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THE INHIBITION BY 2,4-DINITROPHENOL OF THE REMOVAL OF OXALOACETATE FORMED BY THE OXIDATION OF SUCCINATE BY RAT-LIVER AND -HEART MITOCHONDRIA

A. B. OESTREICHER, S. G. VAN DEN BERGH* AND E. C. SLATER

*Laboratory of Biochemistry, B. C. P. Jansen Institute**, University of Amsterdam, Amsterdam (The Netherlands)*

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SUMMARY

1. The effect of 2,4-dinitrophenol on the oxidation of succinate by isolated mitochondria has been studied in the presence and absence of arsenite.

2. The degree of inhibition by uncoupler of succinate oxidation is correlated with the rate of removal of oxaloacetate formed by malate dehydrogenase. Dinitrophenol inhibits aspartate formation in mitochondria of both rat liver and rat heart, and the formation of phosphoenolpyruvate in rat-liver mitochondria.

3. Arsenite increases the inhibition by dinitrophenol of succinate oxidation, because it blocks another pathway for removal of oxaloacetate, namely citrate synthesis.

4. Addition of ATP and oligomycin together with dinitrophenol prevents inhibition of succinate oxidation by the dinitrophenol, as well as inhibition of the conversion of oxaloacetate into phosphoenolpyruvate.

5. Glutamate prevents the inhibition of succinate oxidation by uncouplers, by enhancing the removal of oxaloacetate by transamination.

6. Oxaloacetate formed from added aspartate and α-oxoglutarate does not inhibit succinate oxidation by intact mitochondria unless dinitrophenol is added.

7. It is proposed that dinitrophenol inhibits an energy-requiring removal of oxaloacetate from the vicinity of succinate dehydrogenase.

INTRODUCTION

It has been known for some time that the oxidation of succinate by isolated mitochondria is inhibited by uncoupling concentrations of 2,4-dinitrophenol and other uncouplers[1–3]. This inhibition increases with the time of incubation[2], is prevented by Amytal[1,2], is less in the presence of glutamate or acetate[4], and is reversed by ATP[1,3]. In earlier papers from this laboratory[4–6], it was proposed that the inhibition is caused by an increase in the steady-state concentration of oxaloacetate, which, as a

* Present address: Laboratory of Veterinary Biochemistry, The State University, Utrecht, The Netherlands.

** Postal address: Plantage Muidergracht 12, Amsterdam, The Netherlands.

strong competitive inhibitor of succinate dehydrogenase (EC 1.3.99.1), controls the oxidation of succinate by mitochondria[7,8]. It was proposed that ATP, formed by oxidative phosphorylation in the absence of dinitrophenol, or added as such, promotes the disappearance of oxaloacetate[4]. In detail, it was suggested that, in the case of heart mitochondria, the function of the ATP is to bring about the synthesis of acetyl-CoA from endogenous fatty acids, and that the acetyl-CoA is responsible for removing the oxaloacetate[4]. CHAPPELL[3] proposed that in the case of liver and kidney mitochondria, the important reaction of oxaloacetate is with GTP to form phosphoenolpyruvate, catalysed by phosphoenolpyruvate carboxylase (EC 4.1.1.32). An alternative explanation for the reactivation by ATP proposed by AZZONE *et al.*[9], namely that ATP is specifically required for the interaction of reduced succinate dehydrogenase with the respiratory chain, was shown to be unlikely by the observation[5] that glycerol-phosphate oxidation is insensitive to dinitrophenol, and became untenable when it was found that the reactivation is insensitive to oligomycin[3,10].

