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Effects of 2-Butylmalonate, 2-Phenylsuccinate, Benzylmalonate, and *p*-Iodobenzylmalonate on the Oxidation of Substrates by Mung Bean Mitochondria[1]

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ABSTRACT

The effect of inhibitors of carboxylic acid anion transport on the oxidation of substrates by mung bean (*Phaseolus aureus*) mitochondria was investigated. The oxidation of malate in the presence of either glutamate or cysteine sulfinate was inhibited by 2-butylmalonate, 2-phenylsuccinate, benzylmalonate, and *p*-iodobenzylmalonate in both intact and broken mitochondria. The oxidation of succinate, on the other hand, was inhibited in intact but not in broken mitochondria. The oxidation of reduced nicotinamide adenine dinucleotide was inhibited only by *p*-iodobenzylmalonate. This inhibition occurred only in coupled mitochondria and could be reversed by the addition of adenosine diphosphate.

These results suggest that the compounds studied inhibit the entry of succinate and probably also of malate into mung bean mitochondria. All four compounds inhibit the enzyme(s) which oxidize malate while *p*-iodobenzylmalonate also interferes with oxidative phosphorylation.

The entry of the dicarboxylic acids, succinate and malate, into animal mitochondria is inhibited by certain analogues of these intermediates, *e.g.*, 2-butylmalonate, 2-phenylsuccinate, benzylmalonate, *p*-iodobenzylmalonate (1, 2, 9–12). This, along with other lines of evidence, has led to the belief that the entry of these intermediates is mediated by a carrier system. As yet, few investigations have been made on the carboxylic acid transport systems of plant mitochondria (8).

The present study compares the effect of the above mentioned transport inhibitors on the oxidation of malate and succinate and exogenous NADH.

MATERIALS AND METHODS

Mitochondria were isolated from 7-day-old etiolated mung bean (*Phaseolus aureus*) stems by homogenizing at 4 C in either a mortar and pestle or a Braun juice extractor. The homogenizing medium consisted of 0.3 M mannitol, 0.07 M sucrose, 0.1% (w/v) bovine serum albumin, 3.2 mM cysteine, 1.0 mM EGTA,[2] and 10 mM tris adjusted to pH 7.8 with HCl. The tissue to medium ratio was 1:2 (w/v) and the final pH 7.2. The homogenate was filtered through several layers of cheesecloth and centrifuged according to the scheme of Ikuma and Bonner (5) with one extra centrifugation at 6000*g*. The wash medium was the same as the homogenizing medium except that cystein was omitted and the concentration of EGTA was reduced to 0.1 mM. Oxygen consumption was measured with a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, Ohio) and recorded on a Bausch and Lomb recorder. The measurements were carried out in a 1.5 ml water-jacketed vessel at 25 C stirred with a magnetic stirrer. The reaction medium was the same as the wash medium with 5.0 mM potassium phosphate, 5.0 mM $MgCl_2$, 10 mM KCl, and other additions as described in the legends to the figures and tables. Protein was measured according to the method of Lowry *et al.* (6).

Table I. *Respiratory Characteristics of Mung Bean Mitochondria*
Aliquots of the mitochondrial preparations containing about 0.5 mg of protein were incubated in 1.5 ml of reaction medium as described in "Materials and Methods" with substrates: succinate (0–5 mM), malate (0–10 mM) in the presence of 10 mM glutamate, or NADH (0–0167 mM). ADP (130 μM) was added to determine the state 3 rate of oxygen uptake. Oxygen consumption was measured at 25 C as described in "Materials and Methods."

Substrate	Q_{O_2}	Respiratory Control	ADP/O	Apparent *Km*
	nmoles O_2/min·mg N	*ratio*		*mM*
Succinate	741	2–3	1.3–1.9	0.35
Malate + glutamate	510	3–10	2.3–2.7	0.6
NADH	811	2–4	1.2–1.7	0.05

Broken mitochondria were prepared either by incubation for 5 min with 1.1% Triton X-100 or by sonication for 5 min with an MSE Model 60W ultrasonic disintegrator. Oxygen consumption was measured as described above for intact mitochondria but with the addition of an artificial electron acceptor, PMS which is required because components of the electron transport chain are lost when the mitochondria are ruptured.

[1] Financial support was from the Medical Research Council of Canada.

[2] Abbreviations: EGTA: ethyleneglycol-bis-(β-amino-ethylether)-N,N'-tetraacetic acid; IBM: *p*-iodobenzylmalonate; F_3CCP: carbonyl cyanide-*p*-trifluromethoxyphenylhydrazone; PMS: phenazine methosulfate.

