THE INHIBITION OF MALATE, TRICARBOXYLATE AND OXOGLUTARATE ENTRY INTO MITOCHONDRIA BY 2-n-BUTYLMALONATE

B.H. Robinson and J.B. Chappell

Department of Biochemistry, Medical School, University of Bristol, England

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By following the swelling of mitochondria suspended in isotonic solutions of the ammonium salts of various anions and by measuring the reduction of intramitochondrial nicotinamide nucleotides in substrate-depleted mitochondria it was found that L-malate was required in catalytic quantities for the entry of citrate, cis-aconitate and isocitrate into mitochondria (Chappell, 1964, 1966; Chappell & Haarhoff, 1967). In this paper results are presented showing that in addition to L-malate, 2-hydroxymalonate (see Ferguson & Williams, 1966) and 2-hydroxy-2-methyl-malonate will activate the entry of tricarboxylic acids. It is also shown that 2-oxoglutarate entry is activated by L-malate (see Meijer & Tager, 1966) but the structural requirements for the activator are different in that malonate, maleate, meso-tartrate and succinate will replace L-malate, whereas the hydroxy-substituted malonates are relatively inactive. Both these entry systems are markedly inhibited in a competitive manner by 2-n-butylmalonate. This compound also inhibits the entry of malate into mitochondria. It is suggested that butylmalonate acts in all three systems by preventing the entry of malate.

METHODS AND MATERIALS. Mitochondria were prepared by conventional techniques in a medium containing 0.25M-sucrose, 3.4mM-tris chloride and 1mM-ethylene-glycol-bis-(aminoethyl) tetracetate (EGTA) pH 7.4. The twice-washed mitochondria were stored at 0^{o} in a concentrated

suspension (60-80 mg of protein/ml). Mitochondrial swelling was followed by light-scattering changes as described by Chappell & Crofts (1965). The reduction of intramitochondrial nicotinamide nucleotides was followed either by fluorimetry or double-beam spectrophotometry as described by Chappell & Crofts (1966).

RESULTS. If mitochondria are incubated with an uncoupling agent (e.g. trifluoromethoxy-carbonylcyanide-phenylhydrazone [FCCP]) or with ADP together with phosphate and are then treated with antimycin or rotenone, to block electron transport, the addition of isocitrate, citrate or cis-aconitate does not result in NAD(P) reduction until low concentrations of malate are added. The malate added without tricarboxylic acid does not result in significant reduction (see Fig. 1A). In Table 1 the effect

Table 1. Relative activities of various dicarboxylic acids in activating isocitrate and 2-oxoglutarate entry. All tested at 1mM.

Compound	Isocitrate Entry	Oxoglutarate Entry
L-Malate	(100)	(100)
Maleate	28	74
Malonate	0	56
Mesotartrate	17	46
Succinate	17	46
2-Methylenesuccinate (itaconate)	28	25
2-Methylmaleate (citraconate)	0	9
DL-2-Methylsuccinate	0	9
D-Malate	6	7
D-Tartrate	0	0
L-Tartrate	0	0
2-Hydroxymalonate (tartronate)	50	5
2-Hydroxy-2-methylmalonate (isomalate)	53	8

