Interconversion of Added Adenine Nucleotides during Mitochondrial Swelling*

Jerald L. Connelly and Curtis H. Hallstrom†

ABSTRACT: The effect of rat liver mitochondrial enzymes on the interconversion of added adenine nucleotides, prior to and during the swelling process, has been investigated. The rate of change in concentrations of adenosine mono-, di-, and triphosphate (AMP, ADP, ATP) and of inorganic phosphate under various conditions was determined during swelling experiments. The adenine nucleotide composition was governed by adenylate kinase, ATPase, and oxidative phosphorylation.

The changing pattern of adenine nucleotides permitted an evaluation of the extent of participation of these activities in the swelling process. In the presence of added substrate, ATP synthesis has priority over adenylate kinase for the utilization of ADP. Except in the presence of oligomycin A, swelling is accompanied by an increase in ATPase activity. It is concluded that none of these enzyme activities is responsible for or directly related to the mechanisms which control water uptake by mitochondria. They may, however, influence this process indirectly by causing variations in adenine nucleotide composition.

Since the early studies by Raaflaub (1953a,b) which demonstrated the increase in time of onset (TO[1]) of mitochondrial swelling by the addition of ATP, the chemistry by which ATP provides this "protection" has remained obscure. As in certain of the other energy-requiring functions observed in mitochondria, ATP is considered to act through a reversal of part of the oxidative phosphorylation machinery. This reasoning is based upon the observation that oligomycin inhibits the ability of ATP to increase TO (Connelly and Lardy, 1964b) and to enhance ion accumulation by mitochondria (Brierley *et al.*, 1963; Engstrom and DeLuca, 1963), and also upon the findings of Sanadi and Fluharty (1963) regarding the sensitivity of ATP-supported reduction of NAD to oligomycin A. The acceptance of this explanation was, however, complexed by the observation that ADP, as well as ATP, could provide for an increased TO of swelling (Hunter and Ford, 1955; Fonnesu and Davies, 1956; Lipsett and Corwin, 1959). It would be expected, even in the absence of added substrate, that ADP might be phosphorylated to ATP, but this would necessitate the forward action of the phosphorylation mechanism. Substrate level phosphorylation (Chappell and Greville, 1961) as well as the action of adenylate kinase, shown to be present in liver mitochondria (Kotelnikova, 1948; Barkulis and Lehninger, 1951; Novikoff *et al.*, 1952), are alternative means by which ADP could be converted to ATP.

Integrity of mitochondria may depend on not only the nature of the content of adenine nucleotides but also on the functioning of the various enzymes which influence these compounds. At the present the relative importance of ADP and ATP, and the significance of the mechanisms by which these nucleotides can be interconverted, to the process of mitochondrial swelling is not well established. This work reports the nature of the interconversion of adenine nucleotides in mitochondria during the swelling process.

Experimental Section

The preparation of rat liver mitochondria and the conduct of swelling studies was essentially the same as previously described (Connelly and Lardy, 1964a). For the timed-sampling experiments a trial run was made using the proper reaction mixtures (described in the legends) to establish the nature of the swelling curve. Subsequently, the identical medium was made up to three times its normal volume of 6 ml to allow for sampling during the swelling run. The TO of the untreated swelling media runs (P_i, sucrose, buffer) was from 5 to 9 min, and TO in the absence of P_i was maximal.

Samples (1 ml), taken at timed intervals, were placed in 0.1 ml of ice-cold 30% TCA. This method of deproteinizing was found to be satisfactory by Eggleston and Hems (1952) for the preparation of samples for chromatography of adenine nucleotides. The TCA-precipitated samples were centrifuged in the cold and aliquots of the supernatant were quantitatively analyzed

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† U. S. Public Health Service Predoctoral Fellow.

[1] Abbreviations used in this work: TO, time of onset (Connelly and Lardy, 1964a); AMP, ADP, and ATP, adenosine 5′-mono-, di-, and triphosphate; TCA, trichloracetic acid; NAD, nicotinamide-adenine dinucleotide; P_i, orthophosphate.

