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PHTHALONIC ACID, AN INHIBITOR OF α-OXOGLUTARATE TRANSPORT IN MITOCHONDRIA

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SUMMARY

Phthalonic acid is a powerful inhibitor of α-oxoglutarate transport in mitochondria. This conclusion is based on the following observations:

1. Phthalonic acid inhibits the oxidation of α-oxoglutarate but has no effect on the oxidation of glutamate or *cis*-aconitate.

2. With arsenite present, phthalonic acid inhibits the oxidation of glutamate plus malate and of *cis*-aconitate plus malate. Under these conditions α-oxoglutarate accumulates inside the mitochondria. With glutamate plus malate as substrates the inhibition is competitive with malate with a K_i value of 20 μM.

3. Phthalonic acid inhibits the oxidation of intramitochondrial NAD(P)H by α-oxoglutarate plus ammonia. The inhibition is competitive with respect to α-oxoglutarate with a K_i of 30 μM.

4. Phthalonic acid inhibits the exchange between extramitochondrial α-oxoglutarate and intramitochondrial malate.

INTRODUCTION

Numerous organic compounds have been tested for their ability to inhibit mitochondrial anion transport (see ref. 1 for review). For most anion translocators more or less specific inhibitors are now available [1]. Of the translocators that bring about the transport of citric acid cycle intermediates the tricarboxylate translocator can be specifically inhibited by benzene-1,2,3-tricarboxylate [2, 3] and the dicarboxylate translocator by butylmalonate [4, 5]. However, no specific inhibitor for the α–oxoglutarate translocator has been described so far. Although several compounds are known that interfere with α–oxoglutarate transport, none of these is found to be specific, since they also inhibit the dicarboxylate and tricarboxylate translocators [1, 2, 6, 7].

In this paper we report the results of experiments showing that phthalonic acid is a compound that inhibits α-oxoglutarate transport in mitochondria. Although

this compound is not completely specific for the α-oxoglutarate translocator (it slightly inhibits the dicarboxylate translocator as well) the inhibitor can be useful in metabolic studies using isolated mitochondria.

METHODS AND MATERIALS

Rat liver mitochondria were prepared according to the method of Hogeboom [8] as described by Myers and Slater [9].

The incubation medium contained the following standard components: 15 mM KCl, 5 mM $MgCl_2$, 2 mM EDTA, 50 mM Tris · HCl (pH 7.4) and 10–25 mM sucrose (derived from the mitochondrial suspension). The reaction temperature was 25 °C unless otherwise stated.

Oxygen uptake was measured polarographically with a Clark-type electrode.

Malate-loaded mitochondria were prepared as described previously [10].

Separation of the mitochondria from their suspending medium occurred by centrifugation through silicone oil into a layer of $HClO_4$, using an Eppendorf microcentrifuge (Model 3200) [10].

Mitochondrial protein was determined with the biuret method [11] using egg albumin as standard.

Malate and α-oxoglutarate were measured fluorimetrically using common enzymic procedures [12].

Phthalonic acid was prepared according to the method of Graebe and Trümpy [13] with naphthalene or decaline as starting material (see also ref. 14).

n-Butylmalonate was prepared from the diethyl ester as described by Vogel [15].

All enzymes and cofactors were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp.

RESULTS

We selected phthalonic acid as a possible inhibitor of α-oxoglutarate transport in mitochondria because of its structural resemblance to α-oxoglutarate (Fig. 1). In order to test its ability to inhibit α-oxoglutarate transport we studied its effects on intramitochondrial reactions involving α-oxoglutarate.

The effect of phthalonic acid on intramitochondrial reactions involving α-oxoglutarate

Fig. 2 shows that phthalonic acid inhibited the mitochondrial oxidation of α-oxoglutarate. Since, as will be discussed below, phthalonic acid did not affect the

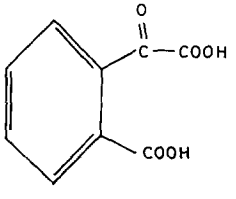

Fig. 1. Phthalonic acid.

