The pathway of inorganic-phosphate efflux from isolated liver mitochondria during adenosine triphosphate hydrolysis

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(Received 28 March 1980/Accepted 8 July 1980)

1. The distribution of P_i between mitochondria and suspending medium during uncoupler-stimulated hydrolysis of ATP by rat liver mitochondria [Tyler (1969) *Biochem. J.* **111**, 665–678] has been reinvestigated, by using either mersalyl or *N*-ethylmaleimide as inhibitors of P_i transport and either buffered sucrose/EDTA or LiCl/EGTA solutions as suspending medium. More than 75% of the total P_i liberated was retained in mitochondria treated with either inhibitor at all ATP concentrations tested (0.2–2.5 mM). With low ATP concentrations and mersalyl-treated mitochondria incubated in sucrose/EDTA, virtually all the P_i liberated was retained in the mitochondria. 2. Larger amounts of P_i appeared in the suspending medium during ATPase activity, despite the presence of *N*-ethylmaleimide, when LiCl/EGTA was used as suspending medium compared with sucrose/EDTA. Two sources of this P_i were identified: (a) a slow efflux of P_i from mitochondria to suspending medium despite the presence of *N*-ethylmaleimide; (b) a slow ATPase activity insensitive to carboxyatractyloside, which was stimulated by added Mg^{2+} , partially inhibited by oligomycin or effraeptin and strongly inhibited by EDTA. 3. It is concluded that liver mitochondria preparations contain two distinct forms of ATPase activity. The major activity is associated with coupled mitochondria of controlled permeability to adenine nucleotides and P_i and is stimulated strongly by uncoupling agents. The minor activity is associated with mitochondria freely permeable to adenine nucleotides and P_i , is unaffected by uncoupling agents and is activated by endogenous or added Mg^{2+} . 4. When mitochondria treated with mersalyl were incubated in buffered sucrose solution, almost all the P_i liberated was recovered in the suspending medium, unless inhibitors of P_i -induced large-amplitude swelling such as EDTA, EGTA, antimycin, rotenone, nupercaine or Mg^{2+} were added. Thus the loss of the specific permeability properties of the mitochondrial inner membrane associated with large-amplitude swelling also influences the extent of P_i retention during ATPase activity. 5. The results confirm the previous conclusion (Tyler, 1969) that the P_i transporter provides the sole pathway for P_i efflux during uncoupler-stimulated ATP hydrolysis by mitochondria. It is concluded that more recent hypotheses concerning the influence of Mg^{2+} on mersalyl inhibition of the P_i transporter [Siliprandi, Toninello, Zoccaroto & Bindoli (1975) *FEBS Lett.* **51**, 15–17] and a postulated role of the adenine nucleotide exchange carrier in P_i efflux [Reynafarje & Lehninger (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 4788–4792] are erroneous and should be discarded.

The incubation of isolated rat liver mitochondria with low concentrations of thiol-reactive reagents such as organic mercurials or *N*-ethylmaleimide causes a strong inhibition of oxidative phosphorylation (Scott & Gamble, 1961) and also induces mitochondrial swelling during uncoupler-stimulated ATP hydrolysis (Azzi & Azzone, 1965). In 1969, I discovered that both these phenomena could be

attributed to an inhibition by thiol reagents of a P_i -transporter system (Chappell, 1968) located in the mitochondrial inner membrane. When this system was blocked by the organic mercurial mersalyl, about 90% of the total P_i liberated during ATP hydrolysis was retained in the mitochondria. It was concluded that the efflux of the products of ATP hydrolysis was mediated by two distinct carrier

systems, the ATP/ADP exchange transporter (Klingenberg & Pfaff, 1966) and the P_i transporter being responsible for the efflux of ADP and P_i respectively.

Reynafarje & Lehninger (1978), by using rather different incubation conditions from those used by Tyler (1969), concluded that only one-half of the total P_i formed during ATP hydrolysis was carried from the mitochondria to the suspending medium via the P_i transporter and that the other one-half was transported by the carboxyatractyloside-sensitive adenine-nucleotide exchange carrier. Siliprandi *et al.* (1975) presented evidence suggesting that the efflux of P_i from mersalyl-treated mitochondria could be restored by the addition of Mg^{2+} . A precise understanding of the transporter systems catalysing anion exchanges across the mitochondrial inner membrane is relevant to more recent studies of the proton stoichiometry of respiratory-chain phosphorylation (Mitchell & Moyle, 1979).