Despite circumstantial evidence in favour of the explanation of dinitrophenol inhibition in terms of accumulation of oxaloacetate (see also refs. 11 and 12), this view has gained acceptance only slowly (*cf.* refs. 13 and 14), mainly because direct analyses of the oxaloacetate present in the mitochondrial suspension at the end of the isolation do not always correlate with the degree of inhibition[9,15]. In 1965, however, PAPA *et al.*[16] demonstrated that prevention of the inhibition of succinate oxidation on adding ATP to rabbit-kidney mitochondria previously incubated with succinate and dinitrophenol was correlated with an increase in formation of phosphoenolpyruvate and a decrease of the level of oxaloacetate. In their recent papers PAPA and co-workers[17,18] have provided convincing evidence that, at least in rabbit-kidney mitochondria, the inhibition of succinate oxidation by uncouplers in the absence of rotenone is caused by oxaloacetate. However, the way in which uncouplers affect the level of oxaloacetate, particularly in rat-liver and -heart mitochondria, still requires clarification. This is the subject of the present paper. A preliminary account of part of this work has appeared[19] (see also ref. 20).

METHODS

Rat-liver mitochondria were isolated by the method of HOGEBOOM[21] as described by MYERS AND SLATER[22]. Rat-heart mitochondria were isolated in 0.25 M sucrose and 0.001 M EDTA (pH 7.4) according to the procedure of HOLTON *et al.*[23].

The standard reaction mixture contained, unless stated otherwise, 15 mM KCl, 50 mM Tris–HCl buffer (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 30–40 mM potassium phosphate buffer (pH 7.5), 60 mM succinate, 30–40 mM glucose, 50 mM sucrose (derived from the mitochondrial suspension) and other additions as specified in the tables. The final volume was 1 or 2 ml. The pH was 7.5. The reaction temperature was 25°. The reaction was carried out in Warburg flasks (gas volume, 6–7 ml or 11–13 ml). The gas phase was air. O_2 uptake was measured with differential manometers with a narrow capillary. The reaction was initiated by the addition of 0.2 ml fresh mitochondria in 0.25 M sucrose, containing 3–8 mg mitochondrial protein, and stopped by the addition of $HClO_4$. After removal of the precipitated protein, the extracts were neutralized with KOH. $HClO_4$ was removed as $KClO_4$ precipitated in the cold. The metabolic products of the oxidation were estimated in the clear supernatant of the

extracts. Pyruvate and phosphoenolpyruvate were assayed with lactate dehydrogenase (EC 1.1.1.27)[24] and pyruvate kinase (EC 2.7.1.40)[25], oxaloacetate with malate dehydrogenase (EC 1.1.1.37)[26], malate with malate dehydrogenase at pH 9.1 (ref. 27) or with malate dehydrogenase, citrate synthase (EC 4.1.3.7) and acetyl-CoA at pH 8.1 (ref. 28), malate *plus* fumarate by including fumarate hydratase (EC 4.2.1.2) in the latter assay, aspartate with malate dehydrogenase and aspartate transaminase (EC 2.6.1.1)[29], and α-oxoglutarate with glutamate dehydrogenase (EC 1.4.1.3)[30]. Depending on the amount of metabolite, the assays were carried out either with a Zeiss PMQ-II or an Aminco-Chance dual-wavelength spectrophotometer.

Citrate was determined with the pentabromoacetone method as described by STERN[31]. Protein was determined by the biuret method as described by CLELAND AND SLATER[32].

Enzymes and special reagents were obtained as follows: lactate dehydrogenase, pyruvate kinase, malate dehydrogenase, glutamate dehydrogenase, aspartate transaminase, alcohol dehydrogenase (EC 1.1.1.1), fumarate hydratase, citrate synthase, ATP, ADP, NAD^+, α-oxoglutarate, pyruvate and succinate from Boehringer, Mannheim, Germany; L-glutamate and L-aspartate from British Drug Houses; rotenone from Penick, New York. Hexokinase (EC 2.7.1.1) was prepared from yeast by the method of DARROW AND COLOWICK[33], omitting the final crystallization step. Hexokinase from Boehringer was also used. In this case the enzyme was diluted in 0.1% serum albumin and dialysed against 2 mM phosphate buffer (pH 7.4) containing 1% glucose to free it from NH_4^+. Activities are expressed in I.U.B. units. NADH was prepared from NAD^+ by reduction with alcohol dehydrogenase and ethanol. Oligomycin was kindly supplied by the Upjohn Chemical Co.

