# THE MECHANISM OF LOW-AMPLITUDE ORTHOPHOSPHATE-INDUCED SWELLING IN ISOLATED RAT LIVER MITOCHONDRIA

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## INTRODUCTION

The swelling of isolated mitochondria in response to the introduction of orthophosphate ($P_i$) to the suspending medium has been studied in a number of works (1–15). In particular, Packer (11) found the $P_i$-induced swelling to be dependent on the presence of state 4 and to be reversed upon the induction of state 3.

In most cases, swelling was monitored in mitochondrial suspensions by following the transmission or scatter of the incident light. Swelling leads to a decrease in the amount of light scattered or an increase in the light transmitted.

Previous work indicates that, at least under certain conditions, $P_i$-induced swellings are quantitatively osmotically reversible (14). The question arises as to whether the volume change induced by $P_i$ could be accounted for by the uptake of ions and the consequent osmotic swelling. It is possible to test this premise experimentally. The mitochondrial osmotically active volume can be estimated quantitatively by a photometric technique previously described (16). It is possible to estimate the uptake of ions in mitochondria after filtration with Millipore filters (Millipore Corp., Bedford, Mass.). For example, using this approach, it has been possible to show that low-amplitude swelling induced by $Ca^{2+}$ in the presence of acetate can be accounted for by the uptake of $Ca^{2+}$ and accompanying anions (17).

## MATERIALS AND METHODS

Male rats of the Holtzman strain, weighing from 170 to 350 g, were sacrificed by a blow on the head and cervical spinal fracture. One to four rats were used for each experiment. The details of the isolation have been previously described (e.g. 16). The mitochondria were isolated in 0.32 M sucrose-0.001 M disodium EDTA solution at pH 7.4. The mitochondrial pellets were resuspended in approximately 12 ml of 0.30 molal sucrose, and 0.01 M Tris, pH 7.4, with the homogenizer operated at a very low speed. The stock suspension was kept on ice and represented about 40 mg protein/ml.

A Coleman Junior Spectrophotometer Model 6D (Coleman Instruments, Maywood, Ill.) was used to record optical density changes. The experimental suspensions were maintained at 21° ± 1°C by circulating water at the appropriate temperature through a special metal adapter. The measurement was carried out with light of 600 or 700 mμ wavelength (see 16). Typically, 0.1 ml mitochondrial suspension was added to 3 ml incubation medium, or 0.25 ml mitochondrial suspension was added to 9 ml incubation medium. The incubation medium contained: 10 mM succinate, 1.6 mM disodium-EDTA, 0.30 molal sucrose, and 0.01 M Tris. The solution was brought to a pH of 7.4 by adding a precise

amount of NaOH from a stock solution. Where $H^+$ ejection from mitochondria was determined, 0.1 mM Tris replaced the usual concentration of buffer. A Vortex-Genie mechanical stirrer (Fisher Scientific Company, Pittsburgh, Pa.) was used to mix the suspension after the addition of mitochondria or of the necessary solutions. 30 sec after the start of an experiment, $Na_2HPO_4$ at pH 7.4 was added. In a typical experiment, the changes in optical density were followed for 6 min. In some experiments, it was monitored for longer periods.

The photometric method of Tedeschi and Harris (16) was used to estimate mitochondrial volume. The optical density measurement of the experimental suspensions can be readily converted to relative osmotically active volume since optical density is inversely proportional to volume. The calibrations necessary to determine the appropriate constants for this conversion require photometric measurements with solutions of varied osmotic pressure but the same refractive index. Relative osmotically active volume can be converted to absolute osmotically active volumes by the determination of mitochondrial water volume under standardized conditions and after correction for extramitochondrial water (18).

The calculated osmolality of the incubation medium was verified by measurement of freezing point depression (using an Osmette, Precision Osmometer, Precision Systems, Framingham, Mass.).

