Studies on the Mechanisms Controlling Time of Onset and Extent of Mitochondrial Swelling*

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ABSTRACT: Volume changes in rat liver mitochondria are influenced by pH and by varying concentrations of swelling agent, adenosine triphosphate (ATP), respiratory substrate, and divalent cations. The response to these agents of selected aspects, extent and time of onset, of the swelling process has been studied in order to further elucidate the mechanisms involved in swelling. Variations in time of onset and extent of swelling from pH 6.2 to pH 8.4 were qualitatively parallel.

Increased concentrations of swelling agent (orthophosphate, calcium, thyroxine) result in decreasing time of onset but have a minimal effect on extent. Added substrate or ATP acts to reverse the effect of swelling agent on time of onset. These same agents reverse the effect of hydrogen ion on both time of onset and extent up to pH 7.6. Increasing concentrations of magnesium or manganese increase the time of onset when orthophosphate is the swelling agent but have no effect when calcium replaces orthophosphate. In marked contrast, magnesium and manganese decrease the extent with either swelling agent. The ability of ATP to increase the extent, but not the ability of ATP to increase the time of onset, is inhibited by pyrophosphate and uridine triphosphate. These observations provide evidence for the differentiation of mechanisms controlling time of onset and extent of swelling and suggest a multiple rather than simple relationship between swelling and other energy-requiring functions of the mitochondrion.

T he studies of the chemical processes involved in changes of mitochondrial gross structure have continued to contribute to an understanding of swelling and contraction and to the associated mechanism of oxidative phosphorylation. Recent reports also link active ion accumulation with at least some facets of swelling (Chappell and Crofts, 1965; Pressman, 1965; Tedeschi and Hegarty, 1965; Judah et al., 1965; Azzi and Azzone, 1965). Because of the complex and poorly understood nature of the swelling process, it has been difficult to correlate the "whole" process of swelling with other energy-linked functions of the mitochondrion. Consequently, the over-all process has been described by three component aspects; time of onset (TO[1]) of swelling, rate of swelling, and extent of swelling (Connelly and

Lardy, 1964b). The last of these has been shown to be related to a high-energy intermediate of oxidative phosphorylation (Connelly, 1964). The general effects of various factors on TO and extent have been summarized by Connelly and Lardy (1964a). This paper provides an extended description of the responses of TO and extent to specific conditions. The data indicate that while in some instances responses are parallel, TO and extent can be differentiated and, in fact, can be varied independently.

Experimental Section

The preparation of rat liver mitochondria and the conduct of swelling studies was essentially the same as previously described (Connelly and Lardy, 1964b). Optical density changes were measured by a Coleman Jr. colorimeter set at 515 mμ. In serial experiments up to thirty runs were begun within a 15-min period in order to minimize variation due to aging. Stock mitochondria, equivalent to 1.0 g of rat liver/ml of 0.25 M sucrose, was kept at 4° prior to beginning experiments. All runs were made at room temperature (24°). The composition of the medium is described in the figure legends. Additions made during the experiments are noted by arrows on the figures. In experiments involving variation of pH, the pH of each run was maintained by buffering and determined immediately after the experiment using a Beckman Model 76 pH meter.

Nucleotides and substrates were obtained from the Sigma Chemical Co. Inorganic chemicals were of reagent or analytical grade and all water was double distilled.