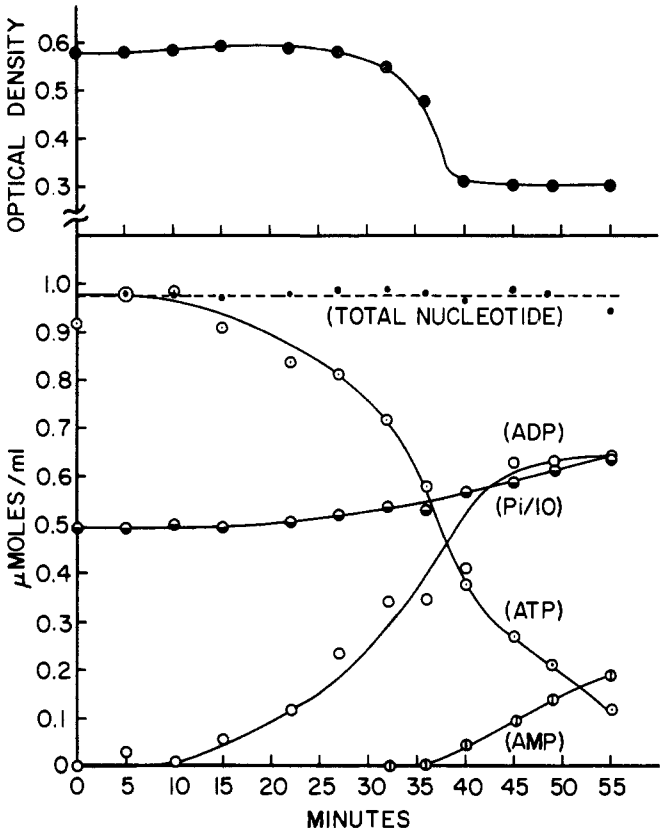

FIGURE 1: The disposition of added ATP and its relationship to mitochondrial integrity. Media contained 0.139 M sucrose, 1.67×10^{-2} M histidine (pH 7.5), 5×10^{-3} M P_i, 10^{-3} M ATP, and 0.3 ml of mitochondria in 18.0-ml volume. Concentration of nucleotides is expressed as micromoles per milliliter of reaction mixture.

for adenine nucleotides and inorganic phosphate as described below.

Separation and Determination of Adenine Nucleotides. TCA-precipitated supernatant (50 μl) was spotted in duplicate on Whatman No. 1 paper in 7–8 applications. Spots, which were located 1 in. apart and 1.25 in. from the lower edge of the paper, were dried with a stream of cool air in order to maintain the diameter at 1 cm. The chromatogram was developed for 30 hr,[2] using the Pabst system no. one (Pabst Circular OR-10, 1956), isobutyric acid–ammonia–water (66:1:33). The observed R_F values in this system were: 0.49 for AMP, 0.31 for ADP, and 0.20 for ATP with the solvent front of about 11 in. Chromatograms were dried and nucleotides were located under a Mineralight Model R-51 lamp and encircled with a soft lead pencil.

Paper strips containing nucleotide spots were cut so that each strip contained about two square inches of paper. A control sample containing all components except the added nucleotide was spotted and developed and appropriate samples were cut out adjacent to the experimental ATP, ADP, and AMP spots. Paper strips were eluted with water for about 10 hr until about 4.9 ml of eluent was collected; 0.05 ml of 1.0 M hydrochloric acid was added and the volume made to 5 ml. The optical density was read at 260 mμ in a Cary 15 spectrophotometer and corrected for absorbance of the control samples.[3] The amount of nucleotides present was obtained by the use of molar absorbancy indexes of 15.1, 14.9, and 14.7×10^3 for AMP, ADP, and ATP, respectively (Bock *et al.*, 1956).

Determination of Inorganic Phosphate. Inorganic phosphate was determined essentially by the method of Lowry and Lopez (1946) in which 0.05-ml samples of the TCA-precipitated supernatants were run in duplicate.

All nucleotides were obtained from the Sigma Chemical Co., St. Louis, Mo. Nucleotides and succinate were neutralized to about pH 7.0. Oligomycin A was obtained from Dr. H. A. Lardy, Enzyme Institute, University of Wisconsin, and antimycin A was obtained commercially. Stock solutions for inhibitors were: 200 μg/ml 100% methanol for oligomycin and 67 μg/ml

[2] The recommended 16 hr of developing time was adequate in the separation of pure nucleotides but inadequate in the separation of nucleotides of biological preparations.

[3] Background absorption of the blanks agreed consistently with that observed for the corresponding areas in the zero-time samples.

