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EVIDENCE FOR A PERMEABILITY BARRIER FOR α-OXOGLUTARATE IN RAT-LIVER MITOCHONDRIA

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SUMMARY

1. Investigation of the oxidation of glutamate (*plus* arsenite) in rat-liver mitochondria has provided direct evidence that a permeability barrier for α-oxoglutarate exists in rat-liver mitochondria, and that malonate increases the permeability of the mitochondria for this oxo-acid.

2. During the oxidation of glutamate (*plus* arsenite), 10–20 nmoles α-oxoglutarate per mg protein accumulate within the mitochondria, and the rate of glutamate oxidation is limited by the rate of efflux of the oxo-acid. When malonate is present in addition to glutamate (*plus* arsenite) the accumulation of α-oxoglutarate within the mitochondria is strongly decreased and this is accompanied by a 2–3-fold stimulation of the oxidation of glutamate.

3. Malonate stimulates hydrogen transfer from α-oxoglutarate or β-hydroxybutyrate to α-oxoglutarate (*plus* ammonia), the reduction of intramitochondrial $NAD(P)^+$ by α-oxoglutarate and the oxidation of intramitochondrial NAD(P)H by α-oxoglutarate (*plus* ammonia). This stimulation is considered to be due to a facilitation of the entry of the added α-oxoglutarate.

4. The effect of malonate on the systems mentioned in the former paragraph can be duplicated by L-malate but not by D-malate. It is suggested that L-malate also affects the permeability of the mitochondria.

5. Half-maximal stimulation of hydrogen transfer from α-oxoglutarate to α-oxoglutarate (*plus* ammonia), of the reduction of intramitochondrial $NAD(P)^+$ by α-oxoglutarate and of the oxidation of glutamate (*plus* arsenite) is obtained with about 0.5 mM malonate and maximal stimulation with 5 mM malonate. The corresponding values for L-malate in the first system are 0.5–1.0 mM and 2 mM.

6. The mechanism of action of malonate or L-malate is unknown. It is suggested that a specific carrier for α-oxoglutarate may exist, analogous to those proposed by CHAPPELL AND HAARHOFF for other anions.

INTRODUCTION

In 1963 it was reported that the Krebs–Cohen dismutation in rat-liver mitochondria, *i.e.* the synthesis of glutamate from α-oxoglutarate (*plus* ammonia) with

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Abbreviation: HQNO, 2-heptyl-4-hydroxyquinoline-*N*-oxide.

α-oxoglutarate as hydrogen donor, is stimulated by malonate[1] or L-malate[2,3]. KLINGENBERG[2,4,5] suggested that this stimulation is due to non-specific anion effects, caused not only by malonate or L-malate but also by P_i, by means of which glutamate dehydrogenase is enabled to react more easily with intramitochondrial NAD(P)H. This suggestion was based on his earlier finding[6,7] that certain anions, particularly L-malate, stimulated the oxidation of intramitochondrial NAD(P)H by α-oxoglutarate (*plus* ammonia). A different explanation, based on the postulate of KLINGENBERG AND SLENCZKA[8] that glutamate dehydrogenase in the mitochondrion reacts with NADP rather than with NAD, was suggested by TAGER[1]. He proposed that malonate brings about a transhydrogenation between NADH, formed during the oxidation of α-oxoglutarate (Reaction 1), and $NADP^+$, and that the NADPH formed in Reaction 2 is utilized for the reductive amination of α-oxoglutarate (Reaction 3). Additional evidence that glutamate dehydrogenase in rat-liver mitochondria is NADP-specific has recently been presented[9–12].

$$\alpha\text{-Oxoglutarate} + NAD^+ + ADP + P_i \rightarrow \text{succinate} + CO_2 + NADH + H^+ + ATP \quad (1)$$

$$NADH + NADP^+ \rightleftharpoons NAD^+ + NADPH \quad (2)$$

$$\alpha\text{-Oxoglutarate} + NH_3 + NADPH + H^+ \rightleftharpoons \text{glutamate} + NADP^+ + H_2O \quad (3)$$

However, as will be discussed in this paper, a study of the effect of malonate and L-malate on the oxido–reduction state of the nicotinamide nucleotides during the Krebs–Cohen dismutation has shown that TAGER's[1] original mechanism is no longer tenable. Further, it has been found that malonate and L-malate stimulate not only the Krebs–Cohen dismutation, but also other systems, such as the synthesis of glutamate with β-hydroxybutyrate as hydrogen donor (*cf.* refs. 5, 11). In all the systems in which malonate or L-malate stimulate, α-oxoglutarate is either a reactant or a product. Evidence will be presented in this paper that rat-liver mitochondria are relatively impermeable to α-oxoglutarate and that malonate and L-malate exert their stimulatory effect on these systems by increasing the permeability of the mitochondrion to α-oxoglutarate. Preliminary accounts of parts of this investigation have been presented[1,11,13,14].

METHODS

Preparation of mitochondria

Rat-liver mitochondria were prepared by the method of HOGEBOOM[15], exactly as described by MYERS AND SLATER[16].

