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SWELLING AND SHRINKAGE PHENOMENA IN LIVER MITOCHONDRIA

VI. METABOLISM-INDEPENDENT SWELLING COUPLED TO ION MOVEMENT

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SUMMARY

1. Water uptake coupled to ion movement has been studied in respiratory-inhibited liver mitochondria, of which the permeability to cations was increased by valinomycin, gramicidin or EDTA, and to anions by raising the pH of the medium. The movement of water was accounted for by the osmotic pressure of the penetrating solutes.

2. The rate of movement of water was inversely proportional to the concentration of solutes in the medium, and was dependent on the presence of permeating cations and anions. The above findings are interpreted within the concept of an osmotic movement of water.

3. The flow of anions through the membrane was inhibited by Ca^{2+} and Mn^{2+}. Mitochondrial swelling was inhibited by sucrose.

4. Ion movement was independent of energy supply from metabolism. The nature of the force driving the ion movement is discussed.

INTRODUCTION

Liver mitochondria undergo a slow but extensive swelling when incubated under aerobic conditions in the presence of isotonic sucrose or saline media. This type of swelling has been denoted as 'large amplitude'[1,2], and 'low energy' or 'irreversible'[3–5] swelling. It has been proposed that this swelling is brought about by relaxation of a contractile mechanism regulating the mitochondrial size[6,7] or by hydration of a gel[8–10]. On the other hand, it has been suggested by TEDESCHI[11] that the mitochondrial swelling is essentially osmotic in nature. On the basis of the studies of JACKSON AND PACE[12], TEDESCHI[11] has calculated that, after prolonged incubation, the experimentally determined increase in mitochondrial volume could be accounted for quantitatively by the osmotic pressure exerted by the penetrating solutes. Further it was also shown by TEDESCHI[11] that the mitochondrial swelling was osmotically reversible through the addition of KCl or of sucrose to the medium. CHAPPELL AND GREVILLE[2] have observed osmotic reversibility of P_i-swollen mitochondria after the addition of sucrose. Movement of solutes together with water has been reported also by AMOORE AND BARTLEY[13], and BARTLEY[14].

An osmotic swelling process in an isotonic medium must be limited by the permeability of the mitochondrial membrane to the penetrating solutes. Slow penetration of sucrose has been observed by TEDESCHI AND HARRIS[15] and by AMOORE AND BARTLEY[13]. AMOORE AND BARTLEY[13] have also shown that K^+, Na^+ and Cl^- enter the mitochondria very slowly.

In view of the low permeability of the mitochondrial membrane to the various solutes, most of the studies referred to above have followed slow movements of solutes. Further, most experiments were done under conditions in which respiration was not inhibited, and thus the contribution of the mitochondrial metabolism to the movement of ions could not be assessed. This contribution may be relevant to understanding the nature of the forces driving the solutes in and out the mitochondrion. Finally, in view of the observation that swollen mitochondria show an increased permeability to solutes, the question arises whether the mitochondrial swelling is due to a primary movement of water before the permeability of the membrane increased, or whether it is due to an increase in the permeability of the membrane before an ion-coupled water movement occurred.

In order to gain further insight into these questions we have studied the swelling in a simplified system in which the permeability of the mitochondrial membrane to ions was increased under controlled conditions. This was achieved by changing the permeability to cations with valinomycin[16], gramicidin[17] or EDTA[18], and to anions[19] by raising the pH of the medium. The results obtained indicate that when the permeability of the mitochondria is increased to both cations and anions, water enters the mitochondrion together with solutes. The characteristics of this process are described below. Preliminary accounts of the present investigation have been reported elsewhere[19].

METHODS

Liver mitochondria were prepared as described previously[20]. The changes in absorbance were followed with an Eppendorf photometer equipped with a recorder. When the rates of absorbance changes are reported, they were been always calculated on the initial rate of swelling. The water content of mitochondria was calculated by subtracting from the centrifuged pellet weight, the dry weight (obtained in vacuum over P_2O_5) and the poly-[^{14}C]carboxyglucose space. The amounts of Ca^{2+}, Rb^+ and Cl^- taken up by the mitochondria during the volume increase were measured by using $^{45}Ca^{2+}$, $^{86}Rb^+$ and $^{36}Cl^-$. The mitochondrial pellet was dissolved in 1 M formic acid, and the counts were measured on aliquots of the formic acid solution. The values for the ion content thus obtained were corrected for the amount of ions dissolved in the poly-[^{14}C]carboxyglucose space.