[1] Abbreviations used in this work: AMP, ADP, and ATP, adenosine 5'-mono-, -di-, and -triphosphate; GMP, GDP, and GTP, guanosine 5'-mono-, -di-, and -triphosphate; UMP, UDP, and UTP, uridine 5'-mono-, -di-, and -triphosphate; TTP, thymidine 5'-triphosphate; CMP, CDP, and CTP, cytidine 5'-mono-, -di-, and -triphosphate; ITP, inosine 5'-triphosphate; TO, time of onset.
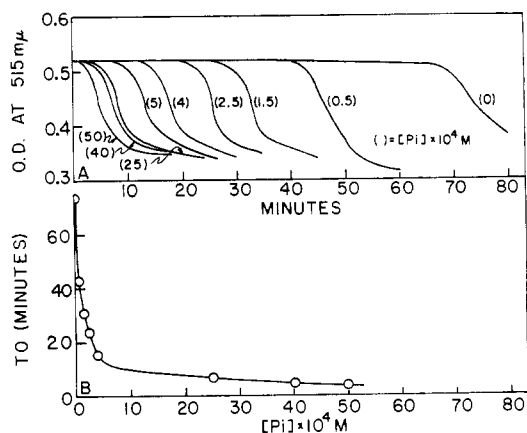
FIGURE 1: Effect of orthophosphate concentration on time of onset of swelling. The medium is 0.14 M sucrose, 0.017 M histidine–HCl, pH 7.4, 0.1 ml of mitochondrial suspension, and sodium potassium phosphate as indicated in a final volume of 6.0 ml. B is the secondary plot of data shown in A.
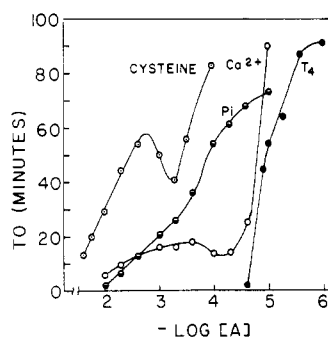

FIGURE 2: Relative potency of various swelling agents (A). Time of onset was determined using the media described in Figure 1. Data on all four agents were collected using the same mitochondrial preparation.

Results

Although the two aspects of mitochondrial swelling denoted TO and extent are affected by a number of agents, this study was limited to using those agents and conditions which could provide information correlative to the mechanism of oxidative phosphorylation. These include variation of pH, swelling agent concentration, divalent ion concentration, and varying conditions involving respiratory substrate and adenosine 5'-triphosphate (ATP).[1]

Effect of Swelling Agent Concentration. Rat liver mitochondria (equivalent to 100 mg of liver) suspended at pH 7.4 in either 0.14 M sucrose or 0.15 M KCl (volume, 6.0 ml) generally remain unaltered for a period up to 3 hr. Thereafter the occurrence of swelling, often referred to as "spontaneous" swelling (Lehninger et al., 1959), proceeds at a laboriously slow rate until full extent is reached. The TO of spontaneous swelling, which is very difficult to estimate, is not consistent among mitochondrial preparations. Variation is apparently dependent on the state of the mitochondria which in turn must be affected by factors such as endogenous substrate concentration, the absolute and relative amounts of adenine nucleotides, and intramitochondrial ionic milieu.

It is well known, however, that this TO can be shortened by a number of swelling agents which appear to act by means of mitochondrial enzymatic machinery rather than by detergent or physical chemical action. Figure 1A shows the regular decrease in TO with increased concentration of orthophosphate. This inverse relationship is described by a hyperbolic curve (Figure 1B). The general nature of the relationship of orthophosphate concentration to TO is also observed when calcium, thyroxine, and sulfhydryl compounds are used
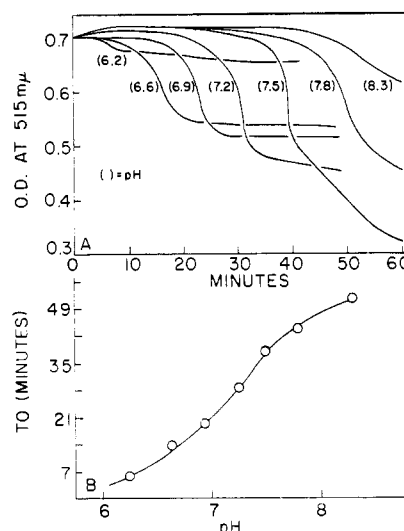

FIGURE 3: Effect of pH on time of onset of orthophosphate-induced swelling. The medium is the same as in Figure 1 where orthophosphate is 5×10^{-3} M. B is the analysis of the effect of pH on time of onset shown in A.

