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The Inhibition of Mitochondrial Dicarboxylate Transport by Inorganic Phosphate, some Phosphate Esters and some Phosphonate Compounds

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1. P_i competitively inhibited succinate oxidation by intact uncoupled mitochondria in the presence of sufficient *N*-ethylmaleimide to block the phosphate carrier, with a K_i of 2.5mM. 2. Of a large number of phosphate esters and phosphonate compounds, phenyl phosphate and phenylphosphonate were found to inhibit competitively uncoupled succinate oxidation by intact but not broken mitochondria. By comparison, benzoate was a relatively weak competitive inhibitor of succinate oxidation by intact mitochondria but a relatively potent inhibitor of succinate dehydrogenase. 3. Phenyl phosphate and phenylphosphonate were non-penetrant, and inhibited P_i-dependent swelling of mitochondria suspended in isosmolar ammonium malate in a manner non-competitive with P_i. The inhibitors did not affect mitochondrial swelling when tested with P_i alone. 4. It is concluded that: (i) phenyl phosphate and phenylphosphonate behaved as non-penetrant analogues of P_i, since their inhibitory properties were in strict contrast with those of benzoate; (ii) phenyl phosphate and phenylphosphonate interacted with the dicarboxylate carrier but not with the phosphate carrier; (iii) P_i was effective as a competitive inhibitor of succinate oxidation because of its being either an alternative substrate for the dicarboxylate carrier or competitive with succinate for the intramitochondrial cations as proposed by Harris & Manger (1968).

Mitochondria from mammalian tissues are believed to possess at least seven anion-transporting systems (Chappell, 1968; Klingenberg, 1970) and recent evidence (Gamble & Lehninger, 1973; Crompton & Chappell, 1973) suggests that in particular instances the number may be greater. With regard to the dicarboxylate carrier, Chappell & Haarhoff (1967), using the ammonium swelling technique, found that catalytic amounts of added P_i were required for mitochondria to swell in isosmolar ammonium succinate or ammonium malate. As a result they proposed that the added P_i has to enter the mitochondria on the phosphate carrier (Chappell & Crofts, 1966; Tyler, 1969) before leaving the mitochondria on the dicarboxylate carrier in exchange for a dicarboxylate ion. The proposal of Chappell & Haarhoff (1967) received support from the work of Fonyo & Bessman (1968) and Tyler (1969), who showed that inhibitors of phosphate transport inhibit dicarboxylate transport as assayed by the ammonium swelling technique, indicating the need for the externally added P_i to enter the mitochondrial matrix.

However, there are further consequences of the proposal of Chappell & Haarhoff (1967), which should be capable of demonstration if their mechanism is correct. Two of these are as follows: that mitochondria can transport P_i, independent of the phosphate carrier, by exchange with penetrant dicarboxylic acids; secondly, that P_i acts as a competitive inhibitor of dicarboxylate transport. The exchange of P_i and other anions has been investigated (Johnson & Chappell, 1970, 1973) and complete agreement with the scheme of Chappell & Haarhoff (1967) was found. The evidence relating to the inhibition of dicarboxylate transport by P_i (Harris & Manger, 1968) and other anions (Quagliariello & Palmieri, 1968) is open to more than one interpretation, however. Both Harris & Manger (1968) and Quagliariello & Palmieri (1968) preferred the thesis that P_i and substrate anions were competing for the internal positive charge of the mitochondrial matrix. It seems equally likely that the competition was for a site on the same carrier, or a consequence of exchange lowering the concentration of dicarboxylate ion in the mitochondrial matrix.

In the present work an attempt has been made to limit the action of P_i to the dicarboxylate carrier by doing experiments in the presence of an inhibitor of the phosphate carrier. Secondly, the action of certain non-penetrant compounds which are potentially analogues of P_i has been examined with regard to dicarboxylate transport. In this way, action at the level of the membrane is discriminated from competition for the intramitochondrial cations.

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Experimental

Methods

Mitochondrial respiration. Mitochondrial respiration was monitored polarographically by using a Clarke-type oxygen electrode (Chappell, 1961). The polarizing voltage was supplied by a commercial polarograph (Polariter PO 4; Radiometer, Copenhagen, Denmark) which had its own recorder. The experimental cell was thermostatically controlled by a water jacket maintained at 25°C.