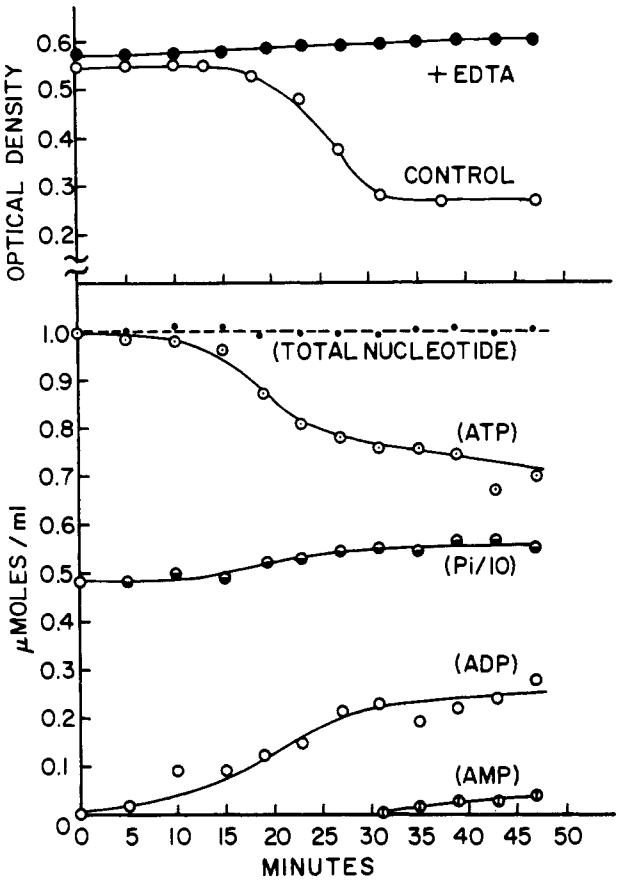

FIGURE 2: The effect of EDTA on the disposition of ATP. Media are the same as in Figure 1 for the control curve. Where added, EDTA is 10^{-3} M. P_i and nucleotide concentrations were determined from samples taken from the "+ EDTA" run.

50% ethanol for antimycin. Water for the preparation of reagents and for dilution was doubly distilled.

Results

In the experiments reported here no effort was made to distinguish between bound and free or endogenous and exogenous adenine nucleotides. Based on the millimicromoles of adenine nucleotides per milligram of mitochondrial nitrogen, as determined by Pressman (1958) and Brenner-Holzach and Raaflaub (1954), it is calculated that mitochondria contribute only 3.5–5.0 mμmoles/ml to the total adenine nucleotides, about 1000 mμmoles/ml, present in each reaction mixture. Thus the variation in the composition of endogenous adenine nucleotides during the experiment was considered to contribute only slightly to the final distribution picture. Furthermore, because the rates of penetration of adenine nucleotides are extremely rapid (Pfaff *et al.*, 1965), measurement of variations in total adenine nucleotide composition reflects a variation in activities of the mitochondrial enzymes which act upon these compounds.

Disposition of Added ATP. Figure 1 indicates that the protective action of ATP on mitochondrial integrity is accompanied by a slow ATPase as shown by the increase in ADP and inorganic phosphate up to about 30 min. At and following TO there is a marked increase in ATPase activity (see Brenner-Holzach and Raaflaub, 1954). The appearance of increased amounts of AMP from 30 min on probably reflects adenylate kinase activity. The possibility that the increased ATPase occurring at TO is a consequence rather than a cause of the swelling condition is consistent with the earlier observations of Potter *et al.* (1953). In studying latent ATPase in rat liver mitochondria these workers observed a lag phase having a low ATPase activity followed by a very rapid ATPase beginning between 5 and 30 min. This change undoubtedly reflects the onset of swelling as is further evidenced by the lack of ATPase activity in the presence of magnesium ion which is known to increase TO.

Essentially the same picture (Figure 2) is observed when mitochondrial swelling is delayed by the presence of EDTA. Furthermore, the increase in ATPase activity at about 15 min in samples taken from the EDTA-treated medium coincides with the TO observed in the control run. This strongly suggests that although mito-

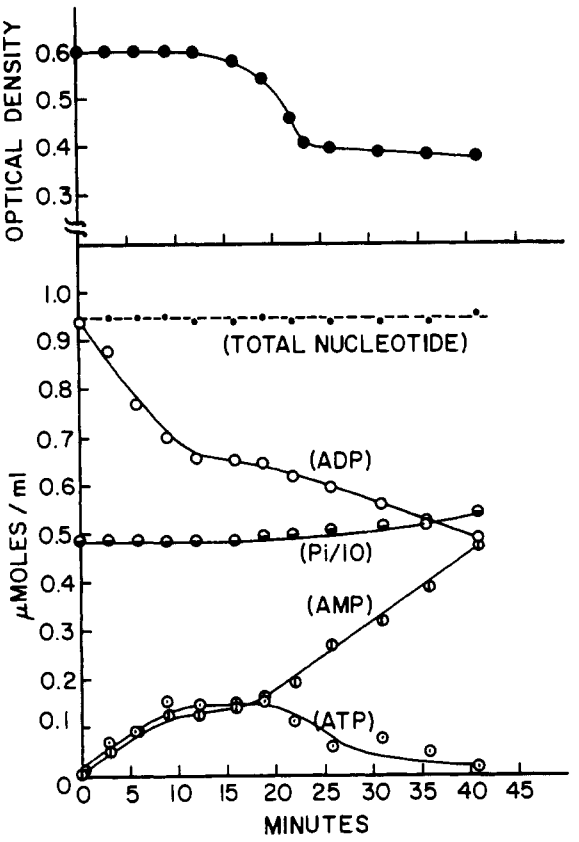

FIGURE 3: The disposition of added ADP and its relation to mitochondrial integrity. Media are the same as in Figure 1 except that 9.5×10^{-4} M ADP replaces 10^{-3} M ATP.