as swelling agents (Figure 2). Although no effort has been made to do exhaustive surveys of these latter agents, several interesting observations have been made. Consistent with the reports of others (Lehninger and Schneider, 1959), thyroxine has been found to be more potent and cysteine less potent than orthophosphate. In view of the biphasic relationship between cysteine and TO, this agent appears to have a multiple influence. Furthermore, because of the high concentrations of sulfhydryl compounds required to effect the TO, these agents were less suitable than orthophosphate, calcium, and thyroxine in many studies. Calcium behaves somewhat similarly to cysteine, as shown by Figure 2, although it is considerably more potent and is able to decrease TO to approximately 10 min at the relatively
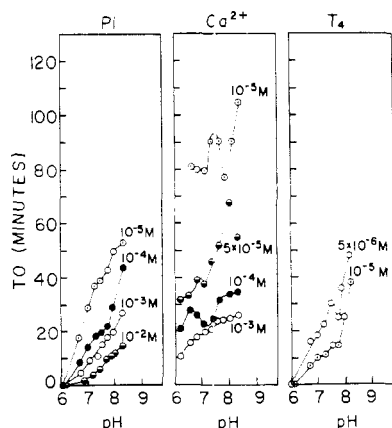
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FIGURE 4: Relative influence of swelling agents on the pH-time of onset relationship. Media are the same as in Figure 1. When orthophosphate was used buffered solutions were prepared to prevent significant changes in the pH of the medium.
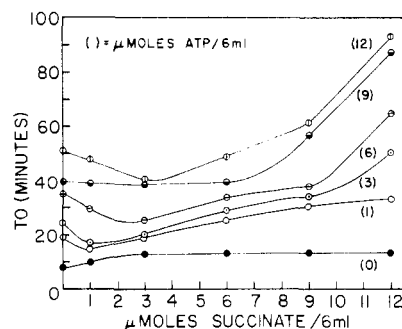

FIGURE 5: Effect of combined ATP and succinate on time of onset of swelling. The medium is the same as in Figure 1. The swelling agent is 5×10^{-3} M orthophosphate. The indicated quantities of ATP and succinate were added before the mitochondria.

low concentration of 10^{-4} M. It is interesting to note, however, that the effect of calcium between 10^{-4} and 10^{-2} M varies only slightly.

In contrast to the regular effect on TO by orthophosphate is the essential lack of influence of this agent on extent. Figure 1A shows that little variation of extent occurs when phosphate concentration is varied 100-fold. Furthermore, this relationship holds at all pH values even though extent varies with pH.

When thyroxine and calcium were used as swelling agents, a slight variation in extent was noted with change in concentration. While this variation was observed with concentrations of thyroxine in the range 10^{-4} to 10^{-5} M, relatively high concentrations of calcium were necessary to cause decreased extent.

Effect of Hydrogen Ion Concentration. Numerous observations have been made regarding the influence of pH on the swelling process (Lehninger *et al.*, 1959; Novelli *et al.*, 1963; Connelly and Lardy, 1964a). The variation in extent with pH has been described in an earlier communication (Connelly and Lardy, 1964a). The monoionic dissociation curve shown with orthophosphate (Figure 2 in Connelly and Lardy, 1964a) has also been observed when the swelling agent is calcium, thyroxine, and sulfhydryl compounds (Connelly, 1964). This similarity in responses[2] suggests that there are very likely common aspects in the machinery stimulated by these swelling agents.

The effect of hydrogen ion concentration on TO is shown in Figure 3. The pH-TO relationship also appears to be that of a monoionic dissociation (see Figure 3B), although this is not a consistent observation, es-

pecially when swelling agents other than orthophosphate are used. Figure 4 compares the responses of TO to pH when orthophosphate, calcium, and thyroxine are swelling agents. pH optima, often observed with calcium and thyroxine, suggest that more than one ionizable factor is involved.[3]

Oxidative Phosphorylation and Swelling. A. TITRABILITY OF TO AND EXTENT BY ATP OR SUCCINATE. It is well established that TO is directly related to ATP concentration and it appears that TO could be increased to a point where other uncharacterized factors (aging) might overcome the "protection" by ATP and allow swelling (Connelly and Lardy, 1964a, Figure 1; see also succinate = zero, Figure 5). On the other hand, when TO is increased by respiratory substrate, the effect reaches a maximum at low concentrations of substrate and TO does not vary with further titration (Figure 5). TO is markedly and continually titrable by respiratory substrate in the presence of ATP (Figure 5) or ADP (J. L. Connelly and C. H. Hallstrom, 1965, unpublished data).

