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ION TRANSPORT IN LIVER MITOCHONDRIA

II. METABOLISM-LINKED ION EXTRUSION

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SUMMARY

Liver mitochondria undergo a passive swelling when the membrane is rendered permeable to ions. When ATP or substrates are added, ions and water are extruded from the mitochondria.

The amount of water extruded is accounted for osmotically by the amount of ions extruded as indicated by direct isotopic measurements of $^{86}Rb^+$ and $^{36}Cl^-$.

The process of water extrusion has been found to be dependent on: (a) the concentration of ions of the suspending medium; (b) the permeability of the mitochondrial membrane to cations and anions of the medium. Furthermore, if the energy supply is interrupted, the shrinkage is followed by a swelling phase provided that the mitochondrial membrane is still permeable to cations and anions of the medium.

It is concluded that mitochondria are capable of extruding cations and anions at the expense of metabolism. The implications of the ion extrusion process to mitochondrial physiology are discussed.

INTRODUCTION

Extrusion of water from the mitochondria has been reported in three instances: (a) after addition of ADP[1], respiratory chain inhibitors or uncoupling agents[2] to mitochondria in 'low amplitude' or 'reversible' swelling[3]; the extrusion of water has been shown to be coupled to the efflux of ions down a concentration gradient[3-5]; (b) after addition of P_i to mitochondria swollen in the presence of Ca^{2+} and acetate[6]; the shrinkage has been proposed to be linked to the formation of insoluble calcium phosphate from soluble calcium acetate; (c) after addition of ATP[7-9], or in the presence of a coupled respiration[10] when the mitochondria have undergone a 'large amplitude' or 'irreversible' swelling[3]; this third type of water extrusion, which is the only linked to metabolism, has been concluded by LEHNINGER[11,12] to be independent

of the ionic environment or ion fluxes and the consequence of a contractile change of the mitochondrial membrane. A similar suggestion has been advanced by CROFTS AND CHAPPELL[10].

In a previous paper[13] we have reported that mitochondria swell when the membrane is rendered permeable to the ions of the suspending medium. When either an oxidizable substrate or ATP is added to the swollen mitochondria an extrusion of ions coupled to a mitochondrial shrinkage is observed[14]. The essential features of this process are described below also in relation to previous systems where metabolism-dependent mitochondrial shrinkage have been observed.

Extrusion of ions from the mitochondria, in a process which does not occur down a concentration gradient (*cf.* ref. 15) and is thus necessarily coupled to metabolism, can presumably take place according to two basic types of mechanisms, operation of a contractile system in the mitochondrial membrane or active extrusion of ions. In the first mechanism increase of the intramitochondrial hydrostatic pressure induces extrusion of water which is followed by efflux of ions down the concentration gradient. In the second mechanism a primary movement of ions against a concentration gradient is coupled with an osmotic movement of water. Experiments were therefore designed to ascertain whether the process of mitochondrial shrinkage is dependent on the presence of ions and quantitatively correlated with the influx and efflux of ions into and from the mitochondria.

EXPERIMENTAL

Liver mitochondria were used in all experiments. Absorbance measurements were carried out with a recording Eppendorf photometer. Measurements of ions and of extramitochondrial water in the pellet, were made with $^{86}Rb^+$, $^{36}Cl^-$ and [^{14}C]-carboxypolyglucose as described in a previous paper[13]. O_2 uptake was measured polarographically.

[^{14}C]Carboxypolyglucose was kindly given by Dr. E. PFAFF.

RESULTS

The metabolism-dependent mitochondrial shrinkage coupled to ion extrusion.

Liver mitochondria were incubated in 50 mM KCl at pH 8.8 in the presence of rotenone. Addition of valinomycin[16] caused a fast swelling. Addition of succinate at pH 8 resulted in a shrinkage which reached about 50–60% of the extent of the swelling. When oxygen was exhausted in the medium again swelling occurred. Therefore in these experiments the swelling phase was independent of metabolism whereas the shrinkage phase was dependent on metabolism.