Reaction conditions

The standard reaction medium contained 15 mM KCl, 5 mM $MgCl_2$, 50 mM Tris–HCl buffer, 25 mM sucrose (derived from the mitochondrial suspension), 0.1–0.5 mM ADP, 10–30 mM potassium phosphate buffer and, where present, 20–30 mM glucose and 5 I.U. hexokinase (EC 2.7.1.1). Further additions are given in the legends to the tables and the figures. The final pH was 7.5. The reaction temperature was 25°. The incubations were carried out in Warburg flasks (gas volume 6–7 ml) attached to differential manometers, except for the short-time incubations and the filtration experiments. The latter were carried out in 5-ml beakers with magnetic stirring, as

described by VAN DAM[17]. The reactions were started by the addition of mitochondria unless otherwise stated. In most cases, the incubations were terminated by the addition of 0.1 ml 35 % $HClO_4$. After removal of the protein by centrifugation, $HClO_4$ was removed in the cold as $KClO_4$ by neutralization with 1 M KOH (see ref. 12). When NAD(P)H was to be estimated, the reaction was stopped by addition of 0.5 ml 1 M KOH in ethanol. The alkaline extracts were neutralized by slow addition of a solution containing 0.5 M triethanolamine, 0.5 M HCl, 0.4 M KH_2PO_4 and 0.1 M K_2HPO_4.

Rapid filtration of the mitochondria

In some experiments the reaction was stopped by rapidly filtering the mitochondria from the incubation medium on a Millipore filter (HA 02500, pore size 0.45 $\pm$ 0.03 μ). The filter was mounted on a pyrex filter holder connected with a 100-ml vacuum flask, which contained a small tube to collect the filtrate.

To stop the reaction, 1.0 ml reaction mixture was pipetted onto the filter and the small vacuum flask was placed under vacuum by opening a 3-way stopcock connecting the flask with a larger vacuum flask (volume, 3 l) at a pressure of approx. 5 mm Hg. It took less than 5 sec for the fluid to be sucked through the filter, provided that not more than about 3 mg mitochondrial protein was applied to the filter.

The mitochondria remaining on the filter were washed twice with 2 ml ice-cold 0.25 M sucrose. The filter with the mitochondria was then placed on a second filter holder and the mitochondria were extracted by adding 1.65 ml of a mixture of 1 volume 6.4 % $HClO_4$ and 1 volume 200 mM Tris–HCl (pH 7.4). After approx. 30 sec, vacuum was carefully applied and the extraction medium was allowed to pass slowly through the filter. The mitochondrial extracts were neutralized with 1 M KOH in the cold and the precipitated $KClO_4$ was removed by centrifugation.

The filtrates and the neutralized mitochondrial extracts, representing the extra- and intramitochondrial spaces, respectively, were assayed for α-oxoglutarate. No corrections were applied for fluid adhering to the mitochondria and the filter. Control experiments were performed to test the error thereby introduced. Mitochondria were incubated in a medium containing 6.7 mM α-oxoglutarate and arsenite (to block the oxidation of α-oxoglutarate) and then filtered. The mitochondria on the filter were either extracted immediately or washed with sucrose before extraction and α-oxoglutarate was estimated in the mitochondrial extracts. It was found that after washing with sucrose once, the content of α-oxoglutarate in the mitochondrial extract decreased from 66 to 6 nmoles, indicating that in our experiments, in which the mitochondria on the filter were washed twice, the error due to adhering reaction mixture must have been less than 5 %.

In parallel incubations carried out under exactly the same conditions, the reaction was stopped with $HClO_4$ (without filtration). This allowed a determination to be made of the total amount of metabolite present (indicated as 'total' in Fig. 5).

Analytical procedures

Glutamate was determined in the earlier experiments with glutamate decarboxylase (EC 4.1.1.15) by the method of GALE[18] and, in more recent experiments, by the spectrophotometric method of BERNT AND BERGMEYER[19] using glutamate dehydrogenase (EC 1.4.1.3). Aspartate was determined by the method of PFLEIDERER,

GRUBER AND WIELAND[20] with malate dehydrogenase (EC 1.1.1.37) and aspartate transaminase (EC 2.6.1.1). α-Oxoglutarate was determined with glutamate dehydrogenase as described by SLATER AND HOLTON[21]. Small amounts of α-oxoglutarate were determined by the same method using the Aminco–Chance double-beam spectrophotometer (wavelength pair 350–375 mμ) as described earlier[12]. Ammonia was determined by a modification[22] of the method of KIRSTEN, GEREZ AND KIRSTEN[23] using glutamate dehydrogenase and ammonia-free NADH. Malate was determined spectrophotometrically with malate dehydrogenase according to HOHORST[24]. Oxaloacetate was determined spectrophotometrically as oxaloacetate *plus* pyruvate (assuming that some decarboxylation of oxaloacetate occurs[25]), with NADH and a mixture of malate dehydrogenase and lactate dehydrogenase (EC 1.1.1.27). Acetoacetate was determined according to WILLIAMSON, MELLANBY AND KREBS[26] using purified β-hydroxybutyrate dehydrogenase (EC 1.1.1.30). Esterified phosphate was determined by the enzymic method of SLATER[27]. NAD^+ and $NADP^+$ were determined in neutralized acid extracts and NADH and NADPH in neutralized alkaline extracts. In the experiments reported in Table V, a fluorimetric method[28] was used. In the other experiments, the methods of KLINGENBERG[29], as modified by VAN DAM[17], were employed, using the Aminco–Chance double-beam spectrophotometer (wavelength pair 350–375 mμ). Protein was determined by the biuret method as described by CLELAND AND SLATER[30], with egg albumin as standard.