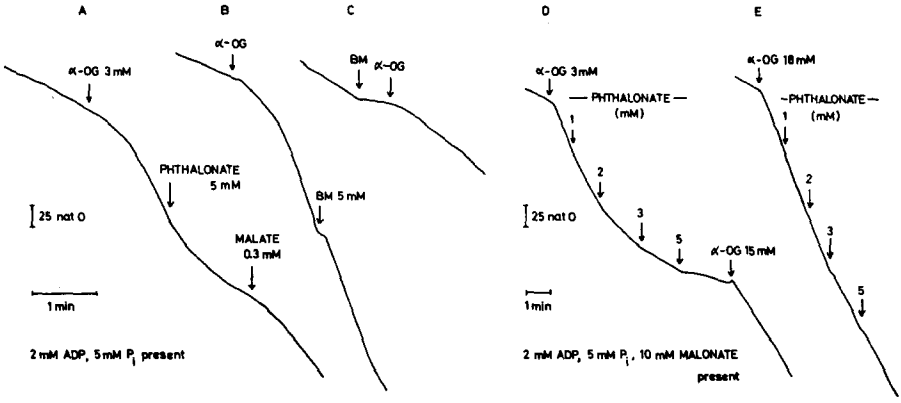

Fig. 2. Effect of phthalonic acid on the oxidation of α-oxoglutarate by rat liver mitochondria. The incubation medium (1.6 ml) contained the standard components plus 2 mM ADP, 5 mM potassium phosphate, 3.2 mg (A–C) or 1.5 mg mitochondrial protein (D and E) and 10 mM malonate (D and E). αOG, α-oxoglutarate; BM, butylmalonate.

oxidation of glutamate and *cis*-aconitate, this effect must have been due to an inhibition of α-oxoglutarate entry into the mitochondria and not to an inhibition of α-oxoglutarate dehydrogenase (EC 1.2.4.2).

When the oxidation of α-oxoglutarate is studied it is important to realize that for entry of α-oxoglutarate into the mitochondria the presence of intramitochondrial malate is required, because α-oxoglutarate exchanges with intramitochondrial malate in a 1 : 1 fashion [16]. Upon the addition of α-oxoglutarate to mitochondria preincubated with ADP and phosphate, the intramitochondrial malate is depleted and entry of α-oxoglutarate is slow. Since α-oxoglutarate oxidation yields malate the intramitochondrial malate pool can be replenished by recycling malate back into the mitochondria via the dicarboxylate translocator (cf. ref. 17). Thus the oxidation of α-oxoglutarate is preceded by a lag period before a maximal rate of oxygen consumption is reached (Fig. 2A). This lag can be removed by addition of malate (not shown). The inhibition of α-oxoglutarate oxidation by phthalonic acid shown in Fig. 2A could, therefore, be due either to direct inhibition of the α-oxoglutarate translocator or to an inhibition of the dicarboxylate translocator. In order to distinguish between these two possibilities the effect of butylmalonate on α-oxoglutarate oxidation was tested. Butylmalonate had no effect on oxygen consumption when added after the lag period (Fig. 2B), but strongly inhibited α-oxoglutarate oxidation when added first (Fig. 2C). This result demonstrates that flux through the dicarboxylate translocator after the lag period was negligible. Consequently, the inhibitory effect of phthalonic acid must have been on the α-oxoglutarate translocator itself.

Of interest is that the inhibition of α-oxoglutarate oxidation by phthalonic acid was partially released by addition of a small amount of malate (Fig. 2A). This effect of malate appeared to be butylmalonate sensitive (not shown). Apparently, intramitochondrial malate protects the α-oxoglutarate translocator against the inhibitory action of phthalonic acid. Because of this protective effect of malate the inhibitory effect of phthalonic acid on α-oxoglutarate oxidation was found to be slightly dependent on the amount of malate present in the mitochondrial suspension at the time the inhibitor was added.