Poly-[^{14}C]carboxyglucose was kindly given by Dr. E. PFAFF.

Protein was measured by the biuret method.

RESULTS

Movement of ions through the mitochondrial membrane

The experimental model used in the present studies is shown in Fig. 1. Liver mitochondria supplemented with a respiratory chain inhibitor such as rotenone were

incubated in KCl medium at pH 8.8. Addition of valinomycin caused swelling. The swelling was not affected by the presence of other respiratory chain inhibitors such as KCN or antimycin A or of energy-transfer inhibitors such as dinitrophenol and/or oligomycin. At the end of the swelling phase succinate was added, and this caused a shrinkage phase which was interrupted by the exhaustion of O_2. Mitochondria underwent a swelling phase under anaerobic conditions, and a shrinkage phase after aeration. A stable swelling followed the addition of antimycin. The properties of the respiration-dependent shrinkage are described elsewhere[21,22].

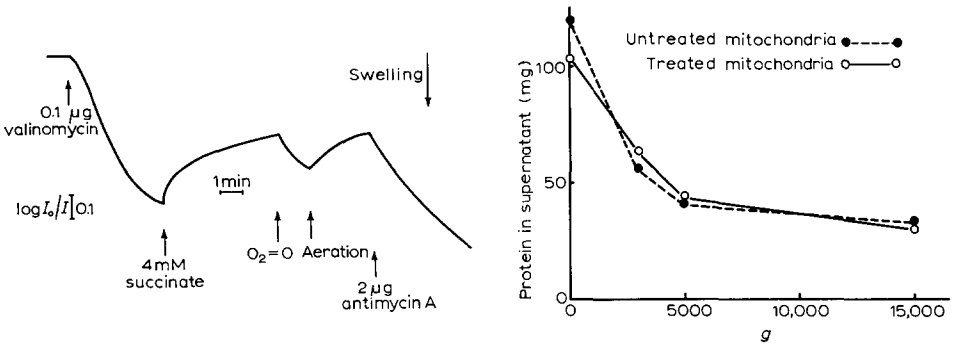


Fig. 1. Swelling and shrinkage of liver mitochondria induced by the addition of valinomycin at pH 8.8. Experimental conditions: 100 mM KCl, 12 mM Tris–HCl (pH 8.8), 2 μM rotenone, 2.8 mg protein. Vol. 2 ml. Temp. 20°.

Fig. 2. Sedimentation pattern of normal and swollen mitochondria. Mitochondria (140 mg protein) were allowed to swell in a medium of the following composition: 50 mM KCl, 12.5 mM Tris (pH 8.8), 2 μM rotenone, 2 μg valinomycin. Swollen mitochondria were centrifuged at 30000 × g for 15 min, and the pellet was resuspended in 0.25 M sucrose. Swollen and unswollen mitochondria were centrifuged at the g indicated in the figure. After each centrifugation the pellets were discarded and the supernatants analyzed for the protein content.

Fig. 2 shows that raising the pH of the incubation medium to 8.8 did not cause the formation of submitochondrial particles. In fact liver mitochondria swollen at pH 8.8 in the presence of valinomycin and then shrunk by the addition of sucrose showed the same sedimentation pattern as fresh mitochondria.

Table I shows that mitochondria swollen in the presence of valinomycin at pH 8.8 increase their water content from 2.5–3.0 to 5.9–6.2 μl/mg dry wt. The increase in mitochondrial water was paralleled by an osmotic equivalent uptake of Rb^+ and Cl^-. In fact the concentrations of Rb^+ and Cl^- in the water that moved into the mitochondria were close to the concentrations of Rb^+ and Cl^- in the external medium. On the other hand, Table I shows that the initial concentration of Rb^+ in the mitochondrial water was higher, and that of Cl^- was lower, than those of the external medium. The movement of Rb^+ and Cl^- during swelling, at a concentration of 40–50 mM, thus caused a decrease in the intramitochondrial concentrations of Rb^+ and an increase in the intramitochondrial concentration of Cl^-. The high initial intramitochondrial concentration of Rb^+ suggests that $^{86}Rb^+$ has exchanged with other intramitochondrially bound cations.

