BBA 43253

Nature of the stimulation by uncouplers of the efflux of α-oxoglutarate from rat-liver mitochondria

When rat-liver mitochondria oxidize glutamate in the presence of arsenite, α-oxoglutarate accumulates within the mitochondria, and the rate of oxidative deamination is limited by the rate of efflux of α-oxoglutarate[1,2] Malonate increases the rate of α-oxoglutarate efflux, and hence of glutamate deamination[1,2] Malonate promotes α-oxoglutarate efflux by an exchange–diffusion reaction (mediated by a specific translocator[2]), the stoicheiometry of which is one to one[3]. Thus the following exchange–diffusion reactions ensure a continuous removal of the α-oxoglutarate, formed during glutamate oxidation, from the intra- to the extramitochondrial compartment:

$$\alpha\text{-Oxoglutarate}^{2-}_{in} + \text{malonate}^{2-}_{out} \rightarrow \alpha\text{-oxoglutarate}^{2-}_{out} + \text{malonate}^{2-}_{in} \quad (1)$$

$$\text{Malonate}^{2-}_{in} + \text{phosphate}^{2-}_{out} \rightarrow \text{malonate}^{2-}_{out} + \text{phosphate}^{2-}_{in} \quad (2)$$

$$\text{Phosphate}^{2-}_{in} + H_2O \rightarrow \text{phosphate}^{-}_{in} + OH^-_{in} \quad (3)$$

$$\text{Phosphate}^{-}_{in} + OH^-_{out} \rightarrow \text{phosphate}^{-}_{out} + OH^-_{in} \quad (4)$$

$$\text{Phosphate}^{-}_{out} + OH^-_{out} \rightarrow \text{phosphate}^{2-}_{out} + H_2O \quad (5)$$

$$\text{Sum: } \alpha\text{-Oxoglutarate}^{2-}_{in} + 2OH^-_{out} \rightarrow \alpha\text{-oxoglutarate}^{2-}_{out} + 2OH^-_{in} \quad (6)$$

The translocators mediating Reactions 1 and 2 may utilize not only malonate, but also other dicarboxylate ions, like malate or succinate[4]. Direct evidence has recently been obtained[5] that phosphate may be transported across the mitochondrial membrane either by the dicarboxylate–phosphate translocator (Reaction 2), which is inhibited by mersalyl and butyl malonate[6], or by a phosphate–hydroxyl translocator (Reaction 4), which is sensitive to mersalyl but not to butyl malonate (ref 6; see also refs. 7–9).

Since the mechanism of glutamate transport is not known (see ref. 10), it is not possible to write down all the individual reactions involved in the sum reaction:

$$\text{Glutamate}_{out} + \tfrac{1}{2}\,O_2 \rightarrow \alpha\text{-oxoglutarate}_{out} + (NH_3)_{out} + H_2O \quad (7)$$

PAPA *et al.*[11] found that if an uncoupler is added to rat-liver mitochondria supplemented with glutamate and arsenite, an effect is observed similar to that obtained by adding malonate: α-oxoglutarate efflux increases, and the rate of oxidation of glutamate is stimulated The nature of this effect of uncouplers has now been examined further.

As observed previously by DE HAAN[12], phosphate is required in order to obtain maximal stimulation by uncouplers of α-oxoglutarate efflux. Fig. 1A shows that, in the presence of dicoumarol, increasing the concentration of phosphate up to 10 mM resulted in a progressive stimulation of glutamate oxidation (measured as α-oxoglutarate formation), and that this effect of phosphate was almost completely abolished by butylmalonate. The stimulation of glutamate oxidation by dicoumarol (in the presence of phosphate) was also inhibited by mersalyl (Fig. 1B). The phosphate requirement and the sensitivity to these two inhibitors suggested that the dicarboxylate–phosphate translocator was involved in the stimulation by dicoumarol of

α-oxoglutarate efflux Since a dicarboxylate ion is required for Reactions 1 and 2, the possibility was examined that some malate might be formed from α-oxoglutarate, in spite of the presence of arsenite. Indeed as shown in Table I, the amount of malate present in the reaction mixture was increased from 5 nmoles in the absence of dicoumarol to 51 nmoles in the presence of the uncoupler This was accompanied by a substantial decrease in the amount of α-oxoglutarate in the mitochondria and an increase in the total amount of α-oxoglutarate formed. The amount of aspartate found was not significantly affected by dicoumarol.