TABLE I

THE EFFECT OF PHTHALONIC ACID ON THE OXIDATION OF GLUTAMATE AND OF *cis*-ACONITATE BY RAT LIVER MITOCHONDRIA

Mitochondria were incubated in a medium containing the standard components, 2 mM ADP, 5 mM phosphate and either 3 mM glutamate plus 3 mM malate or 3 mM *cis*-aconitate plus 3 mM malate. Arsenite, if present, was added at 1 mM.

Additions	Phthalonate (mM)	ΔO (μatom O/min/ per mg protein)	Inhibition (%)
Glutamate	0	106	
	5	107	−1
Glutamate + malate + arsenite	0	142	
	5	21	85
cis-Aconitate + malate	0	92	
	5	97	−5
cis-Aconitate + malate + arsenite	0	72	
	5	21	71

Figs 2D and 2E show that the inhibitory effect of phthalonic acid on α-oxoglutarate oxidation could be overcome by raising the α-oxoglutarate concentration, thus demonstrating the competitive nature of the inhibition. In this experiment the oxidation of α-oxoglutarate was carried out in the presence of malonate to prevent malate formation. Apart from inhibiting succinate dehydrogenase malonate has the advantage of stimulating α-oxoglutarate transport in mitochondria [18].

In Table I the effect of phthalonic acid on the oxidation of glutamate and of *cis*-aconitate was studied. Oxygen consumption with glutamate alone was not affected by phthalonic acid. Since the oxidation of glutamate by rat liver mitochondria in

TABLE II

THE EFFECT OF PHTHALONIC ACID ON EFFLUX OF α-OXOGLUTARATE FROM RAT LIVER MITOCHONDRIA

Mitochondria (3.0 mg protein) were preincubated in a medium containing the standard components, 1 mM ADP, 10 mM phosphate, 20 mM glucose, hexokinase, 1 mM arsenite, 3 mM malate and phthalonic acid as indicated; final volume, 1.5 ml. After 3 min, either 3 mM glutamate or 3 mM *cis*-aconitate was added. 4 min later the mitochondria were separated from their suspending medium by centrifugation through silicone oil into $HClO_4$. The amounts of intramitochondrial α-oxoglutarate were not corrected for α-oxoglutarate present in the sucrose space.

Substrate	Phthalonate (mM)	α-Oxoglutarate$_{in}$ (nmol/mg protein)	α-Oxoglutarate$_{out}$ (nmol/mg protein)
Glutamate + malate	0	5	297
	2	16	89
	10	31	20
cis-Aconitate + malate	0	5	228
	2	32	85
	10	46	49

State 3 proceeds to the extent of 90 % via the transamination pathway [19], this result rules out the possibility that the inhibition by phthalonic acid of oxygen consumption with α-oxoglutarate as the substrate, as described in Fig. 2, was due to an inhibition of α-oxoglutarate dehydrogenase. When the oxidation of glutamate was carried out in the presence of arsenite and malate, phthalonate strongly inhibited oxygen consumption (Table I). Similar results were obtained with *cis*-aconitate in the presence of malate to activate its entry into the mitochondria: in the absence of arsenite no effect of phthalonic acid was observed, but 70 % inhibition was obtained in its presence. With arsenite present, α-oxoglutarate, whether formed by transamination from glutamate or by oxidation of *cis*-aconitate, cannot be oxidized further and must leave the mitochondria. Since oxygen consumption under both conditions was sensitive to phthalonic acid, this result is consistent with inhibition of α-oxoglutarate transport across the mitochondrial membrane. This was confirmed in the experiment depicted in Table II where it is shown that phthalonic acid not only inhibited the appearance of α-oxoglutarate in the supernatant, but also caused an accumulation of α-oxoglutarate within the mitochondria under these conditions.