The radioactive compounds were purchased from New England Nuclear Corp. (Boston, Mass.), or Tracer Labs (Waltham, Mass.). To estimate the uptake of the labeled solutes, the suspension was filtered through a Millipore filter, 47 mm in diameter and 0.45 $\mu$ in pore size. For the experiments with $C^{14}$ or $P^{32}$, the filter from each determination was then placed in a flask with 10 cc methyl Cellusolve (ethylene glycol monoethylether) and extracted with continuous shaking. The radioactivity of the filtrate and the extracts was determined after delivering 50 $\mu$l to a vial containing 10 ml of a prepared fluor solution. The fluor solution consisted of 1000 cc toluene, 1000 cc ethylene glycol monoethylether and 83 cc Liquifluor (New England Nuclear Corp.).

The $P^{32}$ and $C^{14}$ samples were counted for 10 and 40 min, respectively, in a Unilux Scintillation Counter (Nuclear-Chicago Corp., Des Plaines, Ill.) until approximately 5,000–10,000 counts were recorded for $P^{32}$ and about 10,000 for $C^{14}$. For $P^{32}$, the effect of quenching was corrected by comparing the counts with those obtained from unlabeled experimental duplicates to which known amounts of $P^{32}$ had been added. The $C^{14}$ counts were corrected for quenching by the method of ratios (19) and using quenched standards provided by Nuclear-Chicago Corp.

The $Na^{22}$ samples were counted directly after the whole filter or an aliquot of the supernatant was placed in the counting vial (Plastic Gamma Counting Tubes, Nuclear-Chicago Corp.). A Nuclear-Chicago Well Scintillation Counter Model No. 8725 was used.

The uptake of the samples was calculated from the radioactivity of the filters by subtracting the radioactivity of a control filter through which the appropriate labeled incubation medium had been passed. This method was found to be reliable by Tupper and Tedeschi (17). A similar test of the technique can be carried out by comparing the $P^{32}$ uptake estimated directly from the disappearance of the label from the medium to that obtained with the method described. The results are shown in Table I, and they demonstrate that the technique is reliable.

$K^+$ was determined by flame photometry from the filtrate of experimental suspensions with a Beckman 9200 flame photometry attachment (with atomizer-burner assembly) and a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). $K^+$ was present from endogenous sources.

The $H^+$ ions were determined by using a Beckman combination pH electrode and Beckman Expandomatic pH meter. The $H^+$ equivalents corresponding to the pH were determined by a titration of the medium. In these experiments, the Tris buffer was reduced in concentration from $1.0 \times 10^{-2}$ M to $1.0 \times 10^{-4}$ M. Reducing the concentration of the tris did not affect the magnitude of the swelling.

Protein was determined by a modified Biuret method (20).

## RESULTS AND DISCUSSION

Mitochondria swell upon the addition of various concentrations of orthophosphate as shown in

TABLE I

*Moles Phosphate Taken Up* $\times 10^{-7}$

| Exp. | Filter method (F) | Supernatant method (S) | F/S Ratio |
|---|---|---|---|
| 1 | 0.023 | 0.025 | 0.92 |
| 2 | 0.018 | 0.020 | 0.90 |
| 3 | 0.017 | 0.016 | 1.06 |
| 4 | 0.020 | 0.019 | 1.03 |
| | | Mean: 0.98 ± 0.08 | |

$P_i$ taken up. Comparison of calculations from the amount retained in the filter and that remaining in the supernatant. Each experiment represents 8–10 determinations. 0.1 ml stock mitochondrial suspension was added to 3 ml incubation medium. $P_i$ was added to each determination after 30 sec of incubation. Each determination was filtered after 6 min. The deviations are standard deviations.

