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Ion Movements During Energy-Linked Mitochondrial Structural Changes

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Abstract

The structure of isolated rat liver mitochondria has been observed in the electron microscope following incubation of the mitochondria in vitro under a variety of conditions. The results show that ultrastructural changes are only associated with the energization and deenergization of isolated mitochondria if the composition of the incubation medium permits ion movements in or out of the matrix. The mechanism of energy coupling does not appear to depend on these major mitochondrial structural changes. The addition of low levels of valinomycin greatly increases the rate at which the matrix compartment swells and shrinks on energization and deenergization even at low K^+ concentrations.

Introduction

There have been a number of reports demonstrating that isolated mitochondria incubated in vitro undergo structural changes which can be observed in the electron microscope after fixation [1–7]. Except at low osmolarities [8–10], the most obvious changes that appear to occur during incubation involve the swelling or shrinking of the inner compartment rather than changes in the total mitochondrial volume. Hackenbrock, Green, and others [1, 4, 11] have related these changes to the state of energy coupling, whereas some investigators have stressed that changes in the volume of the inner compartment can be related to ion movements [12–17]. More recently, it has been suggested that the changes, whether osmotic or not, may have a role in the regulation of mitochondrial respiration [18–22]. The fact that

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these structural changes have been described as either energy-linked or osmotic in nature has suggested that the structural changes may be of two different types, and this interpretation has been strengthened as a result of the claim by Hackenbrock et al. [23] that energy-linked structural changes can occur in the absence of ion movements. In this paper we report the results of experiments in which we have attempted to resolve this controversy by examining the conditions under which energy-linked structural changes occur during oxidative phosphorylation in isolated mitochondria. These results emphasize the importance of ion movements during any changes in the volume of the mitochondrial inner compartment, and show that the structural changes which are visible in the electron microscope are not obligatory for energy coupling during oxidative phosphorylation.

When we deliberately increased the permeability of the mitochondrial inner membrane to K^+ by the addition of valinomycin, the mitochondrial structural changes occurred more rapidly even at low K^+ concentrations and we observed that both the increases and decreases in matrix volume were associated with the gain and loss of mitochondrial K^+, respectively. These observations suggest that in the absence of ionophore, the permeability of the inner membrane to cations may limit the structural changes which accompany the transition from one respiratory state to another [26].

Materials and Methods

Mitochondria were isolated in 0.25 M sucrose from the liver of male Wistar rats by Kielley and Kielley's modification of Schneider's method [24]. Mitochondrial protein was estimated by a biuret method [6].

Respiration was measured polarographically and ADP:O ratios were calculated from the oxygen electrode recordings by the method of Chance and Williams [26]. Changes in absorbance were measured at 510 nm using a Shimadzu MP50 spectrophotometer.

Potassium ion levels in mitochondria were measured using an EEL digital flame photometer with internal standard. The mitochondria were separated from the incubation medium by centrifugation for 20 sec in an Eppendorf microcentrifuge. The pellet was then washed with 250 mM sucrose at 0°C and treated with 6% perchloric acid to precipitate proteins and release internal K^+. The extract was then diluted to an appropriate concentration for flame photometry. The mitochondrial phosphate content was estimated on the perchloric acid extracts by a modification of the method of Fiske and Subbarow [27].

The levels of K^+ in the incubation medium were followed with a selective

ion-sensitive glass electrode coupled to a vibron electrometer and chart recorder.

