SIZE AND SHAPE TRANSFORMATIONS CORRELATED WITH OXIDATIVE PHOSPHORYLATION IN MITOCHONDRIA

I. Swelling-Shrinkage Mechanisms in Intact Mitochondria

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ABSTRACT

Two types of swelling-shrinkage change manifested by isolated mammalian heart mitochondria have been studied. One type, designated as phase I or "low amplitude" swelling-shrinkage, is estimated to lead to changes in mitochondrial volume of 20 to 40 per cent, to changes in light scattering of about 30 per cent, and to changes in viscosity. These physical changes in mitochondria are brought about rapidly and reversibly by normal reactants of the respiratory chain. Their speed, specificity, and reversibility indicate that they are closely geared to the normal function of the respiratory chain and are a true reflection of a mechanochemical coupling process characteristic of the physiology of mitochondria. A second type of swelling-shrinkage mechanism, designated as phase II or "high amplitude," leads to changes in light scattering, viscosity, and mitochondrial volume which, frequently but not always, are of higher magnitude than the phase I type. Phase II swelling-shrinkage seems to be only partly under the control of the respiratory chain. Prior to the completion of phase II swelling, a stepwise loss of mitochondrial function can be identified, such as changes in the rate of substrate utilization and loss of respiratory control. Reversal of this type of swelling cannot be effected if the swelling change reaches a steady state. This type of swelling may provide cells with a mechanism for destroying mitochondrial substance.

The relationship of oxidative phosphorylation to mitochondrial swelling and shrinkage has been described by various investigators using turbidometric and light-scattering techniques (1–4). Many types of mitochondria exhibit swelling and shrinkage changes which are dependent upon the conditions for oxidative phosphorylation, and indeed all the reactants of the process (*e.g.* substrates, oxygen, and phosphate acceptors) can, under appropriate circumstances, affect mitochondrial volume (2, 5–7). Reagents capable of interacting with the respiratory chain, such a cations (8), arsenate (5), uncoupling agents (9, 10), and various inhibitors of electron transport (11, 12) can also change mitochondrial volume. Because of the ability of these different conditions and reagents to change mitochondrial volume, it has been postulated (5, 9, 13) that they exert their effect on mitochondrial structure indirectly, perhaps through their influence on the levels or activity of intermediates of phosphorylation. These swelling-shrinkage states of mitochondria occur immediately after a change in the metabolic state of mitochondria (2, 5, 7), being completed within seconds after the termination of the functional changes which appear to drive them. This type of mitochondrial volume change reflects the physiology of mitochondria and has been arbi-

trarily designated as phase I (5) to distinguish it from a second type of change (designated as phase II) which can be brought about in mitochondria by a whole series of reagents, both "physiological" and otherwise, which can lead in some instances to an enormous swelling, and that often requires extended time periods for completion. These slower swelling responses of mitochondria may be reversed by the combined addition of ATP, magnesium ions, bovine serum albumin, and a number of unidentified factors (14, 15) if the mitochondria are incubated in the appropriate ionic environments (16). Lehninger and coworkers have extensively studied this type of swelling-shrinkage phenomenon, and designated it "high amplitude swelling," to distinguish it from the swelling changes (phase I) which are driven rapidly by the action of the respiratory enzyme systems. Lehninger has recently extensively reviewed the subject (17).

A third type of swelling-shrinkage change is brought about in a reversible fashion through an osmotic mechanism which depends upon changes in the tonicity of the environment in which the mitochondria are suspended (*cf.* 17). Osmotic swelling-shrinkage changes may be very rapid, with a half-time of only a few seconds, and are generally agreed to be strictly passive in character. They are, indeed, the only one of the three types of mitochondrial swelling-shrinkage mechanisms which has been satisfactorily quantitated in terms of volume changes (18).