RESULTS

Oxidation of succinate by rat-liver mitochondria

Fig. 1A shows that dinitrophenol inhibits the oxidation of succinate by rat-iver mitochondria, the inhibition increasing with time. ATP, in the presence of

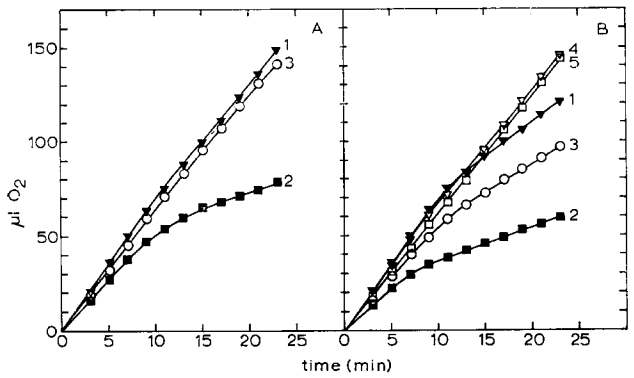

Fig. 1. Effect of arsenite on the time course of O_2 uptake during succinate oxidation in rat-liver mitochondria. Experimental conditions as described under METHODS and in Table I. O_2 uptake in the absence and presence of arsenite are given in A and B, respectively. Curve 1, phosphate acceptor; Curve 2, dinitrophenol; Curve 3, dinitrophenol + ATP + oligomycin; Curve 4, phosphate acceptor + 20 mM glutamate; Curve 5, dinitrophenol + 20 mM glutamate.

oligomycin, prevents the inhibition (Curve 3). In the absence of oligomycin (not shown in the figure), ATP stimulated only the initial rate of succinate oxidation, but had no effect on the final inhibition. In the presence of arsenite (Fig. 1B), the inhibition by dinitrophenol sets in more rapidly, and protection by ATP and oligomycin is incomplete. In the absence of dinitrophenol, arsenite causes a decline of the rate of O_2 uptake after 10 min (*cf.* Curves 1 in Figs. 1A and 1B). This is prevented by the addition of glutamate (Curve 4) which also completely protects against inhibition by dinitrophenol (Curve 5).

The products of oxidation of succinate, measured at the end of the experiment shown in Fig. 1, are listed in Table I. Under all conditions tested, malate was found to be the main product. Fumarate was not measured, but in comparable experiments, amounts equal to 6–10% that of the malate were found. Except in the presence of glutamate, only little oxidation past malate took place. In the control (Line 1), no oxaloacetate and only small amounts of its metabolic products (phosphoenolpyruvate, pyruvate and aspartate) were found. Coupled with the inhibition of the O_2 uptake, dinitrophenol (Line 3) caused a decline in the amounts of phosphoenolpyruvate and aspartate and a slight increase in the amount of oxaloacetate. Arsenite (Lines 2 and 4) caused an accumulation of pyruvate, which was less in the presence of dinitrophenol. The inhibition by dinitrophenol of the levels of phosphoenolpyruvate and aspartate was greater in the presence of arsenite. The protection by ATP (*plus* oligomycin) against inhibition of oxygen uptake by dinitrophenol (Lines 5 and 6) was associated with an increased synthesis of phosphoenolpyruvate, and, in the presence of arsenite, of pyruvate, and an increased oxaloacetate concentration. Aspartate levels were little affected by the addition of ATP and oligomycin. It is noteworthy that only in the absence of arsenite is there any correlation between the levels of oxaloacetate and inhibition of succinate oxidation. In the presence of arsenite, the already higher concentration of oxaloacetate is lowered by the addition of dinitrophenol. In the presence of glutamate, much of the malate was oxidized to oxaloacetate with sub-

TABLE I

PRODUCTS OF OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA IN THE PRESENCE AND ABSENCE OF ARSENITE AND THE EFFECT OF DINITROPHENOL, ATP AND GLUTAMATE

Standard reaction mixture with 4.7 mg mitochondrial protein. Further additions, where indicated were: 0.1 mM 2,4-dinitrophenol, 2.4 mM ATP + 10 μg oligomycin, 1 mM arsenite and 20 mM glutamate. When dinitrophenol was absent, phosphate acceptor (0.9 mM ADP and 6 units of hexokinase) was present. Reaction volume, 1 ml. Reaction for 24 min at 25°. Abbreviations: DNP, 2,4-dinitrophenol; PEP, phosphoenolpyruvate.