chondria are prevented from swelling by the presence of EDTA, changes related to the mitochondrial swelling process and reflected by the increased ATPase activity are, nevertheless, proceeding as observed in the untreated media (Figure 1). It should be noted, however, that the data in Figure 2 differ from the data in Figure 1 in the rates of ATPase and AMP formation. It is evident that the uptake of water by mitochondria resulting in the change in optical density does not cause the ATPase or AMP formation and neither do these activities *per se* cause swelling to occur. They are, however, indicative of the fact that the conditions required for mitochondrial swelling are being or have been established.

The Disposition of Added ADP. ADP added to mitochondria is rapidly converted to AMP and ATP by the adenylate kinase reaction.[4] Figure 3 indicates that there is little change in the inorganic phosphate concentration during this activity. Following TO there is again a marked increase in the ATPase activity as reflected by the rapid changes in ADP and ATP concentrations. The continued increase in concentration of AMP reflects a concurrent and continuing adenylate kinase activity. The disposition of ADP added to mitochondrial medium containing EDTA (Figure 4) is markedly different from that seen in Figure 3. In agreement with the observation of Bruni and Luciani (1962), the presence of EDTA strongly inhibits the adenylate kinase activity (see also Tapley, 1956; Lehninger and Schneider, 1959; Gallager, 1960). It was not surprising then that the primary utilization of ADP in the presence of EDTA was through its phosphorylation to ATP. A slight change in the rate of loss of ADP and in the rate of increase of ATP and inorganic phosphate coincident with TO is consistent with the previously observed increase in ATPase activity at TO. It appears from both Figures 2 and 4 that EDTA acts to decrease the rate of ATPase relative to that observed in its absence. This may very possibly be due to the ability of EDTA to chelate mitochondrial magnesium ions.

The relative importance of the adenylate kinase and phosphorylation mechanisms in the utilization of ADP was investigated by providing added substrate to the mitochondrial medium. The results, shown in Figure 5, indicate that under these conditions, oxidative phosphorylation is the prime activity and adenylate kinase activity is negligible or very slight until 50 min. Especially pertinent from data shown in Figure 5 is the fact that at, or prior to, TO the concentration of ATP

[4] Although these experiments were conducted in the absence of added magnesium, mitochondria contain sufficient bound magnesium for adenylate kinase activity (Siekevitz and Potter, 1953; Novikoff *et al.*, 1952; Wadkins, 1962).

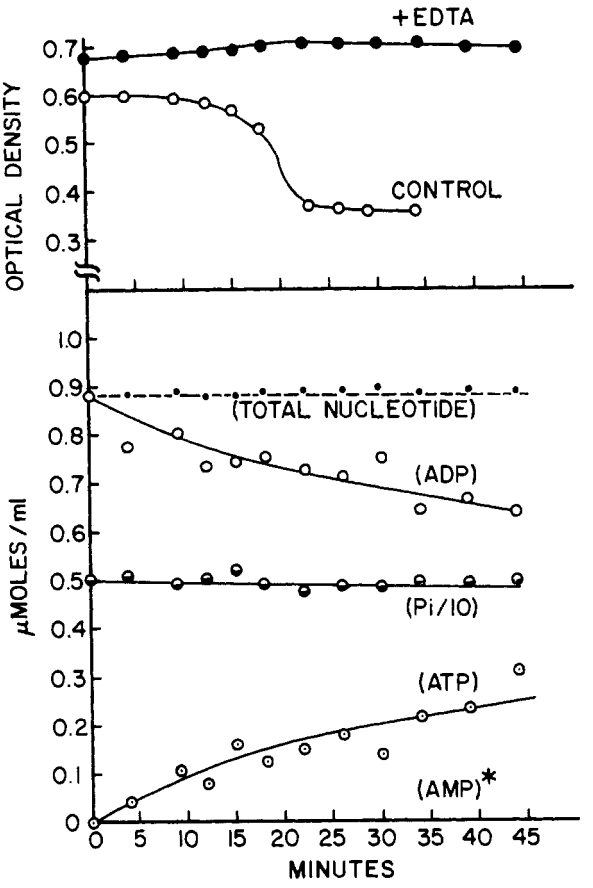

FIGURE 4: The effect of EDTA on the disposition of ADP. Media are the same as in Figure 3 for the control curve except ADP is 9×10^{-4} M. Where added, EDTA is 10^{-3} M. P_i and nucleotide concentrations were determined on samples taken from the "+ EDTA" run. *No AMP could be detected in samples either by absorption of ultraviolet light on the chromatograms or by spectrophotometric measure of the sample eluents.