It is the purpose of the present article to present some quantitative evaluation of the two types of "non-osmotic" swelling-shrinkage phenomena, phase I and phase II, or "low and high amplitude," respectively, in order to bring into focus their similarities and differencies. The findings are examined in terms of their consequences for the physiology and pathology of mitochondrial structure.

METHODS

The isolation of rabbit heart mitochondria in 0.32 M sucrose plus 0.001 M versene was carried out by the differential centrifugation procedure previously described (2). Polarographic assay of respiration and oxidative phosphorylation was also as described earlier, employing a reaction mixture of 0.05 M sucrose, 0.02 M KCl, 0.02 M phosphate buffer at pH 7.5, and mitochondria, in a final volume of 3 ml in the cuvette.

Swelling-shrinkage changes were followed by measuring scattering changes at 90° with 546 mμ light in the Brice-Phoenix Light-Scattering Photometer. The apparatus was modified for time recordings by employing a DC amplifier and a recording milliammeter in conjunction with the scattering apparatus. For light-scattering or turbidity records, the initial level was defined as 100 per cent, and the percentage change obtained under different conditions or by the addition of reagents to the system was then recorded on the chart. Scattering or turbidity increases or decreases refer, therefore, to the relative enhancement or diminution of the scattering from the 100 per cent level.

Direct measurements of mitochondrial volume were performed by the use of the mitocrit technique (19, 20). Samples of mitochondrial suspension were introduced into capillary tubes, and these were centrifuged in the cold in a Capillary Centrifuge. The percentage of volume occupied by the mitochondria was read after 20 minutes' centrifugation time at 10,000 *g*.

The "viscosity" of mitochondrial suspension in different swelling-shrinkage states was determined empirically with a Cannon-Ubbelholde type viscometer, at 20°C (viscometer constant = 0.01392 centistoke/second). The data (outflow times in seconds) were corrected in each case for the outflow time of the corresponding solvent with all the conditions identical except that mitochondria were absent. In those experiments where light-scattering changes were recorded simultaneously with oxygen utilization, a vibrating platinum electrode was inserted into a standard 1-cm cuvette in the light-scattering photometer.

RESULTS

Phase I or "Low Amplitude" Swelling-Shrinkage States

LIGHT-SCATTERING CHANGES WITH METABOLIC STATE: The phase I swelling-shrinkage states of heart mitochondria are illustrated in Fig. 1 as a time recording of the light-scattering and respiration changes in different metabolic states. It has the following characteristics: When mitochondria are incubated in the presence of substrate (glutamate), the addition of phosphate and then ADP at concentrations greater than those necessary to saturate the respiratory chain leads to scattering decreases and increases (swelling and shrinkage changes) respectively. The swelling changes are rapid, often reaching completion within seconds after the functional changes have been set into motion as judged by the rates of O_2 consumption. The swelling by phosphate

required approximately a minute for completion and led to a 20 per cent decrease in light scattering. The shrinkage induced by ADP was completed within approximately 15 seconds. Addition of ADP is seen to accelerate respiration of these "tightly coupled" mitochondria by a factor of 21. Since relatively small aliquots of ADP were added, it was possible for the ADP to be completely phosphorylated to ATP.[1] Its disappearance is accompanied by a swelling to the level seen in the absence of ADP, and so a slowing down of respiration. Multiple ADP additions lead to successive cycles as judged by light scattering and respiration. The principal characteristic of this phenomenon is that the structural parameter follows close on the heels of the functional changes.