The present paper describes a study of P_i distribution between mitochondria and suspending medium during ATP hydrolysis, using a wider variety of incubation conditions than I used previously, and examines sources of error in the measurements. The results indicate that under properly controlled experimental conditions, P_i efflux during ATP hydrolysis stimulated by uncoupling agents is mediated exclusively by the P_i transporter, in agreement with the previous conclusion (Tyler, 1969). They also provide a simple explanation for the observations of Siliprandi *et al.* (1975) and show that the inhibition of P_i efflux by mersalyl is not influenced by added Mg^{2+} .

Materials and methods

Reagents

The following compounds were obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey KT2 7BH, U.K.: antimycin, carbonyl cyanide *m*-chlorophenylhydrazide, carboxyatractyloside, diadenosine pentaphosphate, *N*-ethylmaleimide, oligomycin and rotenone. ATP was from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. Efrapeptin and ionophore A23187 were gifts from Dr. R. L. Hamill, Lilly Research Laboratories, Indianapolis, IN, U.S.A. Nupercaine (cinchocaine hydrochloride B.P.) was a gift from CIBA Laboratories, Horsham, Sussex, U.K. Other reagents were from BDH, Poole, Dorset, U.K. AnalaR grade reagents were used whenever possible.

Enzyme preparations

Rat liver mitochondria were isolated in 0.25 M-sucrose containing 10 mM-Tris/HCl buffer, pH 7.5 (at 1°C), by the method of Schneider (1953). The

mitochondria pellets were washed twice and again homogenized in sucrose/Tris solution to obtain a stock suspension of mitochondria containing about 60 mg of protein/ml, which was stored at 1°C until used. A rapid estimate of the protein content of freshly isolated mitochondria was obtained by measuring the A_{750} of a suspension of 30 μ l of stock mitochondria in 3 ml of sucrose/Tris solution at 20°C. The protein content was read off from a standard curve prepared by measuring the A_{750} of a series of suspensions of mitochondria of known protein content, in the same solution. The protein value of individual mitochondrial preparations determined by measuring A_{750} agreed closely with values subsequently obtained by biuret analysis (Gornall *et al.*, 1949) of the stored suspension. The preparations contained 20.7 ± 3.2 nmol of P_i (mean $\pm$ S.E.M.; 23 preparations).

Enzyme assays

Assays were performed at room temperature (18–20°C). Total ATPase activity was measured either in a reaction mixture containing sucrose (0.2 M), Tris/HCl buffer, pH 7.4 (20 mM), KCl (15 mM) and EDTA (1 mM), which is referred to as 'sucrose/EDTA reaction mixture', or in LiCl (0.12 M), lithium Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer, pH 7.05 (10 mM) and EGTA (1 mM), which is referred to as 'LiCl/EGTA reaction mixture'. The incubation of mitochondria in reaction mixtures was carried out in a glass test tube. After each addition to the bulk incubation mixture, the tube was capped with Parafilm and the contents were mixed by gently inverting the tube. To ensure reproducible results mitochondria were preincubated and additions were made to the reaction mixture according to a strict time schedule. At zero time, a portion of the stock suspension of mitochondria was added to give a protein concentration of 2.3 mg/ml. Other reagents were added in the form of small volumes of concentrated solutions, to give the final concentrations indicated: at 15 s, in all experiments, antimycin (1 μ g/mg of protein) and rotenone (2 μ g/mg of protein); and in some experiments, at 30 s, carboxyatractyloside (6 μ M); at 45 s, oligomycin (2 μ g/mg of protein), efrapeptin (0.5 μ g/mg of protein), EDTA (0.5 mM) or $MgCl_2$ (2 mM); at 1 min, mersalyl (0.05 mM; 20 nmol/mg of protein) or *N*-ethylmaleimide (0.12 mM; 50 nmol/mg of protein); at 4 min, carbonyl cyanide *m*-chlorophenylhydrazide (1 μ M).