is maximal and even under these conditions swelling occurs with a concurrent ATPase activity. This agrees with the data shown in Figure 1 where the ATP concentration at TO is approximately 75% of that present at zero time. It must be concluded then that although the treatment of mitochondria in a swelling medium by ATP increases the TO, the mere presence of ATP is not enough to ensure continued structural integrity of the mitochondria.

In the presence of EDTA and succinate, ADP is rapidly phosphorylated with a marked increase of ATP up to the TO of the control (Figure 6). Thereafter there appeared to be a lowered rate of phosphorylation which could reflect the ATPase activity observed in earlier figures.

The Disposition of Added ADP in the Presence of Oligomycin A and Antimycin A. In the absence of oxidative phosphorylation and ATPase, both of which are blocked by oligomycin, ADP is utilized only by the adenylate kinase reaction (Figure 7). Wadkins (1962) has shown the latter activity to be unaffected by oligomycin. It is noteworthy that in comparison with the picture in Figure 3 the swelling process continues even in the absence of ATPase, thus indicating a related but not dependent relationship between these activities.

The previous experiment was repeated in the presence of respiratory substrate in order to investigate the relationship between adenylate kinase activity and TO under conditions where TO would be relatively large (Chappell and Greville, 1958; Connelly and Lardy, 1964b). Figure 8 shows that TO, in the presence of succinate, ADP, and oligomycin, exceeds the time at which the adenylate kinase reaction approaches equilibrium. Furthermore, it can be seen from Figures 3, 7, and 9 that this activity can continue up to and beyond TO. It is apparent then that swelling and adenylate kinase operate independently. It is perhaps important to consider that the substrate level phosphorylation of ADP, which is not inhibited by oligomycin, could contribute to the interconversion of mitochondrial adenine nucleotides.

Treatment of mitochondria with antimycin should inhibit oxidative phosphorylation but should allow the functioning of adenylate kinase as well as ATPase activities. That this is true is indicated by Figure 9 wherein added ADP, prior to and following TO, is rapidly con-

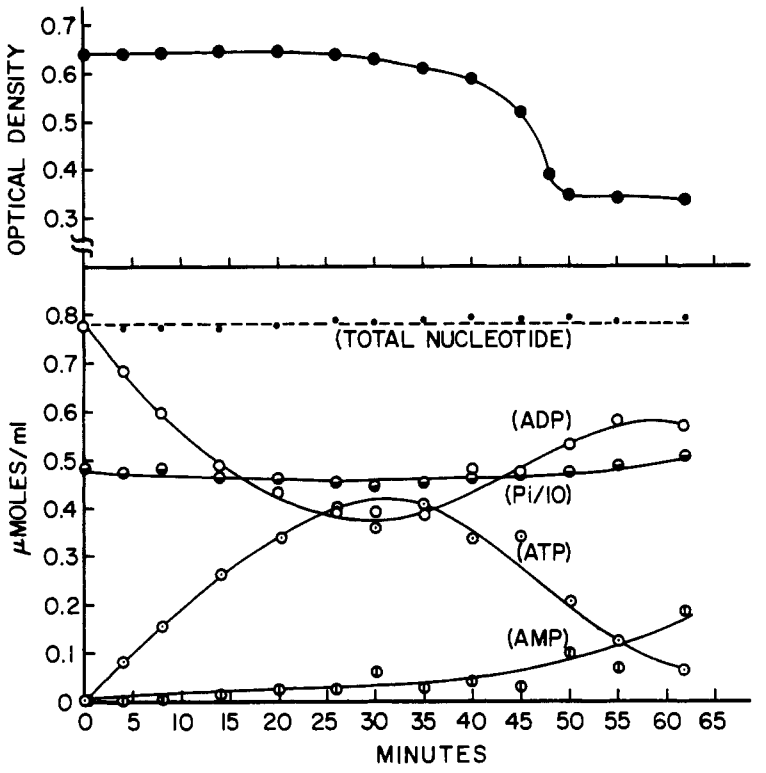

FIGURE 5: The effect of added substrate on the disposition of ADP. Media are the same as in Figure 3 with ADP at 8×10^{-4} M and succinate at 1.67×10^{-4} M.