Fig. 1. The arrow in the figure indicates the time at which $P_i$ was added. As shown by others, the swelling can be rapid and essentially completed in 1–2 min after the addition of relatively high $P_i$ concentrations (e.g. 9.7 mM). In the figure, the swelling is expressed as relative osmotically active volume. The volume of mitochondria in 0.335 osmolar sucrose has been considered arbitrarily as unity. At lower concentrations, the swelling is slower and not so pronounced. Nevertheless, it is essentially completed within 5 min for the cases tested. The swelling taking place during a 5 min incubation is shown in Fig. 2. As shown in the legend of the figure, $P_i$ produces a change in optical density ranging from 7.4 to 15.9% which reflects changes in OD of 0.05 to 0.12. This is comparable to the changes found by others under comparable conditions and considered to be low amplitude (phase I) swelling (e.g. 11, 13, 15). For example, Harris et al. (15) and Packer (11) find a decrease in scatter of approximately 15% with 10 mM $P_i$ (see Fig. 2, reference 15; Fig. 1–2, reference 11). Azzi and Azzone report optical density changes of approximately 0.035 to 0.08 (13) using 2 mM $P_i$. As reported by Packer, phase II swelling is prevented by isolation in a medium containing a low concentration of EDTA (reference 11, p. 218).

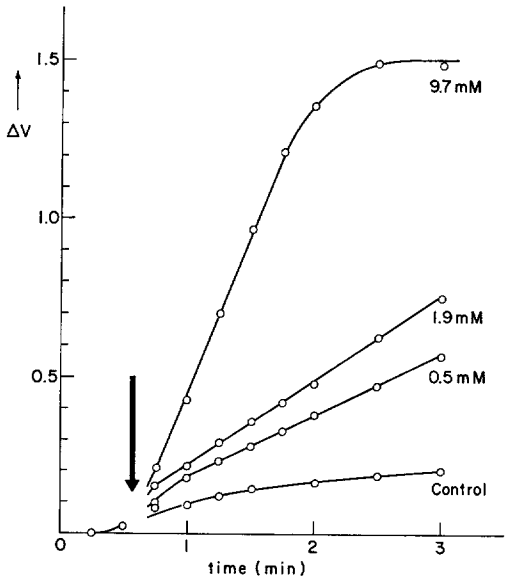


FIGURE 1 Phosphate-induced swelling as a function of time. The degree of swelling corresponds to an increase in relative osmotically active volume (see text).

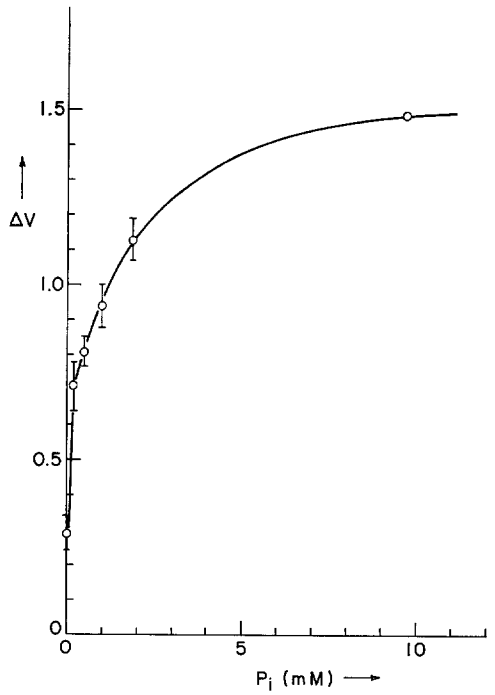


FIGURE 2 Swelling as a function of the phosphate concentration of the medium after a 5 min incubation. The swelling is given in relative osmotically active units (see text). The % optical density changes are as follows: 19.0, 15.6, 13.2, 11.7, 10.5 for the experimental determinations, and 3.1 for the control. ($\Delta OD$ = 0.15, 0.12, 0.10, 0.09, 0.08, and 0.03, respectively)

In order to test whether the effect could be accounted for by the uptake of ions, it is necessary to determine the uptake of osmotically active molecules (osmoequivalents) which would theoretically have produced the measured osmotic volume change (18). This can be readily done from the photometric measurements (see 16, 18). The ions that have penetrated can also be readily estimated by counting the radioactivity or analyzing the filter or the supernatant. A ratio of the ions which are taken up (measured) divided by the osmoequivalents (calculated from the volume changes) should ideally be 1.

