The Transport of Inorganic Phosphate by the Mitochondrial Dicarboxylate Carrier

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1. *N*-Ethylmaleimide inhibited the influx and efflux of P_i in rat liver mitochondria. 2. The efflux was stimulated by either succinate or malate in the presence of *N*-ethylmaleimide, and this stimulation was reversed by 2-*n*-butylmalonate. 2-Oxoglutarate and citrate, even in the presence of low concentrations of malate, were relatively ineffective in stimulating efflux of P_i under these conditions, as was glutamate. 3. By using radioactively labelled P_i and dicarboxylate ions an exchange was demonstrated, the stoichiometry of which was 1.3 ± 0.5 dicarboxylate ions:1 P_i ($n = 10$). 4. An exchange between unlabelled and labelled P_i in the presence of *N*-ethylmaleimide was found which was sensitive to 2-*n*-butylmalonate. 5. It is concluded that the mitochondrial dicarboxylate carrier can transport phosphate by an exchange diffusion with certain penetrant dicarboxylic acids or with phosphate itself. The exchange mechanism is sensitive to 2-*n*-butylmalonate but is unaffected by *N*-ethylmaleimide; the action of mersalyl in this context is commented on.

That mitochondria require a transporting system for P_i was proposed by Chappell & Crofts (1966) on the basis of studies using the ammonium swelling technique and the antibiotics gramicidin and valinomycin. Their proposal received support from the work of Fonyo (1968), Fonyo & Bessman (1968) and Tyler (1968, 1969), who showed that low concentrations of organic mercurial compounds were able to abolish functions dependent on both externally added P_i and the extrusion of P_i from the mitochondrial matrix.

The evidence in favour of a carrier for dicarboxylic acids includes the observation that penetration of such acids is stereospecific (Chappell & Haarhoff, 1967) and the competitive behaviour of certain substituted dicarboxylic acids, for example 2-*n*-butylmalonate (Robinson & Chappell, 1967) and 2-phenylsuccinate (Chappell & Robinson, 1968). Further, mitochondria suspended in isosmolar solutions of ammonium succinate or ammonium malate require catalytic amounts of externally added P_i before swelling is observed (Chappell & Haarhoff, 1967). This effect was interpreted as follows: (i) P_i enters the mitochondria in exchange for OH^- ion via the phosphate carrier; (ii) P_i exits on the dicarboxylate carrier in exchange for dicarboxylate ion entering, thus allowing P_i to recycle and providing an explanation of its catalytic action.

The sequence of events proposed by Chappell & Haarhoff (1967) gained support from the observation that inhibitors of P_i transport inhibit dicarboxylate

transport as assayed by the ammonium swelling technique (Fonyo & Bessman, 1968; Tyler, 1969), indicating the need for externally added P_i to enter the mitochondrial matrix. It should be noted, however, that succinate oxidation is little affected by inhibitors of phosphate transport (Tyler, 1969), since under these conditions succinate may exchange with internal malate produced as a result of its own oxidation (Chappell & Haarhoff, 1967).

Although the exchange of internal P_i for external malate had been measured with a stoichiometry close to 1:1 (Papa *et al.*, 1969), it remained to be demonstrated that the activity of the dicarboxylate carrier was sufficient for this exchange, i.e. that transport of P_i could proceed when the phosphate carrier was inhibited. This is the purpose of the present paper.

Preliminary accounts of this work have been published (Johnson & Chappell, 1970; Chappell, 1970).

Experimental

Methods

Preparation of mitochondria. Rat liver mitochondria were prepared by the method of Chappell & Hansford (1969).

Volume changes of mitochondria. The time-course of the absolute change in mitochondrial volume is reflected in the change in absorbance of the mitochondrial suspension (Tedeschi & Harris, 1955). Continuous monitoring of mitochondrial swelling

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was carried out by using a Hilger-Gilford recording spectrophotometer set at a wavelength of 650nm.