Assay of succinate dehydrogenase activity. Succinate dehydrogenase was assayed in an oxygen electrode by using phenazine methosulphate (Bernarth & Singer, 1962). The mitochondrial suspension was treated with 0.05% Triton X-100.

The preparation of mitochondria and the assay of mitochondrial volume changes and mitochondrial protein were carried out as described by Johnson & Chappell (1973).

Materials

The following were kindly given: phenyl phosphate, phenylphosphonate and *p*-nitrophenyl phosphate by Dr. S. E. Halford; aminomethylenephosphonate by Dr. D. R. Trentham; hydroxymethylenephosphonate by Dr. S. I. Chavin, after its synthesis from the amino compound; carbonyl cyanide *m*-chlorophenylhydrazone by Dr. P. G. Heytler. Most biochemicals were from Boehringer Corp. (London) Ltd. (London W5 2TZ, U.K.) and *N*-ethylmaleimide was from BDH Chemicals Ltd. (Poole, Dorset, U.K.). All other chemicals were of A.R. grade or the highest grade obtainable.

Results

Inhibition of succinate oxidation by P_i

Initially, the inhibition by P_i of succinate oxidation was examined. The incubation mixtures included *N*-ethylmaleimide at a concentration equivalent to 20nmol/mg of mitochondrial protein, which is sufficient to inhibit completely the mitochondrial phosphate carrier (Johnson & Chappell, 1973), and in this way differed from the experiment of Harris & Manger (1968). The results (Fig. 1), presented in the form of a Dixon (1953) plot, showed that P_i was competitive with succinate, the K_i value for P_i being 2.5mM. This is similar to the K_i value of 3.2mM obtained by Harris & Manger (1968).

Inhibition of succinate oxidation by some phosphate esters and phosphonate compounds

A survey of some potential analogues of P_i was undertaken to gain further evidence about the nature of the P_i inhibition of succinate oxidation. The compounds chosen were mainly phosphate esters and were those most readily obtained. The effects of these compounds on the rate of mitochondrial respiration with succinate as substrate are shown in Table 1.

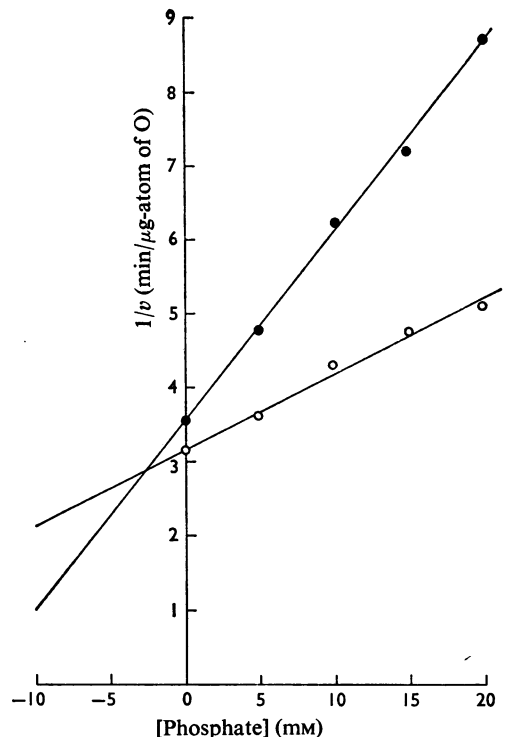

Fig. 1. *Inhibition of succinate oxidation by P_i*

Mitochondria (2mg of protein) were incubated in a medium comprising 120mM-KCl–20mM-Tris–HCl, pH 7.4, and containing 40nmol of *N*-ethylmaleimide. After 1min, 1µg of rotenone was added followed by P_i to the appropriate final concentration and either 1mM (●)- or 2mM (○)-succinate (final concentrations). Rapid respiration was initiated by the addition of carbonyl cyanide *m*-chlorophenylhydrazone to 1µM, the steady-state rate of respiration being measured to give *v*.

The phenyl derivatives appeared to be the most potent inhibitors, and the phosphate or phosphonate moiety appeared to be responsible for the inhibitory property of these molecules. The reasons for thinking that this is the case are two: (i) modification of the benzene ring as in *p*-nitrophenyl phosphate was without effect on the inhibition; (ii) replacement of the phosphate or phosphonate moiety by a carboxyl group, giving benzoate, considerably decreased the efficacy of the inhibitor.