Addition				$-\Delta O$	Δ*Malate*	Δ*PEP*	Δ*Pyruvate*	Δ*Asp*	Δ α-*Oxo-glutarate*	Δ*Oxalo-acetate*
As_2O_3	*DNP*	*ATP**	*Glu*	(μatoms)	(μmoles)	(μmole)	(μmole)	(μmoles)	(μmoles)	(μmole)
—	—	—	—	13.8	10.8	0.048	0.010	0.053	0.039	0.000
+	—	—	—	11.1	8.6	0.059	0.195	0.067	0.092	0.039
—	+	—	—	7.1	4.3	0.017	0.004	0.037	0.072	0.013
+	+	—	—	5.4	4.0	0.008	0.092	0.010	0.039	0.017
—	+	+	—	13.5	9.7	0.102	0.007	0.022	0.058	0.002
+	+	+	—	9.1	8.4	0.082	0.176	0.017	0.084	0.026
+	—	—	+	13.6	5.3	0.007	0.023	2.64	2.48	—
+	+	—	+	13.4	6.6	0.003	0.032	1.88	2.18	—

* + oligomycin.

sequent transamination to aspartate and α-oxoglutarate. Judging by the low levels of phosphoenolpyruvate and pyruvate found, the oxaloacetate preferentially reacts with added glutamate even in the presence of dinitrophenol, which in this experiment somewhat inhibited the aspartate formation. This inhibition was not found in all experiments. The slight excess of α-oxoglutarate over aspartate shown in the last line of Table I is probably due to the stimulation by dinitrophenol of deamination of glutamate by rat-liver mitochondria[34].

Table II shows that arsenite prevents the conversion of pyruvate to citrate, presumably by inhibiting the oxidation of pyruvate to acetyl-CoA, thereby removing a substrate for one of the pathways of oxaloacetate removal. This is probably the explanation of the inhibitory effect of arsenite on succinate oxidation. Relative to the O_2 uptake, more citrate is formed in the presence of dinitrophenol and absence of arsenite; this may be a reflection of the inhibition by dinitrophenol of other pathways for the metabolism of oxaloacetate. The protection by oligomycin against inhibition by dinitrophenol was associated with increased pyruvate in the presence of arsenite and increased citrate in its absence. The small amounts of citrate found in the presence of arsenite might be due to acetyl-CoA derived from the oxidation of fatty acids. The residual citrate was greatly decreased by dinitrophenol, even in the presence of ATP.

Table III summarizes measurements made after oxidation of succinate in the presence of arsenite for 6 and 26 min. The most striking effect of dinitrophenol, apart from inhibition of the oxidation, is the complete inhibition of phosphoenolpyruvate formation between 6 and 26 min. In the absence of dinitrophenol, or in the presence of ATP as well as dinitrophenol, the formation of phosphoenolpyruvate is roughly proportional to time. During the first 6 min, more pyruvate was formed in the presence of dinitrophenol than in its absence. As in the experiment in Table I, there is no correlation between the level of oxaloacetate and the degree of inhibition.

Oxidation of succinate by rat-heart mitochondria

Table IV shows that dinitrophenol also inhibits the oxidation of succinate by rat-heart mitochondria. In contrast to the behaviour with liver mitochondria,

TABLE II

THE EFFECT OF ARSENITE ON THE FORMATION OF CITRATE AND PYRUVATE DURING OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA

Rat-liver mitochondria (4.9 mg) were pre-incubated for 4 min in a medium that contained, in addition to the standard components, 3 mM ATP and 10 mM fluoroacetate. At zero time, arsenite, hexokinase, 2,4-dinitrophenol and oligomycin were added as indicated, and the amounts of pyruvate and citrate measured after 26 min at 25°. Reaction volume, 2 ml.