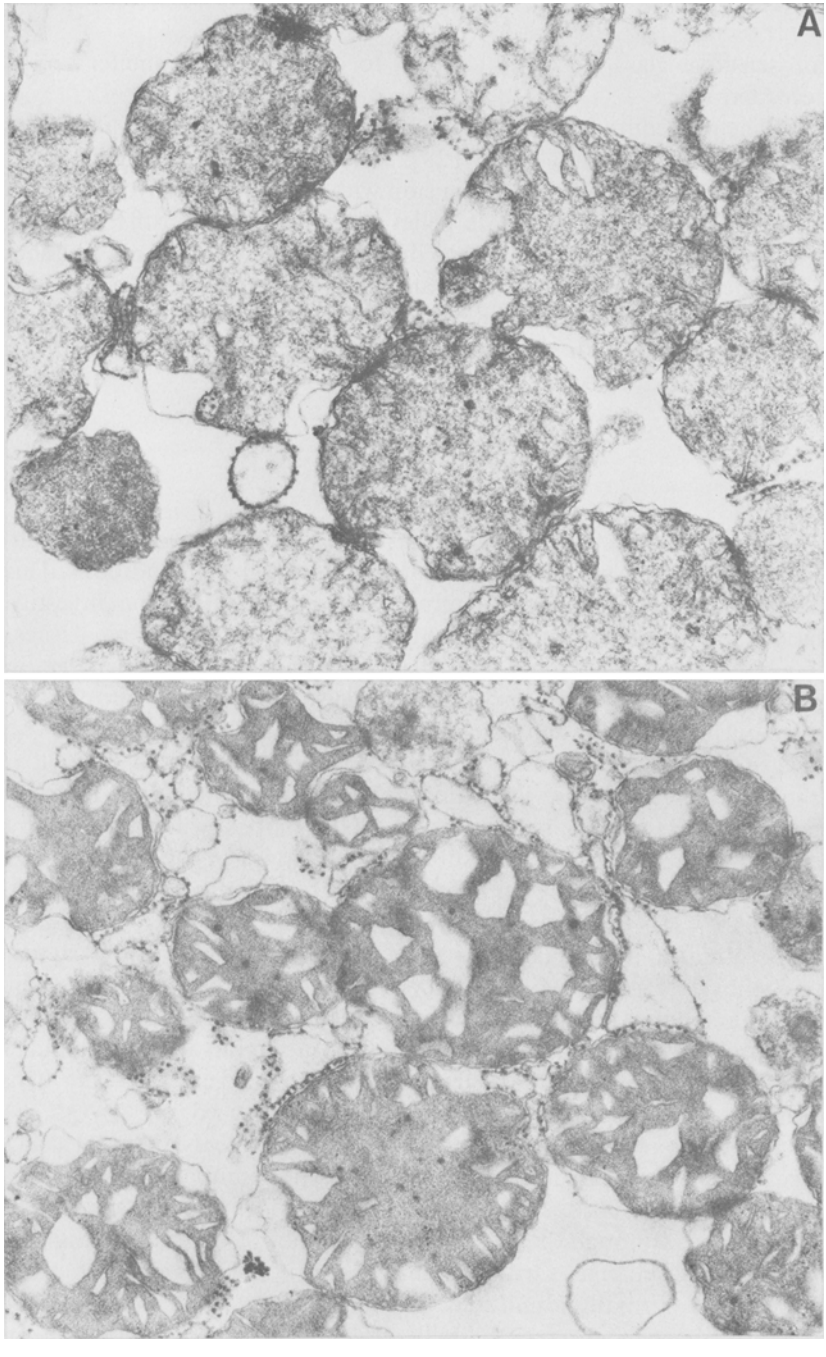
The procedure for electron microscopy was a slight modification of the method of Jasper and Bronk [28]. Except where noted in the text, the mitochondria were fixed in suspension with 3% glutaraldehyde for one hour and then centrifuged to form a pellet which was then postfixed for 1 hour with osmium tetroxide. In order to duplicate the conditions of Hackenbrock [1] some mitochondria were separated from the incubation medium prior to fixation by centrifugation for 30 sec in the microcentrifuge and then the pellet was treated with 3% glutaraldehyde for 1 hour. All fixation procedures were carried out at 0°C.

Results

Previous work [1–7, 11, 29, 30] has led to some confusion over which type of mitochondrial structure is associated with energizing conditions. This has been partly due to differences in terminology and to the fact that investigators have experimented with mitochondria isolated from different tissues and incubated them in a variety of media. Discrepancies in the interpretation of structural changes have also arisen because some investigators have not distinguished between mitochondria deenergized in different ways.

In this paper the term "energized" is only applied to isolated mitochondria when they are incubated in conditions which gives them a high potential for the phosphorylation of ADP; that is, when they have been incubated in the presence of substrate, inorganic phosphate, and oxygen (this is equivalent to the State 4 described by Chance and Williams [26]). In our view it is not particularly helpful to apply the term nonenergized (or deenergized) in an unqualified way to mitochondria with a low capacity to phosphorylate ADP. Mitochondria are obviously deenergized when the membrane potential has been completely collapsed (as will occur following additions of an uncoupler or a high concentration of valinomycin plus K^+), but those incubated with substrate, phosphate, oxygen, and ADP (Chance and Williams State 3) will be only partially deenergized.