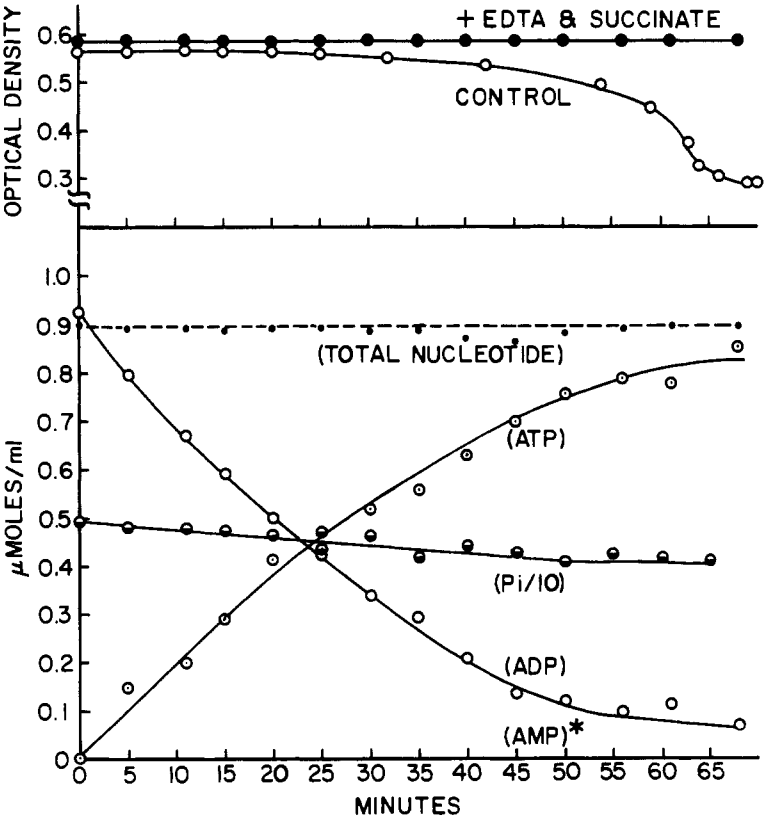

FIGURE 6: The effect of added substrate and EDTA on the disposition of ADP. Media are the same as in Figure 5 for the control curve except ADP is 9×10^{-4} M. Where added, EDTA is 10^{-3} M. P_i and nucleotide concentrations were determined on samples taken from the "+ EDTA and succinate" run. *No AMP could be detected in samples either by absorption of ultraviolet light on the chromatograms or by spectrophotometric measure of the sample eluents.

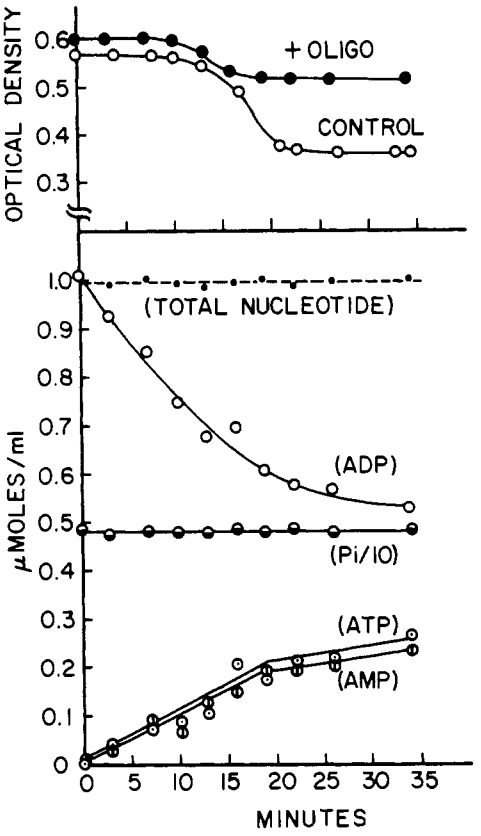

FIGURE 7: The effect of oligomycin on the disposition of ADP. Media are the same as in Figure 3 for the control curve except ADP is 10^{-3} M. Where added, oligomycin is 2 μg/ml. P_i and nucleotide concentrations were determined on samples taken from the "+ oligo" run.

TABLE I: Summary of Adenine Nucleotide Enzyme Activities Observed during Mitochondrial Swelling.