Kinetic constants

In order to study the magnitude of the inhibition of α-oxoglutarate transport by phthalonic acid we measured its effect on the oxidation of intramitochondrial NAD(P)H by α-oxoglutarate and ammonia. The incubations were carried out in the presence of rotenone to block NADH dehydrogenase (EC 1.6.99.3), arsenite to inhibit α-oxoglutarate dehydrogenase and malonate to activate α-oxoglutarate entry into the

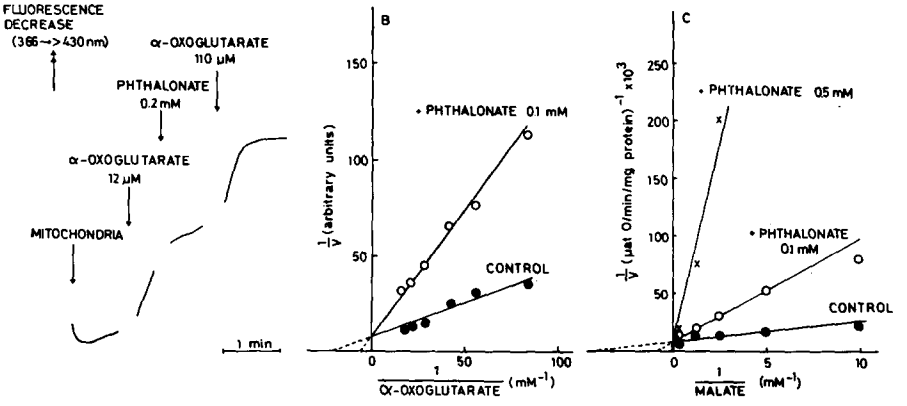

Fig. 3. Effect of phthalonic acid on influx and efflux of α-oxoglutarate in rat liver mitochondria. In A and B the influx of α-oxoglutarate was studied by measuring fluorimetrically the oxidation of intramitochondrial NAD(P)H by α-oxoglutarate and ammonia. Mitochondria (0.72 mg protein were incubated in a medium containing the standard components, 1 μg rotenone, 0.5 % ethanol, 1 mM arsenite, 2.5 mM phosphate, 10 mM NH_4Cl, 5 mM malonate and, if present, 0.1 mM phthalonate; final volume, 2 ml. After 1 min, the oxidation of NAD(P)H was initiated by addition of α-oxoglutarate at different concentrations. In C the efflux of α-oxoglutarate from the mitochondria was studied by measuring oxygen consumption with glutamate plus malate as substrates. The reaction medium (1.6 ml) contained the standard components plus 2 mM ADP, 5 mM phosphate, 1 mM arsenite, 10 mM glutamate, 1.6 mg mitochondrial protein and phthalonate at the concentration indicated. After 2 min, malate was added at different concentrations.

mitochondria. Phthalonic acid strongly inhibited the oxidation of intramitochondrial NAD(P)H (Fig. 3A), the inhibition being competitive with α-oxoglutarate (Fig. 3B). In three experiments, carried out with different mitochondrial preparations, K_i values of 20, 28 and 35 μM, respectively, were found.

In the experiment of Fig. 3C the effect of phthalonic acid on the efflux of α-oxoglutarate from the mitochondria was studied by measuring oxygen consumption in the presence of glutamate, malate and arsenite. Inhibition by phthalonic acid was competitive with malate with a K_i value of 20 μM.

Specificity of phthalonic acid

After having established that phthalonic acid is a rather potent inhibitor of α-oxoglutarate transport the question of its specificity had to be considered. The simple oxygraph experiments discussed above already give a great deal of information on this point. Since the experiments were carried out under State 3 conditions it follows that neither the adenine nucleotide nor the phosphate translocator were affected. Oxidation of glutamate and *cis*-aconitate (in the absence of arsenite) were not inhibited by phthalonate so that this compound does not affect aspartate and tricarboxylate transport to any great extent. We have also found that oxidation of pyruvate plus malate was insensitive to phthalonic acid, even when the inhibitor was present in a 10-fold excess (not shown). Thus phthalonic acid does not affect the pyruvate translocator.

