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Ion Transport Underlying Metabolically Controlled Volume Changes of Isolated Mitochondria*

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Abstract. An earlier study has indicated that the swelling of isolated mitochondria induced by inorganic phosphate (P_i) can be accounted for largely or completely by the accumulation of ions. In the present study, a similar uptake was shown to be induced by a wider range of P_i concentrations. Addition of ADP, KCN, or 2,4-dinitrophenol initiates a shrinkage which can be accounted for by the efflux of ions. The results are consistent with the explanations that (*a*) P_i induces a transport of ions and a concomitant osmotic swelling, and (*b*) the addition of substances which compete or interfere with the energy available for transport results in ion efflux and a corresponding mitochondrial shrinkage. The results are not consistent with proposals that the changes in the light scattered by mitochondrial suspensions with alterations in metabolic states reflect a mechanochemical coupling phenomenon.

Isolated mitochondria have been reported to undergo morphological changes in response to changes in metabolic states (e.g.,[1–5]). These reports, based on electron microscopic observations, differ in detail and in interpretation. Nevertheless, they basically propose that a definite structural organization or configuration corresponds to a particular metabolic state. In most experiments, these morphological changes coincide with changes in the scatter of incident light. Generally, the presence of substrates and P_i has been found to induce a decrease in scatter, whereas ADP, uncouplers, or inhibitors of respiration generally induce an increase in scatter (e.g.,[6–11]). Accordingly, the effect of P_i has been interpreted by some workers to correspond to a swelling, whereas the effect of ADP and substances that interfere with metabolism corresponds to a shrinkage (e.g.,[6–8,11]). This interpretation is supported by the osmotic reversibility of the P_i-induced swelling.[12] Conceivably, the structural changes could be a reflection of a mechanochemical coupling event fundamental to oxidative phosphorylation as proposed by others (e.g.,[2–5]). However, the finding that these changes do not always accompany the appropriate metabolic states strongly argues against this interpretation.[1,13–16] The induction of the appropriate configurational changes by osmotic means[17–20] argues for an osmotic basis for these events. Since in some studies[9,20–23] swelling and the appropriate morphological changes seem to be accompanied by the uptake of ions, the basis for the changes in structural states may well correspond to underlying shifts in intramitochondrial ions followed by appropriate osmotic volume changes.

R. A. Harris *et al.*[10] interpret the changes in scatter that accompany changes in metabolic states to be the result of alterations in the contours of the mitochondrial membrane surface, which is capable of reflecting the incident light. This conclusion is based on the apparent absence of mitochondrial volume changes determined by total mitochondrial pellet volume (in a system regenerating oxygen to maintain the appropriate metabolic state) and by measurements of mitochondrial diameters from electron micrographs.[10] The fact that the optical density of mitochondrial suspensions is unaffected by the drastic transition of the mitochondrial shape from rod to sphere[24] is not consonant with this hypothesis. The dimensions of the mitochondrial outermost contours as seen with electron microscopy need not change when the mitochondrial volume changes, since it is the volume of the mitochondrial lumen that is involved in osmotic volume changes. The thickening or thinning of the lumen lined by the cristae can occur readily at the expense of the spacing between the cristae and external to the inner mitochondrial membrane. The fact that the osomotically induced changes mimic the configurations obtained by metabolic states supports this view. The method used by Harris *et al.*[10] to determine total pellet volume is unable to detect rather large changes in mitochondrial volume (see Fig. 7, ref. 26) that correspond in our hands[25] to changes in absorbance of as much as 10%. The method is unable to detect significant changes in volume in the range of osmolarity between 250 and 150 mM, i.e., approximately a 1.7-fold increase in osmotically active volume. A recent experiment, which made use of a special method of negative staining for the electron microscope, supports the view that the structural changes correspond to volume changes.[27]

Evidence has also been presented to show that the apparent swelling produced in the presence of substrate and P_i can be accounted for by ion uptake.[28]

In the present study, alterations of metabolic states in mitochondria produced small changes in the absorbance of the suspensions similar in magnitude to those reported by others (e.g., ref. 10). The results indicate that the apparent swelling after the introduction of substrate and P_i or the apparent shrinkages brought about by ADP, uncouplers, or metabolic inhibitors correspond quantitatively to osmotic volume changes caused by shifts in internal ions.