Addn	Adenylate Kinase	ATPase	Oxidative Phosphorylation
ATP		+[a]	
ATP + EDTA	−	+	
ADP	+	+	
ADP + EDTA	−	+	+
ADP + substrate	(+)[b]	+	+
ADP + substrate + EDTA	−	+	+
ADP + oligomycin	+	−	−
ADP + substrate + oligomycin	+	−	−
ADP + antimycin	+	+	−

[a] +, observed; −, inactive; blank, not observed (activity could be concurrent but masked). [b] Minor contribution.

verted to AMP and ATP. Maximal ATPase activity noted in earlier figures appears to occur following the TO of the antimycin-treated run. The apparent delay in appearance of this activity with regard to TO (see also Figure 3) may be due to the simultaneous production of ATP by the adenylate kinase reaction.

Discussion

In addition to serving as the locus for the conservation of cellular energy in the form of ATP, mitochondria contain a host of enzymes which are influential in the interconversion of the various adenine nucleotides. The early work of Siekevitz and Potter (1953) and Potter *et al.* (1953) attempted to determine the interrelationship of some of these enzymes. With the more recent observation that adenine nucleotides are influential in the maintenance of mitochondrial integrity, or in controlled changes in mitochondrial structure, it has become important to reexamine the significance of these various enzymatic activities to the chemical processes governing the influx and extrusion of water and electrolytes by mitochondria.

The results of the experiments described in Figures 1–9 are summarized in Table I. Although each of the

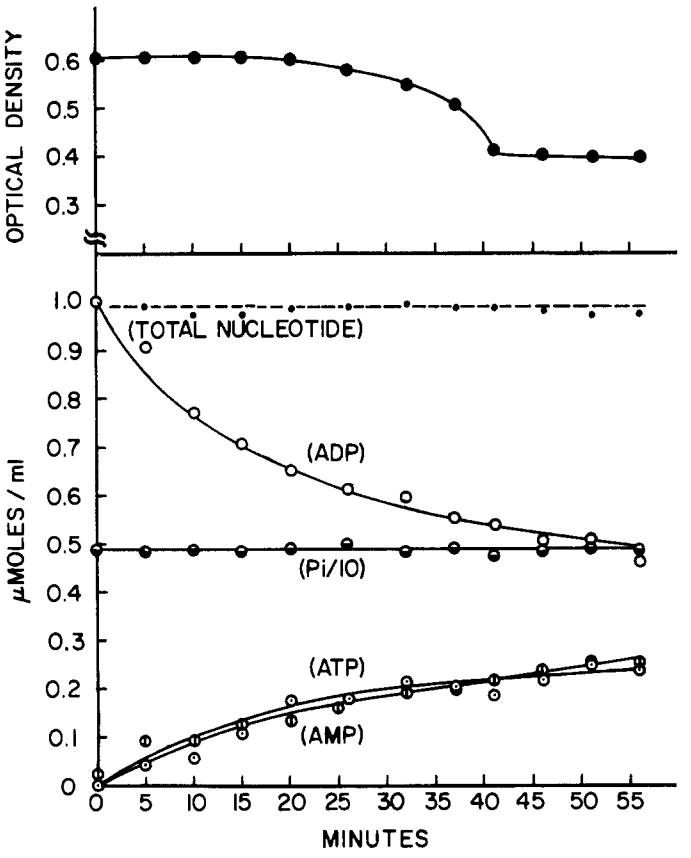

FIGURE 8: The effect of added succinate and oligomycin on the disposition of ADP. Media are the same as in Figure 7 plus 8.3×10^{-5} M succinate.

enzyme activities noted is capable of varying the relative concentrations of the adenine nucleotides, none is directly responsible for the onset of swelling or for the prevention of swelling.

Adenylate Kinase. In view of the fact that EDTA, which inhibits adenylate kinase activity, also delays swelling it might be concluded that this particular activity is a primary regulatory factor. However, as mentioned earlier, this enzyme remained functional up to and beyond TO. The work of Siekevitz and Potter (1953) discloses similar findings. They noted that ADP was rapidly (15 min) converted to ATP plus AMP in a non-swelling situation; *i.e.*, no swelling agent was present and the ATPase activity characteristic of TO was not observed. Furthermore, as shown in Figures 2 and 4, the presence of large amounts of either ATP or ADP, where adenylate kinase activity is blocked by EDTA, does not vary the susceptibility of mitochondria to swelling.

Siekevitz and Potter (1955) have also shown that mitochondria which have been preincubated with orthophosphate, a known swelling agent, retain an active adenylate kinase. Finally, Lipsett and Corwin (1959) found that 2,4-dinitrophenol has a marked influence on TO while adenylate kinase activity is unaffected.