Dependence of swelling on permeability to cations

The swelling described in the present experiments was dependent on an increased permeability of the mitochondrial membrane to univalent cations. Swelling was thus

TABLE I

MOVEMENT OF WATER AND IONS DURING SWELLING

Experimental conditions: mitochondria (48 mg protein) were added to a medium of 50 mM RbCl (labelled either with ^{86}Rb or with ^{36}Cl), 5 mM Tris–HCl (pH 8.5), 5 μM rotenone. Vol. 16 ml. Temp. 22°. After equilibration, a 5-ml sample was withdrawn and valinomycin (5 μg) added. After 10 min a second sample was withdrawn. The samples were sedimented and analyzed. The mitochondrial water was obtained by correcting the total water for the poly-[^{14}C]carboxyglucose space.

Addition	*Wet wt.* (*mg*)	*Dry wt.* (*mg*)	*Mitochondrial water* (μl)	*Ions in the pellet* ($\mu moles$)		*Ions in the mitochondrial water* ($\mu moles$)		*Ionic concn.* (*mM*) *in*	
				^{86}Rb	^{36}Cl	^{86}Rb	^{36}Cl	*mitochondrial water*	*moved water*
None	82.2	16.1	41	4.7		3.5		84	
Valinomycin	179.5	18.3	108	9.2		6.5		60	45
Expt. 1									
None	80.4	16.7	42		2.2		1.0	25	
Valinomycin	180.0	18.2	108		6.4		3.7	34	41
None	89.0	16.2	45.8	4.9		3.6		79	
Valinomycin	173.0	16.7	104.3	8.6		6.0		57	42
Expt. 2									
None	95.0	16.6	50.0		2.6		1.2	18	
Valinomycin	171.3	17.2	102.8		6.4		3.9	38	51

caused by various agents owing to their specific effects on the permeability of the membrane. In fact valinomycin caused swelling when mitochondria were incubated in media containing KCl or RbCl. Gramicidin caused swelling when mitochondria were incubated in media containing NaCl, LiCl, KCl, or RbCl. EDTA caused swelling especially in media containing LiCl or NaCl, and the swelling effect of EDTA was abolished by Mg^{2+}. Neither gramicidin, valinomycin nor EDTA caused swelling when a non-permeating cation such as $Tris^+$, replaced K^+, Na^+, Li^+ or Rb^+.

Dependence of swelling on permeability to anions

The swelling induced by valinomycin or gramicidin in respiratory-inhibited mitochondria was strongly dependent on the pH of the medium. In fact the rate of swelling was high at pH 8.8, and was gradually reduced when the pH of the medium was decreased to 7 (Fig. 3A). As shown in Fig. 3B, the amount of water taken up by the mitochondria also increased with increasing pH, and the water taken up was accompanied by a corresponding amount of $^{36}Cl^-$ (Fig. 3B).