Additions at zero time				*—ΔO* (*μatoms*)	*ΔPyruvate* (*nmoles*)	*ΔCitrate* (*nmoles*)
Arsenite (*mM*)	*Dinitrophenol* (*mM*)	*Oligomycin** (*μg*)	*Hexokinase* (*units*)			
0	0	0	12	20.5	15	270
1	0	0	12	15.6	287	26
0	0.1	0	0	13.7	6	268
1	0.1	0	0	7.8	223	3
0	0.1	10	0	21.0	13	394
1	0.1	10	0	17.2	288	6

* Additional ATP (1 mM) was added together with the oligomycin.

TABLE III

TIME DEPENDENCE OF PRODUCT FORMATION DURING OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA IN THE PRESENCE OF ARSENITE

Standard reaction mixture with 3.2 mg mitochondrial protein, 1 mM arsenite and, where indicated, 0.1 mM 2,4-dinitrophenol (DNP), and 2.7 mM ATP + 20 μg oligomycin. When dinitrophenol was absent, phosphate acceptor (0.9 mM ADP and 12 units hexokinase) was added. Volume, 2 ml. Temperature, 25°.

Addition		*Reaction time*	*ΔMalate*	*ΔPyruvate*	*ΔPhospho-enolpyruvate*	*ΔOxalo-acetate*
DNP	*ATP**	*(min)*	*(μmoles)*	*(nmoles)*	*(nmoles)*	*(nmoles)*
—	—	6	4.5	27	9	1
—	—	26	15.9	144	42	20
+	—	6	2.1	53	3	13
+	—	26	5.4	141	2	31
+	+	6	3.2	70	13	13
+	+	26	11.3	303	53	43

* + oligomycin.

TABLE IV

PRODUCTS OF OXIDATION OF SUCCINATE BY RAT-HEART MITOCHONDRIA IN THE PRESENCE OF ARSENITE

Standard reaction mixture with 2.9 mg sacrosomal protein and, where indicated, 1 mM arsenite, 0.1 mM 2,4-dinitrophenol, 2.4 mM ATP + 20 μg oligomycin, and 20 mM glutamate. When dinitrophenol was absent, phosphate acceptor (0.9 mM ADP + 10–14 units hexokinase) was added. Volume, 2 ml. Reaction for 27 min at 25°. Abbreviations as in Table I.

Addition				*—ΔO*	*ΔMalate*	*ΔPyruvate*	*ΔOxalo-acetate*	*ΔAsp*	*Δ α-Oxo-glutarate*
As_2O_3	*DNP*	*ATP**	*Glu*	*(μatoms)*	*(μmoles)*	*(μmole)*	*(μmole)*	*(μmoles)*	*(μmoles)*
—	—	—	—	9.6	—	—	0.020	0.038	—
—	+	—	—	6.2	—	—	0.017	0.000	—
+	—	—	—	6.7	3.9	0.35	0.044	0.058	0.078
+	+	—	—	4.9	3.0	0.155	0.028	0.011	0.018
+	+	+	—	6.3	3.4	0.28	0.032	0.011	0.051
+	—	—	+	18.8	5.9	0.43	—	5.30	5.30
+	+	—	+	9.8	2.6	0.38	—	2.00	2.03
+	+	+	+	11.3	3.4	0.43	—	2.55	2.65

* + oligomycin.

dinitrophenol also inhibits O_2 uptake in the presence of glutamate. In the presence of arsenite, less pyruvate, oxaloacetate, aspartate and α-oxoglutarate were formed when dinitrophenol was also present. Thus, the latter appears to inhibit all pathways for the breakdown of oxaloacetate. No phosphoenolpyruvate could be detected, as is to be expected from the very low activity of phosphoenolpyruvate carboxylase in rat heart[35]. The addition of ATP and oligomycin counteracted partially the inhibitory effects of dinitrophenol on the O_2 uptake and on the formation of malate, pyruvate, and α-oxoglutarate, but had no significant effect on the amounts of oxaloacetate or aspartate found.

