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MECHANISM OF ACTIVE SHRINKAGE IN MITOCHONDRIA I. COUPLING BETWEEN WEAK ELECTROLYTE FLUXES

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SUMMARY

1. A passive penetration of $(NH_4)_2$ HPO_4 or of K_2 HPO_4+nigericin occurs in respiratory-inhibited liver mitochondria. Addition of succinate at the end of the passive swelling initiates a shrinkage phase which leads to restoration of the initial mitochondrial volume. The rate and time of onset of the active shrinkage depend on the degree of stretching of the mitochondrial membrane. The rate of active shrinkage increases proportionally to the concentration of nigericin while it is strongly inhibited by valinomycin.

2. A number of SH inhibitors such as *N*-ethylmaleimide, *p*-chloromercuribenzoate, *p*-chloromercuriphenylsulphonate, dithiobisnitrobenzoate, exert a marked enhancing effect on the rate of shrinkage. The enhancing effect parallels titration of the phosphate carrier and inhibition of the passive phosphate influx. The above SH inhibitors do not inhibit passive phosphate efflux. In contrast, mersalyl is a powerful inhibitor of the rate of active shrinkage. The inhibition parallels that on phosphate passive efflux and requires higher mersalyl concentrations in respect to inhibition of phosphate influx.

3. The active shrinkage is discussed in terms of (a) a mechanoenzyme, (b) an electrogenic proton pump and (c) a proton-driven P_i pump.

INTRODUCTION

Active transport in mitochondria is generally thought of as consisting of the extrusion of H^+ and uptake of cations. The uptake of weak acids follows passively driven by a transmembrane Δ pH. In most cases the uptake of cations and anions is accompanied by osmotic swelling. However mitochondria are also capable of perform-

Abbreviations: *p*-Cl-HgBzO$^-$, *p*-chloromercuribenzoic acid; *p*-Cl-HgBzSO$^-{}_3$, *p*-chloromercuriphenylsulphonic acid; Nbs_2, 5,5-dithiobis, 2-nitrobenzoic acid; mersalyl, [*O*-3]hydroxymercuri-2-methoxypropylcarbonylphenoxyacetic acid; MalNEt, *N*-ethylmaleimide; TMPD, *N,N,N'N'*-tetramethyl-*p*-phenylenediamine dihydrochloride.

ing the opposite process, namely active shrinkage. In the earlier studies of Lehninger [1–3] the question has arisen whether the active shrinkage involves an increase of hydrostatic pressure, due to a mechanoprotein, or an active extrusion of electrolytes. The question, however, has not been solved [4–6]. An extension of the electrogenic proton pump has been proposed by Brierley [7–9] and by Nichols [10] to account for the inversion of polarity of the ion movements during active shrinkage. Brierley et al. [9] have induced swelling-shrinkage cycles with mercurials.

In the present study the mechanism of active shrinkage has been examined under two experimental conditions: (a) when the coupling of fluxes concerns weak electrolytes and (b) when the coupling of fluxes concerns strong electrolytes [11]. Incubation of respiratory inhibited mitochondria in $(NH_4)_2HPO_4$ or K_2HPO_4 plus nigericin results in a passive swelling. The penetration of the electrolyte consists of two electroneutral fluxes either in the case of P_i or of K^+ via nigericin and of NH_3. Addition of succinate at the end of the swelling initiates a process of energy linked efflux of K_2HPO_4 or $(NH_4)_2HPO_4$. The process of P_i extrusion is markedly enhanced by nigericin and inhibited by valinomycin, while it is markedly dependent on the extent of membrane stretching, phosphate concentration and pH. An interesting feature of the active P_i extrusion is the enhancing effect of several SH inhibitors. While SH reagents which inhibit P_i influx [12–15] increase the rate of shrinkage, mersalyl, which inhibits both influx and efflux of P_i, decreases the rate of shrinkage.

Three alternatives will be considered for the active shrinkage: (a) mechanoprotein [1–3], (b) electrogenic proton pump [7–10] and (c) proton-driven P_i pump.