With the substituted C_3 compounds, the presence of a phosphate group appeared to be of minor significance: glycerate was weakly effective as an inhibitor, but no effect of α-glycerol phosphate could be demonstrated at this or higher concentra-

Table 1. *Inhibition of succinate oxidation by some phosphate esters and phosphonate compounds*

Mitochondria (about 4mg of protein) were suspended in a medium comprising 120mM-KCl–20mM-Tris–HCl, pH 7.4, and containing succinate to a final concentration of 1mM and 1μg of rotenone. Rapid respiration was initiated with 1μM carbonyl cyanide *m*-chlorophenylhydrazone, and was followed by the addition of inhibitor to a final concentration of 5mM. Steady-state rates of respiration were measured.

Inhibitor	Inhibition (%)
Phenylphosphonate	53
Phenyl phosphate	63
p-Nitrophenyl phosphate	61
Benzoate	13
α-Glycerol phosphate	0
3-Phosphoglycerate	50
2,3-Diphosphoglycerate	22
Glycerate	15
Glucose 1-phosphate	0
Glucose 6-phosphate	0
Fructose 1,6-diphosphate	32
AMP	0
Aminomethylenephosphonate	0
Hydroxymethylenephosphonate	0

tions. 3-Phosphoglycerate was much the most active of this class of compound, 2,3-diphosphoglycerate being only marginally more inhibitory than the unsubstituted acid. The most probable explanation of these findings is that the dicarboxylate carrier recognizes two distinct centres of negative charge, as with malonate, a penetrant C_3 dicarboxylic acid (Chappell & Haarhoff, 1967), and that the phosphate moiety is assuming the role of one of these centres. (The carrier would appear to be the most likely site of action, since these compounds were later found to be non-penetrant.) Certainly a single carboxyl or phosphate group was of little consequence under these circumstances.

A similar argument holds for the three phosphorylated sugars, glucose 1-phosphate and glucose 6-phosphate being without effect whereas fructose 1,6-diphosphate caused a significant inhibition. AMP, aminomethylenephosphonate and hydroxymethylenephosphonate were without effect.

It should be noted that in every case where inhibition was observed, the addition of higher concentrations of succinate reversed the inhibition.

Inhibitory properties of phenyl phosphate and phenylphosphonate

The action of phenyl phosphate and phenylphosphonate was examined in greater detail, since their behaviour could not be explained on the basis of their structural similarity to known penetrant dicarboxylic acids. That these compounds were non-penetrant with respect to the mitochondrial inner membrane was shown by suspending mitochondria in isosmotic solutions of potassium phenylphosphonate and potassium phenyl phosphate. After addition of the antibiotic monensin, which promotes a K^+/H^+ exchange (Henderson *et al.*, 1969), or monensin plus an uncoupling agent, there was no change in light-scattering. This finding indicates that the mitochondria were osmotically stable in these solutions and that the phenyl phosphate and phenylphosphonate anions were unable to enter the matrix space by either an electrically neutral or an electrogenic mechanism. Addition of catalytic amounts (2mM) of P_i and malate in the presence of monensin was also without effect, demonstrating that the compounds were not transported by either the dicarboxylate or tricarboxylate carriers (Chappell & Haarhoff, 1967).

Table 2. *Inhibition of respiration and succinate dehydrogenase by phenylphosphonate, phenyl phosphate and benzoate*

All activities were monitored in a medium comprising 120mM-KCl–20mM-Tris–HCl, pH 7.4. Succinate oxidation and succinate dehydrogenase were monitored under equivalent conditions by using 1mM-succinate; the final concentration of β-hydroxybutyrate was 10mM. Intact mitochondria were uncoupled by 1μM-carbonyl cyanide *m*-chlorophenylhydrazone and the inhibitors were present at a final concentration of 10mM. About 4mg of mitochondrial protein was present in each incubation.