Enzymes

Hexokinase was prepared as described by DARROW AND COLOWICK[31], omitting the final crystallization step. In some experiments hexokinase obtained from Boehringer und Söhne GmBH was used, after dialysis against 1 % glucose, 40 mM potassium phosphate (pH 7.4) and 2.5 mM EDTA to remove ammonia. Bovine plasma albumin (0.1 %) was added to the hexokinase before dialysis. Aspartate transaminase was prepared by the method of BORST AND PEETERS[32] as modified by TAGER AND SLATER[33]. β-Hydroxybutyrate dehydrogenase was prepared from *Rhodopseudomonas spheroides* according to the method of WILLIAMSON, MELLANBY AND KREBS[26].

The sources of the other enzymes were: glutamate decarboxylase, Sigma Chemical Co.; glutamate dehydrogenase (in $(NH_4)_2SO_4$ for α-oxoglutarate and NAD(P)H determinations and in glycerol for glutamate and ammonia determinations), malate dehydrogenase, lactate dehydrogenase, alcohol dehydrogenase (EC 1.1.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), Boehringer und Söhne, Mannheim.

Special reagents

The sources of special reagents were: ADP, ATP, oxaloacetate, DL-isocitrate, L-malate, D-malate, Sigma Chemical Co.; α-oxoglutarate, sodium succinate, sodium pyruvate, glucose 6-phosphate, Boehringer und Söhne; glutamate, malonate, β-hydroxybutyrate, British Drug Houses; D-malate, Calbiochem. 2-Heptyl-4-hydroxyquinoline-*N*-oxide (HQNO) was a gift from Dr. J. W. LIGHTBOWN, and oligomycin from the Upjohn Chemical Co. Acetoacetate was prepared from ethylacetoacetate by the method of HALL[34]. Ammonia-free NADH was prepared as described by DE HAAN, TAGER AND SLATER[22]. L_s-(+)-Isocitrate was prepared from *Bryophyllum tubiflorum* by the method of VICKERY AND WILSON[35].

RESULTS

Effect of malonate and L-*malate on the synthesis of glutamate from* α-*oxoglutarate and ammonia*

In Table I, the results of a series of experiments are shown in which the effect of various compounds on the Krebs–Cohen dismutation was studied. HQNO was present to block the respiratory chain, and oligomycin to inhibit respiratory-chain phosphorylation. The esterified phosphate found (Table I, last column) must, therefore, have been synthesized in the substrate-level phosphorylation connected with the oxidation of α-oxoglutarate in the Krebs–Cohen dismutation. When α-oxoglutarate was the only hydrogen donor for glutamate synthesis, for instance in the presence of malonate, the amount of esterified phosphate found was equal to the amount of glutamate synthesized. When other hydrogen donors, such as L-malate or isocitrate, were also present, the amount of esterified phosphate found was less than

TABLE I

THE EFFECT OF MALONATE, L-MALATE, ISOCITRATE AND OXALOACETATE ON THE KREBS–COHEN DISMUTATION IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM glucose, hexokinase, 2–5 μg HQNO, 10 μg oligomycin, 3 % ethanol and 3.6–8.9 mg mitochondrial protein. The values (natoms or nmoles/min per mg protein) given are the means, with the range in parentheses; reaction time, 16–30 min.

Additions	*Number of measurements*	*−ΔO*	*ΔGlutamate*	*−Δ α-Oxo-glutarate*	*ΔEsterifie P*
None	15	9 (5–15)	17 (7–26)	31 (7–75)	12 (4–24
Malonate (20 mM)	19	9 (4–15)	32 (20–58)	72 (15–129)	32 (19–59
L-Malate (20 mM)	3	10 (7–13)	40 (35–44)	66 (46–82)	21 (17–23
L_s-(+)-Isocitrate (4 mM)	11	7 (5–15)	32 (17–40)	36 (20–56)	14 (3–22
Oxaloacetate (10 mM)	1	7	32	47	17

TABLE II

THE EFFECT OF MALONATE, L-MALATE AND D-MALATE ON THE KREBS–COHEN DISMUTATION IN RAT-LIVER MITOCHONDRIA

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM glucose, hexokinase, 5 or 10 μg HQNO, 5 or 10 μg oligomycin, 2 or 3 % ethanol and 8.2 mg (Expt. I) or 4.1 mg (Expt. II) mitochondrial protein. Reaction time, 20 min. Values in μmoles.

Expt.	*Additions*	*ΔGlu*	*ΔAsp*	*ΔOxalo-acetate*	*−Δ α-Oxo-glutarate*	*−ΔMa-late*	*ΔEsterifie P*
I	None	3.3	0.1	0.1	6.1	—	2.0
	Malonate (20 mM)	4.5	0.1	0.2	9.8	—	4.4
	L-Malate (20 mM)	7.0	1.0	0.3	11.5	2.5	3.5
	Malonate *plus* malate	5.9	3.0	0.2	11.3	4.6	4.5
II	None	1.4	—	—	1.7	—	—
	D-Malate (20 mM)	1.7	—	—	2.6	—	—
	Malonate (6 mM)	3.1	—	—	6.8	—	—

the amount of glutamate synthesized and could be used to assess the contribution of α-oxoglutarate as hydrogen donor, *i.e.* of the Krebs–Cohen dismutation.