Methods. Mitochondria were isolated from the livers of male Holtzman rats weighing 210–350 g. Typically, mitochondria were isolated from the homogenates of four livers in 0.32 M sucrose–1 mM disodium EDTA, pH 7.4 (or K_2EDTA for experiments carried out in K^+ media). In a few cases in which mitochondria were washed and resedimented either in the isolation medium or in 0.3 molal sucrose, the experimental results were similar. Mitochondria were resuspended and stored in 0.3 molal sucrose–0.01 M Tris, pH 7.4 at 0°C. Details of the isolation procedure have been reported.[25]

Absorbance changes were followed on a Coleman Junior II spectrophotometer (model 6/20) at 700 nm. The photometric method and the calculations of osmotically active volumes have been described previously.[25,29] The incubation medium was 0.3 molal sucrose–10 mM sodium succinate–1.6 mM disodium EDTA–10 mM Tris (0.1 mM Tris for H^+ determinations), pH 7.4, or the equivalent medium in which K^+ was substituted for Na^+. Generally, swelling was initiated by 1.29 mM Na_2HPO_4 or 5 mM K_2HPO_4 or at higher concentrations as indicated in the figures. The swelling was reversed by the addition of 1.0 mM Tris–ADP, 100 μM 2,4-dinitrophenol (DNP), or 0.026 mM KCN. Other additions are noted in the text. In each experimental determination 0.3 ml of mitochon-

drial stock suspension was added to 9.0 ml of incubation medium. The suspensions were maintained at 20 ± 1°C (see ref. 28).

Polarographic measurements were made with a Clark oxygen electrode and automatically recorded.[30]

Ion uptake was estimated after filtration through 0.65 μm pore size filters, 47 mm in diameter (Millipore Corp., Bedford, Mass.). The procedure for Na^+ made use of ^{22}Na.[28]

For K^+, the filters containing mitochondria, and control filters without mitochondria, were extracted by gentle shaking in 5 ml of 10% Tris–deoxycholate for an average of 12 hr. 1 ml aliquots were diluted in 9 ml of distilled water and their K^+ content was determined by flame photometry (Beckman 9200 Flame Photometry Attachment, Beckman Atomizer-Burner Assembly, and Beckman DU-2 Spectrophotometer, model 109200) at 772 nm. The method of extraction was validated by comparing whole filter counts of ^{22}Na samples with those of 1 ml aliquots from deoxycholate-washed filters of ^{22}Na samples. The extract contained 96 ± 6% of the ^{22}Na (5 determinations).

H^+ concentration was determined by using a Beckman combination pH electrode (39030B7) and a Beckman Expandomatic pH meter.

Protein was determined by the biuret method as modified for mitochondria.[32] The experimental samples contained an average of 0.7 mg/ml protein.

Results. P_i-induced swelling under various conditions: Orthophosphate (P_i) induces swelling in mitochondria. The rate and extent of the swelling is a function of P_i concentration. In a previous study,[28] a Na^+ medium and one P_i concentration (0.22 mM) were selected for detailed study. In this case, the intramitochondrial accumulation of ions accounted for at least 80% of the swelling. Expressed in terms of cation uptake, 0.6 ± 0.03 Na^+ ion was taken up per osmoequivalent gained. The osmoequivalents were estimated from photometric measurements of mitochondrial volumes. One osmoequivalent has been defined as the osmotic volume change ideally corresponding to the net uptake or loss of one mole of a nonelectrolyte or of an ionic species.

In the present study, a wider range of P_i concentration was used to examine swelling in Na^+ or K^+ media. Under the conditions of these experiments no significant H^+ transfer accompanied ion uptake, and therefore it was necessary to follow only cation movements (ΔH^+ was below 0.02 H^+/osmoequivalent). Cations must be accompanied by anions to maintain electric neutrality in the internal mitochondrial compartment.

The results in Table 1 and Fig. 1 show the mean number of cations accumulated per osmoequivalent gained. The results are the same regardless of P_i concentration (1.29–15 mM) or the cation used. The means of several experiments are

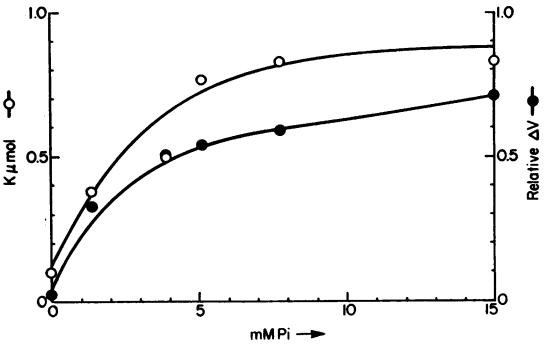

FIG. 1. K^+ uptake and swelling induced by a range of P_i concentrations. The suspension contained 0.48 mg protein per ml. Each point represents an average of 4 determinations. ΔV = 1 change in mitochondrially achieve volume in 0.318 osmolal solution), corresponds to 6.6 μl/experimental determination.

