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Coupling Mechanisms in Anionic Substrate Transport Across the Inner Membrane of Rat-Liver Mitochondria

S. Papa, N. E. Lofrumento, and E. Quagliariello

Department of Biochemistry, University of Bari, Bari, Italy

and

A. J. Meijer and J. M. Tager

Laboratory of Biochemistry, B.C.P. Jansen Institute, University of Amsterdam, Amsterdam, The Netherlands

Abstract

The translocation of P_i, malate, α-oxoglutarate, and citrate across the inner membrane of rat-liver mitochondria has been studied. Investigation on the effect of pH on anionic substrate translocation across the mitochondrial membrane shows that their distribution across the inner membrane can be governed by transmembrane pH difference. However, evidence is presented that the translocation of P_i, but not that of malate, α-oxoglutarate, or citrate can be *directly* coupled to an OH^- counterflux ($H_2PO_4^-$—OH^- exchange-diffusion). The occurrence in rat-liver mitochondria of P_i-dicarboxylate, dicarboxylate-α-oxoglutarate, and malate-tricarboxylate exchange-diffusion reactions is directly demonstrated. The study of the effect of uncouplers on the efflux from mitochondria of substrate anions, in the absence of counteranion, and on the anion exchange-diffusions shows that uncouplers act in at least two ways: they promote the efflux of P_i from mitochondria and inhibit *directly* the exchange-diffusion reactions. The kinetics of this inhibition are described. These results are discussed in the light of previous work on the effect of uncouplers on the distribution of substrate anions across the inner membrane of isolated mitochondria. Coupling mechanisms in substrate anion translocation and aspects of the energetics of anion translocation are discussed.

Introduction

Energy flow in mammalian cells involves the movement of a number of anionic substrates, from the cell sap to the mitochondria and in the reverse direction. The inner membrane of mitochondria is not freely permeable to various anionic substrates, but appears to contain system(s) which mediate the transport of these substrates, thus assuring the operation of those metabolic pathways distributed partly in the mitochondria partly in the cytosol.[1] The studies of Klingenberg *et al.*[2,3] led to the discovery of the adenine translocator and to a detailed knowledge of its function in the translocation of adenine nucleotides across the inner membrane. Investigations on the osmotic behaviour of mitochondria and the kinetics of reduction or oxidation of intramitochondrial NAD(P) by anionic substrates led Chappell and coworkers[1,4,5] to propose the existence in rat-liver mitochondria of three exchange-diffusion translocators,

mediating the transport of P_i, dicarboxylate and tricarboxylate anions, respectively. Evidence has been presented by De Haan and Tager[6] for an α-oxoglutarate translocator in rat-liver mitochondria (see also refs. 7 and 8), and by Azzi *et al.*[9] for glutamate and asparate transporting systems.

From time to time it has been proposed that anionic substrate translocators can mediate an exchange-diffusion of the respective anions with hydroxyl.[5,10,11] According to the chemiosmotic hypothesis of Mitchell, anion translocation occurs either by an OH^-/anion antiport or by an H^+/anion symport (unionized acid uniport).[12,13] Chappell *et al.*[5,8] have, in addition, proposed that the dicarboxylate, tricarboxylate and α-oxoglutarate translocators bring about an exchange-diffusion of phosphate with dicarboxylate, dicarboxylate with tricarboxylate, and dicarboxylate with α-oxoglutarate respectively. These net exchanges could also operate by coupled anion-hydroxyl antiports.[14]

Isolated mitochondria accumulate substrate anions from the medium against a concentration gradient. Uncouplers of oxidative phosphorylation inhibit this accumulation[15–17] and promote the efflux of intramitochondrial anionic substrates.[7] These and related observations have raised the problem of the energetics of anion transport in mitochondria.[5,12,14,18]

In this paper a study of the pH dependence of anion translocation in isolated rat-liver mitochondria is reported. Evidence is presented that the translocation of P_i, but not that of malate, α-oxoglutarate, or citrate, can be directly coupled to an OH^- counterflux. The occurrence of the P_i-dicarboxylate, malate-tricarboxylate, and dicarboxylate α-oxoglutarate exchange-diffusions is directly demonstrated. Data on the mechanism of action of uncouplers on the translocation of various anions involved in the exchanges are reported. Coupling mechanisms in substrate anion translocation and aspects of the energetics of anion transport are discussed. Part of these investigations has been communicated in a preliminary form.[19,20]

Methods

Rat-liver mitochondria were prepared as described by Myers and Slater.[21]

The movement of anionic substrates across the inner mitochondrial membrane was studied with one of the following procedures: 1. The efflux of endogenous substrates or the uptake of added substrates was followed by interrupting the incubation of mitochondria, at various intervals, by rapid centrifugation of the mitochondria from the medium, with an Eppendorf microcentrifuge (Mod. 3200) or with centrifugation-filtration in a Spinco centrifuge (Mod. L.50, rotor SW39) as described by Pfaff.[22] The substrates were directly measured both in $HClO_4$ extracts of the mitochondrial pellet and in the supernatant. The substrate content of the sucrose-impermeable space (matrix space) was calculated by correcting the amount in the mitochondrial extract for that present in the sucrose-permeable space *plus* adherent supernatant; this was determined with [^{14}C]sucrose. 2. Mitochondria were preloaded with labelled substrates by incubation at 0°, washed, suspended again in a second incubation mixture and finally subjected to procedure 1. 3. Mitochondria were preloaded with labelled substrates at 25° in the presence of oligomycin, antimycin, and rotenone. Loaded mitochondria were layered on the top of a second incubation layer and centrifuged through this into $HClO_4$. A

discontinuous density gradient increasing towards the bottom of the centrifuge tube was made by addition of dextran (mol. wt. 60,000–90,000) to the second layer. This was separated from the $HClO_4$, at the bottom of the tube, by a layer of silicone oil. The exposure time of mitochondria to the second incubation layer (0·35 ml) was estimated to be about 15 sec by measuring the oxidation of β-hydroxybutyrate to acetoacetate. The rate of this reaction was determined in separate controls. This procedure is basically similar to that described by Klingenberg *et al.*[23] The substrate content in the matrix space and in the supernatant was determined as in procedure 1.

Where indicated, mitochondrial water was determined gravimetrically, correction being made for that of the sucrose-space plus adherent supernatant.

Assays

Phosphate, malate, citrate, and α-oxoglutarate were determined using $^{32}P_i$ and [^{14}C]-labelled compounds respectively. In addition phosphate was also determined colorimetrically by the method of Wakler and Wollenberger,[24] α-oxoglutarate,[25] malate,[26] and citrate[27] enzymically using the Aminco-Chance dual-wavelength spectrophotometer. Protein was determined by usual biuret method.

Bromothymol Blue Absorbancy

This was monitored directly in the mitochondrial suspension using the Aminco-Chance dual-wavelength spectrophotometer.

Experimental Conditions

The reaction media, reaction temperature, pH, specific additions, and reaction time are indicated in the legends to the tables and figures.

Special Chemicals and Enzymes

ADP, NAD^+, NADH, α-oxoglutarate, and enzymes used in the assays were obtained from Boehringer and Soehne; yeast hexokinase (EC 2.7.1.1.), oligomycin, and antimycin A from Sigma Chem. Co.; dicoumarol, L-malate, citrate, glutamate, malonate, arsenite, rotenone, and mersalyl from British Drug Houses. Butylmalonate from Aldrich Chemical Co.; Valinomycin and nigericin were generously given by Dr. H. A. Lardy.

Results

Effect of pH on the Translocation of Anionic Substrates in Mitochondria

The effect of the pH of the suspending medium on the efflux of intramitochondrial P_i, malate, and citrate is shown in Fig. 1. Freshly isolated mitochondria contain substantial amounts of inorganic P_i but less citrate and malate. In the experiment of Fig. 1B mitochondria were preloaded with [^{14}C]malate or [^{14}C]citrate. Loaded or unloaded mitochondria were then incubated at 25° in media of various pH's in the presence of oligomycin and rotenone. These inhibitors prevented substrate oxidation and energy supply. The incubation was interrupted after 40 sec (Expt. 1A) and 80 sec (Expt. 1B) by rapidly centrifuging the mitochondria from the suspending medium, and the intra- and extramitochondrial level of the anionic substrates was determined. It can be seen that the increase of the pH from 6·5 to 8·5 promoted the efflux from the matrix of P_i, malate, and citrate under these conditions.

Further insight into the mechanism of this pH dependence was obtained by studying the initial phase of anion efflux. In the experiment of Fig. 2 mitochondria were first loaded with one single labelled substrate (P_i, malate, citrate, or α-oxoglutarate), in the presence of oligomycin, rotenone, and antimycin, and then exposed for a few seconds at 0° to a second incubation medium (free of anionic substrates) at various pH's. During the fast exposure to the second medium $^{32}P_i$, accumulated during the first incubation, moved out of the mitochondria. On the contrary there was no significant efflux of [^{14}C]malate, [^{14}C]α-oxoglutarate, or [^{14}C]citrate (see also Fig. 7). It can be seen from Fig. 2 that as the pH of the second layer was increased, the efflux of P_i became greater. In contrast, changing the pH of the second layer from 6·5 to 8·5 had no effect on the intramitochondrial level of malate, α-oxoglutarate, and citrate under these conditions. The OH^- promoted efflux of P_i was blocked by mersalyl (Fig. 2B) but not by butylmalonate (Fig. 2A). Evidence has been presented that mersalyl and other sulphydryl-blocking reagents inhibit the transport of P_i in mitochondria.[28–30]