Table II. *Effect of Transport Inhibitors on Oxidation of Malate and NADH by Mung Bean Mitochondria*

Aliquots of mitochondrial preparations (about 1 mg protein) were incubated in the oxygen electrode vessel as described for Table I. Order of additions to the vessel was: zero time, mitochondria; 1 min, ADP (250 nmoles); 2 min, malate (2.5 mM)/glutamate (5.0 mM) or NADH (1.6 mM); 3 min, inhibitor. Control rates: malate/glutamate: 57.1 ± 4.2; NADH: 195 ± 16 nmoles O_2/min.

Inhibitor	Inhibitor Concentration	Inhibitor of	
		Malate oxidation	NADH oxidation
	mM	%	
2-Butylmalonate	6.7	36	0
	16.7	57	0
	33.2	68	0
Phenylsuccinate	12.5	45	0
	25.0	51	0
Benzylmalonate	12.5	36	0
	18.8	36	0
p-Iodobenzylmalonate	2.0	17	33
	5.0	65	41
	10.0	68	43

Table III. *Effect of Transport Inhibitors on Oxidation of Malate by Intact and Broken Mung Bean Mitochondria*

Aliquots of mitochondrial preparations (about 1 mg of protein) were incubated in the oxygen electrode vessel as described for Table I. Order of additions to the vessel were: zero time, mitochondria; 2 min, malate (5.0 mM) plus cysteine sulfinate (5.0 mM); 3 min, F_3CCP (0.67 mM); 4 min, inhibitor. For the broken preparations, (0.1%) Triton was included in the incubation medium and PMS (0.1 mg/ml) and NAD (0.067 mM) were added at 1 min; other additions were as described for intact preparations. Mitochondria were also broken by sonication as described in "Materials and Methods." The sonicate was centrifuged at 100,000*g* for 60 min, and malate oxidation was measured in the supernatant by monitoring NAD reduction at 340 nm. The incubation medium contained 20 mM tris buffer, pH 7.2, to a final volume of 1 ml; other additions were as for the Triton-treated preparations. Control rates: intact, 64 ± 5.7 nmoles O_2/min; Triton, 56 ± 3.2 nmoles O_2/min; sonicated, 0.147 ± 0.017 (ΔOD/2 min).

Inhibitor	Inhibitor Concentration	Inhibition in		
		Intact	Triton-treated	Sonicated
	mM	%		
2-Butylmalonate	5	...	14	0
	10	50	36	13
	15	...	...	50
	20	75	...	...
Phenylsuccinate	5	24	20	57
	10	35	36	64
	15	...	...	72
Benzylmalonate	5	27	...	32
	10	42	32	47
	15	...	...	52
	20	...	...	69
p-Iodobenzylmalonate	2.5	58	36	40
	5.0	83	64	50
	9.0	...	...	67

RESULTS AND DISCUSSION

Properties of Mung Bean Mitochondria. Five hundred grams of mung bean stems yielded from 15 to 30 mg of protein in the mitochondrial fraction. Electron micrographs revealed that this fraction contained mostly well preserved mitochondria with some contamination by unidentified vesicular structures, possibly plastids. Table I summarizes the respiratory characteristics of these mitochondria which compare favorably with those reported by Ikuma and Bonner (5).

Effect of Transport Inhibitors on the Oxidation of Malate and NADH. Malate must penetrate the inner membrane of the mitochondria in order to be oxidized in the matrix (W. D. Bonner, personal communication). On the other hand, it has been suggested that exogenous NADH may be oxidized without penetrating the inner membrane presumably by an NADH dehydrogenase located in the outer surface of the inner membrane (4) as found for yeast mitochondria (13). Hence, compounds which inhibit dicarboxylic acid transport should inhibit oxygen consumption when malate is a substrate but should not inhibit when NADH is the substrate.

Table II shows that three of the inhibitors, 2-butylmalonate, 2-phenylsuccinate, and benzylmalonate behave as predicted. Malate oxidation was inhibited more than 50% by 2-butylmalonate and 2-phenylsuccinate and to a lesser extent by benzylmalonate. NADH oxidation was not affected.

IBM, on the other hand, inhibited both malate and NADH oxidation. Clearly, the discrepancy between the effects of the first three inhibitors on the one hand and of IBM on the other indicates a more general role for this latter inhibitor than on carboxylic acid transport.