TABLE 1. *Relationship of cation and osmoequivalents in Na^+ and K^+ media; and apparent independence of swelling and cation translocation of respiratory control.*

Expt.	Cation	Mg^{2+}	P_i mM	Micro-osmo-equiva-lents	Cation uptake (μmol)	Cation ÷ osmeq	RCR	ΔV
1	Na	0	1.29	3.20	1.32	0.41		
2	Na	0	1.29	3.80	1.70	0.45		
3	Na	0	1.29	2.95	1.42	0.48	1.8	0.84
4	Na	0	1.29	1.92	1.06	0.55		
5	Na	0	1.29	2.38	1.36	0.57		
6	Na	0	1.29	2.63	1.19	0.45		
7	Na	0	1.29	1.81	1.05	0.58	2.2	0.78
8	Na	0	1.29	3.30	1.10	0.33		
9	Na	0	1.29	3.48	1.39	0.40		
10	Na	0	1.29	3.77	1.15	0.31		
						Mean 0.45 ± 0.09		
11	K	0	5.00	4.87	2.84	0.58		
12	K	0	5.00	3.22	0.92	0.29		
13	K	(No EDTA)						
		3.3 mM	5.00	1.38	0.76	0.55	2.5	0.33
	K	0	5.00	4.11	1.60	0.39	1.8	0.98
14	K	3.3 mM	5.00	2.25	1.31	0.58		
15	K	0	5.00	2.17	1.11	0.51	4.3	0.74
16	K	0	5.00	1.98	1.15	0.58	3.7	0.82
17	K	0	5.00	...	...	...	4.3	1.07
	K	3.3 mM	5.00	...	...	...	5.3	0.65
	K	5.0 mM	5.00	...	...	...	6.2	0.59
						Mean 0.50 ± 0.11		

Before P_i addition the cation concentrations in the media were 24.6 mM Na^+ and 27.1 mM K^+. The medium for Expt. 11 contained 21.5 mM K^+ before P_i addition. In the controls, which had no P_i addition, the osmoequivalents are less than 7% (Na^+ medium) and 10% (K^+ medium) of those in the experimentals. Values for osmoequivalent and cation uptakes are from an average of 4–5 separate determinations per experiment; the mean of all values is given ± SE.

In order of listing, ΔV = 1 (change in mitochondrially active volume in 0.318 osmolal solution) corresponds to 10.0, 12.7, 10.1, 9.3, 9.2, 8.7, 9.1, 10.8, 11.4, 12.4, 12.9, 10.1, 11.8, 10.2, 8.3, 9.4 μl/experimental determination for Expts. 1–16. RCR, respiratory control ratio.

0.45 ± 0.09 (10 experiments) and 0.50 ± 0.11 (7 experiments) for Na^+ and K^+ respectively. The small difference between these figures and those reported previously[28] may be the result of the higher P_i concentrations used and may not reflect a real difference in mechanism. In a Na^+ medium and at high P_i concentrations, we have found a significant uptake of the buffer, Tris. Since either K^+ or Na^+ are transferred against an electrochemical gradient, the uptake corresponds to active transport. The internal concentration of cations, as calculated from our results, corresponds to a concentration 2.5 ± 0.4 (Na^+) and 2.0 ± 0.5 (K^+) times as high as that of the suspending medium. The steady-state distribution of anions[33,34] and direct electrical measurements with micro-electrodes[34–36] indicate that the internal compartment of the mitochondria is electropositive in relation to the outside.

P_i-induced swelling of liver mitochondria has been studied previously in a Mg^{2+}-supplemented K^+ medium.[7] Other reports of mitochondrial swelling in a K^+ medium show inhibition of swelling in the presence of Mg^{2+}.[8] In the present study, additions of Mg^{2+} up to 5.0 mM decrease the magnitude of P_i-induced swelling and the concomitant K^+ uptake. Mg^{2+} concentration and

swelling appear to be inversely related (Table 1). However, a significant swelling does occur in a Mg^{2+}-supplemented medium and the values of K^+ uptake per osmoequivalent gained (Table 1, Expts. 13 & 17) are completely consistent with other values shown for a K^+ medium. In a Na^+ medium supplemented by similar concentrations of Mg^{2+}, 1.29–5 mM P_i does not induce swelling. However, as indicated by previous work,[12] higher P_i concentrations (10 mM) support swelling in a Mg^{2+}-supplemented Na^+ medium. The Mg^{2+} effect on swelling occurs either in the presence or the absence of 3.3 mM EDTA in both Na^+ and K^+ media.