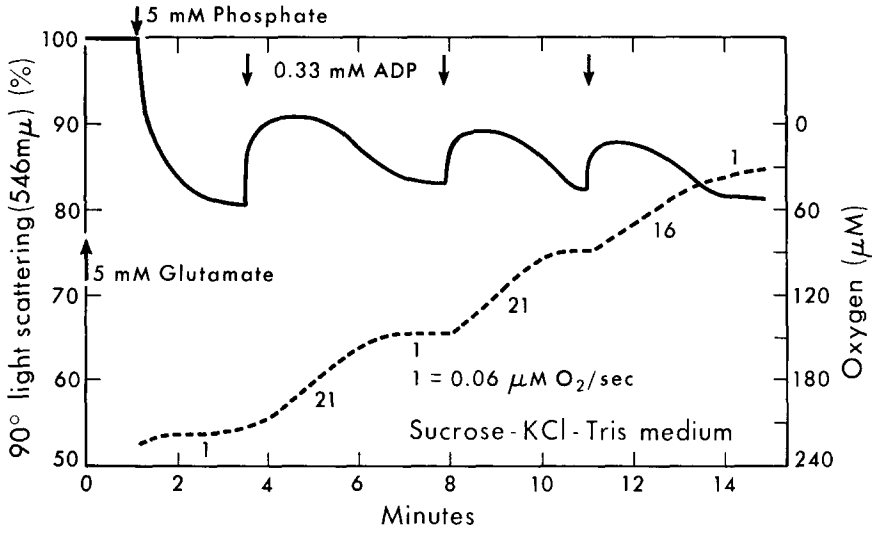

FIGURE 1 Phase I or "low amplitude" swelling-shrinkage changes coupled to respiratory activity in rabbit heart mitochondria. The reaction mixture (3 ml) contained tris(hydroxymethyl)aminomethane buffer (0.02 M, pH 7.5), KCl (0.02 M), sucrose (0.05 M), and mitochondria (1 mg protein per ml). Other additions as indicated. The 5 mM phosphate added during the experiment was at pH 7.5. Explanation in text.

VISCOSITY CHANGES WITH METABOLIC STATE: By using the outflow time of a suspension of mitochondria through a capillary tube as an empirical method, it was possible to demonstrate that the metabolic states of mitochondria discussed above are correlated with changes in "viscosity." The experiment was conducted as follows. The outflow time of the reaction mixture through the capillary viscometer was determined; afterward, an aliquot of mitochondrial suspension (final concentration approximately 1.5 mg protein per ml) was added, and the increase in outflow time over that of the reaction mixture alone was calculated (corrected outflow time). This corrected outflow time was arbitrarily assumed to be the 100 per cent "viscosity" value. In this way changes in corrected outflow time imposed by the various experimental conditions could be compared with the original mitochondrial suspension. As shown in Table I, after addition of substrate (note that the reaction mixture already contained phosphate) an appreciable percentage increase in corrected outflow time was noted. This is shown for three different substrates. This corresponds to the state of decreased light scattering and slow respiration shown in Fig. 1. Upon addition of ADP the mitochondria have the conditions for oxidative phosphorylation. In this state, the corrected outflow time or "viscosity" decreases; it corresponds to the state of increased light scattering or shrinkage of Fig. 1. "Viscosity" variations reflect changes in a structural parameter of either size or shape. Shape changes are highly unlikely since isolated mitochondria in low

[1] This is the principle of polarographic assay of oxidative phosphorylation (21).

sucrose concentrations assume a spherical shape which would not be expected to change. Gotterer *et al.* (3) have shown that the angular light-scattering envelope of liver mitochondria does not change after swelling.

Phase II or "High Amplitude" Swelling Changes

LIGHT-SCATTERING CHANGES: Phase II swelling of heart mitochondria with two types of substances is illustrated in Fig. 2. This type of swelling is characterized by occurring slowly, and frequently leads to higher percentage changes in light scattering (3, 5, 17); thus after approximately 10 minutes, the mitochondria undergo a decline in scattering in the presence of phosphate to the extent of 25 per cent. When Ca^{++} was used as a swelling agent, swelling ensued somewhat more rapidly, requiring approximately 4 minutes after addition of a high concentration of Ca^{++} (5 mM). The light-scattering decrease with Ca^{++} was approximately 60 per cent. This type of swelling does not require steady state conditions of added substrates. This experiment was conducted under the same conditions and with the same mitochondrial concentration as employed in the experiment illustrated by Fig. 1.