The ability of ATP and respiratory substrate to increase the extent of swelling has been described (Connelly and Lardy, 1964a). In an attempt to determine the degree to which extent occurs relative to ATP and succinate concentrations, extent was titrated with these agents. As shown in Figure 6 extent of swelling could be controlled by the quantity of either ATP or succinate. The model experiments depicted in this figure show the titrability of extent both by serial addition and by comparison of the resultant extents of parallel runs where varying amounts of reagent were added initially. For the studies described below the latter procedure was adopted since experiments could be conducted simultaneously, thus minimizing varia-

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[2] It was commonly noted that at pH values below 6.0 agglutination and precipitation of mitochondria occurred. This is in marked contrast to experiments conducted by Arcos and Argus (1964) at pH 4.0 and lower.

[3] The pH optima shown in Figure 4 are representative of several experiments. An optimum at pH 7.6 is consistently observed with thyroxine while optima from pH 6.9 to pH 8.0 are seen with calcium. Generally, as the calcium concentration is lowered optima shift toward higher pH values.
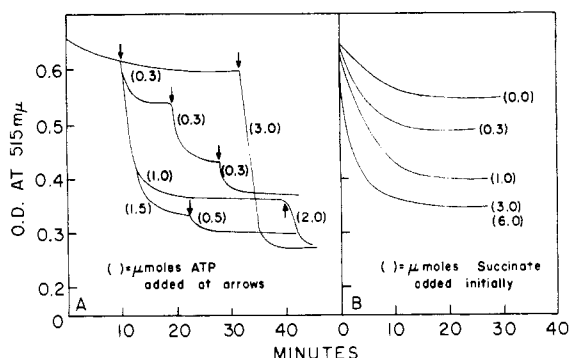
FIGURE 6: Titrability of extent by ATP or succinate. The medium is the same as Figure 1 except that the pH is 6.1. The swelling agent is 5×10^{-3} M orthophosphate. The indicated quantities of ATP and succinate were added as noted in A and B, respectively.

tions due to mitochondrial aging during any one experiment.

B. EFFECTS OF COMBINED SUCCINATE AND ATP on TO AND EXTENT. The parallel influences of succinate and ATP on both TO and extent prompted an investigation of their relative contributions toward these changes. Figure 5 shows that the increase in TO by any particular concentration of succinate is markedly increased by ATP. The relationship is not purely additive but synergistic. The effect of adding succinate to ATP appears to be more complex since low concentrations of succinate are relatively ineffective, whereas high levels (greater than 9 μmoles/6 ml) greatly enhance the ATP effect. These observations as well as the effects of oligomycin on TO (Connelly and Lardy, 1964b; Chappell and Greville, 1959) will receive further attention in a subsequent report (J. L. Connelly and C. H. Hallstrom, 1965, unpublished data).

The effect of ATP and succinate on extent limited by low pH is shown in Figures 7A and 7B. The curves labeled "0.0" show that the change in extent reaches a plateau at about 2.0 μmoles of ATP or succinate. However, on several previous occasions it had been observed that when succinate was added to ATP-swollen mitochondria or when ATP was added to succinate-swollen mitochondria, a significant further increase in extent occurred. As a consequence, experiments were carried out testing varying combinations of substrate and ATP on extent. The results are plotted against both ATP and succinate with the amount of one component shown on the abscissa and the amount of the second component shown in parentheses. It can be seen that the combination of ATP and substrate results in an extent greater than that obtained with either component alone. Thus it appears that respiration or some function involving the electron transport chain *is* required to give swelling to the maximum extent. Second, oligomycin, which acts to block respiratory phosphorylation, enhances the effect of substrate at the lower concentrations (Figure 7B). It should be re-
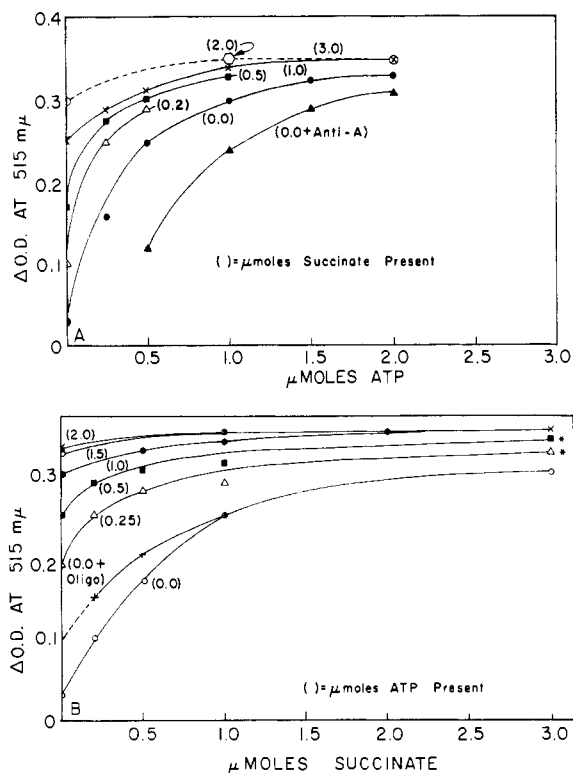