At 6 min, duplicate samples of the mixture (0.3 ml each) were squirted into small plastic tubes containing an equal volume of cold 10% (w/v) trichloroacetic acid and the tubes were transferred to an ice bucket. These samples were used as zero-time samples for the measurement of enzymic activity. Also at 6 min, ATP was added to other samples to

give a final concentration indicated in the Figures. The progress of the reaction was measured by removing further 0.3 ml samples from the reaction mixture at suitable time intervals and terminating the reaction with trichloroacetic acid as before. After all the trichloroacetic acid-treated samples had stood in an ice bucket for at least 5 min, the tubes were centrifuged and the whole supernatant of each tube was transferred to a fresh tube. The P_i content of each supernatant was determined by the method of Lowry & Lopez (1946).

When the distribution of P_i between the mitochondria and the suspending medium was measured, the ATPase reaction was terminated by the addition of carboxyatractyloside ($6 \mu\text{M}$), which inhibits hydrolysis of extramitochondrial ATP. A 1 ml sample of the incubation mixture was spun immediately for 2 min in an Eppendorf model 3200 centrifuge. When the centrifuge had been running for 1 min, samples of the bulk incubation mixture were mixed with trichloroacetic acid to determine total P_i . When the Eppendorf centrifugation had finished, samples were taken from the supernatant obtained and mixed with trichloroacetic acid, to determine the P_i content of the suspending medium. Total incubation mixture samples and supernatant samples were stored in ice for at least 5 min before all samples were centrifuged to remove denatured protein. The supernatants were transferred to fresh tubes before the P_i analysis reagents were added. In some experiments, the supernatant obtained after the Eppendorf centrifugation was further centrifuged at $15000g$ for 10 min. The very small pellet obtained did not contain any detectable P_i . Thus all the sedimentable P_i was removed from the incubation mixture by the 2 min Eppendorf centrifugation.

In most experiments, the value of the mitochondrial P_i content was obtained by calculating the difference between the P_i contents of the total incubation mixture and the suspending medium. In some experiments, the supernatant obtained by Eppendorf centrifugation was removed as completely as possible and the mitochondrial pellet was resuspended in fresh reaction mixture before estimation of the P_i content. In these experiments, there was good agreement between the P_i content of the total incubation mixture and the sum of the P_i contents of the mitochondria and the suspending medium.

The values of P_i content of the total incubation mixture and the suspending medium were corrected to allow for the small amount of P_i present in the stock ATP solution (up to 15 mmol of P_i/mol of ATP). They were also corrected for the amount and the distribution of endogenous P_i between mitochondria and the suspending medium, found in experiments in which ATP was omitted from the reaction mixture. Thus the data presented refer

specifically to the increase in P_i content of the reaction mixtures due to ATP hydrolysis.

Mitochondrial swelling was measured at 750 nm in a Beckman model 25 spectrophotometer. The reaction mixture (3 ml) contained sucrose (0.2 M), Tris/HCl buffer, pH 7.4 (20 mM), KCl (15 mM) and mitochondria (4 mg of protein). The mitochondria were incubated with either mersalyl (20 nmol/mg of protein) or *N*-ethylmaleimide (50 nmol/mg of protein) for 3 min before the addition of ATP (1 mM) and carbonyl cyanide *m*-chlorophenylhydrazone ($1 \mu\text{M}$).

The concentration of mersalyl and *N*-ethylmaleimide in solution was estimated by mixing portions of the solution with portions of standard cysteine solution and measuring the remaining cysteine by the method of Ellman (1959).

Except where indicated, the numerical data presented are the mean values obtained, and the swelling traces are representative of the results obtained, with at least three different mitochondrial preparations.

Results and discussion

The distribution of P_i between mitochondria and suspending medium during uncoupler-stimulated ATP hydrolysis, using a fixed concentration of mersalyl or *N*-ethylmaleimide and various initial ATP concentrations, is shown in Figs. 1 and 2.