TABLE V

THE EFFECT OF OXALOACETATE FORMED BY TRANSAMINATION ON THE OXIDATION OF SUCCINATE BY RAT-LIVER MITOCHONDRIA

Standard reaction mixture except 20 mM succinate instead of 60 mM, and addition of 1 mM arsenite, 2 mM glutamate, 4 μg rotenone, phosphate acceptor (0.9 mM ADP and 12 units hexokinase), and, in Expt. 2, 1 mM malate omitting phosphate acceptor, and, where indicated 0.1 mM 2,4-dinitrophenol, 8 mM aspartate, 10 mM α-oxoglutarate, and 3 mM ATP + 20 μg oligomycin. Reaction volume, 2 ml. In both experiments, 3.6 mg mitochondrial protein were present. Reaction for 20 (Expt. 1) or 22 (Expt. 2) min at 25°. Abbreviations as in Table I. This experiment was carried out in collaboration with Dr. J. M. TAGER and Dr. E. J. DE HAAN.

Expt. No.	*Addition*				$-\Delta O$ (μatoms)	*ΔOxaloacetate* (μmoles)	*ΔPyruvate* (μmole)	*ΔPEP* (μmole)
	DNP	*Asp*	*α-Oxoglutarate*	*ATP**				
1	−	−	−	−	16.1	0.0	0.0	—
	+	−	−	−	15.6	0.0	0.0	—
	−	+	+	−	16.1	0.53	0.11	—
	+	+	+	−	5.7	0.62	0.22	—
	−	+	−	−	16.3	0.003	0.00	—
	+	+	−	−	18.3	0.002	0.00	—
2	+	−	+	+	12.8	0.005	0.006	0.005
	+	+	+	−	0.0	1.05	0.36	0.000
	+	+	+	+	3.4	0.78	0.28	0.033

* + oligomycin.

Effect of oxaloacetate formed by transamination on the oxidation of succinate

In the experiments described to date oxaloacetate was formed by oxidation of malate. In the experiments shown in Table V, it was formed by transamination from aspartate to α-oxoglutarate, and the effect of oxaloacetate formed in this way on succinate oxidation was studied. Rotenone was present to prevent oxidation of malate, and arsenite to prevent oxidation of pyruvate or α-oxoglutarate. Malate and glutamate were added in order to increase the permeability of the mitochondria to α-oxoglutarate[36] and aspartate[37], respectively. In the presence of rotenone, dinitrophenol does not inhibit succinate oxidation[16]. Oxaloacetate formed by transamination also does not inhibit (Line 3 of Table V) except in the presence of dinitrophenol, which caused only a small increase in the amount of oxaloacetate (Line 4). The inhibition by α-oxoglutarate *plus* aspartate *plus* dinitrophenol was less when ATP and oligomycin were also present (last line of Table V). In similar experiments with heart mitochondria, a slight inhibition was found with aspartate *plus* α-oxoglutarate, and this was greatly increased by dinitrophenol.

DISCUSSION

The concentration of succinate (60 mM) was so high in comparison with that of the uncoupler (0.1 mM) that inhibition by the latter of entry of succinate into the mitochondria[38] can be excluded as the explanation of the inhibition of succinate oxidation by dinitrophenol.

The various reactions taking place during succinate oxidation by rat-liver mitochondria are summarized in Fig. 2. The high succinate concentration was chosen

in order to decrease oxidation past malate (*cf.* ref. 39). From the analysis of the products formed in the presence of arsenite it can be calculated that under the conditions used, the oxidation of malate to oxaloacetate amounted to 2–5% of the oxidation of succinate to fumarate in the liver mitochondria. The oxidation of the malate formed under State-3 conditions is controlled by the degree of reduction of