Table I shows that malonate and L-malate stimulate the Krebs–Cohen dismutation markedly. Oxaloacetate also has a stimulating effect, which may be due to L-malate formed by the reduction of oxaloacetate. The small stimulation observed with L_S-(+)-isocitrate, which was also reported in a preliminary communication[1], can be accounted for by the small amount of malate (1–2 %) present as a contamination in L_S-(+)-isocitrate prepared from *Bryophyllum tubiflorum*.

Table II (Expt. I) shows a comparison in a single experiment of the effects of malonate and L-malate on the synthesis of glutamate from α-oxoglutarate and ammonia. It is clear from the amount of esterified phosphate found that the effects of malonate and L-malate on the Krebs–Cohen dismutation are not additive, which suggests that both compounds act at the same point. The stimulatory effect of malate is shown almost exclusively by the naturally occurring L-isomer; D-malate has very little effect (Table II, Expt. II).

TABLE III

EFFECT OF MALONATE AND L-MALATE ON HYDROGEN TRANSFER FROM β-HYDROXYBUTYRATE TO α-OXOGLUTARATE (*plus* NH_3)

Reaction mixture contained, in addition to the basic components, 20 mM β-hydroxybutyrate, 20 mM potassium phosphate buffer, 1 mM arsenite, 20 mM α-oxoglutarate, 20 mM NH_4Cl and 3.48 mg mitochondrial protein. Reaction time, 20 min. Values in μmoles.

Additions	*−Δ α-Oxoglutarate*	*ΔGlutamate*	*ΔAspartate*
None	1.0	0.9	—
Malonate (2 mM)	1.8	2.2	—
L-Malate (10 mM)	3.0	2.9	2.7

Malonate and L-malate also stimulate the reduction of α-oxoglutarate (*plus* ammonia) when β-hydroxybutyrate is the hydrogen donor[5,11]. This is shown in Table III. The reaction was carried out in the presence of arsenite to inhibit α-oxoglutarate oxidation. When L-malate is present, it can also act as hydrogen donor for glutamate synthesis (*cf.* ref. 25). However, the glutamate thus formed transaminates with the oxaloacetate produced during the oxidation of L-malate. Thus when malate is the hydrogen donor for the reduction of α-oxoglutarate (*plus* ammonia) the net result is the amination of malate to aspartate (Reaction 4) and α-oxoglutarate and glutamate do not appear in the sum reaction:

$$\text{Malate} + NH_3 \rightleftharpoons \text{aspartate} \qquad (4)$$

In the presence of L-malate, the amount of glutamate formed or of α-oxoglutarate utilized is, therefore, a measure of hydrogen transfer from β-hydroxybutyrate to α-oxoglutarate (*plus* ammonia). Table III shows that L-malate causes a 3-fold stimulation of this oxido–reduction. The stimulation by malonate is less than that by L-malate (see DISCUSSION).

Effect of malonate and L-*malate on the oxido–reduction state of the nicotinamide nucleotides during the Krebs–Cohen dismutation*

If the mechanism of action of malonate were that suggested in a preliminary account[1] (see INTRODUCTION), NADH would accumulate and NADPH be depleted during the Krebs–Cohen dismutation in the absence of malonate or L-malate. This was tested directly. In the experiment shown in Fig. 1, mitochondria were preincubated with α-oxoglutarate and oligomycin at 25° for 30 sec in order to reduce the nicotinamide nucleotides. At zero time ammonia was added, alone or together with either malonate (Fig. 1A) or L-malate (Fig. 1B). On the addition of ammonia alone

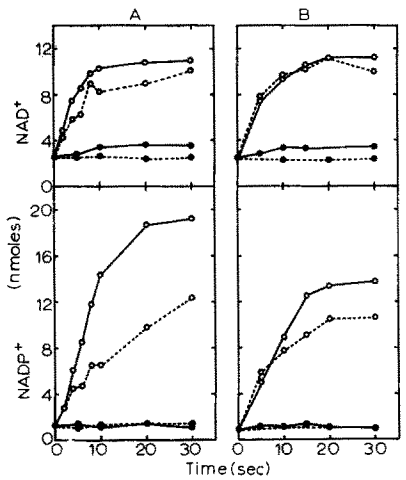

Fig. 1. Effect of malonate and L-malate on the oxidation of NADH and NADPH by α-oxoglutarate (*plus* ammonia) in rat-liver mitochondria. Reaction mixture (1 ml) contained the basic components *plus* 10 mM α-oxoglutarate, 2.5 μg oligomycin and 3.0 mg mitochondrial protein. The mitochondria were preincubated in this reaction mixture for 30 sec at 25°. In A, 10 μmoles NH_4Cl (O---O) or NH_4Cl *plus* 5 μmoles malonate (O—O) were added at zero time. The additions at zero time in B were NH_4Cl (O---O) or NH_4Cl *plus* 10 μmoles L-malate (O—O). In control experiments, no additions were made (●---●), or either malonate (A) or malate (B) was added alone (●—●).

there was an extensive oxidation not only of NADPH, but of NADH as well. When malonate or L-malate was added together with ammonia, it was the oxidation of NADPH that was stimulated; malonate or L-malate had little or no effect on the oxidation of NADH. In other experiments, the percentage reduction of the nicotinamide nucleotides in the steady state during the Krebs–Cohen dismutation was measured. The results (Table IV) confirm that malonate and L-malate stimulate the oxidation of NADPH more than the oxidation of NADH (*cf.* ref. 6). These results show that TAGER's proposed mechanism[1] has to be abandoned.