Incubation and preparation of samples for liquid-scintillation counting. Mitochondria (4–8mg of protein) were incubated at room temperature in 1.0ml of 120mM-KCl–20mM-Tris–HCl, pH7.4, contained in Eppendorf centrifuge tubes of 1.5 ml total volume. They were sedimented in a microcentrifuge (Eppendorf model 3200) by centrifuging for 4min. Most of the supernatant fraction was rapidly removed with a Pasteur pipette and kept for analysis. The small volume adhering to the surface of the pellet was removed with a micropipette, and finally the inside of the tube was wiped dry with a piece of filter paper. The pellet was resuspended by first breaking it up with a Vortex mixer, followed by the addition of 0.4ml of water and remixing. Deproteinization was done by the addition of 0.8ml of 5% (w/v) HClO_4 to 0.4ml of supernatant or to the total pellet dispersed as described above. The denatured protein was sedimented in the microcentrifuge, and 1ml of extract was removed and neutralized to pH7 with 1M-potassium phosphate, pH10.5. The neutralized extract was cooled on ice for 10min to aid precipitation of KClO_4 , which was spun off in the microcentrifuge. A sample (0.5–1.0ml) was assayed for radioactivity.

Determination of radioactivity. Samples were assayed by liquid-scintillation counting in a Nuclear-Chicago mark I spectrometer. Quench curves for determining ^{14}C and ^{32}P in the same sample were constructed according to the operating manual for the counter, and a simple program written for an Olivetti Programma 101 computer by Dr. P. J. England gave a print-out of ^{14}C d.p.m. and ^{32}P d.p.m. from the crude data. The scintillation fluid comprised 4g of 5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole plus 100g of naphthalene in 1 litre of dioxan, and up to 1ml of aqueous sample was added to 15ml of scintillation fluid.

Assay of P_i . P_i was determined in deproteinized extracts of mitochondria and the suspending medium by the method of Martin & Doty (1949).

Assay of mitochondrial protein. Protein was assayed by a modified biuret procedure that included deoxycholate to dissolve the mitochondria. A standard curve was constructed by using bovine serum albumin (fraction V; Sigma Chemical Company, St. Louis, Mo. 63178, U.S.A.).

Materials

Radiochemicals were from The Radiochemical Centre (Amersham, Bucks., U.K.); $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ was synthesized by the method of Glynn & Chappell (1964). Monensin was a gift from Dr. R. L. Hamill

(Eli Lilly and Co., Indianapolis, Ind., U.S.A.) and Dr. P. G. Heytler kindly gave the carbonyl cyanide *p*-chlorophenylhydrazone. 2-Chloromercuri-4,6-dinitrophenol was given by Dr. D. R. Trentham. *N*-Ethylmaleimide was obtained from BDH Chemicals Ltd. (Poole, Dorset, U.K.) and assayed before use by the method of Alexander (1958). Mersalyl [*o*-(3-hydroxymercuri-2-methoxypropyl)carbamoylphenoxylacetate, sodium salt] was from Sigma Chemical Company and 2-*n*-butylmalonate from Aldrich Chemical Company (Milwaukee, Wis. 53233, U.S.A.). All other chemicals were of A.R. grade or the highest grade obtainable.

Results

N-Ethylmaleimide was used extensively in this work. Since no detailed description of its effect on mitochondria has appeared in the literature, a brief examination of its properties is included initially.

Inhibition of P_i influx by *N*-ethylmaleimide

A rapid and convenient way of examining transport in mitochondria is by the ammonium swelling technique (see Chappell & Haarhoff, 1967). However,

Fig. 1. Inhibition of passive mitochondrial swelling in iso-osmolar potassium phosphate by *N*-ethylmaleimide

Mitochondrial suspension (0.2ml) containing about 16mg of protein was incubated for 2min at 0°C with 0.2ml of 145mM-Tris–HCl, pH7.4, containing increasing amounts of *N*-ethylmaleimide. Then 75 μl of incubated sample was added to 2.5ml of 100mM-potassium phosphate, pH7.4, containing 1 μg of rotenone. Swelling was initiated by the addition of 25 μg of monensin in 10 μl of ethanol, and initial rates of swelling were measured to derive the plot. The assay was carried out at 25°C.

when mitochondria are suspended in ammonium phosphate they swell immediately, making initial-rate measurements uncertain. For this reason, transport of P_i was assayed by suspending the mitochondria in iso-osmolar potassium phosphate, obtaining a steady absorbance reading and then initiating swelling with an antibiotic, monensin, which promotes a K^+ - H^+ exchange (Henderson *et al.*, 1969). By preincubating the mitochondria with increasing amounts of *N*-ethylmaleimide and measuring initial rates of swelling, a sigmoid inhibition curve was obtained (Fig. 1) with

Fig. 2. Inhibition of passive mitochondrial swelling in iso-osmolar potassium phosphate by 2-chloromercuri-4,6-dinitrophenol