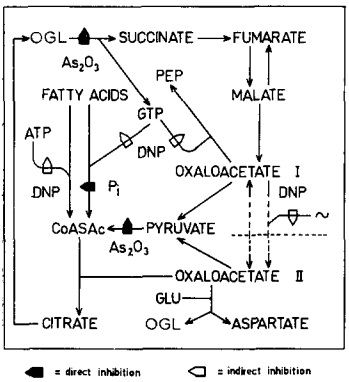

Fig. 2. Pathways for oxidation of succinate in mitochondria. Citrate synthase is placed in Compartment II because uncouplers inhibit the oxidation of pyruvate *plus* malate[34]. DNP, 2,4-dinitrophenol; PEP, phosphoenolpyruvate, OGL; α-oxoglutarate.

the NAD^+ (refs. 40–42). Except at very low $NADH/NAD^+$ ratios the malate dehydrogenase reaction is unfavourable for malate oxidation. Even in the presence of dinitrophenol, when NAD becomes more oxidized, the oxidation past malate was increased only slightly. However, it was markedly increased by glutamate, which, by removing the product oxaloacetate, promotes the oxidation of malate.

The small amount of oxaloacetate formed during succinate oxidation was removed by conversion to phosphoenolpyruvate, pyruvate and citrate, the last reaction taking place with acetyl-CoA mainly formed from pyruvate oxidation. In rat-liver mitochondria the oxaloacetate formed in the absence of dinitrophenol and presence of arsenite seems to be removed sufficiently rapidly to allow succinate oxidation to occur at optimal rate, since the addition of glutamate had no effect on the rate of O_2 uptake. Since the reaction in the presence of glutamate includes the NAD-linked oxidation of malate, the lack of stimulation of O_2 uptake by glutamate indicates that the rate of oxidation is limited by the activity of the respiratory chain. In contrast with liver mitochondria, the oxidation of succinate by heart mitochondria in the absence of dinitrophenol appears to be limited by the activity of the succinate dehydrogenase, since the addition of glutamate, presumably by removing inhibitory oxaloacetate, almost trebles the rate of O_2 uptake. Rotenone also stimulates the rate of O_2 uptake (*cf.* ref. 4).

The inhibition by dinitrophenol of the O_2 uptake can be explained by its effect on two reactions leading to the removal of oxaloacetate, *viz.* phosphoenolpyruvate formation in liver (Table I) and transamination with glutamate in both liver (Table I) and heart (Table IV).

In the presence of arsenite, GTP necessary for the reaction with oxaloacetate catalysed by phosphoenolpyruvate carboxylase must be formed by reaction of GDP

with ATP, catalysed by nucleoside diphosphate kinase (EC 2.7.4.6)[43]. Dinitrophenol, by lowering the concentration of ATP, would inhibit this reaction. The synthesis of phosphoenolpyruvate proceeds normally if ATP and oligomycin are added together with the dinitrophenol (*cf.* ref. 16). Inhibition by uncoupler of the formation of aspartate during glutamate oxidation has been shown by DE HAAN *et al.*[31]. TAGER[44] and DE HAAN[45] propose that oxaloacetate formed by or reacting with malate dehydrogenase and that formed by or reacting with aspartate transaminase are functionally separated from one another and that energy is necessary for the transfer of oxaloacetate from the malate dehydrogenase compartment (I in Fig. 2) to that in the transaminase compartment (II). The data of Table V show that oxaloacetate formed in the transaminase compartment does not inhibit succinate dehydrogenase unless dinitrophenol is present. This can be explained on the basis of the hypothesis of TAGER and DE HAAN if succinate dehydrogenase is also in the malate dehydrogenase compartment. Provided sufficient energy is present to drive oxaloacetate from this compartment to the transamination compartment, the steady-state concentration in the former compartment would be kept low. Dinitrophenol, by inhibiting the oxaloacetate transport, would raise this concentration, thereby inhibiting the succinate dehydrogenase. When oxaloacetate in Compartment II is rapidly removed by the addition of glutamate, the concentration of oxaloacetate in Compartment I would fall, according to the scheme given in Fig. 2, even in the absence of a supply of energy. Glutamate was found to remove the inhibition entirely in liver and to decrease it in heart.