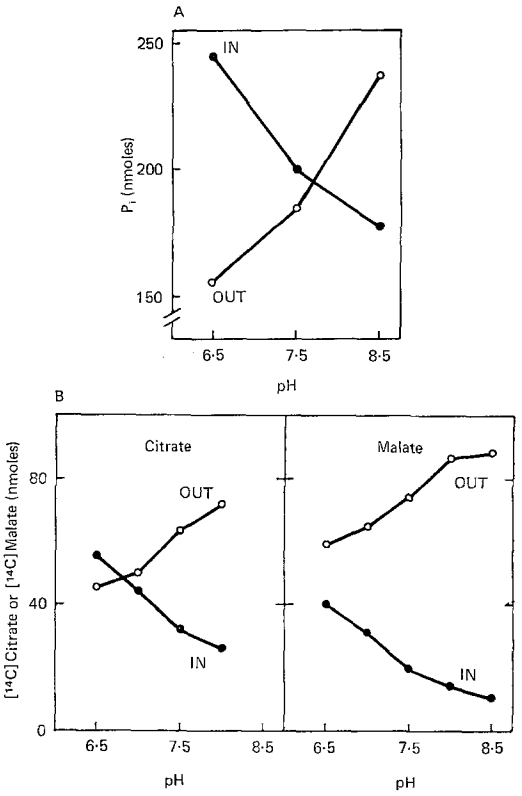


Figure 1. Effect of the pH of the medium on the efflux of anionic substrates from mitochondria. Expt. A: freshly isolated mitochondria (11·7 mg protein) were suspended in a reaction medium, at the pH's shown in the figure, containing: 15 mM KCl, 50 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM EDTA, 1 mM arsenite, 3 μg rotenone, and 15 μg oligomycin. Final volume 1·5 ml, temperature 25°. Expt. B: mitochondria were preincubated 10 min at 0° in 250 mM sucrose with 2 mM malate or 2 mM citrate, washed, and resuspended at 0° in cold 250 mM sucrose containing 100 μM [^{14}C]malate or 100 μM [^{14}C]citrate (about 10^8 counts/min). Loaded mitochondria (6·4 mg protein) were suspended in 1·5 ml of the reaction medium of Expt. A. After 40 sec (Expt. A) or 80 sec (Expt. B) mitochondria were centrifuged from the medium by procedure 1 (see under Methods) and P_i, [^{14}C]malate, and [^{14}C]citrate were determined both in the mitochondrial extract and the supernatant. Substrate content in the matrix ●——●, in the supernatant ○——○.

The experiment of Fig. 3 gives a time-resolution of the events occurring during the efflux of endogenous P_i and malate from mitochondria at pH 8·5 and 25°. In the first 2 sec of incubation a relatively rapid efflux of P_i occurred. During this interval there was no significant movement of malate. The P_i released into the medium then started to move back *into* the mitochondria. At the same time malate started to move *out* of the mitochondria. In Fig. 4 the effect of mersalyl plus butylmalonate on the pH dependence of the efflux of citrate from mitochondria at 25° is shown. The incubation time was 80 sec. It can be seen that the stimulation of the efflux of citrate brought about by increasing the pH (see also Fig. 1) was completely abolished by mersalyl *plus* butylmalonate.

It is known that valinomycin induces an energy-linked uptake of potassium[31,32] by mitochondria that is accompanied by H^+ extrusion and alkalinization of the intramito-

chondrial space.[33] The effect of valinomycin on the uptake of anionic substrates by rat-liver mitochondria is shown in Table I. $^{32}P_i$, [^{14}C]malate, [^{14}C]citrate, or [^{14}C]α-oxoglutarate were added to the mitochondrial suspension in the presence of oligomycin, rotenone and antimycin. Energy was supplied by the oxidation of *NNN'N'*-tetramethyl-*p*-phenylenediamine (plus ascorbate). The induction of K^+ uptake by the addition of valinomycin resulted in a marked stimulation of the uptake of P_i, malate, citrate, and α-oxoglutarate. A similar stimulation has been reported by several authors.[34–37] However, the important point shown by the experiment of Table I is that butylmalonate abolished the stimulating effect of valinomycin on the accumulation of malate, α-oxoglutarate, and citrate. On the other hand butylmalonate potentiated the stimulating effect of valinomycin on the uptake of P_i.

In the experiment of Fig. 5 bromothymol blue was included in the mitochondrial suspension, and its absorbance changes were recorded during the incubation. Evidence has been presented that bromothymol blue measures intramitochondrial pH (Chance and Mela;[38] Harris;[33] see however, refs. 39 and 40). Mitochondria were suspended in a KCl medium and depleted of endogenous P_i by incubation with glutamate and a ~P trap (Figs. 5A and B). The addition of valinomycin, caused a fast and large increase of bromothymol blue absorbance, the kinetics of which were similar to those reported by Pressman *et al.*[32] for the appearance of H^+ in the medium. This absorbance increase indicates, at least in part, mitochondrial alkalinization. The addition of malate gave a small further increase of absorbance; P_i, on the contrary, immediately reversed the absorbance increase. Acetate gave an effect similar to that of P_i. When malate was added to the mitochondrial suspension without prior depletion of P_i (Figs. 5C and D), it caused some reversal of the valinomycin-induced absorbance increase of bromothymol blue. This effect of malate was partly inhibited by 1 mM butylmalonate; the inhibition could be increased by raising the concentration of butylmalonate. That P_i (or acetate) but not dicarboxylate anion can reverse mitochondrial alkalinization, induced by cation accumulation, has also been shown by direct pH measurements on the dissolved mitochondrial pellet[41] or by the distribution of 5,5-dimethyl-2,4-oxazolidinedione

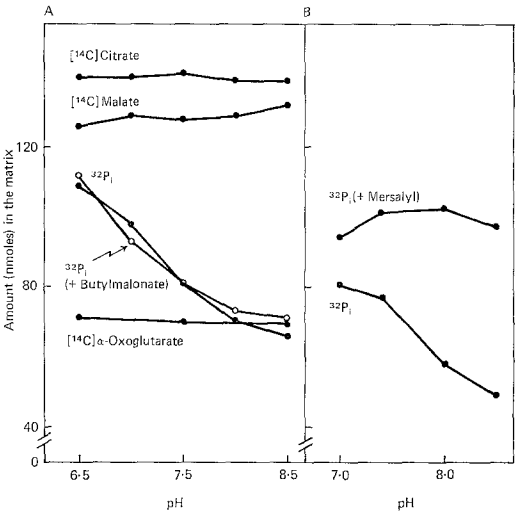


Figure 2. Effect of a rapid exposure of mitochondria to media of various pH's on the intramitochondrial level of anionic substrates. Mitochondria (Expt. A, 7 mg protein; Expts. B, 11 mg protein) were preincubated at 25° in 1 ml of a reaction mixture containing: 150 mM sucrose, 20 mM Tris-HCl (pH 7·4), 0·5 mM EDTA, 1 mM $MgCl_2$, 1·4 μg rotenone, 0·5 μg antimycin, and 10 μg oligomycin. After 1 min 1 mM $^{32}P_i$ ($9 \cdot 10^5$ counts/min), 1 mM [^{14}C]malate ($5 \cdot 10^5$ counts/min), 1 mM [^{14}C]citrate (10^5 counts/min), 1 mM [^{14}C]α-oxoglutarate ($5 \cdot 10^5$ counts/min). Two minutes after the addition of the substrates, mitochondria were centrifuged through a second incubation layer at 0°, and finally into $HClO_4$ (procedure 3, see under Methods). Layer II contained the same components as the preincubation mixture, except the anion substrates, 20 mM Tris-HCl at the pH's shown in the figure and, where indicated, 3 mM butylmalonate or 0·66 mM mersalyl. For the experimental details see under Methods.

(DMO).[42] Furthermore Rossi *et al.*[43] have reported that the uptake of P_i lowers the H^+/K^+ ratio during K^+ uptake by mitochondria.

The Exchange-Diffusion Reactions

In the experiment of Table II, mitochondria were preincubated with ADP, glucose, and hexokinase in order to lower the content of endogenous P_i and respiratory substrates. $^{32}P_i$ was then added in the presence of oligomycin, rotenone and TMPD (plus ascorbate). Mitochondria actively accumulated P_i. $^{32}P_i$-loaded mitochondria were then centrifuged through a second incubation layer free of P_i. The presence of malate in this layer promoted P_i efflux from mitochondria. Concomitantly malate was taken up by mitochondria. P_i-loaded mitochondria took up twice as much [^{14}C]malate as unloaded mitochondria. In the unloaded mitochondria the residual amounts of endogenous anionic substrates must have been responsible for the uptake of [^{14}C]malate. The amount of $^{32}P_i$ driven out of the mitochondria by malate was approximately equal to the extra-uptake of malate. This experiment demonstrates the occurrence of an 1:1 exchange diffusion between malate and P_i. The experiment of Fig. 6 shows that the addition of P_i in the suspending medium promoted efflux from mitochondria of endogenous malate. This effect of P_i was abolished by butylmalonate. Also the efflux of intramitochondrial P_i induced by malate was abolished by this inhibitor (not shown). These data show that butylmalonate inhibits the malate P_i exchange-diffusion.

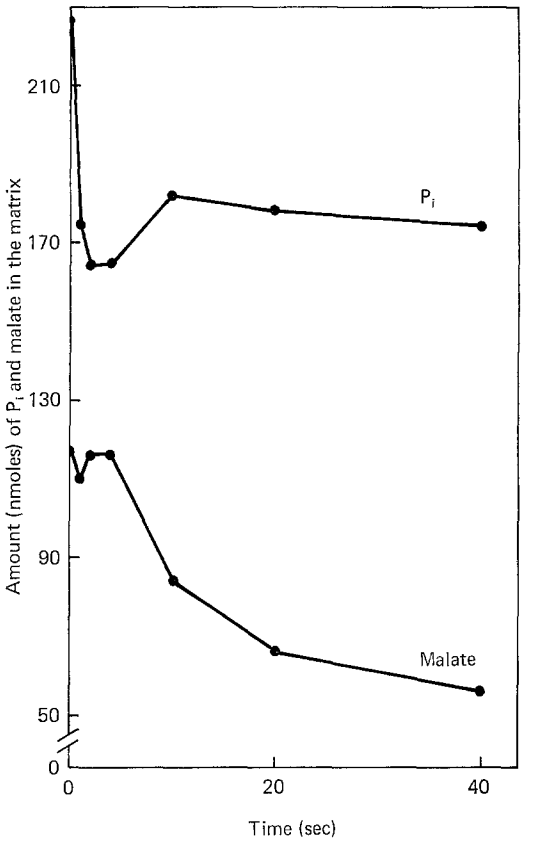


Figure 3. Time course of the efflux from mitochondria of endogenous P_i and malate. The experimental conditions are those described in the legend of Fig. 1A—Mitochondrial protein 11·7 mg. At the times indicated 0·66 mM mersalyl was added and mitochondria were centrifuged by procedure 1 (see under Methods).