Effect of malonate and L-*malate on other α-oxoglutarate-linked reactions*

During a study of glutamate oxidation in rat-liver mitochondria (see ref. 22), arsenite was added in some experiments to block the transamination pathway of glutamate oxidation. In the presence of arsenite, which inhibits the oxidation of α-oxoglutarate, glutamate can be oxidized to α-oxoglutarate *plus* ammonia by glutamate dehydrogenase (Reaction 5).

$$\text{Glutamate} + 1/2O_2 \rightarrow \alpha\text{-oxoglutarate} + NH_3 \qquad (5)$$

TABLE IV

REDUCTION STATE OF NAD(P) IN RAT-LIVER MITOCHONDRIA DURING THE KREBS–COHEN DISMUTATION IN THE PRESENCE AND ABSENCE OF MALONATE OR L-MALATE

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 20 mM glucose, hexokinase, 5 μg HQNO, 10 μg oligomycin, 3% ethanol and 7.7 mg (Expt. 1) or 3.6 mg (Expt. 2) mitochondrial protein. Reaction time, 8 min.

Expt.	*Additions*	$\frac{NADH}{NAD^+ + NADH}$	$\frac{NADPH}{NADP^+ + NADPH}$
1	None	0.90	0.97
	Malonate (20 mM)	0.73	0.98
	NH_4Cl (20 mM)	0.10	0.29
	NH_4Cl *plus* malonate	0.04	0.07
2	None	0.84	1.00
	L-Malate (20 mM)	0.88	1.00
	NH_4Cl	0.38	0.31
	NH_4Cl *plus* L-malate	0.34	0.02

TABLE V

EFFECT OF MALONATE ON THE OXIDATION OF GLUTAMATE IN THE PRESENCE OF ARSENITE

Reaction mixture contained, in addition to the basic components, 10 mM glutamate, 30 mM glucose, hexokinase, 2% ethanol and 3.7 mg mitochondrial protein. Reaction time, 20 min. Values in μmoles.

Additions	*Δ α-Oxoglutarate*	ΔNH_3
Malonate (20 mM)	0.2	1.5
Arsenite (1 mM)	0.5	0.5
Malonate *plus* arsenite	1.3	1.3

Unexpectedly, it was found that malonate stimulated Reaction 5 very markedly (Table V). This effect of malonate is clearly unrelated to its action as an inhibitor of succinate dehydrogenase (EC 1.3.99.1), since no succinate is formed from glutamate in the presence of arsenite. The effect of L-malate on the oxidation of glutamate (*plus* arsenite) could not be tested, because L-malate itself is so rapidly oxidized in the presence of glutamate *plus* arsenite.

All the systems in which a stimulatory effect of malonate or L-malate was found have two components in common, namely α-oxoglutarate and the nicotinamide nucleotides. The effect of the stimulatory compounds on the oxidation of α-oxoglutarate itself was tested. It had already been observed by SLATER AND HOLTON[36] that malonate stimulated the oxidation of α-oxoglutarate by rat-heart mitochondria. This was confirmed by us. Malonate stimulated the oxidation of α-oxoglutarate slightly in mitochondria from both rat heart and rat liver, the effect being more pronounced in experiments of 8–10-min duration (stimulation approx. 20%) than after a longer incubation.

The effect of malonate on the *initial* rate of oxidation of α-oxoglutarate was studied by following the reduction of the intramitochondrial nicotinamide nucleotides (Fig. 2). Mitochondria were preincubated with ADP and P_i in order to oxidize the nicotinamide nucleotides. After the preincubation, rotenone was added to inhibit the respiratory chain, and 5 sec later α-oxoglutarate was added, either alone or together with malonate. In control experiments, α-oxoglutarate was omitted. On the addition of α-oxoglutarate alone, both NAD^+ and $NADP^+$ became reduced, NAD^+ directly (Reaction 1) and $NADP^+$ *via* the energy-linked transhydrogenase[37]. Malonate enhanced the rate of reduction of $NAD(P)^+$ by α-oxoglutarate. Similar results were obtained with L-malate (not shown). In contrast to L-malate, D-malate had only a small effect (not shown).

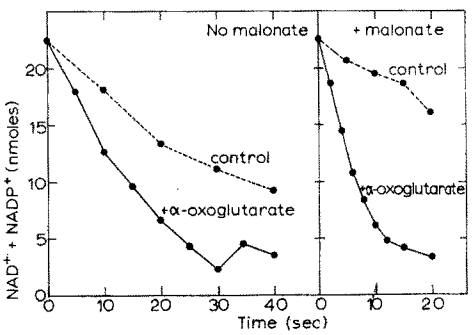

Fig. 2. Effect of malonate on the reduction of intramitochondrial $NAD(P)^+$ by α-oxoglutarate. Reaction mixture (1 ml) contained the basic components, except that 5 mM ADP was added *plus* 4.1 mg mitochondrial protein. After preincubation at 25° for 115 sec in order to oxidize intramitochondrial NAD(P)H, 2 μg rotenone were added and the preincubation was continued for a further 5 sec. At zero time the reactions were started by rapid addition of 10 μmoles α-oxoglutarate or α-oxoglutarate *plus* 10 μmoles malonate. In the control experiments α-oxoglutarate was omitted.