Other reactions leading to the removal of oxaloacetate, namely decarboxylation to pyruvate, and in the absence of arsenite, citrate synthesis are not inhibited by dinitrophenol. Indeed, in the presence of arsenite, the pyruvate concentration after 6 min is increased by dinitrophenol (Table III), probably as a result of the higher oxaloacetate concentration caused by inhibition of the transamination and GTP-dependent decarboxylation reactions.

When ATP and oligomycin were present together with dinitrophenol, the inhibition of the O_2 uptake was either completely prevented or greatly reduced. In rat-liver mitochondria, the promotion by ATP of phosphoenolpyruvate formation is a partial explanation. That ATP does not prevent inhibition of aspartate formation is understandable in view of the evidence[15] that the transport of oxaloacetate from Compartment I to II is driven preferentially by high-energy intermediates (or the high-energy state) which cannot be formed by ATP in the presence of oligomycin. Table V, however, shows some effect of ATP in preventing the inhibition of succinate oxidation by oxaloacetate generated by transamination in the presence of dinitrophenol. However, in rat-liver mitochondria, ATP appears to have an additional effect in either directly or indirectly promoting the decarboxylation of oxaloacetate to pyruvate (Table III). The increased citrate found in the presence of ATP, dinitrophenol, oligomycin and fluoroacetate (Table II) may also be due to an increased decarboxylation of oxaloacetate. Since phosphoenolpyruvate is not formed in heart mitochondria, and the addition of ATP did not increase the amount of pyruvate, it is possible that the relatively small protective effect by ATP with heart mitochondria is due to promotion by ATP of transport of oxaloacetate from Compartment I to II (last line of Table IV), or by activation of fatty acids (Lines 3 and 5 of Table IV).

WOJTCZAK[46] has recently studied the inhibitory effect of added oxaloacetate on succinate oxidation by rat-liver mitochondria in the presence of rotenone and an

uncoupler. She concludes that reactivation by ATP, which is substantial only in the presence of carnitine, and the spontaneous reactivation which sets in after a certain time are due to removal by fatty acids of oxaloacetate from the site of succinate dehydrogenase. Under our conditions, fatty acids play an unimportant role in removing oxaloacetate formed from malate. This is understandable if of the two roles ascribed by WOJTCZAK[46] to the fatty acids, *viz.* generation of NADH and acetyl-CoA, the first is the more important, since fatty acids would not be expected to increase the NADH concentration appreciably in our experiments with a high concentration of malate.

The lack of correlation between the amount of oxaloacetate found at the end of the experiment and the degree of inhibition is not surprising. In the first place, a correlation would not be expected if there are two intramitochondrial compartments, only one of which is concerned in the inhibition of succinate dehydrogenase. Even more important, it was found that most of the oxaloacetate measured at the end of the experiment was extramitochondrial (Table VI).

TABLE VI

EXTRA- AND INTRAMITOCHONDRIAL DISTRIBUTION OF OXALOACETATE

Expt. 1. Standard reaction mixture with 8.4 mg mitochondrial protein and phosphate acceptor (0.9 mM ADP and 12 units hexokinase). Reaction volume, 2 ml. Reaction for 16 min at 25°. The mitochondria were then separated from the reaction mixture by the centrifugation–filtration technique[47] as modified by HARRIS AND VAN DAM[48]. *Expt. 2.* The reaction mixture (1.5 ml) contained the same components as in Table V, except that 10 mM α-oxoglutarate and 8 units hexokinase were present in all incubations. In the zero-time controls, hexokinase was omitted. Mitochondrial protein, 1.9 mg. Reaction for 10 min at 25°. The mitochondria were then separated from the reaction mixture by rapid filtration as described by DE HAAN AND TAGER[36]. This experiment was carried out in collaboration with Dr. E. J. De Haan.

Additions		*Oxaloacetate (nmoles) in*	
Dinitrophenol (mM)	*Asp (mM)*	*Mitochondrial extract*	*Filtrate*
Expt. 1			
0	0	1	17
0.1	0	3	31
Expt. 2			
0	0	0	0
0.1	0	0	2
0	8	2	366
0.1	8	2	470

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