A variety of terms have also been used to describe the different structural forms which can be assumed by a particular type of mitochondrion [e.g., 1, 4]. In this paper we will consider only three different forms: orthodox, condensed, and swollen, although the last two can obviously be qualified to indicate the extent of the change. Orthodox mitochondria are those which have the appearance normally found in sections of tissue fixed with glutaraldehyde [28]; the inner membrane is closely applied to the outer membrane, and in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cross-section the cristae appear as empty invaginations in which the two membranes are closely applied. Condensed mitochondria are those in which the inner compartment is reduced in volume compared with the outer compartment, so that there are obvious spaces opened up between the inner and outer membranes and between opposing cristal membranes; conversion from the orthodox to the condensed form need not cause a reduction in total mitochondrial volume. Swollen mitochondria are obviously those in which there has been an increase in total mitochondrial volume; they are also characterized by the expansion of the inner compartment to the point where few cristae can be seen. The detailed appearance of mitochondria in these different structural forms will depend on the source of the mitochondria: those with a larger area of inner membrane obviously have more cristae and tend to show a greater degree of condensation or swelling.

The Influence of Incubation Conditions on the Changes in Volume of the Matrix Compartment Under Energizing Conditions

In agreement with previous workers, we have found that freshly isolated rat liver mitochondria are in the condensed form when they are examined in the electron microscope after fixation in suspension with glutaraldehyde. As shown in Fig. 1A, expansion of the matrix was observed when the mitochondria were incubated for 15 min in the presence of 2.5 mM succinate, 11 mM phosphate, 6 mM $MgCl_2$, and 18 mM K^+. Under these conditions the matrix compartment expanded to give the mitochondria an orthodox appearance and there was little evidence of intermembrane spaces. Table I shows that, as expected, this structural change was associated with a decrease in absorbance. However, when the K^+ was replaced with 18 mM Na^+ or Tris the absorbance change during 15 min was reduced to less than one quarter, and Fig. 1B confirms that incubation under these conditions did not result in an appreciable expansion of the matrix since the mitochondria remain condensed in appearance. Thus, there were no large changes in matrix volume when K^+ was omitted, despite the fact that energization of the mitochondria occurred in the presence of Na^+, Tris, or K^+ (Table II). The addition of 5 mM ATP greatly reduced the absorbance change in the presence of either K^+ or Tris. It should be noted that the addition of the mitochondria

Figure 1. The influence of incubation conditions on changes in mitochondrial ultrastructure under energizing conditions. Before fixation in suspension the mitochondria were incubated at 28°C for 15 min in 3 ml of a medium containing 115 mM sucrose, 6 mM $MgCl_2$, 11 mM phosphate, 5 mM Tris succinate, and 1.7 mg ml^{-1} mitochondrial protein. A also contained 18 mM K^+, whereas B contained 18 mM Tris (× 60,000).

TABLE I. The Influence of the Composition of the Incubation Medium on the Absorbance of Mitochondria Incubated under Energizing Conditions[a]

Principal cation	Additions	Absorbance change at 510 nm after 15 min incubation
K^+ (18 mM)	—	-0.166 ± 0.007
Na^+ (18 mM)	—	-0.038 ± 0.002
Tris (18 mM)	—	-0.035 ± 0.005
K^+ (18 mM)	5 mM ATP	-0.041 ± 0.002
Tris (18 mM)	5 mM ATP	-0.005 ± 0.001

[a]All incubations contained 115 mM sucrose, 6 mM $MgCl_2$, 11 mM phosphate (pH 7.0), and 5 mM Tris succinate in addition to the constituents indicated in the table; no ADP added. The incubations were carried out at 28°C and contained 1.7 mg ml^{-1} of mitochondrial protein. The change in absorbance is given as the mean of five observations $\pm$ the standard error of the mean.

introduced a small amount of K^+ into the incubations in K^+-free media, since the mitochondrial suspension was found to contain about 42 μg ions of extramitochondrial K^+/g mitochondrial protein. Thus, for the K^+-free incubation reported in Tables I–III and in Figs. 1 and 2 the initial medium K^+ was 0.07 mM.