The requirement for energy supply from metabolism, during the shrinkage phase was further supported by the following observations: (a) the shrinkage phase was inhibited by the addition of respiratory chain inhibitors; (b) respiration as source of energy could be replaced by ATP; oligomycin inhibited the ATP-supported shrinkage; (c) the shrinkage phase was inhibited by uncoupling agents. The sensitivity of the shrinkage to 2,4-dinitrophenol was less than that of other energy-dependent mitochondrial reactions. CROFTS AND CHAPPELL[10] have reported that the respiration-dependent shrinkage induced by the addition of ethyleneglycol-bis-(β-aminoethyl-

ether)-*N,N'*-tetraacetic acid to liver mitochondria is insensitive to dinitrophenol. In our system there was a slight inhibition at 50 μM dinitrophenol and a larger one at 100 μM dinitrophenol. The extent of inhibition was increased by increasing the concentration of KCl in the medium. A large inhibition was obtained when 4 mM succinate was added in the presence of 50 μM pentachlorophenol. Removal of pentachlorophenol by addition of 0.2% bovine serum albumin, resulted in a considerable stimulation of the shrinkage (Fig. 2). Inhibition of shrinkage was also caused by dicoumarol.

In Table I it is seen that addition of succinate to liver mitochondria swollen at pH 8.8 in the presence of valinomycin, resulted in a decrease of the water content from about 6 μl to about 3.4 μl per mg dry weight. Extrusion of water was paralleled

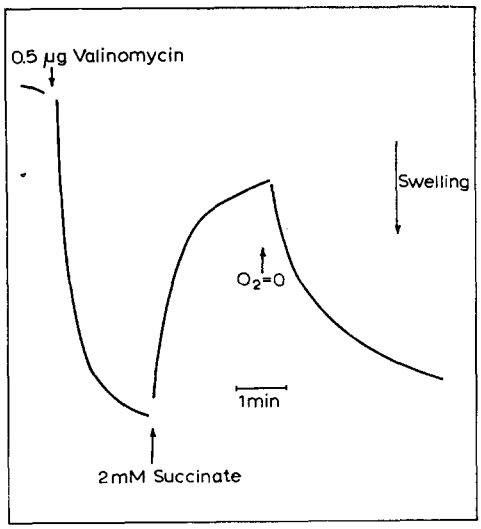
TABLE I

WATER AND IONS MOVEMENT DURING SHRINKAGE

Experimental conditions: mitochondria were added to a medium containing 50 mM RbCl (labeled either with ^{86}Rb or with ^{36}Cl), 5 mM Tris–HCl (pH 8.5), 5 μM rotenone, 5 μg valinomycin. Vol. 11 ml. Temp. 22°. After 10 min of incubation, a sample of 5 ml was withdrawn and 10 mM Tris–succinate (pH 7.0) was added. After 5 min a second sample was withdrawn. The samples were sedimented and analyzed. The mitochondrial water was obtained by correcting the total water for the [^{14}C]carboxypolyglucose space.

Additions	*Wet wt. (mg)*	*Dry wt. (mg)*	*Mitochondrial water (µl)*	*Ions in the pellet (µmoles)*		*Ions in mitochondrial water (µmoles)*		*Ion concn. (mM) in*	
				$^{86}Rb^+$	*$^{36}Cl^-$*	*$^{86}Rb^+$*	*$^{36}Cl^-$*	*mitochondrial water*	*extruded water*
Expt. 1									
None	179.5	18.3	108	9.2		6.5		60	
Succinate	101.5	16.1	55	5.0		3.5		63	57
None	180.0	18.2	108		6.4		3.7	34	
Succinate	83.0	16.0	55		3.8		1.55	27	41
Expt. 2									
None	173	16.7	104.3	8.6		6		57	
Succinate	107	15.4	59.6	5.1		3.5		58	56
None	171.3	17.2	102.8		6.4		3.9	38	
Succinate	114.1	18.1	61.9		3.9		2.2	27	41

by extrusion of osmotic equivalent amounts of ions. The concentration of $^{86}Rb^+$ and $^{36}Cl^-$ in the extruded water was in fact close to the concentration of $^{86}Rb^+$ and $^{36}Cl^-$ of the suspending medium. A proportionality has been shown to exist between changes of absorbance and changes in water content of the mitochondria. The data of Table I indicate that the increases of absorbance observed in the experiments of Figs. 1 and 2 are indeed accompanied by decreases of water content. Furthermore the data of Table I indicate that the extrusion of water is paralleled by the extrusion of osmotic equivalent amounts of ions. Therefore in the experiments reported below the changes of absorbance are taken as expression of movements of water and ions in and out the mitochondrion. A similar technique has been used recently by CHAPPELL AND HAARHOFF[17].