Relationship between concentration of malonate or L-*malate and the degree of stimulation of α-oxoglutarate-linked reactions*

Fig. 3 shows the effect of different concentrations of malonate on three α-oxoglutarate-linked systems: the Krebs–Cohen dismutation (Fig. 3A), the reduction of $NAD(P)^+$ by α-oxoglutarate in the presence of rotenone (Fig. 3B), and the oxidative deamination of glutamate to α-oxoglutarate (Fig. 3C). In all three systems, the maximal reaction rate is reached at a concentration of approx. 5 mM, and the apparent K_m for malonate was calculated to be approx. 0.5 mM. These results suggest that malonate acts at the same point in all three systems.

The effect of the concentration of L-malate on the Krebs–Cohen dismutation was studied in three experiments. Maximal stimulation was obtained at a concentration of approx. 2 mM, and the apparent K_m was 0.5–1.0 mM.

The permeability of rat-liver mitochondria for α-oxoglutarate

As stated above, the components common to all the systems stimulated by malonate or L-malate are the nicotinamide nucleotides and α-oxoglutarate. It seems unlikely that malonate and L-malate act at the level of the nicotinamide nucleotides, since on the one hand they stimulate the oxidation of NAD(P)H (in the Krebs–Cohen

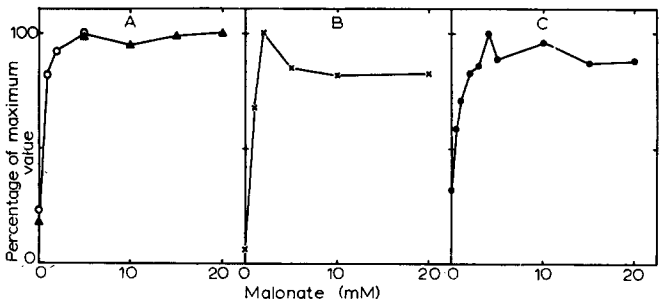

Fig. 3. Rate of α-oxoglutarate-linked reactions as a function of the concentration of malonate. A. Reaction mixture (1 ml) contained the basic components *plus* 20 mM α-oxoglutarate, 20 mM NH_4Cl, 20 mM glucose, hexokinase, 5 μg HQNO, 10 μg oligomycin, 3% ethanol and 6.5 mg mitochondrial protein. Reaction time, 20 min. The circles and triangles represent two separate experiments. B. Reaction mixture and experimental procedure as in legend to Fig. 2, except that 1.9 mg mitochondrial protein was present. The rates of reduction of $NAD(P)^+$ by α-oxoglutarate were expressed as $\Delta NAD(P)^+$ per 10 sec, corrected for the corresponding controls in which α-oxoglutarate was omitted. C. Reaction mixture (1 ml) contained the basic components *plus* 10 mM glutamate, 1 mM arsenite, 20 mM glucose, hexokinase and 3.0 mg mitochondrial protein. Reaction time, 20 min. All the results are expressed as per cent of the maximum values obtained, which were 2.58 μmoles (○—○) and 2.90 μmoles (▲—▲) esterified P in A, 7 nmoles $NAD(P)^+$/10 sec in B and 1.1 μmole NH_3 in C.

dismutation), whereas on the other hand they stimulate the reduction of $NAD(P)^+$ (by α-oxoglutarate).

All the results described above could be explained by assuming that, in the absence of malonate or L-malate, rat-liver mitochondria are relatively impermeable to α-oxoglutarate. The rate of the Krebs–Cohen dismutation or of the reduction of NAD^+ by α-oxoglutarate might be limited by the slow entry of α-oxoglutarate into the mitochondria. On the other hand, during the oxidation of glutamate in the presence of arsenite, α-oxoglutarate is formed; a slow efflux of α-oxoglutarate from the mitochondria would lead to an intramitochondrial accumulation of this compound and hence to product inhibition of the oxidation of glutamate. It seemed possible

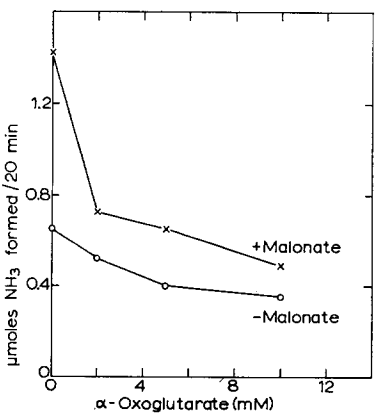

Fig. 4. Effect of added α-oxoglutarate on the rate of oxidation of glutamate (*plus* arsenite) in the presence and absence of malonate. Reaction mixture contained the basic components *plus* 10 mM glutamate, 1 mM arsenite, 30 mM glucose, hexokinase, the amounts of α-oxoglutarate as indicated in the figure, 6.6 mg mitochondrial protein and where indicated, 20 mM malonate. Reaction time, 20 min.

that malonate or L-malate might exert their stimulating effect by increasing the permeability of the mitochondria to α-oxoglutarate.

This hypothesis was tested by studying the effect of added α-oxoglutarate on the oxidative deamination of glutamate (*plus* arsenite). Fig. 4 shows that in the absence of malonate, added α-oxoglutarate caused only a small inhibition of ammonia formation from glutamate. However, in the presence of malonate, which stimulated ammonia production, added α-oxoglutarate was markedly inhibitory. Our interpretation of these results is that an equilibrium between intra- and extramitochondrial pools of α-oxoglutarate is set up only in the presence of malonate.