TABLE II. The Influence of Incubation Conditions on the Efficiency of Phosphorylation and the Rate of Respiration in the Presence of ADP[a]

Principal cation	Addition	Respiration rate in the presence of 250 μM ADP (μg atoms O min^{-1} ml^{-1})	ADP/O
K^+ (18 mM)	—	0.158 ± 0.008	1.80 ± 0.078
Na^+ (18 mM)	—	0.155 ± 0.008	1.76 ± 0.081
Tris (18 mM)	—	0.157 ± 0.008	1.81 ± 0.080
K^+ (18 mM)	5 mM ATP	0.162 ± 0.006	1.75 ± 0.076
Tris (18 mM)	5 mM ATP	0.167 ± 0.004	1.77 ± 0.080

[a]For the composition of the incubation medium see Table I. Each value is the mean of five observations $\pm$ the standard error of the mean.

TABLE III. The Uptake of K^+ and Phosphate by Mitochondria Incubated Under Energizing Conditions[a]

Principal cation	Addition	Mitochondrial K^+ (μg equivalents/g protein) after 1 min	Mitochondrial K^+ (μg equivalents/g protein) after 15 min	Mitochondria phosphate (μmol/g protein) after 1 min	Mitochondria phosphate (μmol/g protein) after 15 min
K^+ (18 mM)	—	83.2	152.8	31	44
Tris (18 mM)	—	61.2	64.0	34	40
K^+ (18 mM)	5 mM ATP	90.2	101.0	38	40

[a]See Materials and Methods section for details of the measurement of K^+ and phosphate. Incubation conditions given in Table I; no ADP added.

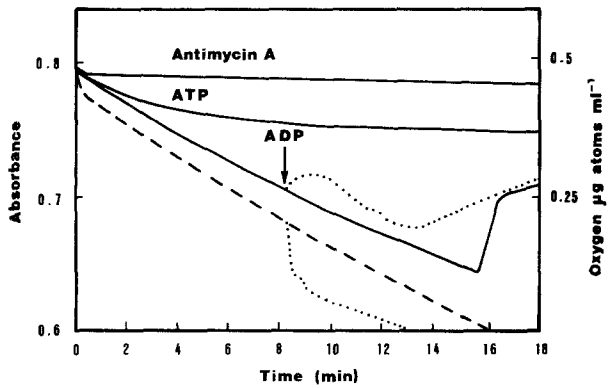


Figure 2. The time course of oxygen uptake and of changes in absorbance during incubation of mitochondria under energizing conditions. The solid lines show absorbance at 510 nm and the broken line shows the oxygen content of the medium. The incubation conditions are the same as those in Fig. 1 except that 2 μg antimycin A/mg mitochondrial protein was present in the incubation represented by the top trace and 5 mM ATP was present in the incubation represented by the second trace; when 250 μM ADP was added at the point indicated by the arrow, the dotted lines show the subsequent changes in oxygen uptake and absorbance where these differed from control incubations in the absence of ADP.

The Time Course of Respiration and Absorbance Changes

Figure 2 shows that the rate of oxygen consumption and change in absorbance are nearly linear when mitochondria are incubated in the K^+ medium. This illustration also shows the absorbance changes in the presence of antimycin A and ATP. The inhibition of respiration by antimycin A prevented the absorbance change completely and the addition of 5 mM ATP was strongly inhibitory, especially after the first 5 min. Both of these inhibitory effects were confirmed by electron microscopy and in both cases the mitochondria remained in a condensed state after a 15 min incubation (data not shown).