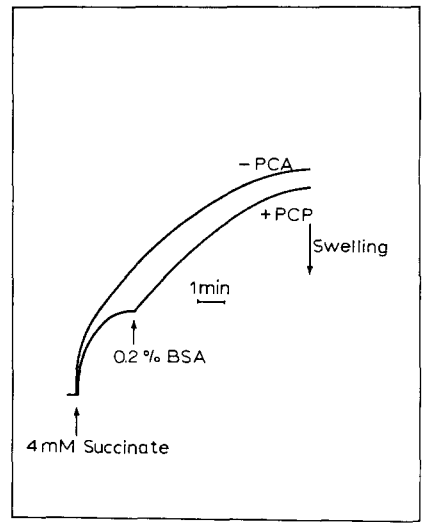


Fig. 1. Passive swelling and active shrinkage of liver mitochondria. Mitochondria (2.8 mg protein) were incubated in a medium containing 50 mM KCl, 12.5 mM Tris–HCl (pH 8.8), 3 μM rotenone. Vol. 2 ml. Temp. 22°.

Fig. 2. Inhibition of shrinkage by pentachlorophenol (PCP). Mitochondria swollen at pH 8.8 in the presence of valinomycin (*cf.* Fig. 1) were sedimented and resuspended in 50 mM KCl, 12.5 mM Tris (pH 7.5). Incubation was carried out in 50 mM KCl, 12 mM Tris–HCl, in the presence and absence of 50 μM pentachlorophenol. Protein 2 mg. Vol. 2 ml. Temp. 22°. BSA stands for bovine serum albumin.

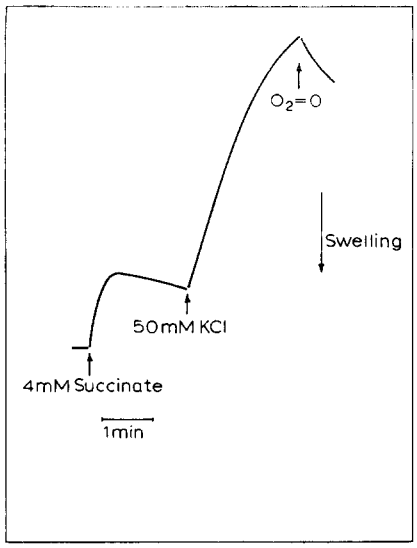
The concentration of Rb^+ in the intramitochondrial water was higher than that of the medium (Table I). This is presumably due to exchange of $^{86}Rb^+$ with bound intramitochondrial K^+. On the other hand the intramitochondrial concentration of Cl^- was lower than that of the external medium. These findings suggest that Cl^- is extruded up a gradient. The same suggestion cannot yet be made in the case of Rb^+ until evidence will be provided that part of the intramitochondrial Rb^+ is bound and thus that its activity is lower than its concentration.

Dependence of water extrusion on the presence of ions

In our experimental system the shrinkage was almost completely dependent on the presence of ions. As shown in Fig. 3, mitochondria swollen in KCl, centrifuged and resuspended in water, underwent a very small shrinkage on addition of 4 mM succinate. The subsequent addition of 50 mM KCl resulted in a large shrinkage phase. The shrinking effect of KCl was not purely osmotic, since it was dependent on the presence of succinate.

In Fig. 4 are reported the extents of shrinkage with various KCl concentrations. It is seen that the rate of shrinkage became progressively reduced below 25 mM KCl and was almost abolished when the mitochondria were incubated in water. It is also shown in Fig. 4 that the extent of shrinkage of valinomycin-treated mitochondria was higher in Na^+ than in K^+ media and was decreased at the higher NaCl concentrations. The relevance of this observation will be discussed in a later section.