In order to test whether the dicarboxylate translocator could be blocked by phthalonic acid we studied its effect on the activation by malate of *cis*-aconitate oxidation. The mitochondria were depleted of malate by preincubation with ADP and phosphate. The oxidation of *cis*-aconitate was then initiated by addition of 0.1

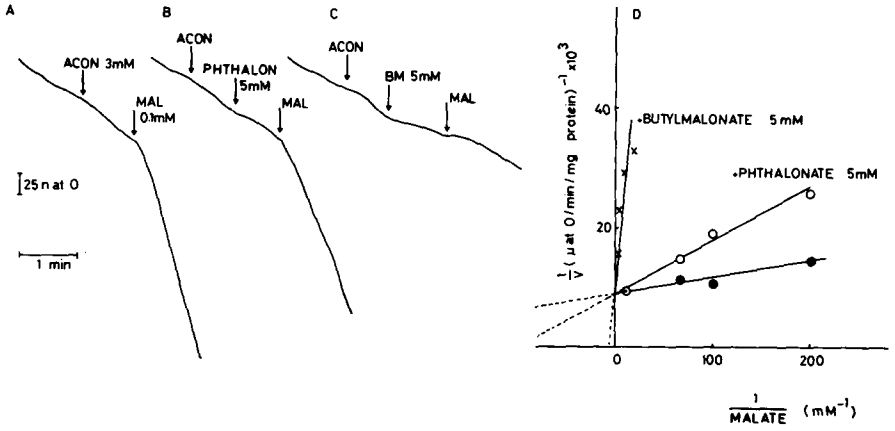

Fig. 4. Effects of phthalonic acid and butylmalonate on the activation by malate of *cis*-aconitate oxidation. Mitochondria (3.2 mg protein) were preincubated in a medium (1.6 ml) containing the standard components, 2 mM ADP and 5 mM phosphate. After 2 min, 3 mM *cis*-aconitate was added, followed by addition of malate as indicated in Fig. 4A. Phthalonate or butylmalonate, if present, were added as indicated in B and C. In D the same procedure was followed but in this case the malate concentration was varied. The rate of oxygen consumption was measured 20 s after the addition of malate. ACON, *cis*-aconitate; MAL, malate; PHTHALON, phthalonate; BM, butylmalonate.

TABLE III

EFFECT OF PHTHALONIC ACID ON MITOCHONDRIAL ANION EXCHANGES

Malate-loaded mitochondria (2 mg protein/ml) were incubated in a medium containing the standard components, plus 2 μg rotenone, 1 % ethanol and the additions shown in the table; temperature, 0 °C. After 1 min, the mitochondria were rapidly centrifuged through silicone oil into $HClO_4$. The mitochondrial suspension (no addition) contained per mg protein 20 nmol intramitochondrial malate and 12 nmol extramitochondrial malate (Experiment 1), 18 nmol intramitochondrial malate and 7 nmol extramitochondrial malate (Experiment 2) or 20 nmol intramitochondrial malate and 5 nmol extramitochondrial malate (Experiment 3). The percentage exchange was calculated from the mean value of the decrease in mitochondrial malate and the increase in extramitochondrial malate upon a certain addition; the amount of mitochondrial malate with no addition was taken as 100 %.