An experiment on the coupling between the flux of malate and that of α-oxoglutarate across the mitochondrial membrane is presented in Table III. Mitochondria were preincubated with ADP, glucose hexokinase. [^{14}C]malate, added in the presence of rotenone and antimycin was accumulated by mitochondria. The uptake of malate reached a plateau value (77 nmoles) after 2 min. In the absence of malate 35 nmoles α-oxoglutarate penetrated the mitochondria. When α-oxoglutarate was added to mitochondria already loaded with malate, 76 nmoles α-oxoglutarate were taken up. Thus there is an extra uptake of 41 nmoles α-oxoglutarate. By comparing the intramitochondrial malate of lines 2 and 4, it can be seen that the extra-uptake of α-oxoglutarate

was coupled with the efflux of an approximately equal amount of malate. We have, therefore, a 1:1 exchange diffusion of extramitochondrial α-oxoglutarate with intramitochondrial malate. It has been possible to demonstrate the occurrence of this exchange-diffusion reaction also in the reverse direction, namely between intramitochondrial α-oxoglutarate and an extramitochondrial dicarboxylate (malate or malonate). This is illustrated in Table IV. In the experiments of the first three lines glutamate was added to the mitochondria in the presence of P_i, P_i-acceptor, and arsenite. The oxidation of glutamate generated α-oxoglutarate inside the mitochondria.[6,7] Glutamate oxidation was then arrested with rotenone plus antimycin. On continuing the incubation much of the α-oxoglutarate produced was retained inside the mitochondria (line 1). The addition of malonate (line 2) promoted efflux of α-oxoglutarate. Concomitantly malonate was taken up by the mitochondria. In the absence of glutamate, no α-oxoglutarate was found inside the mitochondria, and much less malonate was taken up. It can be seen that the amount of

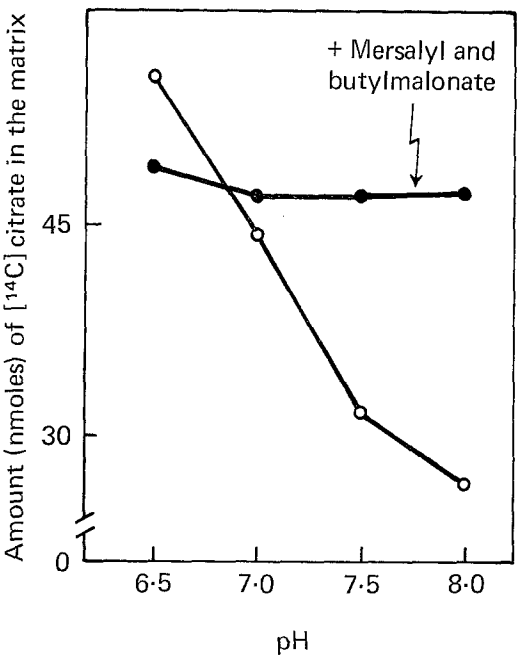


Figure 4. Effect of butylmalonate plus mersalyl on the pH dependent efflux of citrate. Mitochondria were loaded with [^{14}C]citrate as described in the legend to Fig. 1B and subsequently suspended in media at different pH's (6·4 mg protein, final volume 1·5 ml). Reaction temperature 25°, reaction time 80 sec. The composition of the medium and the experimental procedure was that described in Fig. 1. Where indicated 2 mM butylmalonate and 0·66 mM mersalyl were present in the reaction mixture from the beginning of the incubation.

TABLE I. Effect of valinomycin and butylmalonate on the uptake of anionic substrates by rat-liver mitochondria

Additions	Amount (nmoles) in matrix of			
	$^{32}P_i$	[^{14}C]Malate	[^{14}C]Citrate	[^{14}C]Oxoglutarate
None	157	158	125	103
Valinomycin	251	456	192	165
Butylmalonate	127	96	73	45
Butylmalonate + Valinomycin	319	75	56	46

Mitochondria (6 mg protein) were incubated 1 min at 25° in 1·0 ml of the reaction medium described in the legend to Fig. 1, with the addition of 1 mM $^{32}P_i$, 1 mM [^{14}C]malate, 1 mM [^{14}C]-citrate, or 1 mM [^{14}C]α-oxoglutarate; 0·5 μg antimycin, 0·3 mM *NNN'N'*-tetramethyl-*p*-phenylenediamine (TMPD) and 3 mM ascorbate. Where indicated: 0·1 μg valinomycin and 2 mM butylmalonate. Further procedure as in the legend to Fig. 1 (see also under Methods).

α-oxoglutarate driven out of the mitochondria by malonate was practically equal to the extra uptake of malonate by α-oxoglutarate-charged mitochondria. Thus there is a 1:1 exchange-diffusion of intramitochondrial α-oxoglutarate with extramitochondrial malonate. In Table IV the effect of butylmalonate is also shown. It inhibited the uptake of malonate, the extent of the inhibition being greater in uncharged mitochondria than in those charged with α-oxoglutarate. On the other hand, butylmalonate had no effect on the malonate-promoted efflux of α-oxoglutarate. The lower part of the table shows that practically the same amount of intramitochondrial α-oxoglutarate was exchanged with extramitochondrial malonate, in the absence and in the presence of butylmalonate. Thus α-oxoglutarate-charged mitochondria took up malonate in two ways: in a butylmalonate-sensitive reaction (see Table I and Fig. 6), and in exchange-diffusion with α-oxoglutarate (butylmalonate-insensitive).

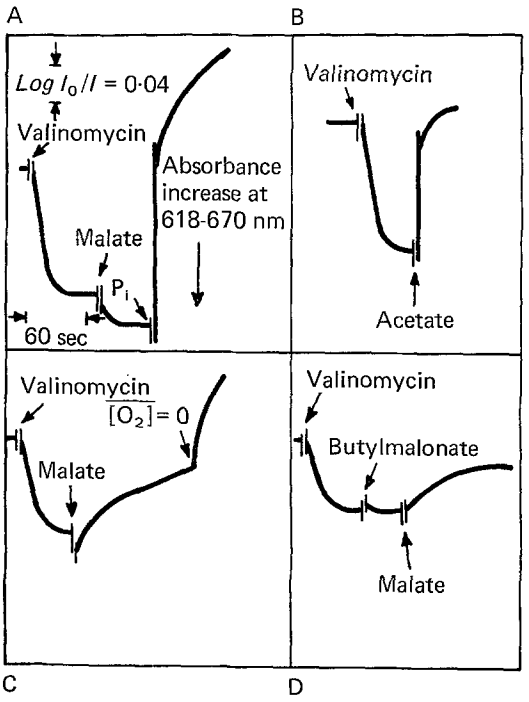


Figure 5. Effect of malate, P_i and acetate on bromothymol blue absorbance changes in mitochondria. In Expts. A and B, rat-liver mitochondria (9 mg protein) were preincubated for 4 min at 25° in the presence of 130 mM KCl, 20 mM Tris-HCl (pH 7·5) 5 mM glutamate, 0·2 mM ADP, 3 μM bromothymol blue, 10 mM glucose and 15 I.U. yeast hexokinase. After 2 min, 15 μg oligomycin were added followed 1 min later by 0·2 μg valinomycin. In Expts. C and D, mitochondria were not preincubated and the medium contained all the components, except glucose and hexokinase. Additions: 3 mM malate, 3 mM P_i, 3 mM acetate and 1 mM butylmalonate. Final volume, 3 ml. For other details see under Methods.

The occurrence in rat-liver mitochondria of an analogous exchange-diffusion between malate and citrate is demonstrated by the experiment of Table V. In Expt. A mitochondria were loaded with [^{14}C]citrate at 0°, washed, and then incubated for 80 sec at 25° in the presence of oligomycin and rotenone. Malate, added to the reaction mixture, was taken up by the mitochondria and, at the same time, promoted citrate efflux. The ratio between the nmoles of malate taken up and those of citrate driven out of the mitochondria was 1·9. The addition of mersalyl plus butylmalonate severely depressed malate uptake but had no significant effect on the malate-driven efflux of citrate. In the presence of these inhibitors the ratio between the nmoles of malate taken up and those of citrate driven out of the mitochondria was 0·8. In Table VB an experiment on the exchange between intramitochondrial malate and extramitochondrial citrate is shown. Mitochondria were preincubated in the presence of P_i, phosphate acceptor, and arsenite to deplete them of endogenous citrate. Mitochondria were then loaded with different amounts of malate in the presence of rotenone: 1 min after the addition of malate, citrate was added (together with butylmalonate to block malate-phosphate exchange). After 30 sec mitochondria were centrifuged from the medium by procedure 1 as described by Pfaff[22] (see under

TABLE II. Exchange diffusion between intramitochondrial $^{32}P_i$ and extramitochondrial [^{14}C]Malate in rat-liver mitochondria

Additions to		Amount (nmoles) in matrix of	
Layer I	Layer II	$^{32}P_i$	[^{14}C]Malate
$^{32}P_i$	—	228	—
—	[^{14}C]Malate	—	68
$^{32}P_i$	[^{14}C]Malate	165	135
Δ $^{32}P_i$		−63	
Δ [^{14}C]Malate			+67

Mitochondria (15·6 mg protein) were preincubated for 3 min at 25° with 130 mM sucrose, 20 mM Tris-HCl (pH 7·5), 10 mM KCl, 3 mM $MgCl_2$, 50 μM ADP, 20 mM glucose, and 15 I.U. yeast hexokinase. 3 mM ascorbate plus 0·3 mM TMPD were then added, followed 1 min later by 30 μg oligomycin, 6 μg rotenone, and 1·5 μg antimycin. After 1 min, 2 mM $^{32}P_i$ was added where indicated. The final volume was 3 ml. The mitochondria were transferred 1 min later to Layer II and from this into $HClO_4$ by centrifugation filtration (see under Methods: procedure 3). Where indicated, 2 mM [^{14}C]malate was present in Layer II. $\Delta^{32}P_i$ is the difference in the P_i content of the matrix in the presence and absence of malate and Δ[^{14}C]malate the difference in the malate content in the presence and absence of P_i.