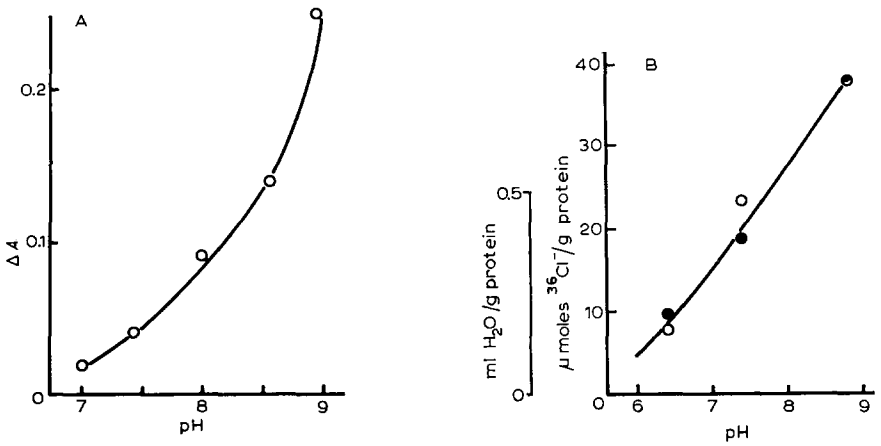


Fig. 3. Effect of pH on the movement of H_2O and $^{36}Cl^-$. In A, 50 mM KCl, 12.5 mM Tris at the pH's indicated, 2 μM rotenone, 0.1 μg valinomycin. Vol. 2 ml. Temp. 22°. B, mitochondria (194 mg protein) suspended in 50 mM $Rb^{36}Cl$ in the presence of 2 μM rotenone, were added to centrifuge tubes containing 0.2 μg valinomycin and 12.5 mM Tris–HCl at the pH's indicated. Time of incubation 3 min. Temp. 20°. Vol. 5.22 ml. After centrifugation the pellet was analyzed for water content, and after dissolution with concentrated formic acid, for radioactivity.

On the other hand, when the time of incubation was prolonged in order to allow complete equilibration of solutes between extra- and intramitochondrial spaces, the extent of swelling was independent of the pH of the medium (Fig. 4).

It was supposed that raising the pH of the medium increased the permeability to the anion. This suggestion was supported by the following findings. Firstly, the increase in pH also increased the rate of swelling when Cl^- was replaced with other anions such as acetate, formate, succinate (*plus* antimycin), ketoglutarate, citrate, lactate, sulphate, nitrate or perchlorate. Only with phosphate was the rate of swelling rather high at pH 7. Secondly, when the above-mentioned anions were replaced with a non-permeating anion such as ribonucleate, no swelling ensued. Thirdly, raising the pH of the medium increased the rate of transfer of the anion through the membrane even in the absence of a net flux of the anion or of water. In fact, as shown

in Table II, the rate of exchange of the internal $^{36}Cl^-$, that entered the mitochondria during the swelling phase, with external Cl^- was much faster at pH 8.8 than at pH 6.5. On the other hand, the rate of exchange of internal $^{86}Rb^+$ with external Rb^+ was about the same at pH 8.8 and 6.5.

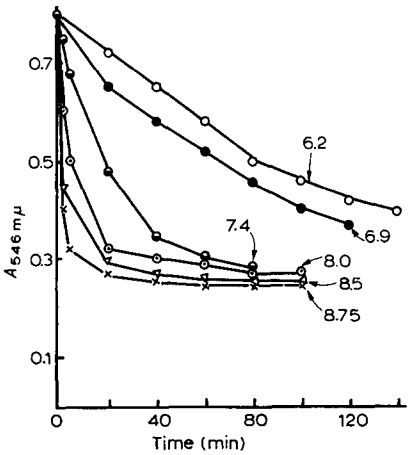


Fig. 4. Rate and extent of swelling at different pH's. Experimental conditions as for Fig. 3A. Amount of mitochondrial protein was 0.61 mg. pH's as indicated.

TABLE II

EXCHANGE BETWEEN INTERNAL $^{36}Cl^-$ AND EXTERNAL $^{35}Cl^-$, AND BETWEEN INTERNAL $^{86}Rb^+$ AND EXTERNAL $^{85}Rb^+$ AT DIFFERENT pH's

Swelling of mitochondria was measured as for Table I. After centrifugation and acidification to pH 6.5 with HCl, the swollen mitochondria (with their contained ions labelled either with $^{86}Rb^+$ or with $^{36}Cl^-$) were added to media of different pH. 5-ml samples were centrifuged, either immediately or after 90 min, at 17000 × *g* for 15 min.

	2 min		*90 min*	
	pH 6.5	*pH 8.8*	*pH 6.5*	*pH 8.8*
$^{36}Cl^-$ in the pellet (counts/min)	12400	3860	4826	2964
Pellet weight (mg)	157.8	157.8	175.8	171.5
$^{86}Rb^+$ in the pellet (counts/min)	5474	5188	5781	6310
Pellet weight (mg)	168.0	160.8	189.0	174.8

Effect on swelling of the concentration of solutes

Mitochondria incubated in a medium containing non-permeating solutes behave like osmometers: as shown in Fig. 5A, when valinomycin was omitted the mitochondrial volume decreased with increasing concentration of KCl in the medium. Maximal volume was observed with mitochondria incubated in H_2O. When valinomycin was added, an increase in the ionic strength of the medium caused no reduction in the mitochondrial volume, the latter being almost unchanged by replacing H_2O with 0.1 M RbCl. The volume of the mitochondria under conditions of permeability of the membrane to solutes was thus independent of the concentration of the solutes

in the medium. On the other hand, the rate of swelling after the addition of valinomycin was inversely proportional to the salt concentration (Fig. 5B). This finding accords with the concept that the rate of movement of water which is linked to the movement of ions must increase with decreasing salt concentrations.