The experiments of LEHNINGER[18] of ATP-induced mitochondrial shrinkage in



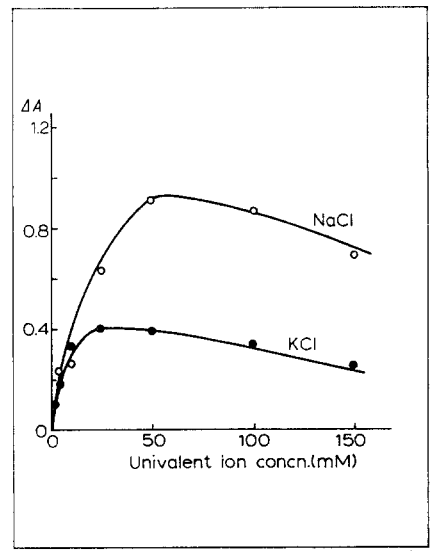


Fig. 3. Succinate-induced shrinkage in the presence and absence of KCl. Mitochondria, pretreated as described in Fig. 2, were added to an ion-free medium. Vol. 2 ml. Temp. 22°.

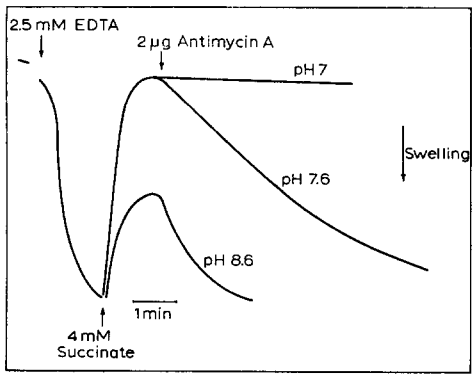
Fig. 4. Effect of ion concentration on succinate-induced shrinkage. Mitochondria, pretreated as described in Fig. 2, were added to media of 12.5 mM Tris–HCl (pH 7.5), 4 mM succinate–Tris and amounts of KCl or NaCl in the concentration indicated in the figure. Protein 2 mg. Vol. 2 ml. Temp. 22°.

salt-free solutions apparently negate that the extrusion of water is linked to the extrusion of ions. The presence of 25 mM Tris–HCl in LEHNINGER's experiments may perhaps explain the discrepancy with the present data.

The reversibility of shrinkage

In Fig. 5, swelling of mitochondria, incubated at pH 8.8 in 50 mM NaCl, followed the addition of EDTA[3,15]. At the end of the swelling phase the pH of the medium was brought to 7, 7.6 and 8.6 by addition of 4 mM Tris–succinate at the appropriate pH. Rate and extent of shrinkage were higher at pH's 7 and 7.6 than at pH 8.6. After the shrinkage phase was completed respiration was inhibited with antimycin. However addition of antimycin did not result in a swelling at pH 7 whereas it caused a slow swelling at pH 7.6 and a fast swelling at pH 8.6. The rate of entrance of water in the mitochondria was parallel to the permeability of the membrane to anions (*cf.* ref. 13).

In Fig. 6 are also shown the effects of the variation of the permeability of the membrane to cations on the rate of swelling after the metabolism-dependent shrinkage. After swelling caused by EDTA, shrinkage followed the addition of succinate at pH 7. Addition of antimycin A caused no swelling at this pH. Alkalinization of the medium to pH 8, by addition of 5 mM Tris buffer, resulted in a swelling. When the cation permeability of the membrane was decreased by 2.5 mM $MgCl_2$, again the swelling phase was inhibited. The ion flux after interruption of the energy supply was thus dependent on the permeability of the membrane not only to the anions but also to the cations of the suspending medium.



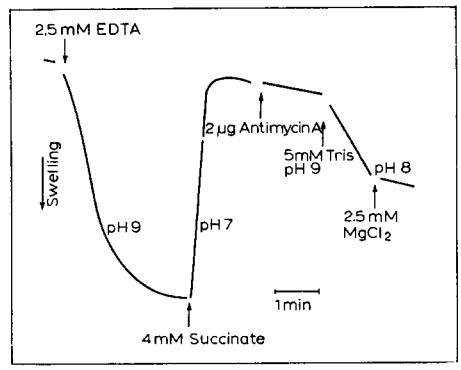
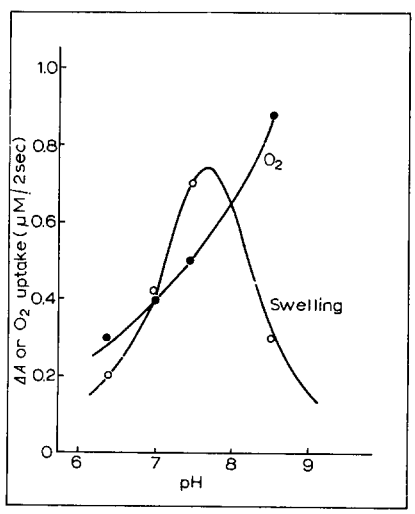