The experiments shown in Fig. 1 used the sucrose/EDTA buffer of Tyler (1969) and those of Fig. 2 used a LiCl/EGTA buffer similar to that of Reynafarje & Lehninger (1978). When no ATP was added, most of the endogenous P_i present in the mitochondrial preparation was recovered in the suspending medium. After incubation with added ATP, at least 75% of the extra P_i liberated was retained in the mitochondria under all conditions tested. More P_i was liberated into the suspending medium when LiCl/EGTA was used compared with sucrose/EDTA buffer and also when *N*-ethylmaleimide was used compared with mersalyl. Since various thiol reagents commonly used to inhibit mitochondrial P_i transport appear not to bind at the P_i -binding site of the transporter (Palmieri *et al.*, 1974) and their binding is influenced by the pH of the reaction mixture (Fonyó *et al.*, 1974; Klingenberg *et al.*, 1974) they should not necessarily be expected to cause complete inhibition of activity or the same extent of inhibition under all conditions. When mersalyl was used to inhibit P_i transport in mitochondria suspended in a sucrose/EDTA medium, almost all the P_i liberated was retained in the mitochondria, showing that under these conditions there was no P_i efflux associated with the adenine nucleotide exchange system. This observation is in agreement with the recent experiments of

Fig. 1. Effect of ATP concentration on the distribution of P_i in sucrose/EDTA mixture

Samples of mitochondria were preincubated as described in the Materials and methods section with either *N*-ethylmaleimide (a) or mersalyl (b) and the reaction was started by the addition of ATP to give the initial concentrations indicated. Incubation was continued for 3 min before the addition of carboxyatractyloside and measurement of the total P_i liberated (●) and the distribution of P_i between mitochondria (▲) and the suspending medium (○).

Fonyó & Vignais (1979). Diadenosine 5-pentaphosphate ($20\mu\text{M}$), an inhibitor of soluble adenylate kinase activity (Feldhaus *et al.*, 1975), which was present in the LiCl mixture used by Reynafarje & Lehninger (1978), had no significant effect on the rate or extent of ATP hydrolysis and was omitted.

The effect of various concentrations of mersalyl and *N*-ethylmaleimide on the distribution of P_i between mitochondria and sucrose/EDTA suspending medium during uncoupler-stimulated hydrolysis, using a fixed ATP concentration, is shown in Fig. 3. The results show that concentrations of thiol-reactive reagents sufficient to block the P_i transporter induce almost total retention of P_i in the mitochondria. Since high mersalyl concentrations (about 60 nmol/mg of protein) block di-

Fig. 2. Effect of ATP concentration on the distribution of P_i in LiCl/EGTA mixture

The conditions were similar to those described in the legend to Fig. 1, except for the use of LiCl/EGTA mixture instead of sucrose/EDTA mixture. (a), *N*-Ethylmaleimide was used; (b), mersalyl was used. ●, Total P_i liberated; ▲, P_i content of mitochondria; ○, P_i content of the suspending medium.

carboxylate anion- P_i exchange via the dicarboxylate carrier system, whereas *N*-ethylmaleimide is without effect (Meyer & Tager, 1969; Johnson & Chappell, 1973), these results eliminate the possibility that significant P_i efflux might have been due to the exchange of external endogenous substrate anions, such as malate, for P_i in the matrix space.

The origin of the P_i appearing in the LiCl/EGTA suspending medium during ATPase activity is suggested by the results of experiments shown in Fig. 4. When mitochondrial ATPase activity was used to accumulate P_i in the matrix space, followed by the addition of carboxyatractyloside to inhibit further ATPase activity, subsequent incubation of the mitochondria revealed two phenomena. First, there was a slow P_i efflux from mitochondria to suspending medium despite the presence of *N*-ethylmaleimide. These results indicate either that the activity of the P_i transporter was not totally inhibited by *N*-ethylmaleimide or that mitochondrial swelling associated with P_i accumulation in the matrix space

had induced a small increase in permeability of the mitochondrial inner membrane to P_i . Secondly, a slow increase in the total P_i content of the reaction mixture was observed, indicating the presence of an ATPase activity insensitive to carboxyatractyloside. The concentration of carboxyatractyloside used in these experiments was at least five times the concentration necessary to produce a maximum effect on ATPase activity. Similar results to those shown in Fig. 4 were obtained when oligomycin ($2\mu\text{g}/\text{mg}$ of protein) was used instead of carboxyatractyloside, except that oligomycin was a less effective inhibitor of ATPase activity.