Methods) and the concentration of citrate and malate in the matrix was determined. It can be seen that citrate added to malate-loaded mitochondria was actively accumulated and, at the same time, promoted malate efflux. The ratio between the amount of citrate taken up and that of malate driven out ranged between 0·87 and 0·97. Thus citrate, like α-oxoglutarate, is transported across the inner mitochondrial membrane by an exchange-diffusion with malate, the stoicheiometry of the reaction being of one to one. The experiment of Table VI shows that butylmalonate had no inhibitory effect on the efflux of intramitochondrial citrate, driven by extramitochondrial malate. Thus also the citrate-malate exchange, like the α-oxoglutarate-dicarboxylate exchange, is butylmalonate insensitive. By exposing [^{14}C]malate loaded mitochondria to a second incubation layer containing increasing concentration of the various anions that exchange with [^{14}C]malate (unlabelled malate, P_i, α-oxoglutarate, and citrate) it was possible to see that the efflux of [^{14}C]malate, driven by the various counteranions, followed saturation kinetics (see Figs. 8 and 9). The Lineweaver-Burk plots of these and similar experiments allowed us to measure the V_{max} for malate efflux and the K_m for the various counteranions tested (see Table VII).

The Effect of Uncouplers

Figure 7 shows the results of an experiment on the effect of added anions and dicoumarol, on the efflux from mitochondria of $^{32}P_i$, [^{14}C]malate, [^{14}C]citrate, and [^{14}C]α-oxoglutarate. Mitochondria were first loaded with these substrates and then transferred by centrifugation filtration to a second medium free of labelled substrates. The anion efflux was followed at 0° in an interval of a few seconds. The efflux of $^{32}P_i$ was increased by unlabelled P_i. Unlabelled malate promoted a rapid and extensive efflux of both

[^{14}C]malate and [^{14}C]citrate. Dicoumarol, in the absence of counteranions, stimulated the efflux of $^{32}P_i$, but had practically no effect on the matrix level of [^{14}C]-malate, [^{14}C]citrate, or [^{14}C]α-oxoglutarate. Other uncouplers too, like 2,4-dinitrophenol (see also ref. 20) and FCCP (see ref. 44), stimulated P_i efflux in the absence of counteranions, but had no effect on the mitochondrial level of malate, citrate, or α-oxoglutarate. The lack of any effect of uncouplers on the mitochondrial level of these last compounds does not, however, exclude the possibility of an effect of uncouplers on the exchange-diffusion reaction *per se*. This was examined directly by studying the effect of uncouplers on the counteranion driven efflux of [^{14}C]malate from mitochondria. The Lineweaver-Burk plot of Fig. 8 shows that dicoumarol inhibits competitively the exchange-diffusion of malate with citrate or with P_i. The data given in Table VII show that dicoumarol inhibited also the exchange-diffusion of [^{14}C]malate with cold malate or with α-oxoglutarate. In the first case the inhibition was purely competitive, in the second partly competitive.

Figure 9 shows that 2,4-dinitrophenol gave a purely competitive inhibition of the malate-P_i and a partly competitive inhibition of the malate–α-oxoglutarate exchange-diffusion. However, this uncoupler had no effect on the malate–citrate or malate–malate exchanges. Probably, the malate-phosphate, malate–α-oxoglutarate, and malate–tricarboxylate systems must all be inhibited in order to inhibit the malate–malate exchange. This appears to be the case with dicoumarol but not with 2,4-dinitrophenol.

Coupling Between the Exchange-Diffusion Reactions

In the experiment of Fig. 10 mitochondria, preloaded with [^{14}C]α-oxoglutarate

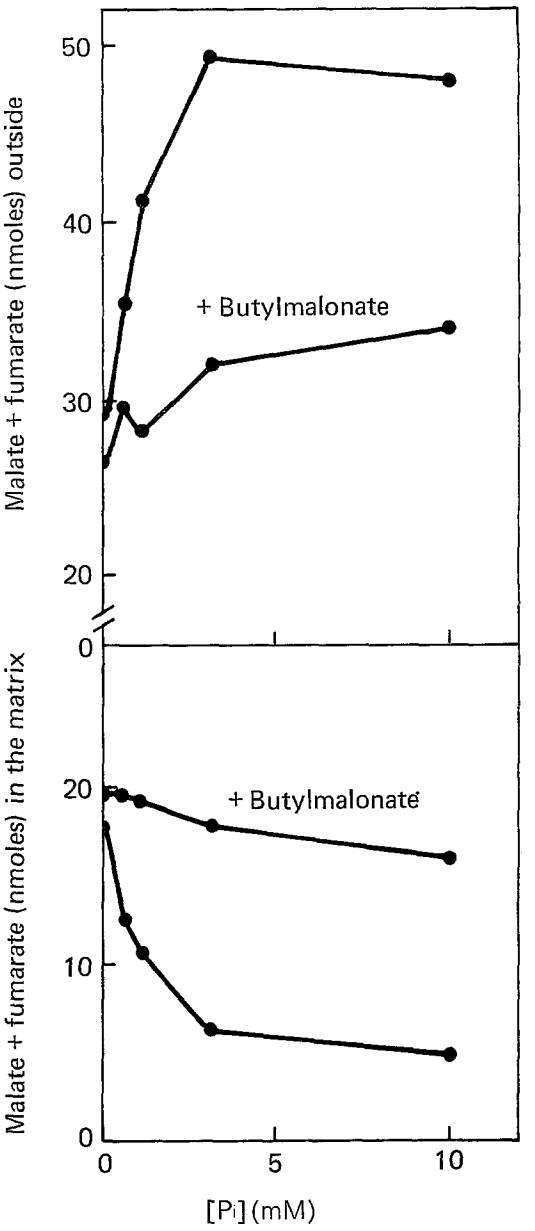


Figure 6. Effect of butylmalonate on the P_i-driven efflux of endogenous malate from mitochondria. Mitochondria (5·9 mg protein) were suspended in 1·5 ml of the reaction mixture described in Fig. 1—Final pH 7·5, reaction temperature 20°, reaction time 1 min. Where indicated P_i, at the concentrations shown in the figure, and 5 mM butylmalonate were present from the beginning of the incubation. For other experimental details see legend to Fig. 1 and Methods.

TABLE III. Stoicheiometric exchange between intramitochondrial malate and extramitochondrial α-oxoglutarate in preincubated mitochondria

First addition	Incubation time (min)	Second addition	Incubation time (min)	Intramitochondrial content (nmoles) of [^{14}C]L-Malate	Intramitochondrial content (nmoles) of α-Oxoglutarate
[^{14}C]Malate	2	None	–	76·7	1·8
[^{14}C]Malate	4	None	–	77·4	0
None	2	α-Oxoglutarate	2	—	35·5
[^{14}C]Malate	2	α-Oxoglutarate	2	39·4	76·2
		Δ Malate		−38·0	
		Δ α-Oxoglutarate			+40·7

Rat-liver mitochondria (12·9 mg protein) were preincubated at 25° with 125 mM KCl, 3 mM $MgCl_2$, 20 mM Tris-HCl (pH 7·5), 0·5 mM ADP, 0·5 mM P_i, 20 mM glucose, hexokinase, and 1 mM arsenite. Final volume, 2·5 ml. Final pH, 7·5. After 2 min, 2·5 μg rotenone and 1·25 μg antimycin were added, followed 10 sec later by the additions given in the table. The incubation was then continued as specified in the table. Where indicated, 1 mM [^{14}C]malate and 3 mM α-oxoglutarate were added. The mitochondria were separated from the suspending medium by procedure 1, as described by Pfaff[22] (see under Methods). The intramitochondrial concentration of α-oxoglutarate and [^{14}C]malate was calculated by correcting the amount found in the mitochondrial extract for that present in the adherent supernatant. For the determination of adherent water, carboxy-[^{14}C]dextran (mol. wt. 60,000–90,000) was included in the reaction mixture.

TABLE IV. Exchange of intramitochondrial α-oxoglutarate with extramitochondrial malate. Insensitivity of the exchange to butylmalonate

Addition during: Preincubation	Addition during: Incubation	Intramitochondrial: α-Oxoglutarate (nmoles)	Intramitochondrial: [^{14}C]Malonate (nmoles)
Glutamate	None	43	—
Glutamate	[^{14}C]Malonate	18	111
Glutamate	[^{14}C]Malonate + Butylmalonate	20	85
None	[^{14}C]Malonate	0·1	93
None	[^{14}C]Malonate + Butylmalonate	0	56
−Butylmalonate	Δ α-Oxoglutarate	−25	
	Δ [^{14}C]Malonate		+18
+Butylmalonate	Δ α-Oxoglutarate	−23	
	Δ [^{14}C[Malonate		+29

Rat-liver mitochondria (20·0 mg protein) were preincubated for 2 min with 125 mM KCl, 20 mM Tris-HCl (pH 7·5), 3 mM $MgCl_2$, 1 mM ADP, 1 mM arsenite, 20 mM glucose and hexokinase. Final volume, 4 ml. Where indicated, 10 mM glutamate was added. After 2 min (±glutamate) 4 μg rotenone, 2 μg antimycin, and 20 μg oligomycin were added, and (where indicated) 1·5 mM [^{14}C]Malonate and 10 mM butylmalonate. The incubation was continued for 2 min and the mitochondria were separated from the suspending medium by procedure 1, as described by Pfaff[22] (see under Methods). For other details see legend to Table III.