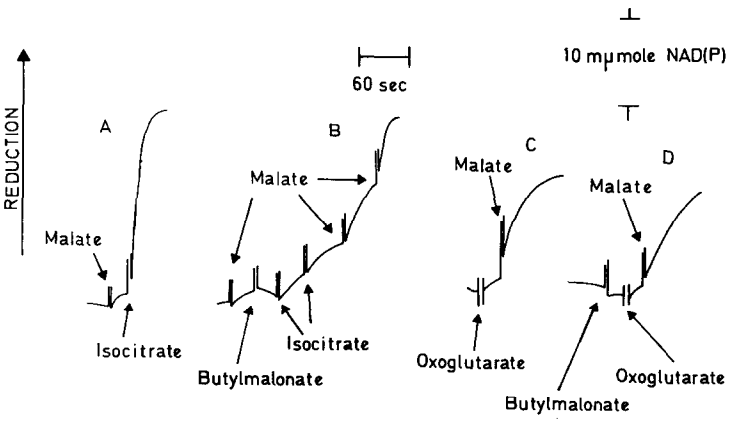

Fig. 1. The effect of 2-n-butylmalonate on the reduction of mitochondrial NAD(P) by isocitrate and oxoglutarate in the presence of L-malate. Measurements were made at 340-373 mμ with a double-beam spectrophotometer. Rat liver mitochondria (6 mg of protein) were added to a medium containing 0.12 M-KCl, 1 mM-phosphate and 20 mM-tris chloride, pH 7.4, total volume 2.5 ml. 1 μM-FCCP was added and the suspension incubated at 30° for 3 min then 0.5 μg of antimycin was added (not shown). Then additions were made at the times indicated as follows:- (A) Malate (final concentration 0.25 mM) followed by isocitrate (0.4 mM). (B) Malate (0.25 mM), butylmalonate (4 mM), isocitrate (0.4 mM), isocitrate (0.4 mM), malate (to 0.5 mM), malate (to 1.5 mM). (C) Oxoglutarate (1 mM) followed by malate (0.25 mM). (D) Butylmalonate (0.5 mM), oxoglutarate (1 mM), malate (0.25 mM). The recorder was reset after oxoglutarate addition which caused a non-specific absorbancy change.

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of replacing L-malate by various analogues is shown. 2-Hydroxymalonate and 2-hydroxy-2-methylmalonate were one-half and maleate and 2-methylenesuccinate one-quarter as effective as L-malate. In experiments in which the swelling of liver mitochondria was followed in a solution containing 84mM ammonium citrate, 2mM-phosphate and 20mM-tris chloride,

pH 7.4, the addition of L-malate was required before swelling occurred (Chappell & Haarhoff, 1967). In this case also 2-hydroxy- and 2-hydroxy-2-methylmalonate would replace L-malate, but less effectively (Fig. 2).

Malate is also required before 2-oxoglutarate will cause reduction of intramitochondrial NAD(P) (Fig. 1C). In this case however the hydroxy-substituted malonates will not replace L-malate, whereas maleate, malonate, meso-tartrate and succinate will (Table 1). It is also possible to follow oxoglutarate entry by the technique of following NAD(P) redox changes since this acid is involved in the glutamate dehydrogenase and transaminase reactions. In both these cases L-malate is required before reaction will occur (Chappell, Henderson, McGivan & Robinson, 1967). The relative effectiveness of analogues in replacing L-malate is the same in these cases.

In Fig. 2 the relative effectiveness of L-malate and 2-hydroxy-2-methylmalonate in inducing swelling of mitochondria suspended in iso-osmotic solutions of ammonium citrate and oxoglutarate is shown. 2-Hydroxy-2-methylmalonate was far more effective with citrate than with oxoglutarate.

2-n-Butylmalonate acts as an inhibitor of tricarboxylic and oxo-glutarate entry as judged from both swelling experiments and those in which intramitochondrial NAD(P) redox changes were followed. In Fig. 1B an experiment is shown in which 4mM-butylmalonate was added to a mitochondrial suspension which had been incubated with FCCP and to which antimycin and L-malate had been added. The addition of isocitrate caused little reduction of nucleotide and increasing the isocitrate concentration had little effect. However increasing the malate concentration markedly increased the rate of NAD(P) reduction. In a series of separate experiments it was shown that the K_m for L-malate was 0.16mM and the K_i for butylmalonate 0.4mM. Butylmalonate also prevents the reduction of