Further properties of the ATPase activity resistant to carboxyatractyloside are shown in Table 1. The activity was partially inhibited by efrapeptin, a peptide inhibitor of mitochondrial ATPase (Susa *et al.*, 1975), or by oligomycin. Sodium azide (0.5 mM) and dicyclohexylcarbodi-imide (0.5 mM), which also inhibit mitochondrial ATPase (Novikoff *et al.*, 1952; Beechey *et al.*, 1967), had similar effects to oligomycin. The activity was strongly inhibited by EDTA and stimulated by added Mg^{2+} , suggesting that the activity observed without added Mg^{2+} was due to activation of the enzyme by endogenous Mg^{2+} . Similar stimulation of the ATPase activity of liver mitochondria by added Mg^{2+} and inhibition by EDTA were described many years ago (Lardy &

Wellman, 1953; Myers & Slater, 1957). All the extra P_i liberated during this ATPase activity was found in the suspending medium. No change in activity was observed when *N*-ethylmaleimide and uncoupling agent were omitted from the reaction mixture or when mersalyl replaced *N*-ethylmaleimide.

Many of these properties are similar to those of liver mitochondria disrupted by various mechanical

Fig. 3. Influence of thiol-reactive reagent concentration on P_i distribution

Samples of mitochondria were incubated in sucrose/EDTA reaction mixture with either mersalyl or *N*-ethylmaleimide at the concentrations shown. The ATP concentration used was 0.2 mM . For other details, see the Materials and methods section. O, ●, With mersalyl; □, ■, with *N*-ethylmaleimide. Open symbols indicate the P_i liberated into the suspending medium and closed symbols indicate the total P_i liberated.

Fig. 4. Effect of incubation on P_i release and distribution in mitochondria treated with *N*-ethylmaleimide

Samples of mitochondria were preincubated with *N*-ethylmaleimide in LiCl/EGTA reaction mixture as described in the Materials and methods section. The reaction was started by the addition of either 2 mM -ATP (a) or 0.2 mM -ATP (b), followed 2 min later by the addition of carboxyatractyloside. Portions of the reaction mixture were then taken immediately and at the other times indicated, to determine the total P_i content (●) and the distribution of P_i between the mitochondria (▲) and the suspending medium (O).

or chemical methods (Kielley & Kielley, 1953; Siekevitz *et al.*, 1958). The results therefore suggest that the mitochondrial preparation contains two types of particulate ATPase activity. The major activity is due to the presence of mitochondria in which the permeability of the inner membrane to adenine nucleotides and P_i is wholly dependent on the activity of specific anion transporter systems. The ATPase activity of these mitochondria is stimulated by uncoupling agents. A minor ATPase activity is also present, whose properties suggest that it is due to mitochondria possessing membranes freely permeable to ions and therefore insensitive to the effect of mersalyl or *N*-ethylmaleimide and carboxyatractyloside or to uncoupling agents. The rate of this activity was almost constant during storage of the mitochondria for at least 30h, suggesting that these particles were either present in the liver *in vivo* or damaged during the isolation procedure. The activity was not removed during additional washing of the mitochondrial preparation by dilution and re-centrifugation. It has been estimated that the outer membrane of approx. 10% of the population of liver mitochondria is stripped away during the standard isolation procedure (Criss, 1970). Thus it would not be surprising to find some mechanically disrupted mitochondria in the stock suspension. Although this explanation seems most probable, it should be remembered that other cells have been found to contain a membrane-bound ATPase activity, sensitive to mitochondrial ATPase inhibitors, which is not associated with mitochondria (Apps & Schatz, 1979).

The observed rate of Mg^{2+} -activated ATP hydrolysis (8nmol/min per mg of protein) may be compared with the rate of ATP synthesis measured

under similar conditions with either succinate or glutamate as substrate (approx. 200nmol/min per mg of protein; Chappell, 1964). The ATPase activity is approx. 4% of the rate of ATP synthesis and ATPase activity would therefore not be expected to strongly affect the P/O and respiratory-control ratios obtained with liver mitochondria preparations, though it should cause some decrease in their true values.