TABLE I

"Viscosity" Changes Accompanying Phase I Swelling-Shrinkage in Different Metabolic States of Heart Mitochondria

The outflow time of the reaction mixture, sucrose (0.05 M), KCl (0.02 M), phosphate (0.02 M, pH 7.5), without mitochondria was subtracted from the outflow time obtained with reaction mixture plus mitochondria (corrected outflow time). The corrected outflow time (which varied between 12.1 and 15.3 seconds) was taken as 100 per cent, and the relative percentage changes were calculated from the change in corrected outflow time after adding substrate and substrate + ADP. See text for further explanation.

	Corrected outflow time (%)		
	Initial condition	After substrate	After substrate + ADP
α-Ketoglutarate	100	207	136
Succinate	100	140	119
Glutamate	100	120	103

VISCOSITY CHANGES: The high amplitude phase II swelling changes were readily amenable to viscosity determinations, because of the slow nature of the changes involved. Table II summarizes three experiments carried out over a range of protein concentrations with heart mitochondria. It may be seen that a very large increase in outflow time occurs after incubation of mitochondria in the presence of phosphate. Even when the mitochondrial protein concentration was varied over a wide range, the percentage increase in outflow time induced by phosphate was approximately the same. These results with different concentrations of mitochondria tend to rule out other factors such as aggregation, shear effects, and release of soluble protein as the preferred explanation of the increased outflow time observed under conditions of swelling. The control experiments showed that the outflow time of a mitochondrial suspension incubated in the reaction mixture in the absence of phosphate remains unchanged over the 30-minute period of incubation.

Comparison of Phase I with Phase II Swelling

LIGHT-SCATTERING CHANGES:[2] A comparison of phase I with phase II swelling is given in Fig. 3. The conditions employed here are identical with those employed in the experiments illustrated by Figs. 1 and 2. Phosphate (5 mM) was added in two separate experiments to mitochondrial suspensions. In the control, substrate was not present, and in the experimental, 5 mmoles α-ketoglutarate was incubated in the reaction system. With α-ketoglutarate present, phosphate causes swelling in a manner similar to that seen before with glutamate present (Fig. 1). The swelling decline is quite rapid, occuring in a matter of several seconds. After a considerable incubation of the system, the addition of 400 mM ADP leads to a cycle of shrinkage and swelling. Thus during the time interval of the experiment, the system had retained its fully reversible character to manifest shrinkage-

[2] This is the method of choice for this comparison, as Gotterer *et al.* (3) have shown a greater sensitivity of light scattering over absorbancy measurements at low mitochondrial concentrations for following volume changes.

swelling changes. With respect to the control, incubated in the absence of substrate, it may be seen that the light-scattering drop began only late in the experiment (*cf.* Fig. 2). However, in this case, the swelling is not reversible by ADP (nor is it reversed if supplemented with substrate). It may be further noted that the total light-scattering changes induced by phosphate in both the control and the experimental cases are approximately the same (also seen with viscosity results of Tables I and II). One, however, is reversible (phase I) and the other is not reversible (phase II) by ADP.[3] It may be concluded from this comparison that the steady state swelling changes following the activity of the respiratory enzymes in phase I are not always of lower amplitude than the phase II type. For clearly, in the case of phosphate in heart mitochondria, the magnitude of the "low amplitude" swelling equals that of the "high amplitude" swelling (phase II swelling). Rather, the distinction may be one of reversibility or functional integrity of the system (see Discussion).