EXPERIMENTAL METHODS

Rat-liver mitochondria were prepared in 0.25 M sucrose, 1 mM EDTA, 5 mM Tris/Cl pH 7.4. After two washings they were resuspended in the same medium devoid of EDTA.

The mitochondrial volume was determined gravimetrically. The mitochondrial suspension was centrifuged in a Sorvall RC2B centrifuge for 10 min at $40\,000 \times g$. The volume of the matrix space was determined by correcting the weight of the mitochondrial pellet for the amount of mitochondrial protein and the volume of a high molecular weight impermeant solute. For this purpose ^{14}C-labeled Dextran, M_r 60 000, was usually employed. The total penetration and extrusion of phosphate was determined with $^{32}P_i$. The kinetics of the penetration and extrusion of solutes into and from mitochondria was followed continuously by recording the absorbance change at 600 nm in a Hitachi Perkin Elmer Spectrophotometer, model 124, equipped with a constant temperature cuvette compartment. The absorbance changes were converted into solute flux rates either according to the procedure of Klingenberg et al. [17] or as follows.

Tedeschi and Harris [16] and Massari et al. [18, 19] have shown that a straight line is obtained by plotting the volume obtained from gravimetric measurements against the reciprocal of osmolarity or against the reciprocal of absorbance. The relation holds when the amount of mitochondrial protein is lower than 1mg/ml, as used here. The initial rate of water translocation is then calculated in $\mu l \cdot s^{-1} \cdot mg\ protein^{-1}$, from the shape of the tangent to the absorbance change trace, according to equation:

$$V = \frac{a \text{ mg protein}}{A_1^2} \frac{\Delta A}{\Delta t}$$

where v is the rate of water translocation, a is the slope of the plot of volume/mg protein vs mg protein/absorbance, A_1 is the initial absorbance and $\Delta A/\Delta t$ is the absorbance change in the time interval Δt. The rates and amounts of water translocation can be converted into rates and amounts of solute translocation on the basis of the equation described by Massari et al. [18, 19].

Titration of SH groups was carried out either with dithiobis, 2-nitrobenzoic acid (Nbs_2) or with *N*-ethylmaleimide. In the former case, the procedure indicated by Ellman [20] was followed. The Ellman reagent was titrated against a standard solution of cysteine. In the latter case the procedure indicated by Klingenberg et al. [17] was followed and *N*-[^{14}C]ethylmaleimide was used. The passive efflux of solutes from the mitochondria was determined by adding a standard amount of high molecular weight solute, such as polyethylenglycol (M_r 1000 or 4000) or sucrose, and by recording continuously the rate of absorbance increase. In parallel experiments the absorbance change was compared with volume changes as determined by gravimetric methods.

RESULTS

Fig. 1 shows schematically the swelling-shrinkage cycles of the system taken into consideration. In Fig. 1A, respiratory inhibited mitochondria were incubated in the presence of K_2HPO_4 and P_i influx initiated by nigericin. At the end of the swelling, addition of succinate initiated active extrusion. In the experiment of Fig. 1B the penetration of P_i was accompanied by that of NH_3 and no nigericin was required.