The experiments were carried out at a $P_i$ concentration of 0.22 mM. This concentration was chosen since the swelling is moderate and the changes are sufficiently slow to permit relatively long filtration times (about 30–40 sec). Typically, the phosphate was introduced as a small volume (about 10 $\mu$l) of a concentrated stock solution.

An equivalent volume of distilled water was added in the controls.

Table II represents the results where the amounts of $P_i$, $Na^+$, and succinate in the mitochondria were estimated. Estimates of $K^+$ indicate that the uptake of this ion is negligible. The results are summarized in Table III.

Under the present conditions, the ions which have been estimated account for approximately 80% of the swelling. It is interesting to note that the anionic charges taken up do not correspond to the $Na^+$ taken up. This is similar to results obtained with $Ca^{2+}$-induced swelling (17). The requirement of electric neutrality in the internal compartment argues strongly for the presence, in the medium, of some endogenous anion unaccounted for in these experiments (17) which is taken up by the mitochondria. There are no significant exchanges of $H^+$ in these preparations (below 0.02 moles $H^+$/osmoequivalents).

TABLE II

*The Relationship between Ion Uptake and Osmoequivalents*

| Ion | Exp. | Conditions | Ion × $10^{-6}$ moles | $V_i$ | $\Delta V$ | Osmoequivalents × $10^{-6}$ | Ions/osmoequivalents |
|---|---|---|---|---|---|---|---|
| | | | | μl | μl | | |
| $P_i$ | 1 | $P_i$ | 0.032 | 13.0 | 4.6 | 1.61 | 0.020 ± 0.005 |
| | | no $P_i$ | | | 1.1 | 0.40 | |
| | 2 | $P_i$ | 0.029 | 17.8 | 5.4 | 1.38 | 0.021 ± 0.003 |
| | | no $P_i$ | | | 0.9 | 0.23 | |
| | 3 | $P_i$ | 0.048 | 12.9 | 5.4 | 1.89 | 0.025 ± 0.008 |
| | | no $P_i$ | | | 1.2 | 0.40 | |
| | | | | | | | Mean: 0.022 ± 0.006 |
| $Na^+$ | 1 | $P_i$ | 1.37 | 12.9 | 6.5 | 2.31 | 0.59 |
| | | no $P_i$ | 0.09 | | 1.3 | 0.47 | 0.19 |
| | 2 | $P_i$ | 0.60 | 11.3 | 2.7 | 0.97 | 0.62 |
| | | no $P_i$ | 0.06 | | 0.4 | 0.17 | 0.35 |
| | 3 | $P_i$ | 1.50 | 14.1 | 6.7 | 2.41 | 0.62 |
| | | no $P_i$ | 0.07 | | 1.0 | 0.37 | 0.19 |
| | 4 | $P_i$ | 1.04 | 14.7 | 5.1 | 1.82 | 0.57 |
| | | no $P_i$ | 0.09 | | 1.1 | 0.38 | 0.24 |
| | | | | | | | Mean: $P_i$ : 0.60 ± 0.03 |
| | | | | | | | Mean: no $P_i$ : 0.24 ± 0.08 |
| Succinate | 1 | $P_i$ | 0.32 | 15.3 | 6.1 | 2.16 | 0.15 |
| | | no $P_i$ | −0.09 | | 1.3 | 0.46 | — |
| | 2 | $P_i$ | 0.31 | 17.9 | 7.2 | 2.56 | 0.12 |
| | | no $P_i$ | 0.00 | | 1.5 | 0.54 | — |
| | 3 | $P_i$ | 0.29 | 9.5 | 4.4 | 1.56 | 0.18 |
| | | no $P_i$ | 0.02 | | 0.7 | 0.25 | 0.08 |
| | 4 | $P_i$ | 0.26 | 13.4 | 5.9 | 2.13 | 0.14 |
| | | no $P_i$ | 0.00 | | 1.6 | 0.57 | — |
| | | | | | | | Mean: $P_i$ : 0.15 ± 0.03 |
| | | | | | | | Mean: no $P_i$ : 0 |