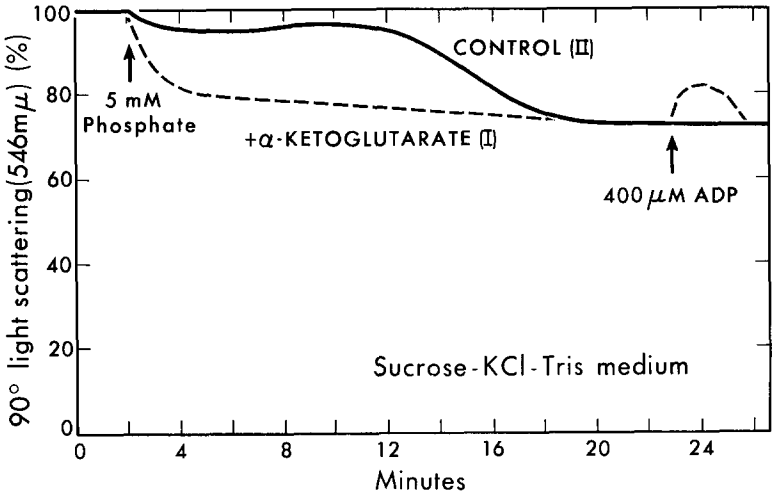

FIGURE 2 Phase II or "high amplitude" swelling in rabbit heart mitochondria. Conditions as in Fig. 1.

TABLE II

"Viscosity" Increase Accompanying Phase II Swelling Induced by Phosphate

The reaction mixture contained sucrose (0.11 M), tris (0.005 M, pH 7.5), and rabbit heart mitochondria. At zero time, phosphate (20 mM, pH 7.5) was added.

Expt.	Protein/ml (mg)	Corrected outflow time (sec.) First reading	Corrected outflow time (sec.) 30-minute reading	Increase of corrected outflow time (%)
321	8.70	58.0	122.0	205
322	1.15	8.5	15.4	181
324	2.18	17.8	36.4	204

[3] Lehninger (14) and Neubert *et al.* (15) have reported that ATP when incubated together with Mg^{++} and bovine serum albumin can reverse phase II swelling induced by phosphate. We have further examined ATP reversal of phase II swelling and find that it is effective only if it is attempted *before* the cessation of light-scattering change (see Discussion).

Passive Osmotic Swelling-Shrinkage

VOLUME CHANGES: A plot of the reciprocal of the sucrose concentration as a function of mitochondrial pellet volume (per cent) is shown in Fig. 4. Some variations are obtained in different experiments, but all runs are seen to give a linear relationship within the range of sucrose molarities examined here. Rabbit heart mitochondria thus manifest volume changes with sucrose in accordance with osmotic law. Heart mitochondria have also been shown to obey osmotic law by the optical density technique when examined in the range of sucrose concentrations used in Fig. 4 (*cf.* reference 2).

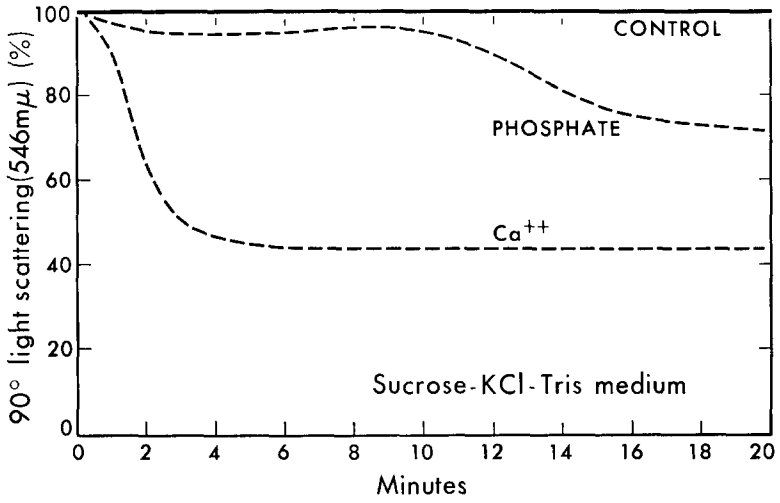

FIGURE 3 Comparison of phase I and phase II swelling induced by phosphate in rabbit heart mitochondria. Conditions as in Fig. 1.