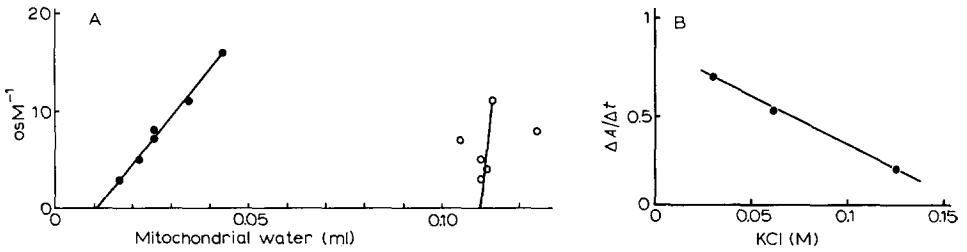


Fig. 5. The effect of various concentrations of KCl on water content of rat-liver mitochondria. Mitochondria were added to a medium containing 5 mM Tris–HCl (pH 8.5), 3 μM rotenone, with 1 μg/ml valinomycin (○—○) or without valinomycin (●—●). B, 25 mM Tris–HCl (pH 8.8), KCl concentrations indicated in the figure, 2 μM rotenone, 0.05 μg valinomycin, 1.8 mg protein. Vol. 2 ml. Temp. 25°.

Inhibition of swelling by divalent cations and by sucrose

A strong inhibition of the swelling was observed after addition of divalent cations such as Ca^{2+} and Mn^{2+} (Figs. 6A, B). The swelling was abolished by 1 mM $CaCl_2$ and reinitiated by 1.3 mM EDTA (Fig. 6A). The swelling was also abolished by 200 μM $MnSO_4$ and reinitiated by 5 mM EDTA (Fig. 6B). The inhibition of swelling

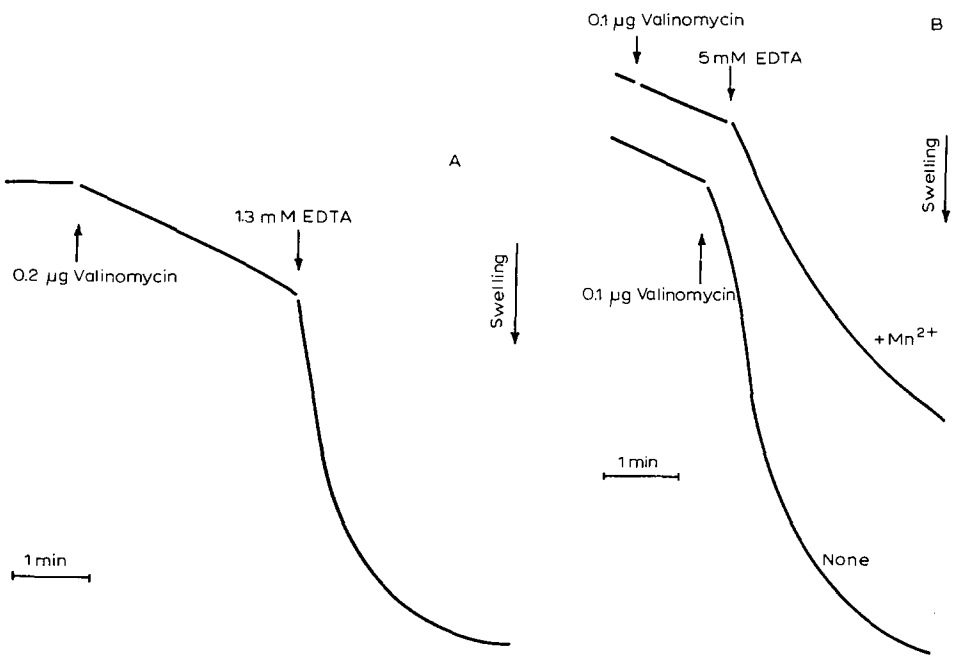
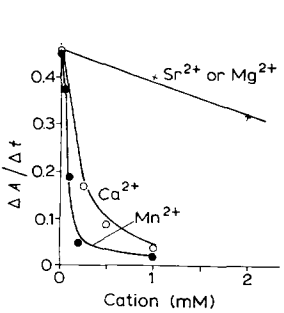