FIGURE 7: Effect of combined ATP and succinate on extent of swelling at pH 6.1. The medium and swelling agent are the same as in Figure 6. The indicated quantities of ATP and succinate were added before the mitochondria. Where noted in A antimycin A is 1.67 $\times$ 10^{-4} M and, in B, oligomycin A is 1.5 $\times$ 10^{-6} M (0.67 μg/ml). Points marked with an asterisk are derived from the upper curve of Figure 7A. Estimated curves are shown by dashed line.

membered that oligomycin blocks completely the ability of ATP alone to increase extent (Connelly and Lardy, 1964a). Finally, antimycin A by blocking respiration appears to deprive ATP of the synergistic effect of endogenous substrate respiration (Figure 7A).

C. NUCLEOTIDE SPECIFICITY OF CHANGE IN TO AND EXTENT. The ability to counter the swelling activity of orthophosphate and thus increase TO is specific for adenine nucleotides, primarily ATP and ADP, as indicated in Table I. A small but significant increase in TO was observed for AMP. All other nucleoside mono-, di-, and triphosphates (with the possible exception of GTP), as well as pyrophosphate, were ineffective. When nucleoside diphosphates were tested for their ability to enhance the effect of succinate, only ADP proved effective.

The nucleotide-stimulated increase in extent of swelling beyond that allowed at pH 6.1 is specific for ATP among nucleoside di- and triphosphates (Figure 8; diphosphates not shown). Neither pyrophosphate nor nucleotides other than ATP, when added at zero time, varied the optical density–time plot from the control
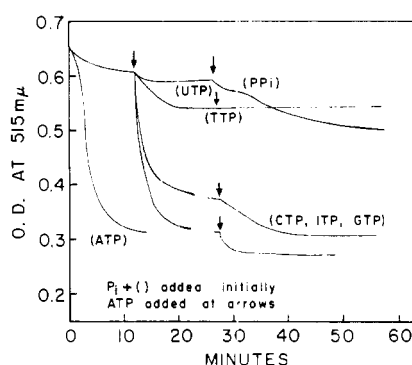
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FIGURE 8: Effect of nonadenosine nucleotides on extent of swelling at pH 6.1. The medium and swelling agent are the same as in Figure 6. Additions made at zero time are shown in parentheses. ATP is added at arrows. Where added, ATP = 1 μmole and pyrophosphate and other nucleotides (including nucleoside diphosphates not shown) = 2 μmoles. Volume is 6 ml.

TABLE I: Effect of Nucleotides and Pyrophosphate on Time of Onset of Swelling.[a]

Addition	Time of Onset (min)	
	− Sub-strate	+ Sub-strate
None	11	14
PP$_i$	5	
ATP	40	
ADP	32	51
AMP	17	
GTP	13	
GDP	9	12
GMP	8	
CTP	4	
CDP	9	12
CMP	9	
UTP	8	
UDP	11	11
UMP	8	

[a] Conditions as in Figure 1 with the swelling agent, orthophosphate, at 5×10^{-3} M. Pyrophosphate, nucleotides, and substrate, where added, are 10^{-3} M.

FIGURE 9: Effect of magnesium on orthophosphate- and calcium-induced swelling. The medium in A is 0.112 M KCl, 0.017 M histidine–HCl, pH 7.4; in B it is the same as in Figure 1. The swelling agents are calcium or orthophosphate at the concentrations indicated. Magnesium, at the concentration noted in parentheses, was added before the mitochondria.

curve. It should be noted here that while ADP is of primary importance in increasing TO, this compound has considerably less ability to increase extent (J. L. Connelly, 1965, unpublished data).