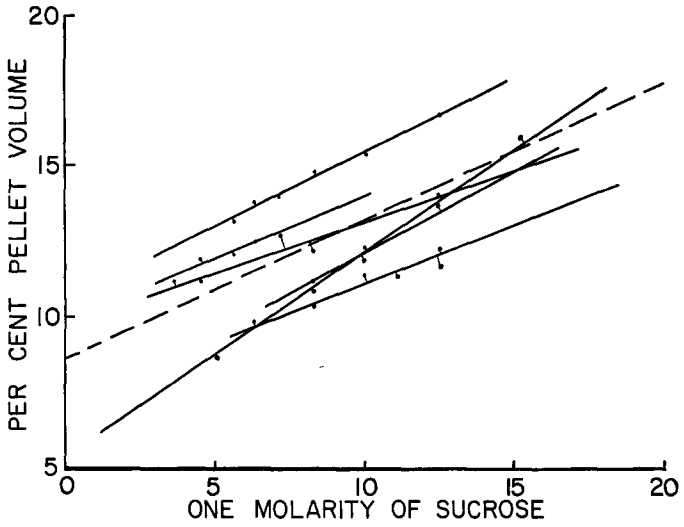

FIGURE 4 Calibration of heart mitochondria volume in different sucrose concentrations.

DISCUSSION

Three types of swelling-shrinkage phenomena may be recognized now in mitochondria. The simplest type is the passive osmotic mechanism (Fig. 4) which can lead to reversible changes in mitochondrial volume in response to changing osmotic pressure (2, 17, 18). The other two mechanisms may be termed active mechanisms, and these appear to be wholly or partly under the control of the respiratory apparatus. It seems important to bring into clear focus the similarities and differences with respect to the origin and nature of these two active mechanisms for controlling mitochondrial volume. This comparison seems warranted if we are to recognize and evaluate their physiological significance. The distinguishing features of these two mechanisms may be discussed under four categories: kinetics, specificity, reversibility, and amplitude or magnitude of the response.

Phase I or "Low Amplitude" Swelling-Shrinkage States

Kinetic studies of this type of mitochondrial volume change (Fig. 1) show that the structural changes are extremely rapid, usually following immediately after the change in metabolic state and ending or reaching a new steady state of shrinkage within a few seconds of their initiation. They are found to lag only a few seconds behind the functional changes which trigger them (5–7). They are highly specific in their occurrence. Variations in concentration of all the reactants of the respiratory chain and the oxidative phosphorylation process cause characteristic states of mitochondrial volume. Two principal states of volume may be recognized, as follows:

State	Substances present	Respiration	Mitochondrial volume
Rest	Substrate, P_i, O_2	Slow	Less contracted or partially swollen
Activity	Substrate, P_i, O_2, ADP	Rapid	Contracted or shrunken

A reversal of the swelling state maintained in the resting condition is best brought about by the addition of ADP, which triggers off rapid shrinkage (*cf.* Fig. 1). The amplitude of the light-scattering changes is generally 30 per cent or less (5), and the corresponding volume changes computed from osmotic calibration curves are of the order of 20 to 40 per cent. Scattering changes are coincident with changes in the "viscosity" of mitochondrial suspensions maintained in these two metabolic states (*cf.* Table I), in confirmation of the interpretation that scattering changes are a measure of mitochondrial volume.[4]

[4] It has thus far not been feasible to carry out direct measurements of mitochondrial volume changes during phase I swelling-shrinkage responses. The difficulty with this determination lies in the necessity for maintaining the mitochondria in a stable swelling-shrinkage state under aerobic conditions during the centrifugation procedure required to separate mitochondria from their medium for a volume determination. A further difficulty arises from the necessity of high mitochondrial concentration in order to obtain an accurate volume (or per cent dry weight) determination; maintenance of the aerobic condition in respiring mitochondria is not feasible under these conditions.