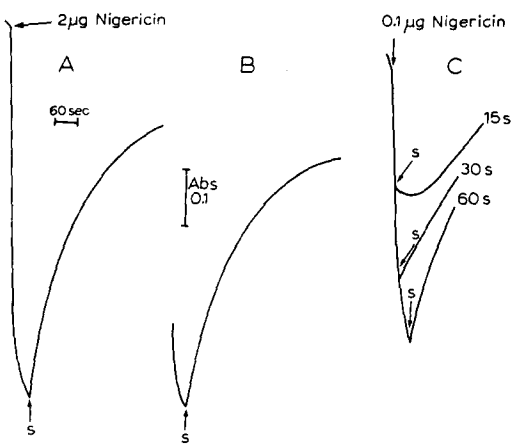

Fig. 1. Schematic representation of swelling-shrinkage cycles in K_2HPO_4 and $(NH_4)_2HPO_4$. The medium contained in A, 30 mM K_2HPO_4, 1 mM EDTA pH 7.5 and 1 μM rotenone. Swelling was initiated by 2 μg nigericin. After swelling, shrinkage was initiated by 1 mM succinate (S). The incubation medium was identical in B, except that K_2 HPO_4 was replaced with $(NH_4)_2HPO_4$. In C, the medium was the same as in A. Swelling was initiated by 0.1 μg nigericin and succinate added after variable periods of times as indicated in the figure. Final volume, 2 ml, 1.6 mg protein.

Again, succinate caused an active P_i extrusion. NH_3 could be replaced by several organic amines. The process of active P_i extrusion always led to almost complete restoration of the initial mitochondrial volume. However, the time of onset of the shrinkage as well as the rate of shrinkage, following the addition of succinate, were markedly dependent on the extent of mitochondrial swelling, Fig. 1C. When succinate was added under conditions of low swelling there was a considerable delay in the initiation of shrinkage and a slowing down of the shrinkage rate. This point will be considered further in the subsequent paper. In the experiments reported below, succinate was always added at the end of the swelling phase.

The rates of penetration of K_2HPO_4 in the presence of 2 μg nigericin, calculated as described in the Methods, were about 120 μmol · min^{-1} · g protein^{-1} at 20 C°. This rate is about 5 times lower with respect to that observed by Klingenberg et al. [17] for the penetration of $(NH_4)_2HPO_4$. However, Klingenberg et al. [17] used 100 mM $(NH_4)_2HPO_4$. The rates of active shrinkage with 1 mM succinate were 20 % lower, say about 100 μmol · min^{-1} · g protein^{-1}. The extrusion of P_i was dependent on the presence of a continuous and coupled respiration. Addition of antimycin or of uncouplers resulted in a complete inhibition of the P_i extrusion and a restoration of P_i penetration. Under the conditions of Fig. 1 the respiratory rate was about 30 μatoms oxygen · min^{-1} · g protein^{-1}. The respiratory rate was constant and was not affected more than 20 %, by changing the concentration of nigericin which affects markedly the shrinkage rate. Rates of shrinkage and of respiration were dependent on the concentration of succinate with an apparent K_m of 1.7 mM. In the present study, most experiments were carried out at 1 mM succinate, which is considerably below the K_m but permits a better evaluation of the kinetics. In other experiments P_i was replaced with acetate in order to test whether the process was of active extrusion of P_i or was a property concerning all weak acids. Addition of an oxidizably substrate

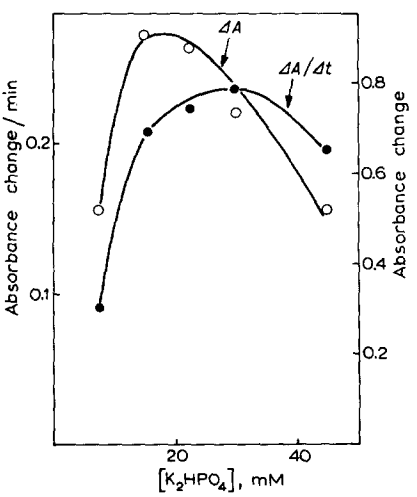

Fig. 2. Dependence of shrinkage on electrolyte concentration. The medium contained 1 mM EDTA pH 7.5, 1 μM rotenone, 2 μg nigericin and variable concentrations of K_2HPO_4 as indicated in the figure. After the attainment of osmotic equilibrium 200 μM *N*-ethylmaleimide (MalNEt) were added and shrinkage initiated by 1 mM succinate. Final volume 2 ml, 1.8 mg protein.