Finally, with regard to the action of nucleotides on extent, it can be seen from Figure 8 that the effect of ATP, added at the times indicated by arrows, is markedly reversed by pyrophosphate, UTP, and TTP. This observation is suggestive of structural analog inhibition and the quantitative aspects of this phenomenon are
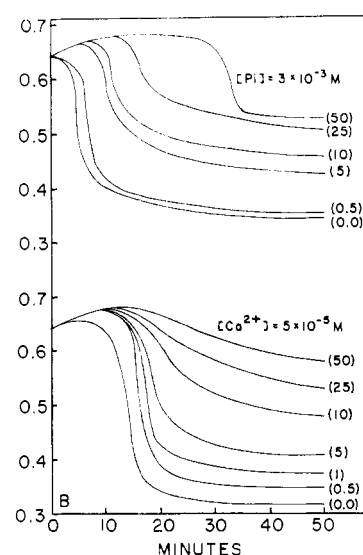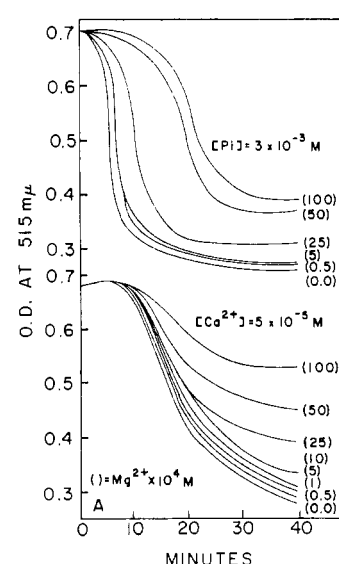
under investigation. In contrast to their effects on ATP-induced increase in extent, UTP (up to UTP/ATP = 6) and pyrophosphate do not affect the action of ATP with regard to TO. This constitutes a major difference in the mechanisms, both of which respond to the presence of ATP, controlling extent and TO.

Effect of Divalent Cations. The participation of magnesium in mitochondrial enzyme reactions, principally those utilizing ATP, is well known. Magnesium ions have also been shown to influence the nature of mitochondrial swelling (Baltscheffsky, 1957; Connelly and Lardy, 1964a; Raaflaub, 1953; Connelly and Lardy,
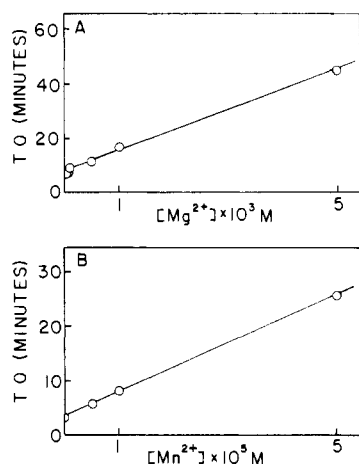
FIGURE 10: Effect of magnesium and manganese on the time of onset of swelling. The media and swelling agent in A and B are the same as in Figure 5.
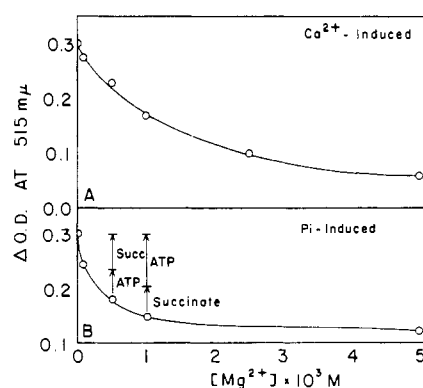

FIGURE 11: Effect of magnesium on extent of swelling and the reversal of this effect by ATP and succinate. A and B are secondary plots of the data shown in Figure 9B. Where employed, 5×10^{-4} M ATP or 5×10^{-4} M succinate was added after swelling had occurred.