System	Inhibition (%) by		
	Phenylphosphonate	Phenyl phosphate	Benzoate
Succinate oxidation	75	75	25
β-Hydroxybutyrate oxidation	10	0	40
Succinate dehydrogenase	15	10	40

Further evidence that the phenyl derivatives were acting at the level of the membrane came from the following observations, which are summarized in Table 2: uncoupled respiration with β-hydroxybutyrate as substrate was relatively unaffected by their presence, indicating that an inhibition of electron transport was unlikely to be responsible for the inhibition of succinate oxidation; secondly, succinate dehydrogenase assayed in mitochondria treated with detergent showed only a small percentage inhibition, again insufficient to account for the effect in the intact system. Table 2 also shows the effect of benzoate, which had a relatively great effect on β-hydroxybutyrate oxidation and succinate dehydrogenase but a relatively minor effect on the oxidation of succinate by intact mitochondria.

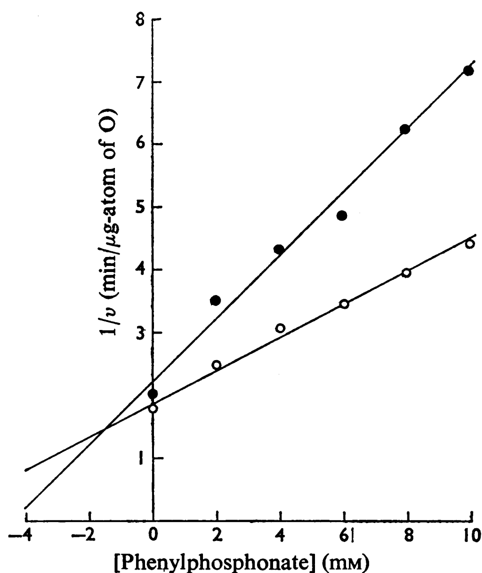

Fig. 2. *Inhibition of succinate oxidation by phenylphosphonate*

Mitochondria (about 4mg of protein) were incubated in a medium comprising 120mM-KCl–20mM-Tris–HCl, pH7.4, and containing 1μg of rotenone and either 1mM(●)- or 2mM(○)-succinate (final concentrations). Phenylphosphonate was added to the appropriate final concentration and rapid respiration was initiated by 1μM-carbonyl cyanide *m*-chlorophenylhydrazone. The steady-state rate of respiration was measured to give v.

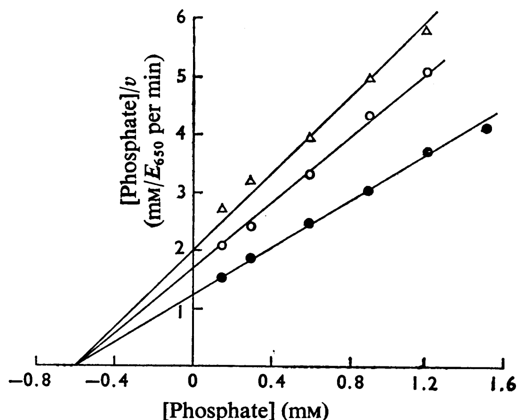

Fig. 3. *Inhibition of P_i-stimulated swelling of mitochondria in ammonium malate by phenyl phosphate and phenylphosphonate*

Mitochondria (about 4mg of protein) were suspended in a medium consisting of 2vol. of 100mM-ammonium malate and 1vol. of 145mM-Tris–HCl (●), or 2vol. of 100mM-ammonium malate and 1vol. of 100mM-Tris–phenylphosphonate (○), or 2vol. of 100mM-ammonium malate and 1vol. of 100mM-Tris–phenyl phosphate (△). In every case, 1μg of rotenone was present and the final pH was 7.4. Swelling was initiated by the addition of P_i to the appropriate final concentration and the initial rate of swelling was measured to give v.

Kinetic plots showed that phenyl phosphate and phenylphosphonate inhibited succinate oxidation competitively, giving K_i values of 1mM and 1.5mM respectively. Fig. 2 shows the data for phenylphosphonate. Benzoate gave a comparatively high K_i value under the same conditions, being estimated as 8mM.