Adenine Nucleotidase. Although TO is consistently marked by an increase in ATPase activity (or decreased rate of ATP production) the finding that swelling proceeds in the presence of oligomycin virtually eliminates any consequence of ATPase activity as a causative factor. Neither is it the direct result of the swelling process. Nevertheless, the onset of this activity coincident with TO reflects the establishment of some condition which corresponds to TO and may indeed be indicative of the cause of swelling. For example, if both respiration and ATP act to provide energy to maintain integrity, increased ATPase would result from the lowering or exhaustion of respiratory substrate. Potter *et al.* (1953) demonstrated that the lag (TO!) in appearance of rat liver mitochondrial ATPase was considerably shorter when mitochondria from fasted animals were used. A role for endogenous substrate is especially suggested by the increase in ATPase in mitochondria protected by EDTA at the TO of the control (−EDTA).

Oxidative Phosphorylation. The phosphorylation of ADP in mitochondria appears to occur only when the forward action of the machinery is operative and is not overcome by equilibrium changes forcing its reversal. Any deterioration of mitochondrial integrity, the reversal of which would use energy, would result in the tapping off of respiratory energy which otherwise would be used in the phosphorylation of ADP to ATP.

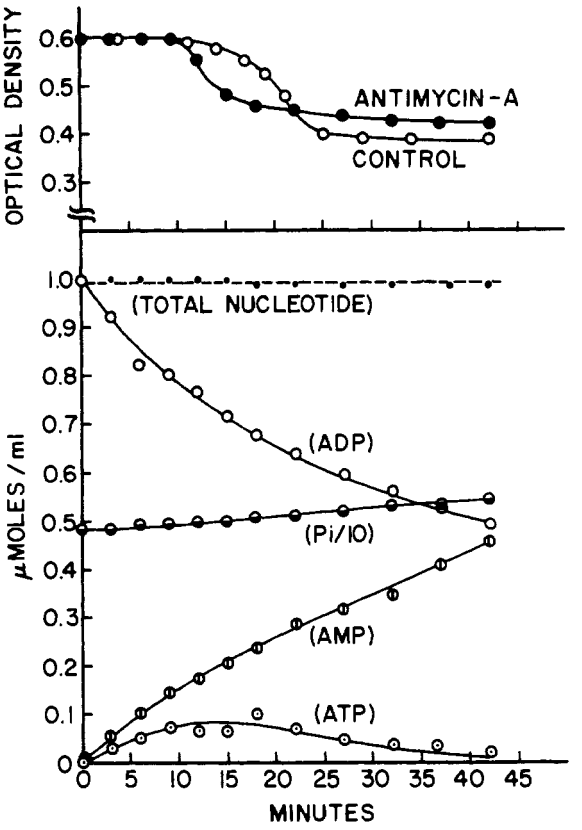

FIGURE 9: The effect of antimycin on the disposition of ADP. Media are the same as in Figure 3 for the control except ADP is 10^{-3} M. Where added, antimycin is 1 μg/ml. P_i and nucleotide concentrations were determined on samples taken from the "+ antimycin A" run.

This additional drain of a share of the respiratory energy would result in lowered or stopped ATP production. In spite of the importance of phosphorylation, the notion that "oxidative phosphorylation protects mitochondria against swelling" (Lipsett and Corwin, 1959) is not compatible with the synergistic action of oligomycin and oxidizable substrate to prolong TO (see also Fonnesu and Davies, 1956).

It appears then that the significance of these activities is not in their functioning *per se* but in their ability to regulate the intramitochondrial levels of adenine nucleotides. In this regard it is highly plausible that variation in mitochondrial structure, and perhaps function, is dependent not on the absolute concentration but instead on the degree of binding of a particular nucleotide. The observations of Brenner-Holzach and Raaflaub (1954) and Lipsett and Corwin (1959) emphasized the importance of bound adenine nucleotides. More recently special attention has been directed toward ADP as being selectively significant in the exchange of mitochondrial adenine nucleotides (Pfaff *et al.*, 1965), in the uptake of adenine nucleotides by mitochondria (E. Carafoli, C. S. Rossi, and A. L. Lehninger, personal communication), and in the maintenance of mitochondrial integrity (J. L. Connelly and C. H. Hallstrom, unpublished data).

It had been suggested earlier (Siekevitz and Potter, 1955) that inactivation of the phosphorylation mechanism was a result of an unbinding of nucleotides, and indeed a loss of adenine nucleotides, including NAD, was observed during "active" swelling (Hunter and Ford, 1955). These workers also provided evidence that EDTA acts by maintaining NAD in the bound form. They and Raaflaub (1955) suggest that ATP may behave in a similar way. The direct involvement of NAD would help explain the beneficial effects of oxidizable substrate (Connelly and Hallstrom, 1966) especially when oxidative phosphorylation is blocked. It can be concluded that while the activities of the enzymes discussed above are incidental, adenine nucleotides, probably bound, act in conjunction with other factors (respiration) to control the condition referred to as mitochondrial integrity.

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