Conditions were exactly as described in Fig. 1, except that the mercurial replaced *N*-ethylmaleimide.

a maximum at about 15 nmol of *N*-ethylmaleimide/mg of mitochondrial protein. There are two possible reasons for the nature of the inhibition curve: (i) transport of P_i was not the limiting feature of the assay system with the result that *N*-ethylmaleimide was able to titrate a proportion of carrier sites before affecting P_i transport; (ii) *N*-ethylmaleimide was able to react with other, more reactive groups before binding the thiol group implicated in P_i transport. From other evidence the second alternative appeared more likely: when the organic mercurial 2-chloromercuri-4,6-dinitrophenol was used instead of *N*-ethylmaleimide, the inhibition curve appeared more hyperbolic than sigmoid (Fig. 2); secondly, when the potassium phosphate was replaced by iso-osmolar potassium acetate, a more rapid rate of swelling was observed, indicating that P_i was a limiting factor in the potassium phosphate swelling system. It should also be emphasized that *N*-ethylmaleimide had no effect on the swelling of mitochondria in potassium acetate, indicating that the mitochondrial inner membrane had not been radically altered with regard to its more general permeability properties.

*Inhibition of P_i efflux by *N*-ethylmaleimide: the effect of added dicarboxylate ions*

Tyler (1969) reported that when mitochondria were incubated in the presence of ATP and an uncoupling agent, most of the P_i liberated was found in the medium. However, in the presence of mersalyl, an inhibitor of P_i influx, the situation was reversed such that most of the P_i was trapped in the mitochondrial matrix. The results in Table 1 are in agreement with Tyler's (1969) findings and show that *N*-ethylmaleimide and mersalyl have a similar effect on the

Table 1. Distribution of P_i after uncoupler-stimulated adenosine triphosphatase activity, and the effect of added succinate

Mitochondria (about 8mg of protein) were incubated in 1ml of 120mM-KCl-20mM-Tris-HCl, pH 7.4, containing 1 μ g of rotenone and 1 μ g of antimycin A for 1min at 20°C. When added, *N*-ethylmaleimide and mersalyl were present at concentrations equivalent to 20nmol and 10nmol/mg of protein respectively. Carbonyl cyanide *p*-chlorophenylhydrazone was added to each sample to a final concentration of 1 μ M, and succinate was present at a concentration of 10mM. Finally, the mitochondria were incubated for 2min in the presence of 2.5mM-ATP, after which they were rapidly sedimented and analysed for P_i .

Additions	P_i (μ mol)		
	Pellet	Supernatant	Total
None	0.26	1.08	1.34
<i>N</i> -Ethylmaleimide	0.80	0.40	1.20
Mersalyl	0.63	0.68	1.31
<i>N</i> -Ethylmaleimide + succinate	0.46	0.67	1.13
Mersalyl + succinate	0.39	0.92	1.31

Table 2. *Effect of added substrate ions on the amount of P_i trapped in the pellet after uncoupler-stimulated adenosine triphosphatase activity*

The conditions used were exactly as described in Table 1. *N*-Ethylmaleimide was present at a concentration equivalent to 20nmol/mg of protein.

Additions	P_i in pellet (μ mol)
None	0.30
<i>N</i> -Ethylmaleimide	0.74
<i>N</i> -Ethylmaleimide+10mM-succinate	0.48
<i>N</i> -Ethylmaleimide+10mM-malate	0.44
<i>N</i> -Ethylmaleimide+10mM-succinate+5mM-butylmalonate	0.70
<i>N</i> -Ethylmaleimide+20mM-citrate	0.72
<i>N</i> -Ethylmaleimide+20mM-citrate+1mM-malate	0.67
<i>N</i> -Ethylmaleimide+20mM-oxoglutarate	0.63
<i>N</i> -Ethylmaleimide+20mM-oxoglutarate+1mM-malate	0.54
<i>N</i> -Ethylmaleimide+20mM-glutamate	0.69

Table 3. *Amount of P_i and added substrates associated with the pellet after uncoupler-stimulated adenosine triphosphatase activity*

The conditions used were exactly as described in Table 1, with *N*-ethylmaleimide present in every case at a concentration equivalent to 20nmol/mg of protein. [γ - 32 P]ATP and 14 C-labelled substrates were used to identify P_i and substrate respectively. 14 C radioactivity in the control was supplied by [14 C]-sucrose and gave a measure of the space in the pellet external to the matrix. In all other cases, by knowing the specific radioactivity of the radioactively labelled substrate it was possible to convert the 14 C space into μ mol of substrate.