Siliprandi *et al.* (1975) observed that swelling during uncoupler-stimulated ATPase activity in mersalyl-treated mitochondria was partially inhibited by added Mg^{2+} and concluded that this effect of Mg^{2+} was due to a stimulation of P_i efflux. As shown in Fig. 5, swelling under their incubation conditions was reversed strongly by cysteine (Tyler, 1969) only in the presence of added Mg^{2+} , indicating that the swelling they had observed in the absence of added Mg^{2+} was not due to P_i retention in the mitochondrial matrix space. This conclusion was confirmed by P_i analyses (Table 2). The addition of various reagents known to inhibit P_i -induced large-amplitude swelling of liver mitochondria (Lehninger, 1962; Tyler, 1977) restored the ability of the mitochondria to retain P_i during ATPase activity and the ability of cysteine to reverse the swelling (Fig. 5 and Table 2). It should be noted that in the experiments performed either in the absence of EDTA or in the presence of added Mg^{2+} , some of the P_i appearing in the suspending medium was due to the ATPase activities described in Table 1. The percentage values given in the last column in these experiments therefore overestimate the amount of P_i efflux occurring during the uncoupler-stimulated portion of the total ATPase activity. Very little P_i efflux occurred when EDTA, antimycin and

Table 1. *Effect of various reagents on ATPase activity and P_i distribution using mitochondria treated with carboxyatractyloside*

Samples of mitochondria suspended in LiCl/EGTA reaction mixture were preincubated with antimycin, rotenone, carboxyatractyloside and with other reagents indicated in the Materials and methods section. After a 10 min incubation, with or without added ATP (0.21 mM), the ATPase activity and distribution of P_i were measured. Values are means $\pm$ S.E.M. obtained for numbers of different mitochondrial preparations shown in parentheses. The initial rate of ATP hydrolysis, observed in the absence of added reagents and carboxyatractyloside, was 49.1 ± 4.4 ($n=9$) nmol/min per mg of protein.

Addition	ATPase activity (nmol/min per mg of protein)	Distribution of P_i (% of total P_i released)	
		Mitochondria	Suspending medium
None	3.0 ± 0.6 (10)	1.0 ± 0.8 (4)	99.0 ± 0.8 (4)
Oligomycin	1.5 ± 0.4 (4)		
Efrapeptin	1.3 ± 0.3 (4)		
EDTA	0.4 ± 0.1 (5)		
$MgCl_2$	8.1 ± 0.8 (10)	1.4 ± 1.2 (4)	98.6 ± 1.2 (4)
$MgCl_2$ + oligomycin	4.8 ± 0.1 (3)		
$MgCl_2$ + efrapeptin	3.6 ± 0.2 (3)		

rotenone were added, thus establishing incubation conditions similar to those used in the experiments of Fig. 1. The addition of Mg^{2+} after a period of swelling due to P_i retention in the presence of EDTA antimycin and rotenone, did not cause either significant contraction of the mitochondria or P_i efflux, though there was some increase in the P_i content of the suspending medium due to Mg^{2+} -activated ATPase activity (Table 2). The exact mechanism of P_i -induced large-amplitude swelling is unknown but this type of swelling is associated with loss of the specific permeability properties of the

Fig. 5. Influence of inhibitors of large-amplitude swelling on ATPase-dependent swelling and cysteine-induced contraction

For incubation conditions, see the Materials and methods section. Mersalyl was present in all experiments except (1), in which *N*-ethylmaleimide was used. The reaction mixtures also contained the following: (2), no further addition; (3), 4 mM- $MgCl_2$; (4), 1 mM-EDTA or 0.2 mM-EGTA; (5), 0.2 mM-nupercaine; (6) and (7), 1 mM-EDTA with antimycin ($1 \mu g/mg$ of protein) and rotenone ($2 \mu g/mg$ of protein). Further additions were made to the reaction mixtures to give the final concentrations indicated: at (a), ATP and uncoupling agent; at (b), 8 μM -carboxyatractyloside; at (c), 0.25 mM-cysteine hydrochloride; at (d), 4 mM- $MgCl_2$. The absorbance of the reaction mixtures before the addition of ATP and uncoupling agent was approx. 1.9.