Fig. 5. Reversibility of the shrinkage phase: dependence on permeability to anions. Mitochondria (corresponding to 2.8 mg protein) were added to media of 50 mM NaCl, 3 µM rotenone, 2 mM Tris–HCl (pH 8.8); 2.5 mM EDTA–Tris (pH 8.8) was added for initiating swelling. 4 mM succinate–Tris at different pH's was added for initiating shrinkage. The pH's indicated over the traces indicate the final pH of the medium after the addition of succinate. Vol. 2 ml. Temp. 22°.

Fig. 6. Reversibility of the shrinkage phase: dependence on permeability to cations. Experimental conditions as in Fig. 5 except that succinate at pH 7 was added.

The interruption of the energy supply seems thus to cause an inversion of the direction of the water flow only when an ion diffusion into the mitochondria is possible. Presumably, when energy from metabolism is not available, a difference in ion concentration at the two sides of the membrane is the driving force for the ion diffusion process.



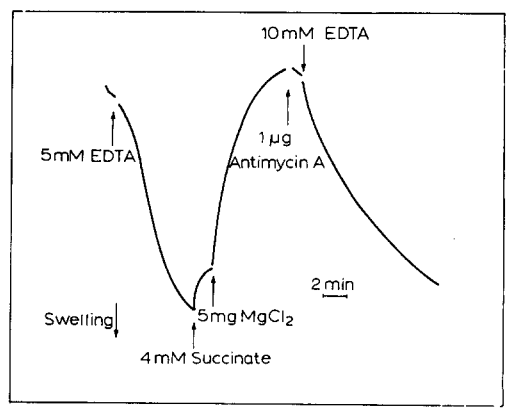


Fig. 7. Effect of the permeability to anions on the shrinkage phase and on O_2 uptake. Mitochondria were pretreated as in Fig. 2 and resuspended in 50 mM KCl. Absorbance and O_2 uptake were measured simultaneously in media of 50 mM KCl, 12.5 mM Tris–HCl at the pH's indicated in the figure. Vol. 4 ml. Amount of mitochondrial protein 10 mg. Temp. 22°.

Fig. 8. Effect of decreasing the permeability to cations by Mg^{2+} on shrinkage. Experimental conditions as in Fig. 5.

Effect of the permeability of the mitochondrial membrane on shrinkage

In the experiments reported below, the influence of the membrane permeability to ions on the water extrusion was studied. Variations of the rate of ion movement through the mitochondrial membrane were obtained in three ways, namely by changing the ion species, by changing the pH of the medium, and by adding Mg^{2+} or removing it by means of EDTA.

As we have reported previously the permeability of the mitochondrial membrane to anions decreases at the acidic pH and increases at the alkaline pH (ref. 13). Rate and extent of absorbance changes and of respiration were therefore measured at various pH. As seen in Fig. 7 the rate of shrinkage was low at pH 6.5, reached a maximum at about pH 7.5 and declined at higher pH. Simultaneous measurements of the respiratory rate revealed that respiration was low at pH 6.5, increased parallel to the rate of shrinkage up to pH 7.5, and continued to increase at variance from the shrinkage rate at the higher pH. Comparison between the oxygen and the absorbance traces indicates that the inhibition of shrinkage at high pH is not due to an inhibition of respiration but more probably to increased permeability to anions.