Direct evidence in favour of the hypothesis that rat-liver mitochondria are relatively impermeable to α-oxoglutarate and that malonate increases the permeability was obtained by studying the oxidation of glutamate (*plus* arsenite) and measuring the amount of α-oxoglutarate within the mitochondria and in the filtrate obtained after separating the mitochondria from the reaction medium on a Millipore filter (see METHODS). In parallel incubations the total amount of α-oxoglutarate present in the complete reaction mixture was measured.

Fig. 5A shows that, in the absence of malonate, the amount of α-oxoglutarate in the mitochondria rose within the first minute to a value of about 40 nmoles (10–20 nmoles/mg protein in different experiments) and then remained approximately constant. Concomitantly, there was a slow increase in the amount of α-oxoglutarate found in the filtrate. In the presence of malonate (Fig. 5B), only about 10 nmoles α-oxoglutarate were found within the mitochondria despite the fact that the reaction rate (as measured by the total amount of α-oxoglutarate formed) was stimulated 2.5 times. Thus malonate prevents an accumulation of α-oxoglutarate in the mitochondrion, thereby releasing the inhibition of glutamate oxidation. Fig. 5C shows that α-oxo-

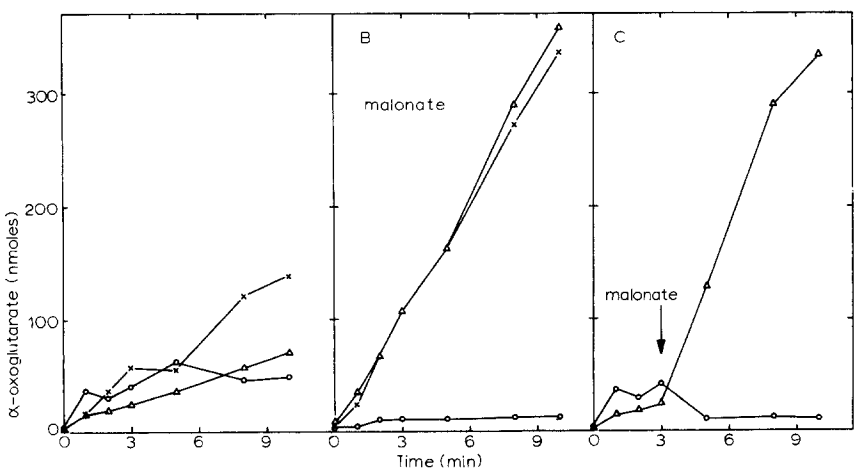

Fig. 5. Effect of malonate on the efflux of α-oxoglutarate from mitochondria during the oxidation of glutamate (*plus* arsenite). Reaction mixture (1.5 ml) contained the basic components *plus* 13 mM glutamate, 1 mM arsenite, 20 mM glucose, hexokinase, 1.9 mg mitochondrial protein and, where present, 10 mM malonate. In the zero-time controls, glutamate and hexokinase were omitted. The filtrate, representing the extramitochondrial space, and the mitochondrial extract were obtained by rapid filtration as described under METHODS. The total amount of α-oxoglutarate present in the reaction medium (indicated as 'total') was obtained from a separate incubation as described under METHODS. △—△, filtrate; ○—○, mitochondrial extract; ×—×, total.

glutarate, already accumulated within the mitochondria, is very rapidly released on addition of malonate; this is accompanied by a sharp increase in the rate of α-oxoglutarate formation.

Results analogous to those shown in Fig. 5 were obtained in an experiment using the centrifugation filtration technique[38,39] to separate the mitochondria from the reaction medium*.

DISCUSSION

At least three methods are available for the study of the permeability of mitochondria for substrate anions. These are (1), determining the rate of the mitochondrial reactions in which the anions take part; (2), measuring the influx into and efflux from the mitochondria of the anions; and (3), following the swelling of the mitochondria (measured as a decrease in light scattering) associated with the uptake of the anions. We have used the first and second methods in our studies and the results presented in this paper provide evidence that a permeability barrier for α-oxoglutarate exists in rat-liver mitochondria. J. B. CHAPPELL AND K. N. HAARHOFF (personal communication), using the third technique[40], have confirmed this (contrast ref. 41). Because of the permeability barrier, the movement of α-oxoglutarate into the mitochondria is the rate-limiting factor in processes depending on added α-oxoglutarate. Similarly, the efflux of α-oxoglutarate is the rate-limiting factor in the oxidative deamination of glutamate in the presence of arsenite.

TABLE VI

EFFECT OF MALONATE ON HYDROGEN TRANSFER FROM α-OXOGLUTARATE TO α-OXOGLUTARATE (*plus* AMMONIA) OR TO ACETOACETATE

Reaction mixture contained, in addition to the basic components, 20 mM α-oxoglutarate, 10 μg HQNO, 20 mM glucose, hexokinase, 2% ethanol, 5 μg oligomycin and 4.1 mg mitochondrial protein. Reaction time, 20 min. Values in μmoles.

