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On the transport of inorganic phosphate and malate in rat-liver mitochondria

A study of the osmotic behaviour of mitochondria and of the kinetics of reduction or oxidation of intramitochondrial NAD(P) by anionic substrates led CHAPPELL *et al.*[1] to propose that P_i and citric-acid-cycle intermediates[2] are transported in mitochondria by specific carriers by an exchange–diffusion process. According to MITCHELL[3], anion translocation occurs either by an OH^-/anion antiport or by an H^+/anion symport. By examining the movement of ^{14}C-labelled substrates from the extra- to the intramitochondrial water and *vice versa*, we have directly demonstrated the occurrence in rat-liver mitochondria of exchange–diffusion reactions between a tricarboxylic acid or α-oxoglutarate and a dicarboxylic acid[4,5]. In this paper, the occurrence of an exchange–diffusion reaction between P_i and malate across the inner membrane of rat-liver mitochondria is shown. Furthermore, evidence is presented that the translocation of P_i, but not that of malate, is directly coupled to an OH^- counterflux (or an H^+ symport).

In the experiment of Table I, mitochondria were preincubated aerobically with ADP, glucose and hexokinase to lower the content of endogenous P_i and citric-acid-cycle intermediates. $^{32}P_i$ was then added in the presence of oligomycin and *N,N,N',N'*-tetramethyl-*p*-phenylenediamine (TMPD) (*plus* ascorbate) as respiratory substrate. Under these conditions, mitochondria accumulated P_i. After having been loaded with $^{32}P_i$, the mitochondria were centrifuged into a second incubation layer free of P_i and from this layer into $HClO_4$. The presence of [^{14}C]malate in the second layer promoted

TABLE I

EXCHANGE DIFFUSION BETWEEN INTRAMITOCHONDRIAL $^{32}P_i$ AND EXTRAMITOCHONDRIAL [^{14}C]-MALATE IN RAT-LIVER MITOCHONDRIA

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[6]. Where indicated, 2 mM [^{14}C]malate was present in Layer II. The $^{32}P_i$ and [^{14}C]malate 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 medium; this was determined with [^{14}C]sucrose. $\Delta^{32}P_i$ 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.

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
$\Delta^{32}P_i$		−63	
Δ[^{14}C]Malate			+67

Abbreviation: TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine.

P_i efflux from the mitochondria. Concomitantly, [^{14}C]malate was taken up. P_i-loaded mitochondria took up twice as much [^{14}C]malate as unloaded mitochondria. Table I shows that the decrease in the P_i content of the mitochondria brought about by malate (63 nmoles) was approximately equal to the amount of malate taken up by the P_i-loaded mitochondria (67 nmoles). Thus, extramitochondrial malate pulls intramitochondrial P_i out by entering the mitochondria in exchange for P_i. The stoicheiometry of the exchange–diffusion is one to one.

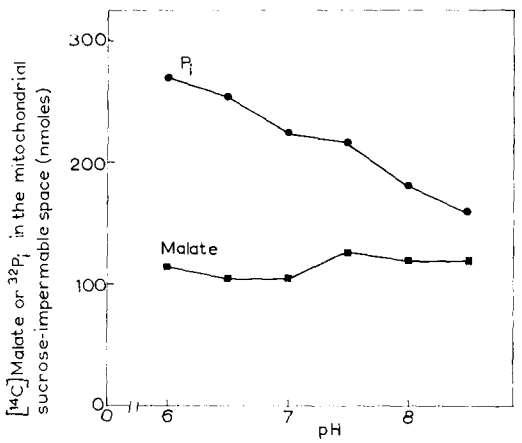

Fig. 1. Effect of pH on the efflux of P_i and malate from rat-liver mitochondria. Rat-liver mitochondria (7.2 mg protein) were incubated in Layer I (final volume, 1.5 ml) with 180 mM sucrose, 10 mM KCl, 0.3 mM TMPD, 3 mM ascorbate, 2 μg rotenone, 15 μg oligomycin and 2 mM [^{14}C]-malate or 2 mM $^{32}P_i$. After 1 min the mitochondria were transferred to Layer II, containing 20 mM Tris–HCl, at the pH shown in the figure, and all the components of the first layer except TMPD, ascorbate, P_i and malate. The experimental procedure was that described in the legend to Table I.

In the experiment of Fig. 1, the pH dependence of the efflux of P_i or malate was examined. Mitochondria were loaded with $^{32}P_i$ or [^{14}C]malate and subsequently transferred to a second incubation layer free of P_i or malate. 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 6.0 to 8.5 had practically no effect on the efflux of malate.