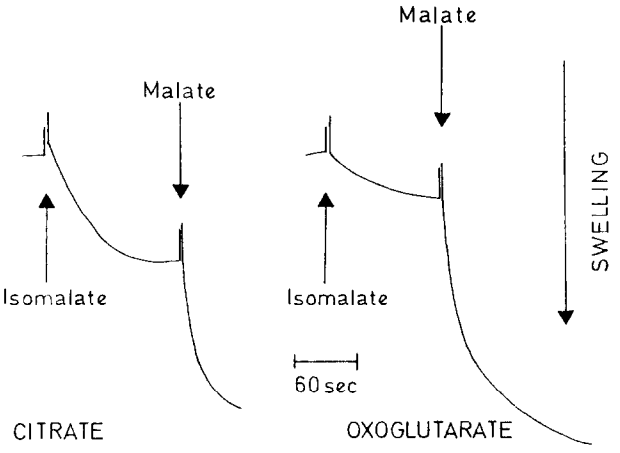

Fig. 2. Light scattering changes of liver mitochondria suspended in citrate and oxoglutarate on addition of 2-hydroxy-2-methylmalonate (isomalate) and malate. Mitochondria (5 mg of protein) were added to a medium containing either 84 mM ammonium citrate or 100 mM ammonium oxoglutarate with 2μM-rotenone, 2 mM-phosphate, 1 mM-EGTA and 20 mM-Tris-Cl. The pH was 7.4, temperature 30° and total volume 2.5 ml. Light scattering changes were followed at 620 mμ at 45° to the incident beam. Additions were made at the times indicated as follows:- isomalate (4.5 mM), malate (2 mM).

NAD(P) by citrate and cisaconitate; the inhibitor was in each case competitive with malate. In Triton-treated or sonicated mitochondria butylmalonate did not cause inhibition of aconitate hydratase or NADP-isocitrate dehydrogenase activities.

2-n-Butylmalonate also inhibited the reduction of NAD(P) by 2-oxoglutarate (Fig. 1D) and the malate-activated swelling in ammonium oxoglutarate solutions. Again the inhibition was competitive with malate. Butylmalonate also inhibited the reduction of intramitochondrial NAD(P) by malate in the presence of cysteinesulphinate (reaction malate + NAD + cysteine-sulphinate $\longrightarrow$ aspartate + $NADH_2$ + sulphinopyruvate). The effect of varying the butylmalonate concentration on NAD(P) reduction

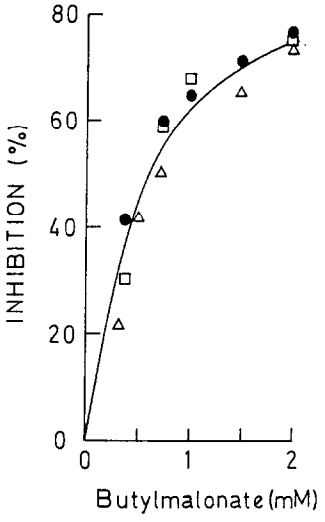

Fig. 3. The effect of varying butylmalonate concentration on the inhibition of reduction of intramitochondrial NAD(P) by isocitrate with malate (□ —— □), oxoglutarate with malate (o —— o) and malate with cysteinesulphinate (Δ —— Δ). Conditions were as described in Fig. 1. The following final concentration of reactants were used:- isocitrate, 0.4 mM; oxoglutarate, 1 mM.

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resulting from addition of (a) isocitrate with malate, (b) oxoglutarate with malate and (c) malate with cysteinesulphinate is shown in Fig. 3. In each case the malate concentration was 1.5mM.

CONCLUSIONS. Butylmalonate inhibits the entry of malate into mitochondria and prevents malate from activating the entry of tricarboxylic acids and 2-oxoglutarate. One possible model for the activating effect of malate on these last two transporting systems is that malate must first enter the mitochondrion on its own carrier before it can exchange for tricarboxylic acids or 2-oxoglutarate by exchange diffusion. The effect of butylmalonate would then be explained simply by its inhibiting malate entry.

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