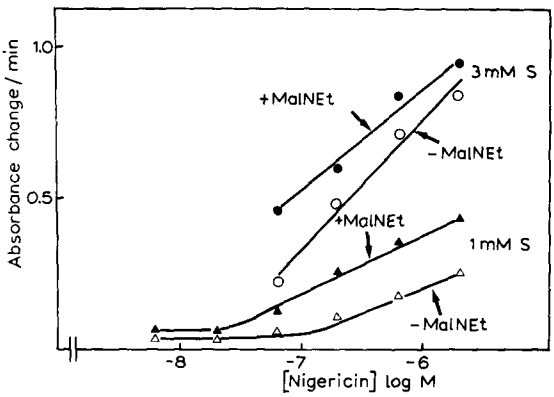

Fig. 3. Effect of nigericin on shrinkage rate. Experimental conditions as in Fig. 2 with 30 mM K_2HPO_4 except that the concentration of nigericin was varied as indicated in the figure. The shrinkage was always started after the attainment of equivalent degree of swelling with 1 or 3 mM succinate, with or without 200 μM *N*-ethylmaleimide (MalNEt). Final volume, 2 ml, 1.8 mg protein.

after swelling, did start a shrinkage phase. However the rate was much lower than that observed with P_i. This may be due in part to the lack of inhibition of the anion influx (see below).

Fig. 2 shows that the active shrinkage following the addition of succinate to mitochondria incubated in K_2HPO_4 plus nigericin was dependent on the electrolyte concentration. Optimal rate and extent of shrinkage were obtained around 20–30 mM electrolyte while a marked inhibition occurred both at higher and lower electrolyte concentrations. Both the rates of swelling and shrinkage were also dependent on the pH of the medium. Below pH 7 the rate of shrinkage was negligible. Fig. 3 shows the effect of the nigericin and succinate concentrations and of *N*-ethylmaleimide on P_i extrusion. The rate of extrusion increased proportionally to the increase of the nigericin concentration, *N*-ethylmaleimide potentiated the rate of P_i extrusion, and rendered possible the extrusion also at low nigericin. The rate of extrusion was also dependent on the succinate concentrations. Table I shows the determinations of

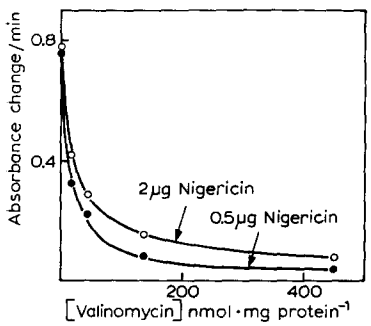

Fig. 4. Effect of valinomycin on shrinkage rate. Experimental conditions as in Fig. 2 with 30 mM K_2HPO_4 and amounts of nigericin as indicated in the figure. Valinomycin was added before succinate, and shrinkage started with succinate preceded by 200 μM *N*-ethylmaleimide (MalNEt). Final volume, 2 ml, 1.6 mg protein.

TABLE I

ACTIVE EXTRUSION OF P_i

The medium contained 30 mM $(NH)_2HPO_4$, 1 mM EDTA, 2 μM rotenone pH 7.5 and 7.6 mitochondrial protein. Final volume 2 ml. The mitochondria were washed in 30 mM $(NH_4)_2HPO_4$. After completion of swelling the samples were bubbled with oxygen, supplemented with 4 mM succinate, 1 mM ATP and various amounts of *N*-ethylmaleimide, and centrifuged after 120 s. The water content was measured gravimetrically and the values corrected for the dextrane permeable space with ^{14}C-labeled Dextran. P_i was measured with $^{32}P_i$.

Additions	H_2O (μl · mg protein^{-1})			P_i (nmol · mg protein^{-1})		
	−succ · ATP	+succ · ATP	Δ	−succ · ATP	+succ · ATP	Δ
none	1.70	1.13	0.57	56	39	17
20 μM *N*-ethylmaleimide	1.70	1.10	0.60	56	38	18
80 μM *N*-ethylmaleimide	1.70	1.06	0.64	56	31	25
200 μM *N*-ethylmaleimide	1.70	0.80	0.90	56	25	31

water and P_i content of the mitochondria after the phase of passive swelling and active shrinkage. Since exhaustion of oxygen results again in swelling, ATP was usually added together with succinate in order to avoid erroneous results due to anaerobiosis of the suspension during centrifugation. However, the extent of absorbance and volume changes were very similar whether or not ATP was added together with succinate. The active shrinkage phase resulted in an extrusion of 17–31 nmol $P_i \cdot$ mg protein^{-1}. This was accompanied by the extrusion of an osmotically equivalent amount of water, say 0.57–0.9 μl · mg protein^{-1}.