mitochondrial inner membrane, perhaps due to the activation of mitochondrial Ca^{2+} -dependent phospholipase (Bjørnstad, 1966; Scarpa & Lindsay, 1972). When experiments were performed by using mitochondria treated with *N*-ethylmaleimide instead of mersalyl, some P_i was retained in the mitochondria and a slower swelling rate occurred even during incubation in the absence of reagents known to protect against large-amplitude swelling (Fig. 5), possibly due to a partial inhibition of phospholipase activity by *N*-ethylmaleimide.

The present results indicate that P_i liberated in the matrix space during ATPase activity is equally as effective as added P_i in inducing large-amplitude swelling and changes in membrane permeability to P_i and other ions under suitable experimental conditions, including those used by Siliprandi *et al.* (1975). Such changes may have contributed to other phenomena described in studies on P_i transport (e.g. Leblanc & Clauser, 1974; Goldschmidt *et al.*, 1976) and may also be responsible for the slow efflux of P_i observed in the experiments of Fig. 4. The rate of this slow efflux (approx. 2 nmol/min per mg of protein) together with the rate of carboxyatractyloside-insensitive and EDTA-sensitive release of P_i from ATP into the suspending medium (approx. 3 nmol/min per mg of protein) is sufficient to account for the rate of liberation of P_i into LiCl/EGTA suspending medium during uncoupler-stimulated ATPase activity (approx. 5 nmol/min per mg of protein), using mitochondria treated with *N*-ethylmaleimide. There is therefore no need to postulate a role for the adenine nucleotide exchange carrier in P_i efflux during ATP hydrolysis.

Although it might be anticipated that the protein catalysing P_i transport will be found in close proximity to that catalysing adenine nucleotide exchange, or even identical with it, current attempts to isolate the mitochondrial anion transporters suggest that the transporters for P_i and adenine nucleotides are distinct proteins (LaNoue & Schoolwerth, 1979). There is therefore no reason at present to modify the previous conclusion (Tyler, 1969) that the products of ATP hydrolysis by uncoupled mitochondria are transported from the matrix space by two distinct carrier systems, one for P_i and the other for adenine nucleotides. On the basis of their conclusion that P_i efflux occurs in part via the adenine nucleotide transporter, Reynafarje & Lehninger (1978) postulated a newly-discovered pathway of P_i efflux due to ATP/(ADP + P_i) exchange, involved in ATP-driven reversed electron flow in the respiratory chain *in vivo*. The present results and conclusions offer no encouragement to these ideas. Although reversed electron flow seems generally accepted as an experimental observation it has long been considered of dubious physiological significance (Krebs, 1967).

Table 2. *Influence of inhibitors of large-amplitude swelling on ATPase activity and P_i distribution*

Incubation conditions were those used in the experiments shown in Fig. 5. Samples were removed before the addition of cysteine, to estimate ATPase activity and P_i distribution. The concentration of ionophore A23187 used was 0.5 μ mol/mg of protein.

Addition	ATPase activity (nmol/min per mg of protein)	Distribution of P_i (nmol/mg of protein)		P_i released in suspending medium (% of total)
		Mitochondria	Suspending medium	
None	68.7	29	177	85.9
None*	63.3	85	105	55.3
MgCl ₂	51.3	83	71	46.1
EGTA	60.7	96	86	47.3
EGTA + ionophore A23187	60.0	141	39	21.7
Nupercaine	58.3	114	61	34.9
EDTA	62.3	110	77	41.2
Antimycin, rotenone	60.3	141	40	22.1
EDTA, antimycin, rotenone	55.0	158	7	4.2
EDTA, antimycin, rotenone†	66.3	160	39	19.6 (see the text)

* *N*-Ethylmaleimide used instead of mersalyl.

† MgCl₂ added after carboxyatractyloside.

I am very grateful to Miss Elizabeth Henley for her excellent assistance and to Mrs. Margaret Howes for her help in some of the experiments.

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