Fig. 6. Effect of Ca^{2+} and Mn^{2+} on swelling. Experimental conditions as for Fig. 1, except that in A 0.2 μg valinomycin and 2 μmoles $CaCl_2$ were added; in B, 0.2 μg valinomycin and 0.4 μmole $MnSO_4$ were added and the protein content was 0.94 mg. Vol. 2 ml. Temp. 20°.

by various concentrations of Mn^{2+} and Ca^{2+} is reported in Fig. 7. Half inhibitory concentrations were lower than 100 μM for Mn^{2+}, and lower than 250 μM for Ca^{2+}. Only a low inhibition of swelling was observed after the addition of Sr^{2+} or Mg^{2+}. The inhibition of the swelling by Ca^{2+} was dependent on the protein concentration: half inhibition was obtained at about 500 mμmoles Ca^{2+}/mg protein.



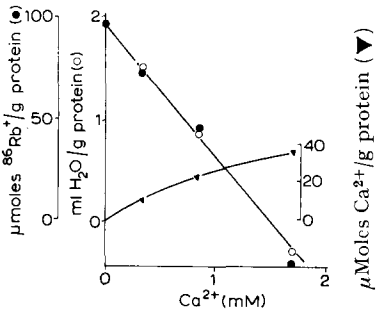


Fig. 7. Effect on swelling of various concentrations of divalent cations. Experimental conditions: 50 mM KCl, 12.5 mM Tris–HCl (pH 8.8), 2 μM rotenone, 0.1 μg valinomycin, 0.94 mg protein. Vol. 2 ml. Temp. 20°.

Fig. 8. Inhibition by Ca^{2+} of univalent ion and water movement. Experimental conditions: 50 mM RbCl (labelled with $^{86}Rb^{+}$), 12.5 mM Tris–HCl (pH 8.8), 2 μM rotenone, 8.5 mg mitochondrial protein. Vol. 5 ml. Temp. 20°. Time of incubation 7 min. The binding of $^{45}Ca^{2+}$ to mitochondria was measured in parallel samples.

The inhibitory effect of Ca^{2+} on the swelling was presumably due to a binding of Ca^{2+} to the mitochondrial membrane which decreased the flow of solutes through the mitochondrion. As shown in Fig. 8, the inhibitory effect of Ca^{2+} on swelling occurred parallel with an inhibition of the entrance of $^{86}Rb^{+}$ and with a binding of $^{45}Ca^{2+}$ to the mitochondrion. The properties of this metabolism-independent binding of Ca^{2+} are reported in a separate paper[23].

Inhibition of the ionic movement and thereby also of the water movement was obtained by the addition of sucrose (Fig. 9). Half-inhibitory concentrations of sucrose were in the range of 25 mM.

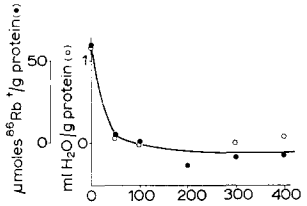


Fig. 9. Inhibition of swelling by various sucrose concentrations. Mitochondria (9.25 mg protein), suspended in 50 mM RbCl (labelled with $^{86}Rb^{+}$) containing 25 mM Tris–HCl (pH 8.8), and 2 μM rotenone, were added to centrifuge tubes containing 0.5 μg valinomycin and sucrose at the concentrations indicated. H_2O and $^{86}Rb^{+}$ were analyzed as for Fig. 4.