1964b). These findings have been confirmed and extended in order to compare the responses of TO and extent to divalent cations. The qualitative effect of magnesium on swelling induced by orthophosphate or calcium in sucrose and KCl media is shown in Figure 9. Extent of swelling is inversely related to the concentration of added magnesium with either calcium or phosphate as swelling agent. This effect parallels that seen with added hydrogen ion. Investigation of the effect of magnesium on TO, however, provided two interesting observations. First, in contrast to the response of TO to hydrogen ions, added magnesium increased TO nearly linearly (see Figure 10A) in a sucrose–phosphate or KCl–phosphate medium. Second, when calcium replaced phosphate, magnesium was essentially without effect on TO in sucrose or KCl but still influenced extent.

Manganese also proved to be a potent agent in delaying TO. Figure 10 shows that while magnesium was effective in concentrations of about 10^{-3} M, manganese was about 100 times as effective and also linear.

Variation in extent by added magnesium, in both calcium- and phosphate-induced swelling, was not linear (Figures 11A and 11B). As in the case of pH-limited extent, the influence of magnesium could be reversed by the addition of ATP or succinate (Figure 11B). It is noteworthy that maximal reversal required both agents.

Discussion

Elucidation of the mechanism and significance of mitochondrial structural changes is both aided and complicated by the fact that these changes are associated with a multiplicity of enzymatic functions in mitochondria. It is now apparent that swelling and shrinking (Connelly, 1964), oxidative phosphorylation, ion accumulations (Brierley et al., 1963; Vasington and Murphy, 1964), triphosphopyridine nucleotide (TPN)

reduction by DPNH₂ (Danielson and Ernster, 1963), succinate reduction of diphosphopyridine nucleotide (DPN) (Chance and Hollunger, 1961), and incorporation of DPN into mitochondria (Greenspan and Purvis, 1965) are all related, however indirectly (Lehninger, 1964; Racker, 1965). The existence of enzymatic machinery common to certain of these, for example, swelling and oxidative phosphorylation (Connelly and Lardy, 1964a), provides valuable tools for indirect study of these mechanisms. On the other hand, the high number of variables which can affect the energy-linked reactions of mitochondria make these studies difficult to control and evaluate.

It follows, nevertheless, that a thorough understanding of the chemistry of, and the factors which influence, the swelling phenomenon would expedite the elucidation of any associated mechanism.

If the over-all process of mitochondrial swelling reflects the operation of a single mechanism, it would be expected that responses to a given stimulus measured by various criteria of swelling would be similar. On the other hand a variation of response, especially in direction, would indicate that the swelling phenomenon is comprised of more than one mechanism. Such mechanisms, although closely associated, must be able to operate independently. It has been shown here that certain agents or conditions affect TO and extent of swelling in a qualitatively parallel manner. However, the responses of TO and extent to other agents are not only quantitatively different but opposite in direction. In addition, each aspect can be qualitatively controlled independent of the other. These observations provide evidence which link TO and extent to the same enzymatic machinery and yet also serve to clearly differentiate them.

The concept of "extent" does not exist unless swelling, and thus TO, has occurred. Consequently, it is apparent that both aspects of this process are dependent upon the presence of the swelling agent or the condition

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which allows swelling. Observations reported here and summarized in Table II indicate that under conditions in which swelling can occur, both TO and extent are independently controllable. Specifically, the time of onset may be decreased by increasing the concentration of the swelling agent with no change in extent. ATP or ATP plus substrate acts antagonistically toward swelling agent in this regard, again with no change in extent. Likewise with regard to variation in extent, at constant TO, it has been observed that extent can be titrated with ATP or substrate to a maximum level after TO has occurred. Extent can also be varied in a regular fashion by the addition of magnesium or manganese to a calcium swelling system having a constant TO.

TABLE II: Comparison of Responses of Time of Onset and Extent to Various Agents.

Increased	Time of Onset	Extent
[H⁺]	↓	↓
[Swelling agent]	↓	. . .ᵃ
[Mg²⁺] when A = Pᵢ	↑	↓
[Mg²⁺] when A = Ca²⁺	. . .	↓
[Substrate]	↑	↑ ᵇ
[ATP]	↑	↑ ᵇ
[PPᵢ]	Does not reverse ATP effect	Reverses ATP effect

ᵃ Slight decrease in extent with thyroxine and calcium. ᵇ Increase in extent occurs only below pH 7.6 or when extent is limited by the presence of magnesium or manganese.