Preliminary observations with P_i-stimulated swelling of mitochondria in isosmolar ammonium malate showed that both phenyl phosphate and phenylphosphonate were inhibitors. However, when the concentration of P_i was varied the degree of inhibition remained constant, indicating that the inhibition was non-competitive with respect to P_i. This was confirmed in an experiment in which high concentrations of inhibitors were used, Cl^- taking their place in the control, with increasing amounts of P_i to stimulate swelling. The results (Fig. 3) indicated that the inhibitors were non-competitive with respect to P_i, since the lines intersect at the abscissa, the point of intersection giving the K_m value for P_i in the ammonium malate swelling system as 0.6mM. However, it is possible that the inhibitors were apparently acting non-competitively in this system owing to a competitive effect at both the phosphate carrier and the dicarboxylate carrier. Competition at the level of the phosphate carrier was investigated by suspending mitochondria in isosmolar NH_4Cl and adding 1mM-P_i in the presence and the absence of 10mM inhibitor. Light-scattering changes were monitored in a split-beam spectrophotometer with mitochondria suspended in isosmolar NH_4Cl as a reference. No inhibitory effect was observed on the rapid low-amplitude swelling after the addition of P_i.

Discussion

The results presented in the present paper indicate that phenyl phosphate and phenylphosphonate are non-penetrant competitive inhibitors of the mitochondrial dicarboxylate carrier. The evidence includes the observations that the swelling of mitochondria in isosmolar ammonium malate and the oxidation of succinate by intact mitochondria are inhibited by these compounds, whereas succinate oxidation by broken mitochondria is relatively unaffected. In the ammonium malate swelling system it was also demonstrated that these inhibitors had a non-competitive action with respect to P_i, and indepen-

dently it was shown that they had no effect on the swelling of mitochondria in ammonium phosphate. It is concluded that they have no effect on the mitochondrial phosphate carrier. That they are non-penetrant was shown by the stability of mitochondria in isosmolar solutions of their K^+ salts in the presence of monensin, with and without uncoupling agent, and catalytic amounts of P_i and malate.

As a first approximation, the inhibitors appear to act in a manner similar to that of 2-*n*-butylmalonate (Robinson & Chappell, 1967) and 2-phenylsuccinate (Chappell & Robinson, 1968). However, unlike these substituted dicarboxylic acids, the evidence presented here suggests that phenyl phosphate and phenylphosphonate owe their effectiveness to their structural similarity to P_i, an alternative substrate of the dicarboxylic carrier (Chappell & Haarhoff, 1967; Johnson & Chappell, 1970, 1973). This proposal is supported by the finding that replacement of the phosphate or phosphonate moiety by a carboxyl group to give benzoate decreased the degree of inhibition of succinate oxidation in intact mitochondria and increased the inhibition in broken mitochondria.

P_i itself was shown to inhibit competitively succinate oxidation in intact mitochondria, with a K_i of 2.5mM under conditions in which the transport of P_i on the phosphate carrier was prevented by *N*-ethylmaleimide. In view of the evidence from the non-penetrant inhibitors, it seems most probable that the inhibitory action of P_i was at the level of the dicarboxylic carrier. However, Harris & Manger (1968) found a K_i value for P_i of 3.2mM with respect to succinate oxidation in the absence of an inhibitor of phosphate transport, and ascribed to this effect a competition for the intramitochondrial cations. Although their interpretation cannot be denied, in view of the similar K_i values in the presence and the absence of *N*-ethylmaleimide, it seems likely that competition for the dicarboxylate carrier is a significant factor. Alternatively, the P_i inhibition might arise from a lowering of the intramitochondrial succinate concentration as a result of an exchange catalysed by the dicarboxylate carrier.

There is some evidence to suggest that the dicarboxylate carrier has separate binding sites for P_i and dicarboxylate ions (Johnson, 1972). However, 2-*n*-butylmalonate, which presumably interacts with the dicarboxylate-binding site, prevents the exchange between internal and external P_i catalysed by the dicarboxylate carrier (Johnson & Chappell, 1970, 1973). Phenyl phosphate and phenylphosphonate show complementary behaviour in inhibiting succinate oxidation when the exchange involves only dicarboxylate ions, succinate and malate (see Chappell & Haarhoff, 1967). A possible explanation concerning the mechanism of action of all these non-penetrant inhibitors is that they bind to the carrier when it is available at the external face of the inner membrane, thereby making it unavailable for transport. In this way, such an inhibitor interacting with either binding site is effective in preventing the transport of both types of anion.

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