In the experiment of Fig. 2, bromothymol blue was included in the mitochondrial suspension, and its absorbance changes were recorded during the incubation. According to CHANCE AND MELA[7], bromothymol blue measures intramitochondrial pH (see, however, refs. 8 and 9). Mitochondria were suspended in a KCl medium and depleted of endogenous P_i by incubation with glutamate and a $\sim$P trap (Figs. 2a and 2b). The addition of valinomycin, which promotes K^+ uptake in exchange for H^+ (ref. 10), caused a fast and large increase of bromothymol blue absorbance, the kinetics of which were similar to those reported by PRESSMAN *et al.*[10] for the appearance of H^+ in the medium. This absorbance increase indicates, at least in part, mitochondrial alkalinisation. The addition of malate gave a small further increase of absorbance (Fig. 2a). P_i, on the contrary, immediately reversed the absorbance increase. Acetate gave an effect similar to that of P_i (Fig. 2b). ROSSI *et al.*[11] have reported that the uptake of P_i lowers the H^+/K^+ ratio during valinomycin-induced K^+ uptake by mito-

chondria. When malate was added to the mitochondrial suspension without prior depletion of P_i, it caused some reversal of the valinomycin-induced absorbance increase (Fig. 2c). This effect of malate was partly inhibited by 1 mM butylmalonate (Fig. 2d). This inhibition could be increased by raising the concentration of butyl malonate. These results, in agreement with those of Fig. 1, indicate that the transport of P_i, but not that of malate, may be directly coupled to an OH^- antiport (or H^+ symport).

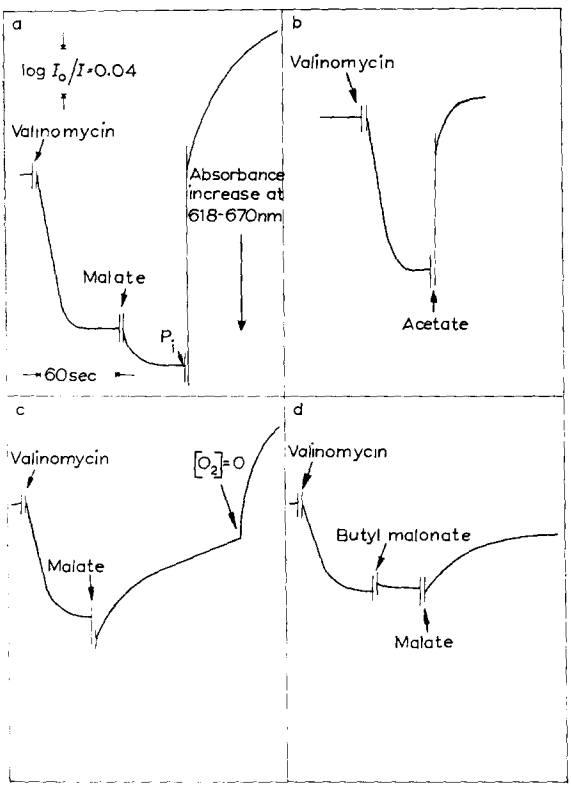

Fig. 2. Effect of malate, P_i and acetate on bromothymol blue absorbance changes in mitochondria. In Expts. a and b, rat-liver mitochondria (9 mg protein) were preincubated for 4 min at 25° in the presence of 130 mM KCl, 20 mM Tris–HCl (pH 7.5), 5 mM glutamate, 0.2 mM ADP, 3 μM bromothymol blue, 10 mM 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 butyl malonate. Final volume, 3 ml. The absorbance changes were continuously recorded in the Aminco-Chance dual-wavelength spectrophotometer.

CHAPPELL AND HAARHOFF[2] have shown that P_i promotes the penetration of dicarboxylic acids into the mitochondria (see ref. 12). They have explained this by assuming that dicarboxylic acids can exchange with either OH^- or P_i. The data presented here directly show the occurrence of an exchange–diffusion reaction between P_i and malate across the membrane of rat-liver mitochondria. There is evidence that this reaction is inhibited by butyl malonate[5,13]. On the contrary, butyl malonate has

no effect on the exchange-diffusion of α-oxoglutarate or of a tricarboxylic acid with a dicarboxylic acid[5]. These facts, together with the different specificity exhibited by the latter two antiport reactions towards the dicarboxylic acid, indicates that the three reactions are mediated by three different translocators (see ref. 14). The pH dependency of P_i efflux and the effect of P_i on the bromothymol blue response provide evidence for the occurrence of a P_i/OH^- antiport (or P_i/H^+ symport) across the inner membrane in rat-liver mitochondria. On the contrary, no direct coupling seems to exist between malate and OH^- (or H^+) translocation. This is also supported by the different sensitivity of the various antiports to inhibitors[13]. Although the four antiport reactions discussed in this paper seem to be mediated by separate translocators, each one can be coupled with the other through circulation of the substrates they share. Such coupling allows an efficient flow of the energy, given by the concentration gradient of the various anions across the membrane, from one system to the other.

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