TABLE V. Citrate-malate exchange in rat-liver mitochondria

Expt. A

Additions	Amounts (nmoles) in the matrix of					
	[^{14}C]Citrate	Malate	Δ Malate	Δ Citrate	Δ Malate	Δ Citrate
None	87·5	12·9				
Malate	42·0	100·0	+87·1	−45·5		
Mersalyl + Butylmalonate	92·0	10·2				
Malate + Mersalyl + Butylmalonate	53·7	39·5			+29·3	−38·3

Expt. B

[Malate$_{out}$] mM (added)	[Malate$_{in}$] mM (recovered)	[Citrate$_{out}$] mM (added)	[Citrate$_{in}$] mM (recovered)	Δ Citrate / Δ Malate
0·16	1·6	—	—	—
0·32	2·7	—	—	—
0·84	5·6	—	—	—
0·16	0·08	1·18	1·3	0·87
0·32	0·33	1·18	2·2	0·97
0·84	1·3	1·18	4·0	0·93

Expt. A: mitochondria were loaded with [^{14}C]citrate and subsequently suspended in the reaction medium at pH 6·5 (see legend to Fig. 1). Mitochondrial protein 6·0 mg, final volume 1·5 ml, reaction temperature 25°, reaction time 80 sec. Where indicated, 1 mM malate, 0·66 mM mersalyl, and 2 mM butylmalonate were added. Further procedure as in the legend to Fig. 1 (see also under Methods). Expt. B: Mitochondria (3·3 mg protein) were preincubated at 20° in a medium containing 15 mM KCl, 50 mM Tris-HCl, pH 7·5, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM ADP, 1·5 mM P_i, and 1 mM arsenite. After 2 min, 2 μg rotenone were added and 30 sec later the mitochondria were loaded with the different concentrations of malate, as indicated. One minute later citrate (in the concentration indicated) +5 mM butylmalonate were added. After 30 sec mitochondria were centrifuged through silicon oil in a Coleman microcentrifuge (see procedure, 1). Malate and citrate levels were already constant after 30 sec. Citrate influx was measured by using [^{14}C]citrate and unlabelled malate. Malate-outflow was measured in parallel incubations by using [^{14}C]malate and unlabelled citrate.

or [^{14}C]citrate were incubated in a KCl-Tris medium for 1 min at pH 7·5 and 25° in the presence of oligomycin, rotenone, and antimycin. P_i caused an efflux of both α-oxoglutarate and citrate from the mitochondria. Concomitantly $^{32}P_i$ was taken up by mitochondria. Butylmalonate abolished the effect of P_i on the efflux of α-oxoglutarate and citrate. It also depressed $^{32}P_i$ uptake. Since butylmalonate inhibits the malate-P_i antiport (Fig. 6) without having any effect on the α-oxoglutarate-dicarboxylate (Table IV) and citrate-dicarboxylate (Tables V and VI) antiports, the following sequence of events can be thought to explain the induction of α-oxoglutarate and citrate efflux by external P_i. Extramitochondrial P_i drives intramitochondrial malate out by an exchange-diffusion; malate, once outside, drives α-oxoglutarate or citrate out of the mitochondria by exchange-diffusion. The partial insensitivity of P_i uptake to butylmalonate is evidently due to

TABLE VI. Insensitivity of the malate driven efflux of citrate from mitochondria to butylmalonate

Additions	Amount (nmoles) in the matrix of [^{14}C]Citrate	Δ
None	64·2	
Malate	15·8	−48·4
Butylmalonate	69·1	
Butylmalonate + Malate	9·7	−59·4

Mitochondria (18·2 mg protein) were incubated for 3 min at 29° with 175 mM sucrose, 20 mM Tris-HCl (pH 7·5), 10 mM KCl, 3 mM $MgCl_2$, 3·5 μg rotenone, 1·75 μg antimycin, and 1 mM [^{14}C]citrate. Final volume, 3·5 ml. Final pH, 7·5. After this incubation mitochondria were washed and resuspended in a second incubation mixture containing all the components of the first except citrate. Where indicated 3 mM malate and 5 mM butylmalonate were added. After an incubation of 2 min at 29° mitochondria were transferred by centrifugation filtration in $HClO_4$ (see procedure 1, under Methods).

the insensitivity to this inhibitor of the P_i/OH^- antiport (Fig. 2) and of the exchange of endogenous P_i with added $^{32}P_i$ (A. J. Meijer, unpublished observation). The Expt. of Table VIII shows that in a long-term incubation the addition of dicoumarol to the suspending medium caused efflux from mitochondria of P_i, malate, and α-oxoglutarate. The stimulating effect of dicoumarol on the efflux of malate and α-oxoglutarate was, however, abolished by butylmalonate, showing that a P_i-malate exchange diffusion is involved (see also ref. 45). The following sequence of reactions can explain the effect of dicoumarol. Dicoumarol promotes *directly* efflux of P_i. This reaction, which is butylmalonate insensitive, is followed by exchange-diffusion of external P_i with internal malate. Malate, once outside, can exchange with intramitochondrial α-oxoglutarate.

TABLE VII. Effect of dicoumarol on the V_{max} of malate efflux from rat-liver mitochondria and the K_m for the counter anion

Expt.	Counter anion	V_{max} (nmoles/15 sec) Control	+Dicoumarol	K_m (μM) Control	+Dicoumarol
1	P_i	45	48	131	400
2	Malate	67	67	23	40
3	*Citrate	57	51	102	217
4	α-Oxoglutarate	51	40	56	84

* Note that the $MgCl_2$ concentration was 1 mM and no account was taken of Mg^{2+} chelation by citrate in calculating the K_m.

Mitochondria (8·0 mg in Expts. 1 and 3; 7·0 mg in Expts. 2 and 4) preloaded with [^{14}C]malate were centrifuged through the second incubation layer containing 0–1 mM counter anion with or without 100 μM dicoumarol. The counter anion-induced efflux of [^{14}C]malate, i.e. the difference in the amount of [^{14}C]malate remaining in the matrix after passage of the mitochondria through Layer II in the absence and presence of counter anion, was determined. These values were used to construct Lineweaver-Burk plots, from which the value for K_m and V_{max} were obtained. For the experimental procedure see the legends to Figs. 8 and 9. Other details under Methods.

The slight inhibition of the efflux of malate and α-oxoglutarate caused by dicoumarol, in the presence of butylmalonate, can be accounted for by the direct inhibitory effect of the uncoupler on the malate-P_i and malate-α-oxoglutarate exchanges (see Table VII).

It should be recalled that in a long-term incubation experiment an increase of the pH of the medium causes efflux not only of P_i but also of malate, and citrate (Figs. 1 and 4).