DISCUSSION

The permeability of the mitochondrial membrane

A low permeability of the mitochondrial membrane to ionized solutes such as KCl and NaCl, or non-ionized solutes such as sucrose and other polyols, has been

reported by several authors[13,15]. (For a review see LEHNINGER[1].) Therefore under conditions of integrity of the membrane, the mitochondrial swelling, coupled with diffusion of solutes into the mitochondria is a slow process. These slow processes have been analyzed in previous studies on mitochondrial swelling such as those of TEDESCHI[11], and BARTLEY[14], in which changes in membrane permeability were not induced. On the other hand, high rates of swelling were obtained in our studies when the permeability to cations was increased with specific antibiotics and to anions by raising the pH of the medium.

The increase in movement of K^+, caused by valinomycin in respiratory-inhibited mitochondria, substantiates the suggestion of CHAPPELL AND CROFTS[17] that the effect of valinomycin is that of rendering the membrane more permeable to univalent cations. The entrance of anions was increased by raising the pH of the medium, which suggests a relationship between the amount of charged groups on the mitochondrial surface and the rate of diffusion of anions. In agreement with this suggestion are the inhibitory effects of Ca^{2+} and Mn^{2+} on the movement of the anions. We have shown previously[5] that the binding of Ca^{2+} to the mitochondrion has no effect on the rate of movement of K^+. The binding of Ca^{2+} or Mn^{2+} may alter the electrical characteristics of the mitochondrial surface thereby influencing the flow of anions. The inhibitory effect of sucrose on swelling, we suggest, may be due to its acting as an osmotically active, non-permeating, solute outside the mitochondrion.

The osmotic nature of the water movement in mitochondria

Two types of force, hydrostatic and osmotic, lend themselves for consideration as being the cause of the movement of water through the mitochondrial membrane. In the first case water flows from the side of higher to the side of lower hydrostatic pressure. In the second case the movement of water is from the side of lower to the side of higher osmotic pressure. Movement of H_2O due to osmotic pressure can occur either through membranes impermeable to solutes, or parallel with the movement of solutes when the membranes are permeable to the solutes.

As mentioned in the INTRODUCTION, TEDESCHI[11] has produced evidence that the increase in mitochondrial volume can be accounted for by the osmotic pressure of the permeating solutes. This result, which is confirmed by the present data, is, however, not sufficient to exclude a movement of water due to hydrostatic forces. In fact, a movement of water due primarily to hydrostatic forces may be followed by movement of solutes to produce osmotic equilibration.

The experiments reported in the present paper strongly suggest that the flow of water was secondary to a flux of solute particles. The rate of water uptake was inversely proportional to the concentration of solutes in the medium. Furthermore the movement of water was dependent on an increased permeability to both cations and anions.

The mechanism of osmotic swelling of liver mitochondria

Movement of a substance can be defined as 'active' if it results in an increase in free energy of the system, or 'passive' if it results in a decrease in free energy. Passive movements may occur spontaneously whereas active movements must be coupled to metabolic processes capable of furnishing energy. In the 'high energy' mitochondrial swelling, studied by CHAPPELL AND CROFTS[17] and by ourselves[18,24], the

entrance of water is coupled to the metabolism-dependent uptake of cations. The movement of cations results in an increase in free energy of the system. The mitochondrial shrinkage, after addition of respiratory chain inhibitors or uncouplers, is coupled to the efflux of cations driven by the concentration gradient formed during the 'active' swelling phase.

In previous experiments on large amplitude swelling where movements of ions were measured in parallel with the movement of water, the 'passive' or 'active' nature of the ionic movement was not considered. In the 'low energy' swelling studied here, the entrance of ions, following the increased permeability of the mitochondrial membrane, occurs in respiratory-inhibited mitochondria and is thus independent of energy supply from metabolism. The movement of ions results in a decrease in free energy of the system. The force driving the movement of ions under these conditions is presumably the presence of a concentration gradient of anions *plus* cations outside the mitochondrion. In agreement with this suggestion is the experiment of Fig. 1 where it is shown that interruption of energy supply, after a respiratory-dependent shrinkage, results in a passive swelling. As will be proposed in a subsequent paper[22], the metabolism-dependent shrinkage of mitochondria involves an extrusion of ions. After inhibition of the extrusion process, ions may re-enter the mitochondrion driven by a concentration gradient at a rate that is dependent on the permeability of the mitochondrial membrane to the ions.