Effect of Transport Inhibitors on the Oxidation of Malate and Succinate in Intact and Broken Mitochondria. In the experiments described above, the transport inhibitors may be preventing the entry of substrate or inhibiting the enzymes

Table IV. *Effect of Transport Inhibitors on Oxidation of Succinate by Intact and Broken Mung Bean Mitochondria*

Aliquots of mitochondrial preparations (about 1 mg of protein) were incubated in the oxygen electrode vessel as described for Table I. Order of additions to the vessel was: zero time, mitochondria; 2 min, succinate (5.0 mM); 3 min, FCCP (0.67 mM), 4 min, inhibitor. For the broken preparations, Triton (0.1%) was included in the incubation medium and PMS (0.1 mg/ml) was added at 1 min; other additions as described for intact preparations. Control rates: intact, 78.6 ± 5.1; Triton, 52.0 ± 4.1 nmoles O_2/min.

Inhibitor	Inhibitor Concentration	Inhibition in	
		Intact	Triton-treated
	mM	%	
2-Butylmalonate	5		0
	10	50	0
	20	75	10
Phenylsuccinate	5	50	0
	10	67	0
	15	75	0
Benzylmalonate	5	50	0
	10	67	0
	15	67	0
	20		0
p-Iodobenzylmalonate	2.5	61	0
	5.0	83	0
	9.0		9

responsible for the oxidation of substrate or both. If the entry of substrate is the only process affected, then substrate oxidation should be inhibited in intact, but not in broken mitochondria.

Table III shows that malate oxidation is inhibited in both intact and broken mitochondria by all four inhibitors. Therefore, these compounds are able to inhibit the enzymes responsible for malate oxidation and nothing can be concluded about their effect on the entry of malate into the mitochondria.

Table IV shows that succinate oxidation is inhibited to a considerable extent by all four inhibitors in intact mitochondria but hardly at all in the broken preparations. In this case, it is clear that these compounds are preventing succinate from reaching the succinate dehydrogenase and provides further evidence for the location of this enzyme on the inside of the inner membrane (4). This suggests that a dicarboxylate transporting system is present in these mitochondria. This conclusion has been confirmed by ammonium swelling experiments similar to those of Chappell and Haarhoff (3), the details of which will be reported elsewhere.

Reversal of IBM Inhibition. The inhibitory effect of IBM on NADH oxidation shown in Table II requires further investigation. Robinson and Williams (11) showed that IBM was a competitive inhibitor of malate transport in rat liver mitochondria.

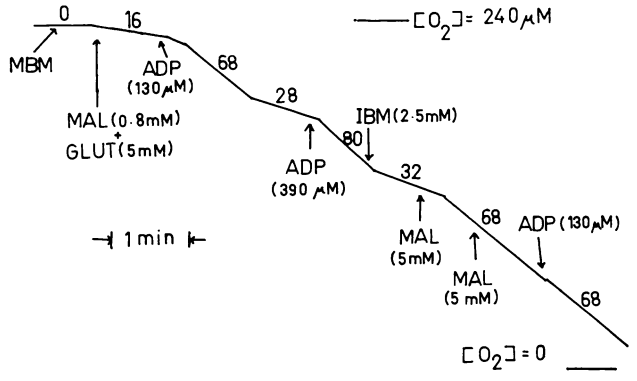

FIG. 1. An O_2 electrode trace showing inhibition of malate/glutamate oxidation by IBM and reversal by additional malate. Numbers along the trace are rates of O_2 consumption expressed as nmoles O_2/min. MBM (mung bean mitochondria): addition of 1 mg of mitochondrial protein. Reaction medium was as described in "Materials and Methods."

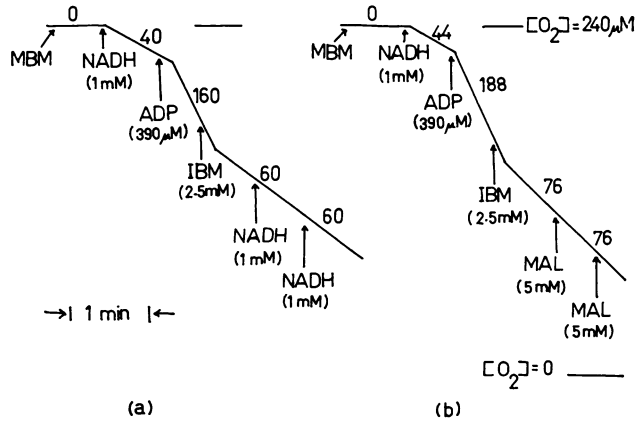

FIG. 2. Oxygen electrode traces showing inhibition of NADH oxidation by IBM and lack of reversal by (a) additional NADH or (b) additional malate. Other conditions as for Figure 1.