The Influence of ADP Addition on the Structure of Energized Mitochondria

Figure 2 shows that the addition of 250 μM ADP after 8 min of incubation under energizing conditions produces a small increase in absorbance as well as the expected stimulation of respiration. Following the phosphorylation of the ADP the rate of respiration decreases and a steady decrease in absorbance is reestablished. After the system has become anaerobic the absorbance begins a steady increase. The change in absorbance on ADP addition suggested that there was a small decrease in matrix volume and we have also examined this point with the electron microscope. Figure 3A shows mitochondria which had been incubated for 15 min in the presence of succinate, phosphate, and K^+, and for a further 30 sec after ADP had been added. If the ADP had no effect the mitochondria should be identical with those shown in Fig. 1A, but comparison of the two figures indicates that some slight shrinkage of the matrix may have occurred. This result is at variance with the observations of Hackenbrock [1] who has reported that mitochondria become fully condensed immediately following ADP addition. However, in the experiments of Hackenbrock, the mitochondria were centrifuged out of suspension before the addition of fixative. When we repeated the treatment used to produce the micrograph shown in Fig. 3A but fixed the mitochondria in a pellet following a 30-sec centrifugation, we obtained the structure shown in Fig. 3B. The mitochondrial profiles in this micrograph are highly condensed and resemble those published by Hackenbrock after ADP addition. Consequently we conclude that the addition of ADP to energized mitochondria probably causes some shrinkage of the matrix, but this change is greatly increased if the mitochondria are spun down prior to fixation.

When ADP was added to mitochondria incubated for 15 min in the K^+ medium with added ATP there was no appreciable absorbance change. Also, when ADP was added to mitochondria incubated for 15 min in the Na^+ or

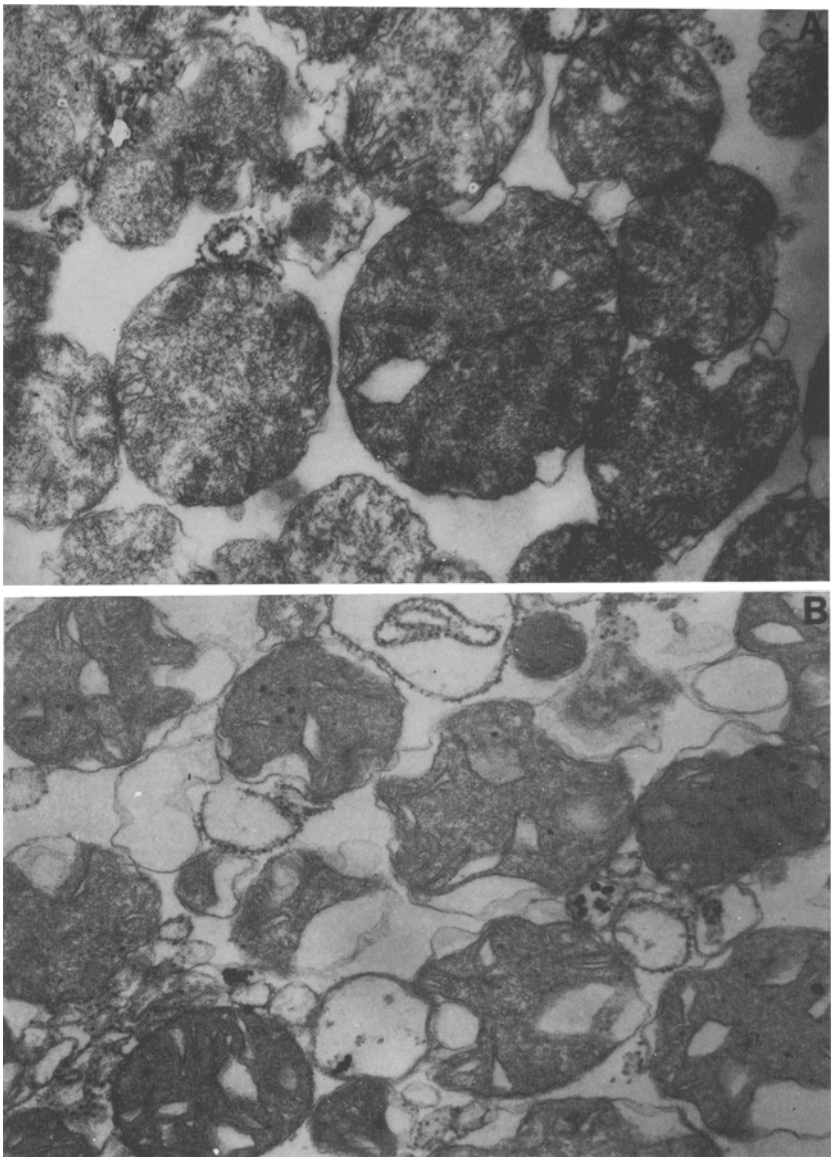


Figure 3. Changes in mitochondrial structure following the initiation of oxidative phosphorylation by the addition of ADP. The micrographs show mitochondria which had been incubated for 15 min under energizing conditions (see Fig. 1) and then fixed after incubation for a further 30 sec after 250 μM ADP was added. A, mitochondria fixed in suspension, B, mitochondria fixed as a pellet after 30 sec centrifugation ($\times$45,000).