Phase II or "High Amplitude" Swelling-Shrinkage Changes

Mitochondrial volume changes of this type occur slowly (*cf.* Fig. 2, and refs. 5, 17). Many reagents are capable of inducing this type of swelling response (*cf.* Introduction and review by Lehninger). Reversal of this type of swelling is not a difficult matter if the swelling is not allowed to proceed to completion, and a suitable combination of ATP, divalent cation such as Mg^{++} or Mn^{++}, bovine serum albumin, and one of a number of factors is provided (*cf.* 17). The amplitude of the swelling response varies with the concentration of the reagent used to induce it, but is generally much greater than in the phase I type, frequently leading to volume increases in excess of 200 per cent and to light-scattering decreases of 80 to 90 per cent. In certain circumstances, however, the amplitude of the swelling response is no greater than that in phase I swelling. There is considerable evidence that during the time course of phase II swelling, many of the normal constituents of the mitochondria leak out and a virtually complete destruction of mitochondrial organization occurs. A qualitative examination (L. Packer, A. L. Tappel, and P. Savant, unpublished results) of the changes preceding the termination of a light-scattering decline has led to the following observations on the sequence of functional changes by mitochondria: (*a*) loss of reversed electron transport with ATP; (*b*) loss of respiratory control; (*c*) an increased oxidation of mitochondrial substrates (presumably through removal of permeability barriers); (*d*) a leakage of pyridine nucleotide out of the mitochondria (*cf.* 17); (*e*) a decline of the rate of oxidation of substrates to that below the initial respiration rate; and (*f*) completion of the light-scattering decline. Reversibility with ATP, Mg^{++}, and bovine serum albumin proceeds only in stages (*a*) through (*e*). After the light-scattering change reaches completion no condition has been found which leads to a reversal of phase II swelling.

Although the two types of active swelling-shrinkage mechanisms examined in this work both manifest light-scattering and "viscosity" changes, the available evidence indicates that they are fundamentally different. Their similarity

appears to reside in the fact that their occurrence is dependent upon the state of the respiratory enzymes. In the case of phase I swelling, the respiratory enzyme system is closely geared to changes in mitochondrial volume with respect to speed of response, specificity of reagents, and reversibility. Phase II swelling is only indirectly under the control of the respiratory system. If the respiratory apparatus remains intact, it appears that phase II swelling will not occur; having been initiated, however, it leads to the loss of many of the normal functions of mitochondria. If these pathological changes have not proceeded too far, reversal of the swelling reaction may be effected. It is difficult at this time to ascertain the physiological significance of either of these mechanisms, since clear *in vivo* or *in vitro* model experiments have not yet been executed. Since mitochondria undergo some change in morphology during the procedures required for their isolation, it is difficult to equate the magnitude of the reversible swelling changes observed in the *in vitro* system with the rapid changes in mitochondrial volume observed in living cells by cinematography (*cf.* Frederic (22)). It seems possible that both types of swelling-shrinkage mechanisms could, by virtue of controlling mitochondrial volume, affect the uptake and release of substances by mitochondria, thus providing a regulatory mechanism in metabolism. Studies with ascites tumor cells manifesting the Crabtree effect have indicated that changes in the structure of mitochondria in the living cell may play a role in this unusual control phenomenon (23). Also *in vitro* experiments with muscle mitochondria models (6, 24) have revealed a shortage of extramitochondrial ATP under conditions where mitochondria are in a high shrinkage state. Similarly, experiments with phase II type of swelling carried out by Tapley (25) indicate that changes in the physiological state of the organism may promote or retard phase II swelling of isolated mitochondria. These findings may have considerable bearing on the physiological role of phase II swelling. A normal function of phase II swelling may also be as a mechanism for loss of mitochondrial substance in cells.

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