Addition	P_i in pellet (μ mol)	Substrate in matrix space (μ mol)
None (sucrose)	0.60	0
10mM-Succinate	0.28	0.35
10mM-Malate	0.27	0.25
20mM-Glutamate	0.58	0.06

distribution of P_i . Table 1 also shows the effect of adding succinate, a penetrant dicarboxylic acid, to the mitochondria with a high P_i content. With both *N*-ethylmaleimide and mersalyl it was found that the distribution of P_i altered so that a greater proportion appeared in the medium. Respiratory activity was excluded in these experiments by the presence of rotenone and antimycin A in the incubation medium.

The basic design of this experiment was repeated by using a variety of substrates of mitochondrial metabolism. The results are presented in Table 2. Both succinate and malate were effective in releasing P_i from the pellet fraction, whereas 2-*n*-butylmalonate added with them had an inhibitory action (shown

Table 4. *Effect of *n*-butylmalonate on the phosphate-phosphate exchange*

The conditions used were as described in Table 1 with the following modifications. *N*-Ethylmaleimide was present in each sample at a concentration equivalent to 20nmol/mg of protein, [γ - 32 P]ATP was used, and [14 C]sucrose was present in the incubation medium. The final concentrations of added unlabelled P_i and *n*-butylmalonate were 10mM and 5mM respectively. The results are means $\pm$ S.D. for six observations.

Additions	32 P retained in the sucrose-impenetrable space (%)
None	100 $\pm$ 5
P_i	35 $\pm$ 5
P_i + <i>n</i> -butylmalonate	80 $\pm$ 10

with succinate). Other substrates were relatively ineffective even at higher concentrations, and the changes found in the presence of an additional low concentration of malate, an activator of entry of citrate and oxoglutarate (Chappell & Robinson, 1968), were those expected of that concentration of malate alone. Glutamate was similarly ineffective despite reports that its entry is weakly activated by P_i (see, e.g., Chappell *et al.*, 1968).

Exchange of P_i and dicarboxylate ion

To demonstrate whether an exchange was responsible for the release of P_i from the mitochondria, [γ - 32 P]ATP was used to generate [32 P] P_i within the matrix space, and the added substrates were 14 C-labelled. The control sample contained [14 C]sucrose

and gave a measure of the space in the pellet external to the matrix. Dicarboxylate uptake was assessed relative to this space (see Table 3). It was found that ^{14}C radioactivity in the pellet was greatly increased with succinate and malate, although hardly at all with glutamate. At the same time, ^{32}P radioactivity in the pellet, over 90% of which could be recovered as P_i by extracting the phosphomolybdate complex into butan-1-ol-benzene (1:1, v/v), was decreased correspondingly. Over ten experiments, the ratio of increase in mol of ^{14}C -labelled substrate to decrease in mol of $[\text{}^{32}\text{P}]\text{P}_i$ was $1.3 \pm 0.5:1$, i.e. mean $\pm$ s.D.

Phosphate-phosphate exchange

The results so far have been consistent with the proposal that the dicarboxylate carrier can exchange dicarboxylate ion and P_i . The activity of the phosphate carrier has been discounted since the experiments have included *N*-ethylmaleimide and rely on *N*-ethylmaleimide in order that P_i may be accumulated in the matrix space. If the dicarboxylate carrier is responsible for transporting P_i , then it should be possible to demonstrate a phosphate-phosphate exchange sensitive to inhibitors of dicarboxylate transport. This was tested by generating $[\text{}^{32}\text{P}]\text{P}_i$ within the matrix space, as described above, but instead of ^{14}C -labelled substrate, unlabelled P_i was added externally. The results (Table 4) showed that the radioactivity in the pellet fell in consequence, indicative of a phosphate exchange; the inclusion of $[\text{}^{14}\text{C}]$ -sucrose in each sample allowed the results to be referred to the sucrose-impenetrable space. When *n*-butylmalonate was added as well as the unlabelled P_i , however, the effect was much decreased in size.