The active shrinkage in K_2HPO_4 plus nigericin was severely inhibited by the addition of valinomycin, Fig. 4. The inhibitory effect of valinomycin was higher at the low than at the high nigericin concentration, suggesting that nigericin was counteracting the valinomycin induced inhibition. The inhibitory effect of valinomycin was about the same on the rate and extent of shrinkage.

Effect of SH inhibitors

Fig. 3 indicates that the inhibition of the P_i carrier enhances the active shrinkage, particularly when the rate of shrinkage is low. This is also supported by the observation that the effect of *N*-ethylmaleimide was more marked (not shown) when the shrinkage occurred in $(Tris)_2HPO_4$ in respect to $(NH_4)_2PO_4$, the reason being that the rate of Tris permeation is presumably lower than the rate of NH_3 permeation. Fig. 5 shows the effect of three SH inhibitors, *N*-ethylmaleimide, *p*-Cl · $HgBzO^-$ and *p*-Cl · $HgBzSO_3^-$ on rate and extent of active shrinkage. It is seen that there was practically no shrinkage in the absence of SH inhibitors while maximal enhancement of rate and extent of shrinkage occurred between 100 and 200 μM inhibitor concentration. A similar curve was also obtained with Nbs_2. Fig. 6 shows a correlation between amount of SH groups titrated by *N*-ethylmaleimide and Nbs_2 and effect on the rate of active shrinkage. Maximal enhancement was obtained with *N*-ethylmaleimide between 12 and 15 μmol bound · g protein^{-1}. This corresponds to the amount of SH group required to titrate the P_i carrier. In the case of Nbs_2 the maximal enhancement was obtained between 5 and 8 μmol · g protein^{-1}.

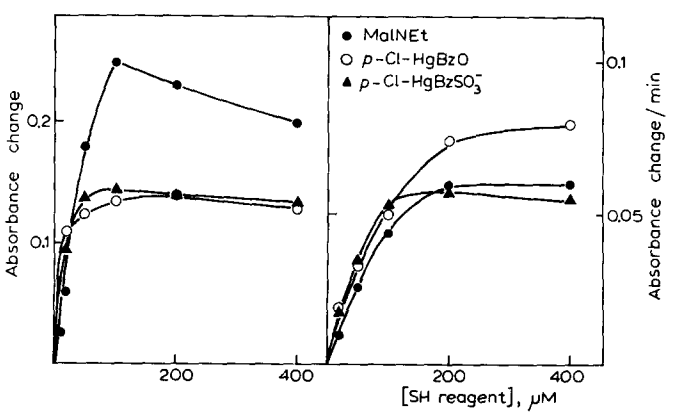

Fig. 5. Effect of SH inhibitors on shrinkage. Experimental conditions as in Fig. 2 except that K_2HPO_4 was replaced with $(Tris)_2HPO_4$ pH 7.5. The SH inhibitors were added before 1 mM succinate. Final volume 2 ml, 0.8 mg protein.

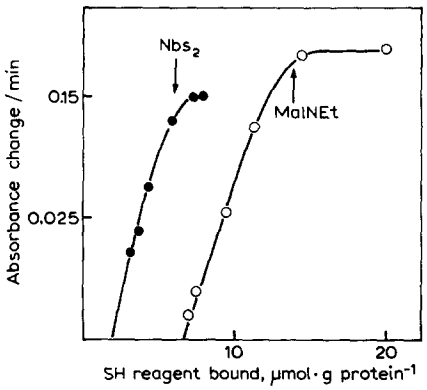

Fig. 6. Relationship between titration of SH groups and enhancement of shrinkage rate. Titration of SH groups and effect of SH inhibitors on shrinkage were done in two parallel experiments. The titration of the SH groups was carried out as described in the Methods. Measurement of shrinkage was carried out under the conditions of Fig. 5. Final volume, 2 ml.