In previous papers we have reported that by removing the membrane-bound Mg^{2+} by the addition of EDTA-liver mitochondria were able to take up aerobically large amounts of Na^+, Li^+ or K^+ (refs. 3, 15). The process was inhibited by the addition of Mg^{2+} whereby the normal low permeability of the mitochondrial membrane to univalent cations was restored. In the experiment reported in Fig. 8, respiratory, inhibited mitochondria were incubated at pH 8.8 in 50 mM NaCl. Addition of EDTA caused a large swelling phase. At this point addition of succinate at pH 8.5 caused a small shrinkage phase, whereas a large shrinkage followed the addition of 5 mM $MgCl_2$. Thus it would seem from these experiments that the extent of shrinkage was scarce under conditions where the membrane was fully permeable to Na^+ and Cl^- and was much larger when the low permeability of the membrane to Na^+ was restored.

In Fig. 4 we have shown that the extent of shrinkage increased, parallel to the increase of the KCl concentration in the medium up to 50 mM. It was however also evident from Fig. 4 that when KCl was replaced by NaCl the extent of shrinkage was considerably increased also up to a concentration of about 50 mM NaCl. At higher concentrations of NaCl a decrease of the extent of shrinkage was observed, and this decrease would have been much more apparent from the data of Fig. 4, should the total extent of shrinkage be subtracted from the initial osmotic shrinkage following the addition of mitochondria to a NaCl medium.

The formation of a difference in ion concentration at the two sides of the membrane can easily explain all the above results. The inhibition of the shrinkage may thus be due to a back flow of ions driven by the concentration gradient, at a rate increasing as the permeability of the mitochondrial membrane increases.

In fact when the permeability of the mitochondrial membrane is increased also to Na^+ by the addition of gramicidin[4] the extent of shrinkage of mitochondria incubated in a NaCl medium was severely inhibited (Fig. 9).

The inhibition of the metabolism-dependent shrinkage by sucrose

A large body of evidence has been collected by LEHNINGER[18] on the inhibitory effect of sucrose upon the mitochondrial shrinkage supported by ATP. Consistent results in studies of mitochondrial shrinkage were indeed obtained only when sucrose

was eliminated from the incubation medium. An inhibitory effect of sucrose on the mitochondrial coupling system was suggested[12].

As seen in Fig. 10 when mitochondria swollen in the presence of P_i and Ca^{2+} were incubated in the presence of various KCl and sucrose concentrations, the percentage inhibition of the shrinkage process, observed at each sucrose concentration, was decreased by increasing the concentration of KCl. For example 50 mM sucrose caused almost 90% inhibition at 10 mM KCl and only 30% inhibition at 100 mM

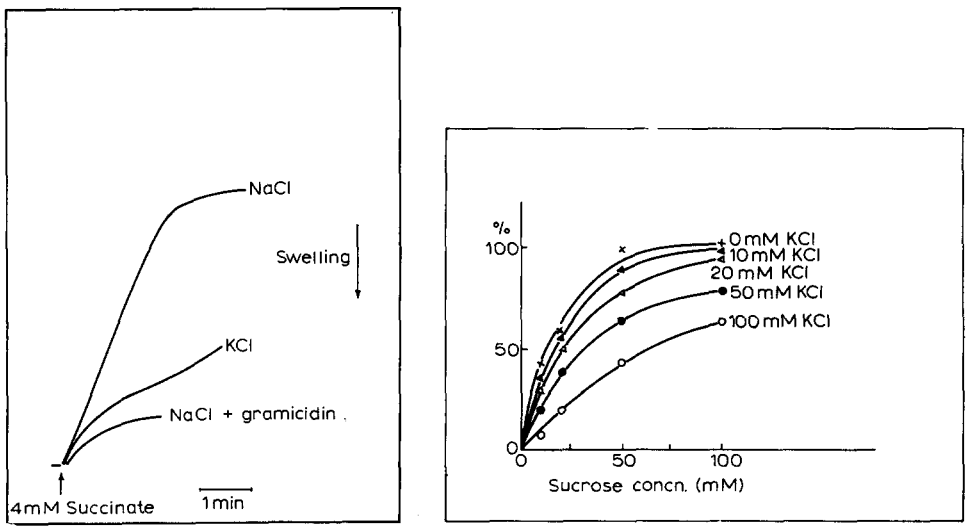


Fig. 9. Effect of increasing the permeability to cations by gramicidin on shrinkage. Mitochondria, pretreated as in Fig. 2 were added to media containing 12.5 mM Tris (pH 7.5) and either 50 mM NaCl or 50 mM KCl or 50 mM NaCl *plus* 1 μg gramicidin. Vol. 2 ml. Temp. 22°.