Additions	*−Δ α-Oxoglutarate*	*ΔGlu*	*−ΔAcetoacetate*
Acetoacetate (7.1 mM)	2.0	—	1.5
Acetoacetate *plus* malonate (6 mM)	3.6	—	1.5
NH_4Cl (20 mM)	1.7	1.4	—
NH_4Cl *plus* malonate	6.8	3.1	—

The experiment shown in Fig. 5 provides direct evidence that malonate makes the mitochondrion more permeable for α-oxoglutarate. A similar experiment could not be performed with L-malate, since in the presence of glutamate and arsenite, L-malate is very rapidly oxidized. However, the effects of malonate and L-malate on the other systems are so similar that it seems justifiable to conclude that L-malate, like malonate, increases the permeability of the mitochondria for α-oxoglutarate.

In preliminary accounts[1,11] of this investigation, it was reported that malonate does not stimulate the reduction of acetoacetate with α-oxoglutarate as hydrogen donor. Table VI shows a comparison of the effect of malonate on hydrogen transfer

* E. J. DE HAAN, S. PAPA AND A. FRANCAVILLA, unpublished results.

from α-oxoglutarate to the two acceptors, α-oxoglutarate (*plus* ammonia) and acetoacetate. In confirmation of the results reported earlier[1,11], it was found that malonate did not stimulate the reduction of acetoacetate. However, in the presence of acetoacetate, malonate did increase the amount of α-oxoglutarate which disappeared. This extra α-oxoglutarate must have been used for reactions other than the reduction of acetoacetate. It is possible that the lack of stimulation by malonate of the reduction of acetoacetate is due to an inhibition of β-hydroxybutyrate dehydrogenase by malonate (*cf.* refs. 26, 42). It is perhaps significant that when the transfer of hydrogens from β-hydroxybutyrate to α-oxoglutarate (*plus* ammonia) is studied, L-malate brings about a greater stimulation than malonate (Table III).

Permeability barriers in mitochondria exist for other Krebs-cycle intermediates. For instance, it has been known for some years that mitochondria are often impermeable to the tricarboxylic acids[43–45]. CHAPPELL AND HAARHOFF[40] have shown that rat-liver mitochondria are impermeable to fumarate, and the existence of a permeability barrier for oxaloacetate has often been suggested (see, *e.g.* refs. 22, 46). The extreme case is found in mitochondria from the flight muscle of the housefly, which, as VAN DEN BERGH[47,48] has shown, are impermeable to all intermediates of the Krebs cycle.

CHAPPELL AND HAARHOFF[40] have proposed the existence of three specific anion carriers in liver mitochondria, necessary to transport substrate anions across a semipermeable membrane. The proposed carriers are an exchange-diffusion carrier for phosphate, a carrier for succinate and L-malate, and an L-malate-activated carrier for the tricarboxylic acids[49]. The stimulation by L-malate of the permeability of rat-liver mitochondria for the tricarboxylic acids has been confirmed by MEIJER AND TAGER[50] and by FERGUSON AND WILLIAMS[51]. Our results suggest that a fourth carrier exists, specific for α-oxoglutarate and activated either by L-malate or (unlike the carrier for the tricarboxylic acids[50,51]) by malonate. The nature of these proposed anion carriers, and the mechanism of the effect of the activators, are unknown. An attractive possibility is that the carriers are enzymes, analogous to the 'permeases' in bacteria; in this respect, the specificity of the activators (see also ref. 51) is suggestive.

The question arises of the physiological function of the permeability barriers for substrate anions in mitochondria. In the extreme case of mitochondria isolated from the flight muscle of the housefly, where there is a permeability barrier for all intermediates of the Krebs cycle, VAN DEN BERGH AND SLATER[47,48] have suggested that the function of the barrier may be to prevent loss of these intermediates from the mitochondria.

Mitochondria from the flight muscle of the housefly apparently lack carriers for the tricarboxylic acid cycle intermediates[41]. In rat-liver mitochondria, the presence of anion carriers may represent a means of controlling the entry into and exit from the mitochondria of these anions. It is possible that the degree of permeability of the mitochondria for the tricarboxylic acids and for α-oxoglutarate may be controlled by the concentration of L-malate present in the cell. In freshly isolated rat-liver mitochondria in the absence of inhibitors, isocitrate, citrate and α-oxoglutarate are oxidized rapidly and there is relatively little stimulation by L-malate or malonate (refs. 14, 46, 52 and this paper). This suggests that sufficient L-malate may be present in the mitochondria to activate the carriers for the tricarboxylic acids and α-oxoglutarate. Furthermore, L-malate is formed during the oxidation of these substrates. CHAPPELL[46] has shown that preincubation of mitochondria leads to loss of permeability

to the tricarboxylic acids, and has suggested that this is because the mitochondria become depleted of endogenous L-malate. MEIJER AND TAGER[52] have shown by direct measurement that the loss of permeability that occurs during preincubation of rat-liver mitochondria is directly correlated with a loss of endogenous L-malate (and its precursors) from the mitochondria.

In freshly prepared rat-liver mitochondria, the concentration of L-malate (calculated from the amount of this acid present[53] and the content of mitochondrial water[39,54]) is 2–3.5 mM; this is sufficient to activate the carriers for the tricarboxylic acids and α-oxoglutarate maximally, assuming that this L-malate remains within the mitochondrion. It is conceivable that fluctuations in the concentration of L-malate *in vivo* may be a means of regulating the movement of other Krebs-cycle intermediates between the mitochondrial and extramitochondrial compartments.

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