Fig. 7 shows the effect of *N*-ethylmaleimide on the rate of passive P_i influx and efflux. The rate of P_i influx was measured from the absorbance change following the addition of nigericin to mitochondria incubated in 30 mM K_2HPO_4. The rate of P_i efflux was measured after addition of an impermeant solute. Addition of an impermeant solute causes an efflux of water which is then followed by matrix solute efflux. In the present system sucrose, polyethyleneglycol M_r 1000 and 4000 all cause osmotic shrinkage. However, rate and extent of shrinkage increased with the molecular weight of the solute indicating an inverse correlation between degree of solute permeation and molecular weight [21]. In mitochondria swollen in K_2HPO_4 plus nigericin, the rates of solute efflux are so high that the absorbance change does not discriminate between efflux of water and of solutes and the kinetics is monophasic. On the other hand, when solute efflux becomes inhibited (for example by mersalyl, see below) the kinetics is biphasic and the extent of shrinkage is greatly reduced. In order to obtain comparable values, the rate of solutes efflux was measured on the extent of absorbance change after a time interval of 60 sec. *N*-ethylmaleimide caused an

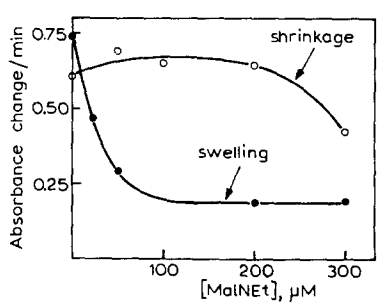

Fig. 7. Effect of *N*-ethylmaleimide on passive swelling and shrinkage. Passive swelling was carried out in 30 mM K_2HPO_4, 1 mM EDTA pH 7.5, 1 μM rotenone and was initiated by 2 μg nigericin. The SH inhibitors were always added a few seconds before nigericin. Passive shrinkage was initiated by the addition of 20 mM sucrose. Final volume, 2 ml, 1.8 mg protein.

almost 50 % inhibition of the swelling rate around 30 μM, while a maximal inhibition of about 80 % was obtained around 100 μM. On the other hand, the shrinkage rate was practically unaffected by *N*-ethylmaleimide up to 200 μM. Only at higher concentrations did a slight inhibition become apparent. Thus the enhancing effect of *N*-ethylmaleimide on active shrinkage is correlated with an inhibition of the P_i influx, while the passive efflux of P_i is insensitive to *N*-ethylmaleimide.

Effect of mersalyl

At variance from the compounds analyzed in the preceding section, addition of mersalyl resulted in a marked inhibition of active shrinkage. However the inhibition of active shrinkage ran parallel to the inhibition of succinate oxidation due to the inhibitory effect of mersalyl on the dicarboxylate carrier. Fig. 8 shows the effect of mersalyl on the rate of active shrinkage as studied in the presence of ascorbate + *N,N,N',N'*, tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) as respiratory substrate whose oxidation is mersalyl insensitive. It is seen that the rate of active shrinkage was first markedly enhanced by the addition of low mersalyl concentrations, up to about 10 nmol · mg protein^{-1}. Higher mersalyl concentrations brought about 80 % inhibition of the shrinkage rate. In Fig. 8 it is also shown that at the mersalyl concentrations affecting shrinkage there was no inhibition of the respiratory rate. That the inhibitory effect of mersalyl is at the level of the anion extrusion mechanism was ascertained by testing whether the same concentrations of mersalyl were unable to decrease either the rate or the extent of the safranine response [22] driven by ascorbate+TMPD. Thus energy coupling is not affected in this range of mersalyl concentrations. Fig. 9 shows the effect of mersalyl on P_i influx and efflux. The influx of P_i was about 90 % inhibited around 10–15 nmol · mg protein^{-1}. In contrast with *N*-ethylmaleimide, mersalyl inhibited also the efflux of P_i, although at slightly higher concentrations. About 90 % inhibition of P_i efflux occurred around