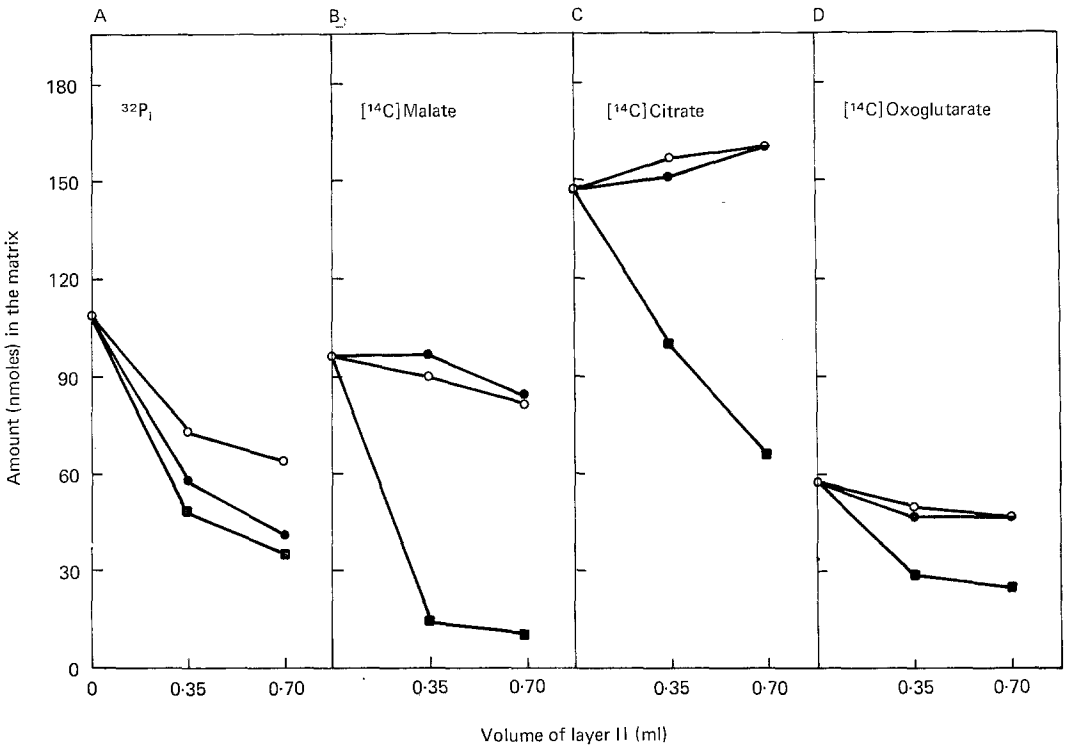


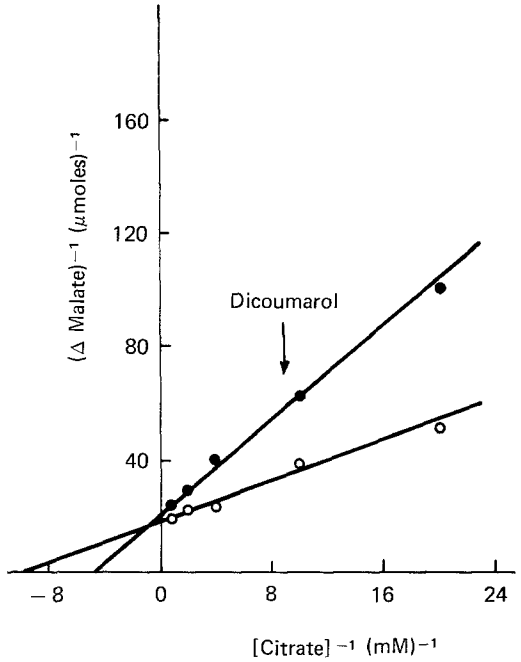
Figure 7. Effect of counteranions and dicoumarol on the efflux of anionic substrates from rat-liver mitochondria. Mitochondria (Expt. A, 9·2 mg protein; Expts. B–D, 7 mg protein) were preincubated in 1 ml of a reaction mixture containing: 150 mM sucrose, 20 mM Tris-HCl (pH 7·4), 0·5 mM EDTA, 1 mM $MgCl_2$, 1·4 μg rotenone, 0·34 μg antimycin, and 10 μg oligomycin. After 1 min, 1 mM $^{32}P_i$ ($9 \cdot 10^5$ counts/min; Expt. A), 1 mM [^{14}C]malate ($5 \cdot 10^5$ counts/min; Expt. B), 1 mM [^{14}C]citrate ($1 \cdot 10^5$ counts/min; Expt. C), or 1 mM [^{14}C]α-oxoglutarate ($5 \cdot 10^5$ counts/min, Expt. D) were added. Two minutes after the addition of the substrates, mitochondria were centrifuged through a washing layer containing the same compounds as the preincubation mixture (except labelled substrate), through a second incubation layer, and finally into $HClO_4$. Layer II contained the same components as the preincubation mixture (except labelled substrate). With no additions (○——○); 40 μM dicoumarol (●——●); 5 mM unlabelled P_i (Expt. A, ■——■) or 5 mM unlabelled malate (Expts. B–D, ■——■). For other details see procedure 3 under Methods.

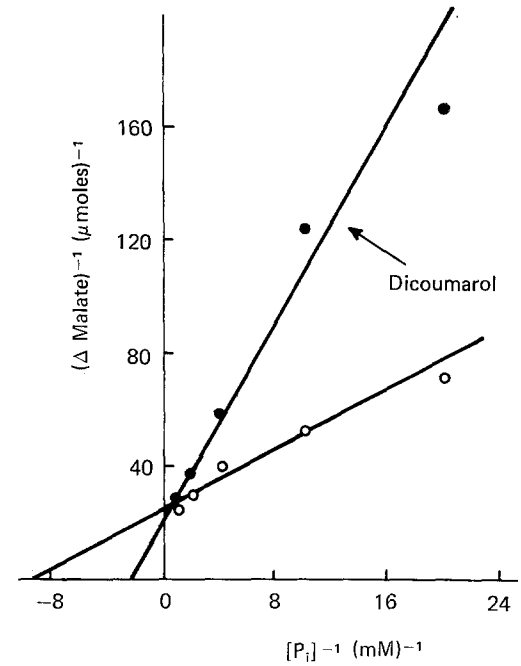
This shows that the increase of the transmembrane ΔpH (outside more alkaline), by promoting an initial efflux of P_i, puts into action all the other exchange-diffusion reactions described.

Effect of Ion Transporting Antibiotics on the Citrate Malate Exchange-Diffusion

In the experiment of Table IX, mitochondria were loaded with [^{14}C]citrate. Butylmalonate and mersalyl were then added and the mitochondria transferred to a second

incubation medium free of potassium. The presence of malate in this medium promoted an efflux of citrate and a stoicheiometric amount of malate was taken up by the mitochondria (see also Table V). The addition of nigericin, which in the absence of malate caused only a slight efflux of intramitochondrial citrate and intramitochondrial (endogenous) malate, caused when added malate was present, a marked stimulation of the efflux of citrate and the uptake of an approximately equal amount of malate. The stoicheiometry of the citrate-malate exchange remained approximately 1:1. Thus nigericin stimulates, under these conditions, the malate-citrate exchange reaction. The data of Table X show that in contrast, valinomycin had no significant effect on the efflux of intramitochondrial citrate both in the absence and presence of external malate.





Discussion

In the absence of energy supply, an external pH increase promotes the efflux from mitochondria of P_i, malate and citrate (Figs. 1 and 4). Conversely it inhibits the uptake by mitochondria of these anionic substrates.[46] The induction by valinomycin of energy-linked uptake of K^+ by mitochondria, which is accompanied by proton ejection[31,32] and alkalinization of the intramitochondrial phase (Figs. 5; see also ref. 33) results in a stimulated accumulation by mitochondria of P_i, malate, citrate, and α-oxoglutarate (Table I, see also refs. 34–37). These and related

Figure 8. Lineweaver-Burk plot of the inhibition by dicoumarol of the exchange-diffusion of [^{14}C]-malate with P_i or citrate in rat-liver mitochondria. Mitochondria (8 mg protein) were loaded with [^{14}C]-malate as described in the legend to Fig. 2 (see also under Methods). Loaded mitochondria were centrifuged through a second incubation layer (0·35 ml), at 0°, containing 0–1 mM P_i or citrate with or without 100 μM dicoumarol. The counteranion induced efflux of [^{14}C]malate, i.e. the difference in the amount of [^{14}C]malate remaining in the matrix after passage of the mitochondria through Layer II in the absence and presence of counteranion, was determined. These values were used to construct Lineweaver-Burk plots. For the details see legend to Fig. 2 and Methods.

experimental results[37] show, in agreement with Mitchell's proposal[12] (contrast ref. 47), that the distribution of substrate anions across the cristae membrane of mitochondria can be governed by a transmembrane pH difference. According to Mitchell[12, 13] this is due to substrate anions being translocated across the mitochondrial membrane by anion-OH^- exchange-diffusion reactions (see also refs. 5, 10, 11) or, what amounts to the same, by anion-H^+ symports. A closer examination of the translocation of anionic substrates in mitochondria shows, however, that the translocation of P_i, but not that of malate, citrate, and α-oxoglutarate, can be directly coupled to an OH^- counterflux. This is supported by the following evidence: (i) When mitochondria, loaded with one single anion, are exposed at 0° for a few seconds only to a medium of pH 7·5 free of anionic substrates, there is a significant efflux of P_i but not of malate, citrate, or α-oxoglutarate. Increasing the pH of the medium from 6·5 to 8·5 promotes the efflux of P_i, but not of the other anionic substrates (Fig. 2); (ii) The stimulation of citrate efflux from mitochondria, observed (in long-term incubation) on increasing the external pH is abolished by butylmalonate *plus* mersalyl (Fig. 4). Butylmalonate abolishes the stimulation of the uptake of malate, α-oxoglutarate, and citrate caused by the valinomycin-mediated active uptake of K^+. In contrast butylmalonate has no inhibitory effect on the OH^- promoted P_i-efflux (Fig. 2) or on the stimulation of P_i uptake by valinomycin. Butylmalonate inhibits specifically the exchange-diffusion of malate with P_i across the mitochondrial membrane (see Fig. 6 and Tables IV and VI). As will be discussed below this last reaction is the link that allows the change in the transmembrane ΔpH to affect the distribution of α-oxoglutarate and citrate across the mitochondrial membrane; (iii)

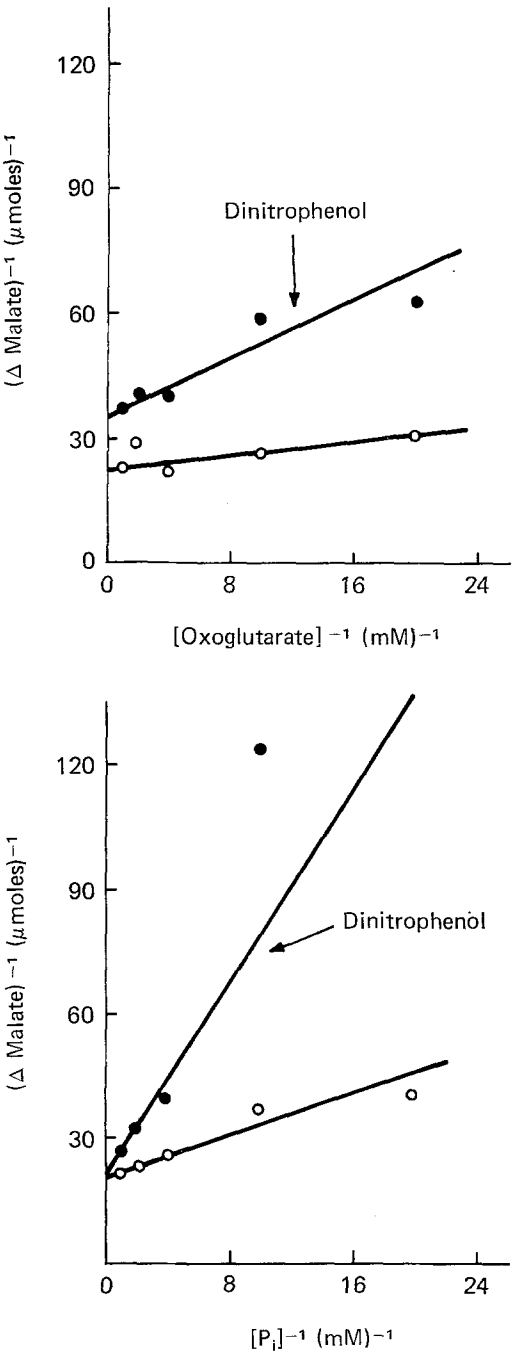


Figure 9. Lineweaver-Burk plot of the inhibition by 2,4-dinitrophenol of the exchange-diffusion of [^{14}C]malate with P_i or α-oxoglutarate in rat-liver mitochondria. Mitochondria (6·5 mg protein). Dinitrophenol: 500 μM. The experimental procedure was exactly that described in the legend to Fig. 8.