Additional evidence for the differentiation of mechanisms controlling TO and extent arises from experiments involving the inhibition of effects of ATP by pyrophosphate or UTP. Although ATP acts on TO and extent in the same direction, the effectiveness of ATP in increasing extent was completely blocked by pyrophosphate or UTP while these agents do not reverse the effect of ATP on TO.

Finally, it is important to note that in the case of hydrogen ion, swelling agent, ATP or substrate concentrations, the reactions of TO and extent are in the same direction while the responses of these two aspects of swelling to added magnesium, in a phosphate medium, are opposite in direction.

In spite of these differences, it is very probable that these two aspects are not only closely related but interdependent. This is suggested by the regularity in variation of both TO and extent with change in pH, and of course, also by the fact that TO must occur before extent is real. Furthermore, the fact that substrate-induced increases in TO and in extent are both enhanced by the

presence of oligomycin testifies to the existence of pathways common to both mechanisms.

On the basis of these observations, it is important that a reevaluation of various aspects of the swelling phenomena be considered separately. First, regarding TO, it should be noted that the uptake of water by rat liver mitochondria which are exposed to swelling agents does not proceed gradually with time but occurs precipitously only after necessary conditions are established. The rate of obtaining this situation, as measured by TO, is influenced by pH, concentration of swelling agent, ATP, respiratory substrate, and various divalent cations.

If continued mitochondrial integrity depends on the presence of adequate amounts of certain high-energy intermediates associated with respiration, the condition required for swelling could result from the loss of the metabolic pressure acting to maintain the necessary concentrations of these intermediates. Such a change in metabolic pressure might be expected with the exhaustion of endogenous respiratory substrate. The influence of the various factors which affect TO might then be explained in terms of their effect on the rate of utilization of endogenous or added substrate. Thus ATP, which represents a product of respiratory phosphorylation, would provide a back pressure against the use of substrate. Relative to this point it is important to emphasize that the addition of oligomycin to mitochondria, treated with substrate, greatly enhances the protective effect of the substrate presumably by diverting the respiratory-generated energy away from ATP production and toward intermediates involved with TO (see Scheme 1 and discussion, Lardy et al., 1964). Second, with regard to pH, the rate of utilization of substrate would be markedly decreased up to pH of about 7.6 by the direct effect of hydrogen ion in any of the reactions associated with substrate utilization. Finally, swelling agents might effect the rate of utilization of substrate by their acting to provide for the use of high-energy intermediates in alternative mechanisms such as ion accumulation and simple hydrolysis.

While the magnitude of TO appears to depend on the continued maintenance of a minimum level of high-energy compounds, the degree to which mitochondria swell following TO is a function of the utilization of high-energy intermediates. Whether or not the two functions involve the same intermediates is not known; nevertheless, it is certain that the extent to which mitochondria can swell following TO is dependent upon the availability of energy derived from the oxidation of respiratory substances or directly from ATP. The use of this energy might control the extent of swelling in two ways. First, generated high-energy intermediates might be used in a process which effects a change of intramitochondrial osmotic pressure resulting in the transference of water. An alternate and additional explanation involves the possibility that the influence of osmotic pressure may be counteracted by contractile elements in the mitochondrial membranes. Particularly applicable to this idea is the observation (J. L. Connelly, 1964, unpublished data; Suranyi and Avi-Dor, 1965)

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that ATP added to mitochondria in a KCl medium first increases extent of swelling and subsequently allows a contraction. It would seem most likely that these changes would result from two separate phenomena. Furthermore, the influence of pH on extent appears to be manifest in the concentration of a particular ionic species rather than in the variation of a reaction rate since extent, at any particular pH, does not vary with time. Thus, in systems where extent of swelling is limited by low pH, high-energy intermediates generated either from ATP or from respiration could condition contractile proteins stoichiometrically to allow increased swelling to fixed extents.

In addition to providing further direct evidence for the participation of high-energy intermediates in the regulation of mitochondrial structural changes, this work partly distinguishes mechanisms involved in controlling TO and extent of swelling. It is suggested that the swelling process begins upon the exhaustion of substrate or substrate-generated high-energy compounds and proceeds to a degree of swelling which is quantitatively influenced by osmotic factors as well as contractile components of the mitochondrial structure.

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