Relation with other types of swelling

Swelling of 'large amplitude' has been obtained by a great variety of agents, such as P_i, arsenate, thyroxine, Ca^{2+}, oleic acid, *etc.* As reviewed by LEHNINGER[1], most of these swelling processes require respiration and are denoted as 'active'. If the definition proposed by LEHNINGER is accepted, the above-mentioned 'active' types of swelling should be considered as basically different from the swelling analyzed in the present paper where the increase in water content of the mitochondria is the result of a 'passive' flow of solutes. We wish however to suggest that the requirement for energy in many of the so-called 'large amplitude' 'active' swellings may not be related to the phase of the process connected with the movement of ions and water but rather with the preceding steps which lead to a permeability change in the mitochondrial membrane[4, 20].

CHAPPELL AND CROFTS[25] have also suggested on the basis of measurements of absorbance that swelling occurs when anions and cations penetrate the mitochondrion.

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REFERENCES

1 A. L. LEHNINGER, *Physiol. Rev.*, 42 (1962) 467.
2 J. B. CHAPPELL AND G. D. GREVILLE, *Biochem. Soc. Symp. Cambridge*, 23 (1963) 39.
3 G. F. AZZONE AND A. AZZI, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 1084.
4 A. AZZI AND G. F. AZZONE, *Biochim. Biophys. Acta*, 113 (1966) 445.

5 G. F. AZZONE AND A. AZZI, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 332.
6 J. RAAFLAUB, *Helv. Physiol. Pharmacol. Acta*, 11 (1953) 142.
7 C. A. PRICE, A. FONNESU AND R. E. DAVIES, *Biochem. J.*, 64 (1956) 754.
8 J. W. HARMAN AND A. KITIYAKARA, *Exptl. Cell Res.*, 8 (1955) 411.
9 R. L. KLEIN AND R. J. NEFF, *Exptl. Cell Res.*, 19 (1960) 133.
10 S. I. HONDA AND A. MUENSTER, *Arch. Biochem. Biophys.*, 88 (1960) 118.
11 H. TEDESCHI, *Biochim. Biophys. Acta*, 46 (1961) 159.
12 K. I. JACKSON AND N. PACE, *J. Gen. Physiol.*, 40 (1956) 47.
13 J. E. AMOORE AND W. BARTLEY, *Biochem. J.*, 69 (1958) 223.
14 W. BARTLEY, *Biochem. J.*, 80 (1961) 46.
15 H. TEDESCHI AND D. L. HARRIS, *Arch. Biochem.*, 58 (1955) 52.
16 C. MOORE AND B. C. PRESSMAN, *Biochem. Biophys. Res. Commun.*, 15 (1964) 562.
17 J. B. CHAPPELL AND A. R. CROFTS, *Biochem. J.*, 95 (1965) 393.
18 A. AZZI, E. ROSSI AND G. F. AZZONE, *Enzymol. Biol. Clin.*, 7 (1966) 25.
19 A. AZZI AND G. F. AZZONE, *Proc. 3rd Meeting Fed. European Biochem. Soc., Warsaw, 1966*, p. 161.
20 A. AZZI AND G. F. AZZONE, *Biochim. Biophys. Acta*, 105 (1965) 253.
21 A. AZZI AND G. F. AZZONE, *Biochim. Biophys. Acta*, 120 (1966) 466.
22 A. AZZI AND G. F. AZZONE, *Biochim. Biophys. Acta*, in the press.
23 C. ROSSI, A. AZZI AND G. F. AZZONE, *Proc. 3rd Meeting Fed. European Biochem. Soc., Warsaw, 1966*, p. 156.
24 A. AZZI AND G. F. AZZONE, *Biochim. Biophys. Acta*, 105 (1965) 265.
25 J. B. CHAPPELL AND A. R. CROFTS, in J. M. TAGER, S. PAPA, E. QUAGLIARIELLO AND E. C. SLATER, *Regulation of Metabolic Processes in Mitochondria*, BBA Library, Vol. 7, Elsevier, Amsterdam, 1966, p. 293.

Biochim. Biophys. Acta, 131 (1967) 468–478