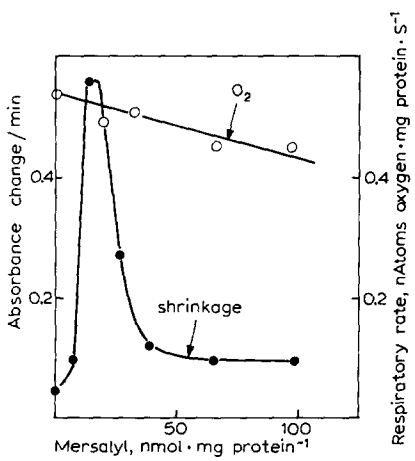

Fig. 8. Effect of mersalyl on active shrinkage and oxygen consumption rates. The swelling was carried out in 30 mM K_2HPO_4, 1 mM EDTA pH 7.5, 1 μM rotenone and was initiated by 2 μg nigericin. At the end of the swelling mersalyl was added. The shrinkage was initiated by 1 mM ascorbate+100 μM TMPD. In a parallel experiment and under identical conditions the oxygen consumption was measured with a Clark oxygen electrode. Final volume, 2 ml, 1.8 mg protein.

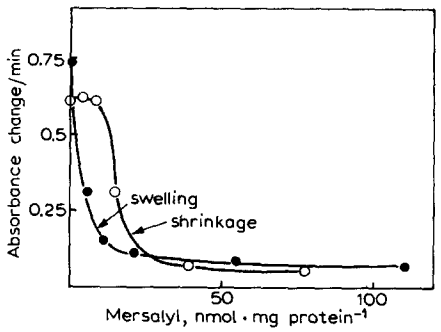

Fig. 9. Effect of mersalyl on passive swelling and shrinkage rates. Passive swelling was carried out in 30 mM K_2HPO_4, 1 mM EDTA, 1 μM rotenone and was initiated by 2 μg nigericin after titration with mersalyl. Passive shrinkage was carried out in 30 mM $(NH_4)HPO_4$, 1 mM EDTA, pH 7.5 and was initiated by addition of 20 mM sucrose after titration with mersalyl. Final volume, 2 ml, 2 mg protein.

20 nmol · mg protein^{-1}. The data of Fig. 9 correlate well with the titration of Fig. 8 since the enhancing effect occurs in the region where there is inhibition of P_i influx but not of P_i efflux while the inhibitory effect on active shrinkage occurs parallel to the inhibition of the passive P_i efflux. The other SH inhibitors tested such as Nbs_2, *p*-Cl-$HgBzSO_3^-$ behaved like *N*-ethylmaleimide rather than like mersalyl.

DISCUSSION

Passive and active ion movements

Since the mitochondrial membrane is highly permeable to water, movement of ions across the membrane leads to osmotic swelling and shrinkage. The ion movements may be denoted as "passive" when driven by ion concentration gradients and "active" when driven by metabolism [23]. In the past, the cycle involving passive swelling and active shrinkage has been denoted as large amplitude swelling and the cycle involving active swelling and passive shrinkage as low amplitude swelling [3]. The passive swelling has also been denoted as low energy, or irreversible, whereas the active swelling as high energy or reversible [24]. The present investigation involves a passive swelling driven by ion concentration gradients and an active shrinkage driven by respiration.