P_i but not dicarboxylate anion reverses intramitochondrial alkalinization caused by cation accumulation (Fig. 5; see also refs. 41 and 42); (iv) Mitochondrial swelling caused by active uptake of Ca^{2+}, in the presence of succinate or other anionic respiratory substrates, requires P_i (arsenate or acetate).[48]

The transport of dicarboxylate, α-oxoglutarate, and tricarboxylate, across the inner membrane of rat-liver mitochondria occurs by specific exchange-diffusion reactions. The present study gives a direct demonstration of exchange-diffusion of dicarboxylate with P_i, dicarboxylate with α-oxoglutarate, and malate with tricarboxylate (cf. refs. 5 and 8). The fact that these substances are transported by an antiport mechanism, together with the fact that at physiological pH the concentration of H_3PO_4 (pK_1 2·12) in equilibrium with $H_2PO_4^-$ is very small, argue in favour of a P_i-OH^- antiport mechanism (rather than a H^+ symport) for the translocation of phosphate across the inner mitochondrial membrane. Similarly it is conceivable that anions and not unionized acids are involved in the exchange-diffusion reactions of malate, α-oxoglutarate, and citrate. The stoicheiometry of the P_i-dicarboxylate, dicarboxylate-α-oxoglutarate, and

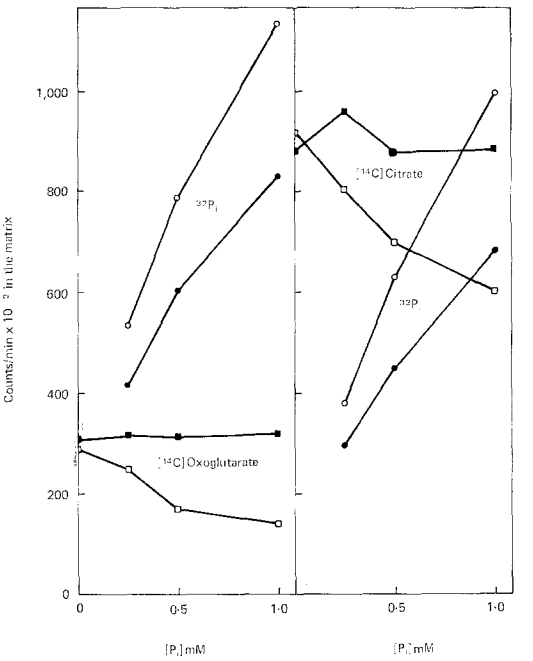


Figure 10. Efflux of intramitochondrial α-oxoglutarate and citrate promoted by P_i-uptake in rat-liver mitochondria. Effect of butylmalonate. Mitochondria were loaded with [^{14}C]α-oxoglutarate or [^{14}C]citrate as described in the legend to Fig. 1—Loaded mitochondria (5·3 mg protein) were then suspended in the reaction mixture described in the legend to Fig. 1. Where indicated $^{32}P_i$, at the concentrations shown in the figure (10^6 counts/min), and 2 mM butylmalonate (■——■; ●——●), were present from the beginning of the incubation. After 1 min mitochondria were centrifuged by procedure 1 (see under Methods) and the intramitochondrial level of $^{32}P_i$, [^{14}C]α-oxoglutarate, and [^{14}C]citrate was determined. For other experimental details see the legend to Fig. 1 and Methods.

TABLE VIII. Effect of dicoumarol and butylmalonate on the efflux from mitochondria of anionic substrates

Additions	Counts per min in the matrix of		
	$^{32}P_i$	[^{14}C]Malate	[^{14}C]Oxoglutarate
None	7,100	38,100	15,500
Dicoumarol	5,700	21,600	8,500
Butylmalonate	5,700	62,700	21,300
Dicoumarol + Butylmalonate	4,600	72,400	31,100

Mitochondria were loaded with $^{32}P_i$, [^{14}C]malate and [^{14}C]α-oxoglutarate. Loaded mitochondria (5·6 mg protein) were incubated 1 min at 25° in 1·5 ml of the reaction mixture described in the legend to Fig. 1. Final pH 7·5. Loading and centrifugation procedure as in the legend to Fig. 1. Additions: 40 μM dicoumarol, 2 mM butylmalonate.

TABLE IX. Effect of nigericin on the exchange of intramitochondrial citrate with extramitochondrial malate

Additions	Amounts (nmoles) in the matrix of	
	[^{14}C]Citrate	Malate
None	92·0	10·2
Nigericin	71·5	8·2
Malate	53·7	54·5
Malate + Nigericin	13·0	81·0
−Nigericin Δ-Citrate	−38·3	
Δ-Malate		+44·3
+Nigericin Δ-Citrate	−58·5	
Δ-Malate		+72·8

Mitochondria were loaded with [^{14}C]citrate and subsequently suspended in the reaction medium at pH 6·5 containing in addition 0·66 mM mersalyl and 2 mM butylmalonate. Mitochondrial protein 6·0 mg, final volume 1·5 ml, reaction temperature 25°, reaction time 80 sec. Where indicated 1 mM malate and 0·2 μg nigericin were added. Further procedure as in the legend to Fig. 1 (see also under Methods).

malate-tricarboxylate exchanges is one to one (Tables II–V). Thus these reactions assure specific changes in the concentration of the various substrate anions in the two spaces, separated by the membrane, without affecting the total concentration of anions. There is evidence indicating that the P_i-OH^- [1], P_i-dicarboxylate [2], dicarboxylate-α-oxoglutarate [3], and malate-tricarboxylate [4] exchange-diffusion reactions are mediated by separate translocators: (i) reactions [2], [3], and [4] exhibit a different specificity spectrum towards the dicarboxylic acids;[1] (ii) the activity of the systems mediating reactions [3] and [4] varies differently in mitochondria of different tissues;[1] (iii) mersalyl inhibits both reactions [1] and [2] but has no effect on reactions [3] and [4];[30] butylmalonate inhibits only reactions [2]; (iv) reactions [1]–[4] have a different sensitivity to uncouplers of

TABLE X. Insensitivity of the exchange of intramitochondrial citrate and extramitochondrial malate to valinomycin

Additions	Counts per min in the matrix of [^{14}C]Citrate
None	85,600
Valinomycin	92,500
Malate	57,700
Malate + Valinomycin	51,100

The experimental procedure was that of the legend to Table IX. Where indicated 0·2 μg valinomycin and 1 mM malate were added. Mitochondrial protein 4·5 mg.

oxidative phosphorylation, and in the case of inhibition the kinetics of inhibition differ from system to system.

Although the four antiport reactions described appear to be mediated by four different translocators, each one can be coupled to the other through circulation of the substrates they share.[19] Such coupling allows the energy, given by the concentration gradient of the various anions across the membrane, to flow efficiently from one system to the other. In this way the transmembrane ΔpH can govern the distribution across the inner mitochondrial membrane not only of P_i but also of the anions whose translocation is not directly protoncoupled (see Figs. 1 and 4.) It also follows that the free energy of the concentration gradient of the substrate anions can be derived from that made available by the flux of electrons along the respiratory chain. It is known that in mitochondria electron flow can be coupled to ejection of protons in the surrounding medium. Various mechanisms have been proposed to explain this proton translocation.[4,18,49–51] Thus, the energy made available by oxidoreductions can be, at least in part, converted to that of a proton gradient across the membrane. Since the translocation of P_i is protoncoupled, the free energy represented by the ΔpH can be converted into that of a P_i gradient and this in turn can flow into the other exchange-diffusion systems, all of these being connected (see Fig. 11). Evidence has been recently obtained, by Papa, Quagliariello, and Chance,[52] that the uptake of P_i by rat-liver mitochondria affects the energy pressure built up by the oxidoreductions of the respiratory chain.

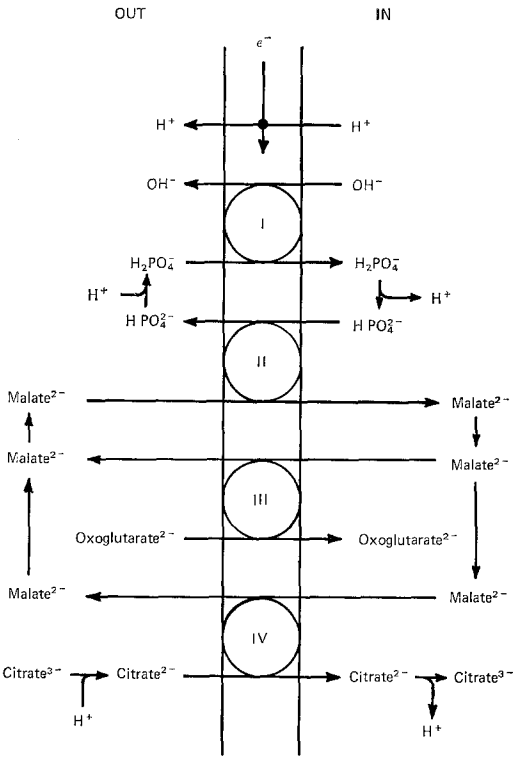


Figure 11. Substrate anion translocation across the inner membrane of rat-liver mitochondria.