The relationship between ion uptake and osmoequivalents presumed to have penetrated the mitochondria both in experiments with $P_i$ and in the absence of $P_i$ (the controls). Each of the four experiments shown below represents 8–10 determinations. The concentration of $Na^+$ in the medium corresponded to 24.3 mM. At 30 sec after incubation, a small volume of $P_i$, typically 10 μl, was introduced in the experimental determinations, while an equal amount of distilled water was introduced in the control determinations. $V_i$ corresponds to the μl of the mitochondria present per tube just before the addition of $P_i$ or water. $\Delta V$ is the change in volume taking place after the additions. The summary gives the means of the four experiments. The standard deviations are given.

TABLE III

*Summary of Ion/Osmoequivalent*

| Ion | Ion/osmoequivalent |
|---|---|
| $P_i$ | 0.022 ± 0.006 |
| Succinate | 0.15 ± 0.03 |
| $Na^+$ | 0.60 ± 0.03 |
| Total | 0.77 |

The uptake of $Na^+$ is against a concentration gradient. The internal concentration of $Na^+$ is approximately three times that present in the external medium (3.5 ± 1.1). The apparent gradient for succinate is about 2 (2.2 ± 0.6).

The uptake of $C^{14}$-succinate deserves some discussion. It is likely that some of the $C^{14}$-uptake corresponds in part to products of the succinate oxidation. However, since the swelling is not rotenone sensitive (the inhibitor blocks below the malate dehydrogenase step), any product of the reaction must have been produced on a one-to-one basis (with 0.82 mM rotenone, the $\Delta V$ was found to be 1.44 ± 0.07; without rotenone, 1.49 ± 0.07; and for the controls (no $P_i$), 0.29 ± 0.09 and 0.27 ± 0.09, respectively).

As shown in Table II, $P_i$ is involved only slightly in providing internal anions. The results are consistent with the interpretation that $P_i$ acts as a triggering effector. The effects of $P_i$ in permitting the penetration of other ions have been previously reported by others (21–23).

The preparations used have been found to phosphorylate. Under the conditions of these experiments, they exhibit a respiratory control ratio of 2.0–4.1. Values in this range are to be expected since the experimental conditions have induced an energy-requiring transport of ions against a concentration gradient. Subsequent work has shown essentially the same results in preparations ranging in respiratory control ratios from 1.8 to 6.2, and in media containing $Mg^{2+}$ (24). Baltscheffsky (25) has previously reported a decrease in respiratory control ratios induced by P in the absence of $Mg^{2+}$.

R. A. Harris et al. (26) have interpreted changes in scatter accompanying changes in metabolic states as alterations in the configuration of the mitochondrial membrane surfaces and not changes in mitochondrial volume. They base this conclusion on (*a*) the lack of changes in mitochondrial diameter measured from the outermost mitochondrial contours seen with the electron microscope, and, (*b*) their inability to detect volume changes in mitochondrial pellets maintained in the appropriate metabolic state. However, the osmotically active space of mitochondria corresponds to the space enclosed by the internal membrane and not the outermost contour. In addition, the gravinometric method used fails to show significant volume changes for the appropriate range of osmotic pressure (between 150 and 250 milliosmolar, see reference 27, Fig. 7). Significant changes in light scatter can be observed in this range (e.g. see reference 16).

## SUMMARY

It has been known for some time that, in the presence of substrate, inorganic phosphate induces swelling in isolated mitochondria. Evidence from the present work indicates that approximately 80% or more of the swelling can be accounted for by an osmotic swelling brought about by the internal accumulation of ions. The data are not consistent with the notion that the decrease in scatter obtained under these conditions reflect a mechanochemical coupling.

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