Asymmetry of inhibitor effects on passive influx and efflux of P_i

Guerin et al. [14] found that the efflux of P_i was inhibited completely by mersalyl and to a lesser extent by other SH inhibitors. Klingenberg et al. [17] found that the efflux of P_i during FCCP-induced ATP hydrolysis was inhibited by all SH inhibitors. Lo Frumento et al. [25] found that 200 μM *N*-ethylmaleimide completely inhibited the P_i efflux induced by nigericin. The effect of SH inhibitors has here been measured on passive influx, passive efflux and active efflux. An asymmetric effect of the inhibitors has been found in two instances: (a) several SH inhibitors inhibit the passive influx but not the passive efflux of P_i; (b) mersalyl inhibits both the passive P_i influx and the passive P_i efflux; the latter occurs at slightly higher mersalyl concentra-

tions. The appearance of SH inhibitor-insensitive P_i efflux, under conditions where the influx is SH inhibitor-sensitive, may be taken as an indication that, after extensive swelling, P_i has gained accessibility for more deeply embedded sites at the matrix side of the membrane. These sites would not be reached by SH reagents acting at the surface, but would be reached by mersalyl where Hg is situated at the end of a five carbon atom side chain. What remains to be established is whether the SH inhibitor-insensitive P_i efflux goes through the P_i carrier.

Mechanism of active P_i extrusion

Active P_i extrusion is markedly enhanced by SH inhibitors and by nigericin. The effect of the SH inhibitors parallels the inhibition of the influx and is due to an inhibition of the P_i carrier. It may be conceivable that the active P_i extrusion leads to formation of a ΔpH. This in turn drives either the extrusion of K^+, in exchange with H^+, or a passive reequilibration of P_i through the P_i carrier. Inhibition of the P_i carrier abolishes the utilization of the ΔpH through P_i influx. Similarly, increasing concentrations of nigericin accelerate H^+/K^+ exchange and thereby favour the utilization of the ΔpH for extruding K^+ rather than for taking up P_i. The inhibitory effect of valinomycin parallels a stimulation of active K^+ uptake. The K^+ actively taken up is released via nigericin causing a K^+ cycling across the membrane and an energy dissipation. The sharp dependence of the active P_i extrusion on the electrolyte concentrations may be attributed to various reasons: variable degree of membrane stretching and thus different site accessibility, variable extent of energy coupling due to stretching, dependence of the extrusion on the dimension of the anion gradient.

Three alternatives may be considered for the active P_i extrusion: (a) a mechanoprotein; (b) an electrogenic proton pump and (c) a proton-driven P_i pump. Operation of a mechanoprotein [1–3] is the simplest mechanism. The increase of hydrostatic pressures leads to extrusion of water, increase of matrix osmotic pressure and efflux of ions down the concentration gradients. The electrolyte efflux would be analogous to the osmotic shrinkage due to impermeant solutes. However a passive solute efflux due to a mechanoenzyme should not be influenced by inhibition of solute influx. The mechanoprotein model is therefore in contrast with the fact that agents inhibiting P_i influx enhance markedly the net P_i extrusion.

An electrogenic proton pump has been proposed by Brierley et al. [7–9] to explain the extrusion from the matrix of Cl^- and P_i, in the presence of mercurials, and by Nichols [10] for the extrusion of Cl^- in brown-adipose-tissue mitochondria: anions diffuse electrophoretically down a $\Delta\psi$; cations are extruded down a ΔpH either via a natural H^+/Cat^+ antiporter, or via nigericin or as un-ionized NH_3. The anion flux is microscopically electrical but it becomes macroscopically electroneutral since electrically coupled to the H^+ flux. The cation flux is also electroneutral microscopically because of the nature of nigericin and NH_3 transport.

The proton-driven P_i pump, depicted schematically in ref. 11, assumes that the primary proton pump is coupled directly with the movement of P_i. The difference between the electrogenic proton pump and the proton-driven P_i pump resides in the molecular relationship between H^+ and anion fluxes. In the former, it is assumed that the H^+ efflux is coupled to the P_i efflux through the membrane potential. In the latter it is assumed that the H^+ efflux drives the P_i efflux through short range interactions and thus the energy-linked transport of P_i is electroneutral also at the micro-

scopic level. The subsequent movements of K^+ via nigericin or of NH_3 are identical in the two models. The data reported in the present paper do not permit a distinction between the two models.

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