It is apparent that mitochondrial swelling depends on metabolism since it is blocked by metabolic inhibitors and in some cases uncouplers.[6,8] The rate of respiration and respiratory control ratios (RCR) were estimated in determinations parallel to photometric measurements for many of the present experiments (see Fig. 2 and Table 1). Although different mitochondrial preparations showed RCR ranging from 1.8 to 6.2, the K^+ uptakes per osmoequivalent gained were not significantly different from one another or from the values shown in Table 1. Comparison of different experiments shows no simple correlation between RCR and volume changes. However, there are indications that within each individual preparation, swelling and RCR vary inversely with the Mg^{2+} concentration

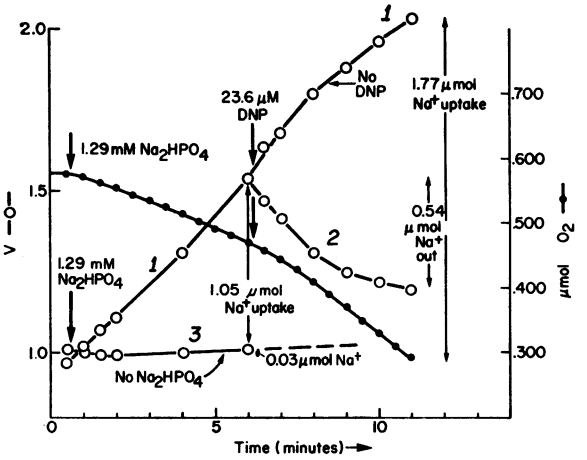

FIG. 2. Respiration, volume changes, and Na^+ uptake or efflux. The incubation medium was the same as in Fig. 1 except that Na^+ replaced K^+. The Na^+ concentration in the medium was 25 mM before P_i addition. Additions are indicated by the arrows. *Curve 1:* Swelling induced by 1.29 mM Na_2HPO_4 added after 30 sec of incubation. *Curve 2:* P_i-induced swelling reversed by the addition of 23.6 μM 2,4-dinitrophenol at 6 min. *Curve 3:* Control—no Na_2HPO_3 added. At times indicated by the last point of the curves (except for curve 1, where some of the samples were filtered at 6 min) the suspensions were filtered and ^{22}Na was estimated. The uptake or release of Na^+ (in micromoles) is indicated. The curve obtained from polarographic measurements (—●—) is typical of several for this experiment and shows successive increases in respiratory rate (decreases in oxygen tension in the medium) when P_i and the uncoupler DNP were added. Protein in the suspensions was 0.62 mg/ml. Points in curves 1–3 and the amounts of uptake or efflux of Na^+ are averages of 4 determinations. $\Delta V = 1$ corresponds to 9.1 μl/experimental determination.

(Expts. 13 & 17, Table 1). In agreement with other studies,[8] we have found that the addition of P_i invariably increases respiration (e.g., Fig. 2). This would be expected from the energy demands of the active transport triggered by P_i. Preliminary evidence indicates that, under some conditions, coupling to oxidative phosphorylation may be increased at the expense of the transport system. As a consequence, RCR values increase with increased Mg^{2+} while ion transport and swelling decrease, as will be reported elsewhere.

The addition of ADP, metabolic inhibitors, or uncouplers would be expected to interfere with the active transport of ions either by competing for the energy available or by blocking the necessary reaction of the cytochrome chain. In accordance with several earlier studies[1,6,8] we find that a reversal of swelling (expressed as an increase in absorbance or scatter) occurs when ADP, KCN, or DNP is added. A typical experiment in which mitochondrial volume and respiration have been followed in parallel determinations is shown in Fig. 2. The osmotically active volume in relative units (the mitochondrial osmotically active volume in a 0.318 osmolal solution was taken as unity) is shown at the left ordinate and the oxygen utilization in micromoles is shown at the right ordinate. 1.29 mM Na_2HPO_4 was added to the suspending medium 30 seconds after the mitochondrial incubation had begun. At 6 min DNP was added to a concentration of 23.6 μM; when this happens the respiratory rate is doubled and the mitochondria shrink with a half time of about 1 min (*curve 2*). The shrinkage is accompained by the exit of 0.54 μmol of Na^+. Samples which received no DNP at 6 min continued to swell and to accumulate Na^+ (*curve 1*). *Curve 3* represents a control to which no P_i was added. The net influx or efflux of Na^+ is indicated at the appropriate time of filtration. Shrinkages initiated by KCN and ADP show similar kinetics although the extent of reversal varies in different experiments.