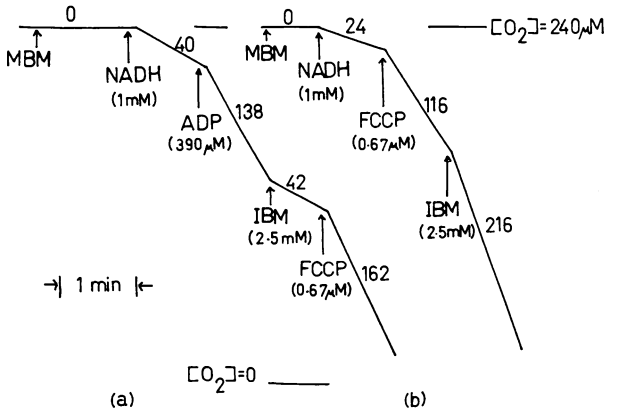

FIG. 3. Oxygen electrode traces showing (a) inhibition of ADP-stimulated NADH oxidation by IBM and reversal by F_3CCP; and (b) enhancement of F_3CCP-stimulated NADH oxidation by IBM. Other conditions are the same as for Figure 1.

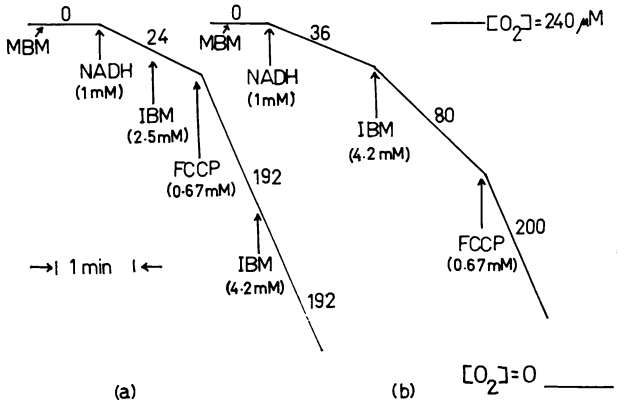

FIG. 4. Oxygen electrode traces showing the effects of IBM alone or in the presence of F_3CCP on NADH oxidation: (a) 2.5 mM IBM added before and 4.2 mM IBM added after F_3CCP; (b) 4.2 mM IBM added before F_3CCP. Other conditions are the same as for Figure 1.

Therefore, the inhibition could be reversed by adding more malate.

Figure 1 shows that the inhibition of malate oxidation by mung bean mitochondria could be largely overcome by additional malate. The inhibition of NADH oxidation, conversely, could not be reversed by either additional NADH (Fig. 2a) or additional malate (Fig. 2b).

Three possible sites of inhibition of NADH oxidation by IBM are (*a*) transport of NADH into mitochondria, (*b*) inhibition of the enzyme responsible for NADH oxidation, or (*c*) interference with phosphorylation since the inhibitions were observed with coupled mitochondria in the presence of ADP and P_i.

The third possibility could be tested by determining the effect of an uncoupler on the IBM-inhibited oxidation of NADH. Figure 3a shows that F_3CCP released the inhibition due to IBM and in fact stimulated the rate of oxidation over the state 3 rate. Figure 3b shows that IBM (2.5 mM), when added after F_3CCP, stimulated the uncoupled rate of oxidation. Figure 4, a and b, shows that at a concentration of 2.5 mM, IBM by itself had no effect, but at a higher concentration (4.2 mM), it stimulated the rate although not to the same extent as the combination of F_3CCP and IBM. IBM, therefore, does not inhibit NADH transport or oxidation but rather interferes with the

processes of phosphorylation. In addition, it appears to act as a weak uncoupler at higher concentrations.

Effect of IBM on NADH Oxidation in Phosphorylating Mitochondria. Figure 5 shows that addition of excess ADP can largely reverse the inhibition of coupled NADH oxidation by IBM. Figure 6 shows the relationship between IBM concentration and percentage of inhibition of the rate of NADH oxidation at different concentrations of ADP. It is clear that the higher the ADP concentration the higher the IBM concentration necessary for maximal inhibition.