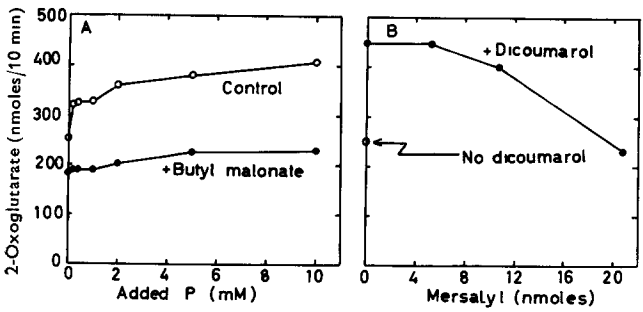

Fig 1 A Effect of phosphate and butyl malonate on the stimulation by dicoumarol of the formation of α-oxoglutarate from glutamate (+ arsenite) Rat-liver mitochondria (4 9 mg protein) were preincubated with 25 mM sucrose, 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 20 mM glucose, 50 mM Tris, 1 mM ADP, and 15 I U hexokinase, in order to deplete them of endogenous phosphate After 5 min, 1 mM arsenite, 10 mM glutamate, the concentrations of P_i indicated, 25 μM dicoumarol and (where indicated) 0 5 mM butyl malonate were added Final pH, 7 5 Temperature, 25° After 10 min the reaction was stopped with $HClO_4$, and α-oxoglutarate determined enzymically in the neutralized extracts B Effect of mersalyl on the stimulation by dicoumarol (+ phosphate) of the formation of α-oxoglutarate from glutamate (+ arsenite) Rat-liver mitochondria (8 mg protein) were incubated at 25° in a final volume of 1 ml with 25 mM sucrose, 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris, 20 mM P_i, 20 mM glucose, 1 mM ADP, 10 mM glutamate, 1 mM arsenite, 50 μM dicoumarol (where indicated) and the amount of mersalyl indicated Final pH, 7 5 Temperature, 25° Other details as in A

TABLE I

EFFECT OF DICOUMAROL ON α-OXOGLUTARATE EFFLUX AND ON THE LEVEL OF MALATE AND ASPARTATE DURING GLUTAMATE OXIDATION IN THE PRESENCE OF ARSENITE

The reaction mixture (final volume, 1 ml) contained 15 mM KCl, 2 mM EDTA, 5 mM $MgCl_2$, 50 mM Tris, 10 mM glutamate, 1 mM arsenite, 1 mM ADP, 20 mM glucose, 20 mM P_i, 15 I U hexokinase, 25 mM sucrose, and rat-liver mitochondria (5 3 mg protein) Final pH, 7 5 Temperature, 25°. After 15 min, the mitochondria were separated from the reaction mixture by centrifugation–filtration[13] and α-oxoglutarate determined in the mitochondria and the extramitochondrial fluid See refs 6, 11, 12 for other experimental details

Additions	*α-Oxoglutarate (nmoles)*			*Total malate (nmoles)*	*Total aspartate (nmoles)*
	Inside	*Outside*	*Total*		
None	26	113	139	5	80
Dicoumarol (50 μM)	13	235	248	51	92

In the experiment of Fig. 2, the effect of dicoumarol on malate formation from added α-oxoglutarate in the presence of different concentrations of arsenite was examined. In the absence of dicoumarol, 1 mM arsenite (the concentration used in the

experiments of refs 1, 2, 5 and 12 and of Fig. 1 and Table I) was sufficient to give maximal inhibition of α-oxoglutarate oxidation and hence of malate formation. Dicoumarol markedly decreased the effectiveness of arsenite in inhibiting malate formation Since it has been shown that uncouplers inhibit the accumulation of anionic substrates[14–16] and of azide[17] by mitochondria, it seems justified to conclude that the decreased effectiveness of arsenite in inhibiting α-oxoglutarate oxidation is due to an inhibition by the uncouplers of arsenite uptake.