The correlation between reversal of swelling and cation efflux initiated by a number of agents is shown in Fig. 3. The extent of reversal is expressed as % reversal (V_R, the volume after reversal/V_S, the swollen volume, $\times 100$). Cation efflux is expressed as the molar ratio of cations lost during shrinkage to cations accumulated during swelling. Values for ions taken up or released can be readily calculated from the estimates of amounts of internal cations after rapid

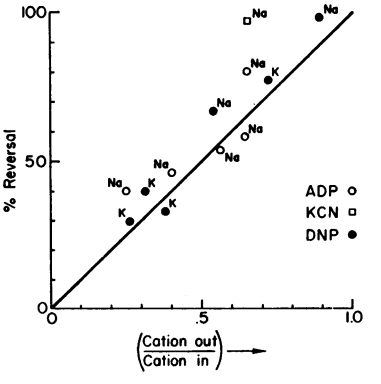

FIG. 3. Correspondence of cation efflux to the reversal of P_i-induced swelling. The line represents the expectations assuming the shrinkage to be entirely osmotic. The incubation media are the same as those reported in Figs. 1 and 2. The % reversal induced by the agents indicated was calculated from volume changes. Each point is an average of 4 determinations. The points represent 12 independent experiments.

filtration, which is complete in less than 5 sec (see *Methods*). The results of 12 independent experiments are shown in Fig. 3. Ideal correspondence of the % reversal to the cationic efflux should conform to the line drawn in the figure. The close fit of most of the experimental points to the line indicates that the major portion of the reversal is accounted for by the efflux of ions. The ratio, cation efflux/influx divided by V_R/V_S, is 0.88 ± 0.16 rather than 1.

Discussion. The results presented are consistent with the idea that the changes in the light scattered by mitochondrial suspensions during changes in metabolic state correspond to osmotic volume changes. Swelling is the result of a P_i-induced entry of ions; shrinkage results from interference with or competition for the energy supply for ion transport and the subsequent efflux of ions. At high P_i concentration the swellings are similar in their time constants to those measured by Harris *et al.*,[10] Packer,[6] and Azzi and Azzone.[8] For example, at a concentration of 9.8 mM P_i[28] and in a Na^+ medium, the half time of swelling is considerably less than 1 min. The half time of shrinkage induced by a number of agents is also rapid, 1 min or less. The swelling reported by Hackenbrock is considerably slower than that observed by other authors.[1] This corresponds to our experience with liver mitochondria in Mg^{2+}-supplemented medium.[12] As previously noted[28] the amplitude of the photometric changes corresponding to the swelling are of the same magnitude as those reported in refs. 6,8, and 10.

The physiological conditions of the preparations used by different investigators are of some interest. Although the degree of coupling was not reported for all the experiments of Green *et al.*, some of the published results permit calculation of a respiratory control ratio of 3–4 (Fig. 10 of ref. 5). Hackenbrock reports RCR values ranging from 2.8 (Fig. 7 of ref. 1) to 6.8 (Fig. 11 of ref. 1). In our preparations we have obtained RCR values of 1.8–6.2, depending on the composition of the incubation medium or the preparation. However, among the different preparations, there is no simple correlation between swelling or transport and the degree of coupling (see Table 1).

We have noted (see *Introduction*) that work carried out with the electron microscope does not support the contention that configurational changes are a reflection of mechanochemical coupling. In particular, (*a*) the appropriate metabolic states are frequently not accompanined by the appropriate configurations, (*b*) evidence has accumulated that the configurational changes correspond to volume changes, and (*c*) configurational changes can be produced by osmotic means.

Some of the work carried out with liver mitochondria by Hackenbrock does not show consistently conspicuous changes in scatter when shifted from state 4 to state 3.[1] However, the variation in internal lumen volume leaves little doubt that there is a change of the space enclosed by the mitochondrial semipermeable membrane. The electron micrographs of muscle mitochondria are different in appearance from those of liver mitochondria.[1–5] However, the interpretation need not be any different. The thickening of the cristae reported to accompany state 4 (e.g.ref. 4) may well correspond to an increase in the volume of the lumen.

Conversely, the thinning of the cristae observed in state 3 or induced by uncouplers may well reflect a shrinkage of the internal space.

The proposal that the configurational changes are a reflection of osmotic volume changes accompanying transport offers a reasonable alternative explanation for the results obtained (*a*) with the electron microscope or (*b*) by the estimation of mitochondrial volume from measurements of the light scattered. The present data support this view and, as noted, there is considerable experimental support from other published works.

Abbreviations: DNP, 2,4-dinitrophenol; RCR, respiratory control ratio.

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