IBM could be inhibiting adenine nucleotide transport (like atractyloside) or preventing the interaction of phosphate with the respiratory chain phosphorylating system at the enzymatic level (like oligomycin). In the first case arsenate-stimulated respiration should be inhibited only in the presence of added ADP; in the second instance arsenate-stimulated respiration should be inhibited in both the presence and absence of added ADP (1). Figure 7 shows that IBM inhibited arsenate-stimulated respiration only in the absence of ADP, *i.e.*, exogenous ADP can overcome the inhibition. Hence, IBM does not behave like either atractyloside or oligomycin. Detailed speculation concerning the molecular interaction of IBM would be premature. Robinson *et al* (12), in discussing the action of this and related compounds on the citrate transport system of mammalian mitochondria, point out that aryl halides are unlikely to bind in a covalent manner, *e.g.*, by sulfhydryl substitution. Further work is necessary to determine precisely how IBM interferes with phosphorylation.

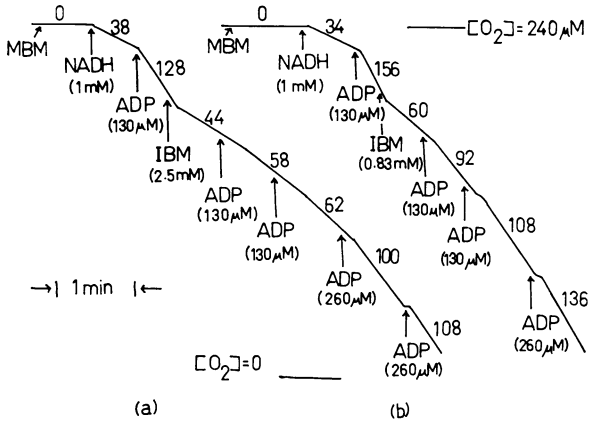

FIG. 5. Oxygen electrode traces showing the effect of ADP on the IBM-inhibited rate of NADH oxidation (a) 2.5 mM IBM and (b) 0.83 mM IBM.

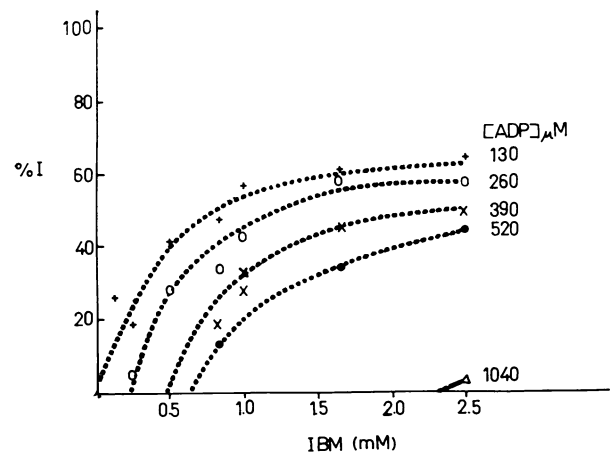

FIG. 6. The percentage of inhibition of NADH oxidation by concentrations of IBM from 0 to 2.5 mM in the presence of concentrations of ADP from 130 to 1040 μM.

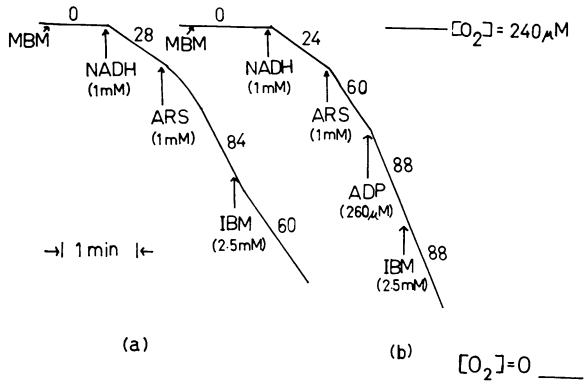

FIG. 7. Oxygen electrode traces showing the effect of IBM on arsenate stimulated NADH oxidation: (a) in the absence of ADP and (b) in the presence of ADP.

CONCLUSION

Butylmalonate, phenylsuccinate, benzylmalonate, and *p*-iodobenzylmalonate inhibit the entry of succinate into mung bean mitochondria. They probably also inhibit the entry of malate into these mitochondria but this phenomenon was obscured by the fact that they also inhibit the enzymes which oxidize malate. Iodobenzylmalonate is much less specific than the other three compounds, since it also interferes with oxidative phosphorylation. It is not clear whether this effect is on the transport of adenine nucleotides or on the phosphorylating enzymes. At high concentrations it also acts as a weak uncoupler.

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