Expts.	Exchanging anion	Exchange (%)		
		Control	Phthalonate (5 mM)	Butylmalonate (5 mM)
1	None	0	−1	—
	P_i (10 mM)	92	86	—
	α-Oxoglutarate (2 mM)	70	8	—
	Citrate (2 mM)	88	74	—
2	None	0	—	0
	P_i (10 mM)	87	—	2
	α-Oxoglutarate (2 mM)	66	—	58
	Citrate (2 mM)	82	—	75
3	None	0	4	—
	P_i (2 mM)	62	28	—
	α-Oxoglutarate (2 mM)	59	13	—

mM malate (Fig. 4A). Phthalonic acid inhibited the activation by malate very slightly (Fig. 4B). When the malate concentration was varied it was found that phthalonic acid competitively inhibited malate entry into the mitochondria with a K_i of about 2 mM (Fig. 4D). For comparison the inhibitory effect of butylmalonate is also shown (Fig. 4C); its K_i value was 0.15 mM (Fig. 4D).

In Table III the effect of phthalonic acid on the exchange of intramitochondrial malate with extramitochondrial phosphate, α-oxoglutarate or citrate was investigated. For comparison the data obtained with butylmalonate are also given. Phthalonate at 5 mM had no significant effect on the efflux of malate induced by 10 mM phosphate or 2 mM citrate. The efflux of malate induced by 2 mM α-oxoglutarate on the other hand was 90 % inhibited by phthalonic acid. Butylmalonate, at 5 mM, did not affect the efflux of malate induced by α-oxoglutarate or citrate, but totally inhibited the exchange induced by phosphate, in agreement with previous observations [5, 20]. When the external phosphate concentration was lowered to 2 mM phthalonic acid inhibited the phosphate-malate exchange by about 50 %.

These simple experiments confirm our conclusions from the metabolic data discussed above, viz. that phthalonate is a rather specific inhibitor of the α-oxoglutarate translocator, although some inhibitory effect is also observed with the dicarboxylate translocator.

Finally, it should be noted that phthalonic acid by itself did not cause any extrusion of malate from the mitochondria (Table III), even when the inhibitor

concentration was raised to 30 mM (not shown). It is likely, therefore, that phthalonic acid does not penetrate the mitochondrial membrane.

DISCUSSION

The results presented demonstrate that phthalonic acid inhibits α-oxoglutarate transport across the mitochondrial membrane. Although the inhibitor is rather specific for α-oxoglutarate transport (K_i approx. 30 μM) it also slightly reacts with the dicarboxylate translocator (K_i approx. 2 mM). This specificity pattern is the mirror picture of that displayed by butylmalonate (cf. also Table III): this compound inhibits the dicarboxylate translocator with a K_i of about 0.4 mM [4] or lower (cf. Fig. 4D) but in addition inhibits α-oxoglutarate transport with a K_i of about 1 mM, according to Palmieri et al. [6].

It is very likely that phthalonic acid inhibits α-oxoglutarate transport because of its structural similarity to α-oxoglutarate. The keto group appears to be important for its specificity since neither phthalic acid nor homophthalic acid showed any preference for the α-oxoglutarate translocator and inhibited the dicarboxylate, tricarboxylate and α-oxoglutarate translocators equally (results not shown).

With regard to its applicability in metabolic studies using whole cells phthalonic acid is probably of limited value. Using isolated rat liver cells we have tested the inhibitor in various metabolic systems in order to elucidate the role of α-oxoglutarate transport across the mitochondrial membrane in cellular processes. However, it was found that processes dependent on the activity of lactate dehydrogenase (EC 1.1.1.27) were all inhibited by phthalonic acid; direct inhibition of lactate dehydrogenase appeared to be responsible for this (2 mM phthalonate inhibited lactate dehydrogenase from rat liver in the presence of 10 mM lactate by 80 % when assayed at pH 8.4). Furthermore, phthalonic acid appeared to be a rather potent inhibitor of phosphoenolpyruvate carboxylase (EC 4.1.1.32). This was concluded from a series of experiments in which it was found that gluconeogenesis from glutamine, alanine and proline, but not from dihydroxyacetone or glycerol, was inhibited by phthalonic acid. This conclusion was confirmed by testing its effect on the activity of phosphoenolpyruvate carboxylase purified from rat liver (Jolaim-Baum, M. and Hanson, R. W., personal communication).

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