The more detailed knowledge of the mechanism of transport of anionic substrates across the inner mitochondrial membrane has made possible a better understanding of the mechanism of action of uncouplers on anion translocation in mitochondria, a problem which has been a matter of considerable debate. The experiments presented in this paper show that uncouplers can act in at least two ways on the transport of anionic substrates. Firstly they promote the efflux of P_i from mitochondria, but not *directly* that of a dicarboxylate or tricarboxylate (Fig. 8; see also ref. 44). Secondly uncouplers inhibit the exchange-diffusion reactions of substrate anions (Table VII). The inhibition of the exchange-diffusion reactions by uncouplers is evidently due to an interaction of the uncoupler with the translocators, that does not involve translocation of the uncoupler molecule. If the latter were the case, one would in fact expect *stimulation* by the uncoupler of the efflux of substrate anions, in the absence of counteranions. The inhibition of the

exchange-diffusion by uncouplers is, on the other hand, reminiscent of the inhibition by uncouplers of some dehydrogenases, like β-hydroxybutyrate dehydrogenase,[53] which are bound to the inner mitochondrial membrane. Uncouplers inhibit the uptake of phosphate by mitochondria[52] and conversely promote its efflux. Thus they tend to dissipate any phosphate gradient across the membrane. This effect does not remain limited to the distribution of P_i but also causes, through the link of the various exchange-diffusion reactions, dissipation of the gradient of the citric-acid cycle intermediates (see Table VIII). The pattern of the effect of uncouplers on the translocation of substrate anion across the membrane will in addition also be determined by the direct inhibitory effects exerted by uncouplers on the various exchange-diffusion translocators. Other experiments, reported elsewhere,[44] indicate that the stimulation by uncouplers of the efflux of phosphate from rat-liver mitochondria and, consequently, their tendency to dissipate substrate anion gradients is a consequence of equilibration of protons across the membrane brought about by the uncouplers.[12, 54]

To give an example of how some of the substrate-anion antiports can be secondarily coupled with the fluxes of other ions across the mitochondrial membrane it is worth examining the case of the citrate–malate exchange. At the physiological pH malate anion carries two negative charges, on the other hand, the concentration of $citrate^{2-}$ in equilibrium with $citrate^{3-}$ is small. If the reaction consisted of an exchange of $citrate^{3-}$ with $malate^{2-}$ it would be electrogenic and should be stimulated by a compensatory flux of cation. On the other hand a neutral $citrate^{2-}$–$malate^{2-}$ exchange would result in a translocation of H^+ across the membrane accompanying the citrate anion. In this case the reaction should be stimulated by promoting H^+ translocation in the direction opposite to that of the citrate flux. The experiment of Table IX seems to distinguish between these two mechanisms. Mitochondria loaded with ^{14}C citrate were suspended in a potassium free medium, and any energy supply was blocked. The experiment shows that the exchange of intramitochondrial citrate with extramitochondrial malate was (in the presence of mersalyl and butylmalonate, to inhibit the other anion exchanges) greatly stimulated by the addition of nigericin. Valinomycin on the contrary had no effect (Table X). Nigericin mediates an electrically-neutral K^+–H^+ exchange diffusion.[32] In a potassium-free medium it causes an influx of H^+ in exchange for mitochondrial K^+. Valinomycin on the other hand mediates under these conditions an electrogenic efflux of K^+.[12, 32, 54] The fact that the $citrate_{in}$–$malate_{out}$ exchange is stimulated by nigericin, but not by valinomycin would indicate that this antiport consists of an electrically neutral exchange of $citrate^{2-}$ against $malate^{2-}$. A summary of this and the other mechanisms of anion translocation across the inner membrane of rat-liver mitochondria is given in Fig. 11.

References

1. J. B. Chappell, *Brit. Med. Bull.*, **24** (1968) 150.
2. M. Klingenberg and E. Pfaff, in: *Regulation of Metabolic Processes in Mitochondria*, J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (eds.), Elsevier, Amsterdam, 1966, p. 180.
3. E. Pfaff and M. Klingenberg, *European J. Biochem.*, **6** (1968) 66.
4. J. B. Chappell and A. R. Crofts, in: *Regulation of Metabolic Processes in Mitochondria*, J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (eds.), Elsevier, Amsterdam, 1966, p. 293.
5. J. B. Chappell and K. N. Haarhoff, in: *Biochemistry of Mitochondria*, E. C. Slater, Z. Kaniuga, and L. Wojtczak (eds.), Academic Press and Polish Scientific Publishers, London and Warsaw, 1967, p. 75.

6. E. J. De Haan and J. M. Tager, *Abstr. 3rd Meeting Federation European Biochem. Soc.*, Warsaw, 1966, Academic Press and Polish Scientific Publishers, London and Warsaw, 1966, p. 159.
7. S. Papa, E. J. De Haan, A. Francavilla, J. M. Tager, and E. Quagliariello, *Biochim. Biophys. Acta*, **131** (1967) 14.
8. B. H. Robinson and J. B. Chappell, *Biochem. Biophys. Res. Commun.*, **28** (1967) 249.
9. A. Azzi, J. B. Chappell, and B. H. Robinson, *Biochem. Biophys. Res. Commun.*, **29** (1967) 148.
10. J. B. Chappell, *Biochem. J.*, **100** (1966) 43.
11. J. B. Chappell, P. J. F. Henderson, J. D. McGivan, and B. H. Robinson, in: *The Interaction of Drugs and Subcellular Components on Animal Cell*, P. N. Campbell (ed.), J. A. Churchill Ltd., London, 1968, p. 71.
12. P. Mitchell, *Chemiosmotic Coupling and Energy Transduction*, Glynn Research Ltd., Bodmin, Cornwall, 1968.
13. P. Mitchell, *Advances in Enzymology*, **29** (1967) 33.
14. E. C. Slater, in: *The Energy Level and Metabolic Control in Mitochondria*, S. Papa, J. M. Tager, E. Quagliariello, and E. C. Slater (eds.), Adriatica Editrice, Bari, Italy, 1969, p. 15.
15. J. L. Gamble Jr., *J. Biol. Chem.*, **240** (1965) 2668.
16. E. J. Harris, K. Van Dam, and B. C. Pressman, *Nature*, **213** (1967) 1126.
17. E. Quagliariello and F. Palmieri, *European J. Biochem.*, **4** (1968) 20.
18. K. Van Dam and K. Kraanijenhof, in: *The Energy Level and Metabolic Control in Mitochondria*, S. Papa, J. M. Tager, E. Quagliariello, and E. C. Slater (eds.), Adriatica Editrice, Bari, Italy, 1969, p. 299.
19. S. Papa, N. E. Lofrumento, M. Loglisci, and E. Quagliariello, *Biochim. Biophys. Acta*, **189** (1969) 311.
20. N. E. Lofrumento, A. J. Meijer, J. M. Tager, S. Papa, and E. Quagliariello, *Biochim. Biophys. Acta*, **197** (1970) 104.
21. D. K. Myers and E. C. Slater, *Biochem. J.*, **67** (1957) 558.
22. E. Pfaff, Ph.D., Thesis, Marburg, 1965.
23. M. Klingenberg, E. Pfaff, and A. Kroger, in: *Rapid Mixing and Sampling Techniques in Biochemistry*, B. Chance, R. H. Eisenhardt, Q. H. Gibson, and K. K. Longernholm (eds.), Academic Press, London, 1964 ,p. 333.
24. B. E. Wakler and A. Wollenberger, *Biochem. Z.*, **329**, (1958) 508.
25. J. M. Tager, S. Papa, E. J. De Haan, R. D'Aloya, and E. Quagliariello, *Biochim. Biophys. Acta*, **172** (1969) 7.
26. S. Ochoa, J. R. Stern, and M. C. Schneider, *J. Biol. Chem.*, **193** (1951) 691.
27. S. Dagley, in: *Methods of Enzymatic Analysis*, H. U. Bergmeyer (ed.), Academic Press, New York and London, 1963, p. 313.
28. D. D. Tyler, *Biochem. J.*, **111** (1969) 665.
29. A. Fonyo, *Biochem. Biophys. Res. Commun.*, **32** (1968) 624.
30. A. J. Meijer and J. M. Tager, *Biochim. Biophys. Acta*, **189** (1969) 136.
31. C. Moore and B. Pressman, *Biochem. Biophys. Res. Commun.*, **15** (1964) 562.
32. B. C. Pressman, E. J. Harris, W. S. Jagger, and J. H. Johnson, *Proc. Natl. Acad. Sci. U.S.A.*, **58** (1967) 1949.
33. E. J. Harris, *FEBS Letters*, **5** (1969) 50.
34. W. S. Lynn and R. N. Brown, *Biochim. Biophys. Acta*, **110** (1966) 459.
35. E. J. Harris, in: *Mitochondria Structure and Function*, L. Ernster and Z. Drahota (eds.), Academic Press, London, 1969, p. 347.
36. E. J. Harris and K. Van Dam, *Biochem. J.*, **106** (1968) 759.
37. F. Palmieri and E. Quagliariello, *European J. Biochem.*, **8** (1969) 473.
38. B. Chance and L. Mela, *Proc. Natl. Acad. Sci., U.S.A.*, **55** (1966) 1243.
39. P. Mitchell, J. Moyle, and L. Smith, *European J. Biochem.*, **4** (1968) 9.
40. N. E. Saris and A. J. Seppala, *European J. Biochem.*, **7** (1969) 267.
41. A. Fonyo, in: *Electron Flow and Energy Conservation*, J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (eds.), Adriatica Editrice, Bari, Italy, 1970, in press.
42. S. Addanki, F. Cahill, and J. F. Sotos, *J. Biol. Chem.*, **243** (1968) 2337.
43. C. Rossi, A. Scarpa, and G. F. Azzone, *Biochemistry*, **6** (1967) 3902.
44. S. Papa, M. A. Zanghi, G. Paradies, and E. Quagliariello, *FEBS Letters*, **6** (1970) 1.
45. A. J. Meijer, S. Papa, G. Paradies, M. A. Zanghi, J. M. Tager, and E. Quagliariello, *Biochim. Biophys. Acta*, **197** (1970) 97.
46. F. Palmieri, E. Quagliariello, and M. Klingenberg, *Biochem. J.* **119** (1970) 36P.
47. E. J. Harris and B. C. Pressman, *Biochim. Biophys. Acta*, **172** (1969) 66.
48. J. B. Chappell and A. R. Crofts, *Biochem. J.*, **95** (1965) 378.
49. P. Mitchell, *Biol. Rev.*, **41** (1966) 445.
50. B. Chance and L. Mela, *Nature*, **212** (1966) 369.
51. R. J. P. Williams, in: *Electron Flow and Energy Conservation*, J. M. Tager, S. Papa, E. Quagliariello, and E. C. Slater (eds.), Adriatica Editrice, Bari, Italy, 1970, in press.
52. S. Papa, E. Quagliariello, and B. Chance, *Biochemistry*, **9** (1970) 1706.
53. P. Jurtshuk Jr., I. Sekuzu, and D. E. Green, *J. Biol. Chem.*, **238** (1963) 3595.
54. P. Mitchell and J. Moyle, in: *Biochemistry of Mitochondria*, E. C. Slater, Z. Kaniuga, and L. Wojtczak (eds.), Academic Press and Polish Scientific Publishers, London, Warsaw, 1967, p. 53.