenases and the permeability of mitochondrial membranes.

It has been shown recently[28,29] that the oxidation of anionic substrates can be limited by the rate of their uptake by mitochondria from the medium and that this process is energy-dependent. This observation may explain the fact that pyruvate is less effective in reactivating succinate oxidation when added to uncoupled mitochondria than is palmitoyl-carnitine or palmitate *plus* carnitine *plus* ATP whose

penetration through mitochondrial membranes is controlled by a different mechanism and is most probably independent on the supply of energy.

The uptake of succinate by mitochondria is also an energy-requiring process and, if the concentration of succinate in the medium is low, it can be a limiting factor in its oxidation[29]. This may be another explanation for the energy-dependence of succinate oxidation as postulated by AZZONE AND ERNSTER[7]. In the present investigation, however, the concentration of succinate was high enough (5 mM) to make its oxidation independent of the energy-requiring uptake.

The second mechanism by which oxidation of fatty acids removes oxaloacetate is the generation of acetyl-CoA which condenses with oxaloacetate to form citrate. This mechanism, suggested by SLATER AND HÜLSMANN[26], should play an important role in removing oxaloacetate, especially in uncoupled state and in the absence of rotenone or amytal, *i.e.* under conditions when the respiratory chain is maximally active and nicotinamide nucleotides are highly oxidized.

It has been observed in the present investigation that in the presence of rotenone and an uncoupler, a complete inhibition of succinate oxidation produced by oxaloacetate is usually followed by a spontaneous reactivation. It is shown that substrate-level phosphorylation plays an important part in this reactivation. Here again the energy is needed for the activation of endogenous fatty acids. In the spontaneous reactivation a certain role is also presumably played by pyruvate which is present, as an impurity, in oxaloacetate preparations and which is also formed from oxaloacetate by its decarboxylation during the incubation. Although pyruvate oxidation itself is not very effective in removing oxaloacetate (Table IV), it initiates the tricarboxylic acid cycle and, consequently, gives rise to the generation of GTP in the substrate-level phosphorylation.

We have also noticed that arsenate potentiates the effect of ATP, although to a lower extent than carnitine. Arsenate has probably a sparing effect on mitochondrial CoA by releasing it from succinyl-CoA and thus stimulating the oxidation of fatty acids. Most of the authors who studied the effect of ATP on the reversal of oxaloacetate inhibition used mitochondria pretreated with an uncoupler (2,4-dinitrophenol or dicumarol) *plus* arsenate[6,7,30] and therefore the effect of arsenate escaped their attention. The others[3,14] noticed, however, that ATP alone had little effect on the reversal of the spontaneous inhibition of succinate oxidation in the presence of 2,4-dinitrophenol.

In a previous paper[15] a reactivating effect of serum albumin, if present together with ADP and P_i, was described. It was suggested that albumin binds an inhibitor of succinate oxidation other than oxaloacetate. At present, we rather postulate that the action of serum albumin can be explained by the stimulation of fatty acid oxidation. Besides, small amounts of fatty acids are introduced into the reaction medium in a form bound to the albumin, and this again could have a beneficial effect. Finally, serum albumin can bind 2,4-dinitrophenol (ref. 31) and re-couple the phosphorylation of added ADP to form ATP. This seems to be the most satisfactory explanation of the observed effect of serum albumin.

The rate and the extent of the reactivation depends on the content of the endogenous fatty acids. Under certain conditions (Fig. 3D) an addition of a small amount of fatty acids is necessary to produce a reactivation. ÅKERBLOM *et al.*[5] have shown that succinate oxidation in rat-liver mitochondria depleted of endogenous NAD^+ is very

susceptible to low concentrations of oxaloacetate. The inhibition can be, however, abolished by the addition of ATP *plus* NAD^+ *plus* fatty acid, *i.e.* under conditions when the oxidation of fatty acids is favoured. The authors suggest that an oxidized derivative of fatty acids can regulate the sensitivity of succinate dehydrogenase towards oxaloacetate. However, these results can also be explained in the sense that the oxidation of fatty acids can effectively remove oxaloacetate from inside of the mitochondria, as postulated in the present paper.

The present investigation also provides an explanation of cyclic fluctuations in the long-term oxidation of succinate in rat-liver mitochondria[32]. An increase in the oxidation rate can be interpreted as the effect of the oxidation of endogenous substrates, mostly fatty acids, on the removal of oxaloacetate. However, when the oxidation of succinate proceeds at maximal rate, the endogenous formation of oxaloacetate exceeds the rate of its removal and the inhibition starts. The accumulation of oxaloacetate enhances the synthesis of citrate and the operation of the tricarboxylic acid cycle which is coupled to the substrate-level phosphorylation, which in turn stimulates the oxidation of new portions of fatty acids. As a result, the oxidation of succinate is restored until an increased production of oxaloacetate again causes an inhibition. The oscillations are most probably based on a high turnover number of succinate dehydrogenase and a great difference between the values of K_i of this enzyme in respect to oxaloacetate ($0.2 \cdot 10^{-6}$–$1.5 \cdot 10^{-6}$ M; refs. 33, 34), and K_m of citrate synthase (EC 4.1.3.7) in respect to oxaloacetate (saturation concentration $4 \cdot 10^{-4}$ M; ref. 35). The diminution of the amplitude of these cycles is the result of decreasing the amount of endogenous substrates.

PAPA, LOFRUMENTO AND QUAGLIARIELLO[36] have postulated that the energy-dependent decarboxylation of oxaloacetate to phosphoenolpyruvate is a factor controlling succinate oxidation in rabbit-kidney mitochondria. The formation of phosphoenolpyruvate has also been observed in rat-liver mitochondria in long-term experiments by SCHOLTE AND TAGER[37]. However, it appears unlikely that in rat-liver mitochondria ATP promotes the reversal of the inhibitory action of oxaloacetate by this reaction since the phosphoenolpyruvate carboxykinase (EC 4.1.1.32) has only a very low activity in these mitochondria. In contrast to kidney, this enzyme in rat liver is mainly located in the cytoplasm[38]. In agreement with this, preliminary measurements carried out in the system used in this investigation, with ATP added, showed a very slow formation of phosphoenolpyruvate as compared to a relatively high oxidation of endogenous fatty acids. Nevertheless, in kidney mitochondria which possess much higher activity of phosphoenolpyruvate carboxykinase than rat-liver mitochondria this enzyme may play a substantial role in removing oxaloacetate.

In conclusion, the present investigation seems to indicate that the oxidation of fatty acids (endogenous or added) is the main factor controlling the level of oxaloacetate in rat-liver mitochondria and, consequently, the rate of succinate oxidation. However, this conclusion may not apply to mitochondria of liver from other species, like pigeon, rabbit or guinea pig, and of other organs, which differ from rat-liver mitochondria in their enzyme pattern and the content of endogenous substrates.

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