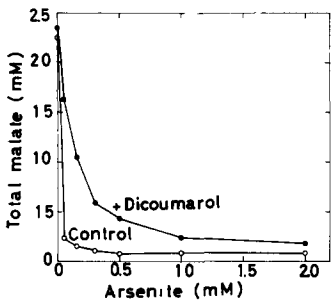

Fig 2 Effect of dicoumarol on the inhibition by arsenite of α-oxoglutarate oxidation in rat-liver mitochondria. Reaction mixture as in Table I, except that 5 4 mg mitochondrial protein were used, 20 mM α-oxoglutarate was the substrate, and the amount of arsenite was varied as indicated. Dicoumarol was used at a concentration of 50 μM. Temperature, 25° Reaction time, 10 min

The data presented show that the dicarboxylate–phosphate translocator is involved in the stimulation by uncouplers of α-oxoglutarate efflux from mitochondria; they also exclude a direct stimulatory effect of the uncouplers on the dicarboxylate–α-oxoglutarate translocator in these experiments. In the presence of dicoumarol, a small amount of malate is formed from glutamate intramitochondrially. This malate must first be transported out of the mitochondria by an exchange with extramitochondrial phosphate. An exchange of extramitochondrial malate with intramitochondrial α-oxoglutarate follows, and the cycle begins once more. The uncouplers promote P_i efflux from mitochondria[18]; this, in turn, promotes the exchange of intramitochondrial malate with extramitochondrial P_i.

This investigation was supported in part by a grant from the Consiglio Nazionale delle Ricerche, Italy to the Department of Biochemistry, University of Bari, and by grants to the Laboratory of Biochemistry, University of Amsterdam, from the Life Insurance Medical Research Fund and from the Netherlands Foundation for Chemical Research (S.O.N.) with financial assistance from the Netherlands Organization for the Advancement of Pure Research (Z.W.O). A.J.M. is grateful to the Consiglio Nazionale delle Ricerche for a Research Fellowship.

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Received August 29th, 1969

Biochim. Biophys Acta, 197 (1970) 97–100

BBA 43254

Effect of oligomygin on proton translocation in submitochondrial particles

In 1965 LEE AND ERNSTER[1] reported that in certain submitochondrial particles critical concentrations of oligomycin restore oxidative phosphorylation, and also cause an inhibition of respiration which is released by uncouplers. The nature of this effect of oligomycin is still not understood (*cf.* refs. 2 and 3). Recently PAPA *et al.*[4,5] found that chloride salts of monovalent cations stimulate respiration and uncouple oxidative phosphorylation in submitochondrial particles (see also ref. 6). Valinomycin potentiates the effect of NH_4Cl and KCl. It has also been observed that valinomycin *plus* nigericin, in the presence of K^+, release the respiratory control induced in submitochondrial particles by oligomycin[7] or dicyclohexylcarbodiimide[3]. Interestingly enough the induction of respiratory control by these substances is accompanied by an energy-linked, nigericin-facilitated, inward translocation of K^+ (refs. 3 and 8). In submitochondrial particles obtained by sonication, respiration (as well as ATP hydrolysis) is accompanied by an inward translocation of protons[9–11]. MITCHELL AND MOYLE[9] have stated that the extent of proton translocation is stimulated (about 25%) by oligomycin. In this paper, it is shown that oligomycin greatly enhances the respiration-linked uptake of protons in submitochondrial particles and depresses the rate of the passive release of protons on the exhaustion of oxygen. The results of this study give further support to the concept that the rate of electron transfer in submitochondrial particles in the absence of phosphate acceptor is controlled by an energy-linked turnover of cations and H^+ across the particle membrane[4,5,12].