Discussion

Although Chappell & Haahrhoff (1967) had proposed that penetrant dicarboxylate ions could exchange for phosphate across the mitochondrial inner membrane, and Papa *et al.* (1969) had measured the stoichiometry of the exchange, direct evidence that the activity of the dicarboxylate carrier was sufficient has been lacking. By using *N*-ethylmaleimide to inhibit the phosphate carrier it has been possible in this work to examine the function of the dicarboxylate carrier independently. Scheme 1 summarizes the interpretation placed on the results. Carrier I represents the adenine nucleotide carrier that catalyses an exchange between ATP and ADP (Klingenberg & Pfaff, 1966). Once in the matrix space, the ATP may be hydrolysed by the uncoupler-stimulated adenosine triphosphatase to produce ADP and P_i . Normally, P_i can leave the matrix in exchange for OH^- ion via carrier II, the phosphate carrier. In the presence of inhibitors of phosphate transport such as *N*-ethyl-

Scheme 1. Proposed mechanisms for the transport of P_i

The left-hand phase, marked 'out', represents the compartment external to the matrix and the right-hand phase, marked 'in', represents the matrix itself. The central phase is the mitochondrial inner membrane containing carriers of type I-III. Carrier I is the atractyloside-sensitive adenine nucleotide carrier; carrier II is the phosphate carrier (inhibited by *N*-ethylmaleimide); carrier III is the dicarboxylate carrier (inhibited by *n*-butylmalonate). The reaction $\text{ADP}-^{32}\text{P}_i \rightarrow \text{ADP}+^{32}\text{P}_i$ is catalysed by the uncoupler-stimulated adenosine triphosphatase. Although the inhibitors, *N*-ethylmaleimide and *n*-butylmalonate, are shown as approaching the inner membrane from the matrix side, it is not meant to imply that this is their actual mode of inhibition.

maleimide and mersalyl, however, the efflux of P_i is prevented and it is retained in the matrix. There exists another mechanism whereby P_i can be released, and this is carrier III, the dicarboxylate carrier. This remains inoperative until suitably penetrant dicarboxylate ions are present in the external medium, whereupon an exchange can take place. Alternatively, if the internal P_i is radioactively labelled, a release of radioactivity by unlabelled P_i may be observed. The dicarboxylate carrier is sensitive to *n*-butylmalonate and the exchanges taking place through the postulated activity of carrier III were inhibited by this compound.

In view of the substrate requirements and inhibitor specificity, it would appear that the dicarboxylate

carrier can transport P_i quite independently of the phosphate carrier. By contrast, the transport of citrate, oxoglutarate and glutamate does not seem to be directly phosphate-coupled, i.e. P_i is not transported on any of the three separate carriers for these ions. However, an exchange between malate and P_i followed by an exchange between malate and either citrate or oxoglutarate is not excluded by these results.

A possible criticism of the adopted approach arises from the observations of Tyler (1969), using mersalyl, and Guérin *et al.* (1970), using *N*-ethylmaleimide, that the mitochondria swell on incubation with ATP and uncoupling agent when P_i efflux is prevented. When swelling was monitored under the conditions employed in the present work, it was found to be of low amplitude over the 5 min incubation period. Also Meijer *et al.* (1970), using a different method, produced results which agree very well with those presented here. They measured the exchange between [32 P] P_i , added externally, and intramitochondrial anions, and found that *N*-ethylmaleimide required *n*-butylmalonate to block the exchange completely. Mersalyl was sufficient by itself to inhibit the exchange reactions, however, in agreement with their earlier work (Meyer & Tager, 1969), although the amounts required were greater than those needed to inhibit P_i transport; significantly, in the presence of *n*-butylmalonate, the concentration of mersalyl necessary for 100% inhibition was decreased. The original criticism, therefore, seems unjustified.

The significance of a phosphate-dicarboxylate exchange system has been discussed in general terms by McGivan & Klingenberg (1971). They have pointed out that by coupling transport of P_i and dicarboxylate a net movement of anions is made possible: this is of physiological significance in lipogenesis and gluconeogenesis, where a net export of anions may be expected (see Greville, 1969, for review). The maintenance of electrical neutrality in ion transport is of general importance and the expectation of exchange-diffusion carriers is one of four postulates of Mitchell's chemiosmotic hypothesis (Mitchell, 1961, 1966). Finally, McGivan & Klingenberg (1971) note the possibility of an energy-linked accumulation of anions brought about at the expense of a pH gradient across the mitochondrial inner membrane. The pH difference is responsible for the uptake of P_i , which can then drive dicarboxylate uptake, and ultimately uptake of tricarboxylate and oxoglutarate. Whether this is of significance with regard to the redox-potential difference between the mitochondria and the cytosol, as suggested by McGivan & Klingenberg (1971), remains to be demonstrated.

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