Fig. 10. Effect of KCl on the inhibition by sucrose of succinate-dependent shrinkage. Mitochonchondria were swollen in a medium of the following composition: 100 mM KCl, 12.5 mM Tris (pH 7.5); 1 mM P_i, 0.5 mM $CaCl_2$. The swollen mitochondria were sedimented and resuspended in 100 mM KCl. Amounts of swollen mitochondria were added to media containing 2.5 mM Tris–HCl (pH 7.5), 2 mM ATP–Tris, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N'*-tetra-acetic acid–Tris. Amount of protein 0.9 mg. Vol. 2 ml. Temp. 22°.

KCl. Depending upon the permeability changes of the mitochondrial membrane, conditions could be obtained where the inhibition of the shrinkage by sucrose was completely abolished by a suitable concentration of KCl. Similar results were obtained whether the shrinkage was supported by respiration or by ATP.

These findings suggest that sucrose acts as an inhibitor of mitochondrial shrinkage because it is an unionized molecule which cannot be moved through the mitochondrial membrane by means of the active processes which cause a redistribution of the charged molecules.

DISCUSSION

The mechanism of ion extrusion

Mitochondrial shrinkage due to the addition of ATP was first observed by

CHAPPELL AND PERRY[7] and then analyzed by LEHNINGER[8–12]. General agreement exists about the dependence on metabolism of this process. The data reported here are in agreement with this conclusion. The mechanism of the metabolism-dependent shrinkage has remained elusive although the proposal has been advanced that the extrusion of water is independent of ion movements and due to contractile changes.

At the beginning of this paper we have characterized an experimental system where the extrusion of water from the mitochondria was coupled to the efflux of ions. Three parameters were investigated in order to discriminate between a mechanism of primary water extrusion and one of primary ion extrusion: (a) the dependence of water extrusion on the presence of ions; (b) the reversibility of the shrinkage phase; (c) the influence of the permeability of the mitochondrial membrane to ions on the shrinkage phase.

Theoretically a process of water extrusion due to a contractile change should be independent in rate and extent of the ion concentration and should be facilitated by a high permeability of the mitochondrial membrane to ions.

On the other hand a water efflux based on a primary ion extrusion mechanism should be dependent in rate and extent on the ion concentration, should become inhibited parallel to the increase of permeability of the membrane to the ions and should be dependent in its reversal, after interruption of energy supply, on the permeability of the membrane to the extruded ions.

Acceptance of the above criteria for deciding between the two alternative mechanisms should however be made with caution. In fact the presence of fixed negative charges within the mitochondria may lead, during a contractile change, to the creation of a difference in concentration of the transported anions at the two sides of the membrane and thus render the process dependent, at a certain extent, on the ion environment. Furthermore the water reuptake after a contractile change would probably be facilitated by an increased permeability of the membrane to the solutes in the same way as a reuptake after a primary ion extrusion.

Therefore we conclude that the experiments reported in the present paper, although do not settle the question as to the primary mechanism of ion extrusion in mitochondria, indicate that the dynamic of the shrinkage process is closely dependent on, and correlated with, the rates of ion movements across the mitochondrial membrane.

Relation between ion extrusion and ion uptake

Increasing evidence has been produced in recent years that mitochondria can accumulate large amounts of univalent and divalent cations. The ion accumulation leads to the formation of concentration gradients and it requires energy supply from metabolism. Two types of mechanisms have been proposed. According to one hypothesis, ions are taken up by the mitochondria through the utilization of high-energy intermediates of oxidative phosphorylation[19]. According to another hypothesis ions are moved into the mitochondria by a transmembrane electrical potential[18–21]. Although the present data suggest that an ion extrusion mechanism is present in mitochondria, they give no indication as to how this mechanism might operate. It will therefore be matter of future investigations to understand whether the ion extrusion process represents the opposite side of the process of cation uptake or is expression of an additional energy-linked mitochondrial activity.

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