Tris media there was only a very small increase in absorbance followed by a return to the original rate of absorbance decrease when the phosphorylation period was completed. Under these conditions (i.e., in the presence of ATP or in the Na^+ or Tris media) the structural changes were so small that no clear differences in the micrographs could be detected before or after ADP addition, and in both cases the mitochondria were in the condensed configuration.

Phosphorylation Efficiency in Relation to Structural Changes

Table II shows the respiration rates and the ADP/O ratios obtained after incubating mitochondria in K^+, Na^+, and Tris media. It is clear that although the nature of the cation in the medium affects the absorbance change, it does not alter the phosphorylation efficiency.

Phosphate and K^+ Uptake by Energized Mitochondria

Our observation that the changes in the composition of the incubation medium can affect the extent to which the mitochondrial matrix expands under energizing conditions suggested that mitochondria energized in the K^+ medium may accumulate ions more readily than in the other media. Table III gives measurements of the K^+ and phosphate content of mitochondria incubated with succinate and phosphate in the various media. As expected, the mitochondrial K^+ only increased following incubation in the K^+ medium and this increase was largely prevented by the addition of 5 mM ATP. Some increase in the phosphate content of mitochondria occurred in all the media tested but this was most marked in the K^+ medium in the absence of added ATP.

Table II shows that in the presence of ADP the respiration rate in the K^+-containing medium was no greater than that in the Na^+ or Tris media, but in the absence of ADP we found that mitochondria energized in the K^+ medium consumed 0.028 μg atoms O min^{-1} ml^{-1}, whereas those energized in the Tris medium consumed 0.025 μg atoms O min^{-1} ml^{-1}. If we assume that this 10% difference is due solely to the extra K^+ accumulation in the former case, then we can use the data in Table III to calculate an approximate K^+/H^+ ratio for K^+ uptake over 15 min as follows: after 15 min the difference in K^+ content in the two media was 89 μg ions g^{-1} mitochondrial protein; since the incubation medium contained 1.7 mg mitochondrial protein ml^{-1} this corresponds to an extra K^+ uptake of 0.151 μg ions K^+ ml^{-1} in 15 min or 0.010 μg ions min^{-1} ml^{-1} and the difference in respiration (0.003 μg atoms O min^{-1} ml^{-1}) would generate 0.012 μg ions H^+ min^{-1} ml^{-1} if it is

assumed that 4 protons are translocated across the inner membrane per oxygen consumed with succinate as substrate [31, 32]. This gives a K^+/H^+ ratio of 0.83, which agrees fairly well with previous estimates [33] of about one.

Structural Changes, Respiration, and Phosphorylation Efficiency in the Presence of Valinomycin

In view of the association between K^+ movements and mitochondrial structural changes suggested by the observations reported above, we examined the effects of increasing the K^+ permeability of the inner membrane with valinomycin. Table IV shows, as expected, that for mitochondria incubated in a Tris phosphate medium there was virtually no matrix expansion in 3 min despite an initial K^+ concentration in the medium of 0.1 mM, which was the concentration produced by the extramitochondrial K^+ associated with the 2.4 mg ml^{-1} mitochondrial protein added. However, valinomycin at a concentration of 75 μg/g mitochondrial protein increased the rate of mitochondrial respiration in the absence of ADP by about 50% and increased the absorbance change in 3 min by a factor of 4. Experiments with the K^+-sensitive electrode showed that this decrease in absorbance in the presence of valinomycin was associated with the uptake of virtually all

TABLE IV. The Influence of Valinomycin Addition on the Respiration, Phosphorylation Efficiency, and Absorbance of Mitochondrial Suspension[a]

Additions	[K+] (mM)	Respiration rate (μg atoms O min^{-1} ml^{-1}) −ADP	Respiration rate (μg atoms O min^{-1} ml^{-1}) +ADP	ADP/O	Δ Absorbance at 510 nm (over 3 min without ADP)
None	0.10	0.025	0.157	1.75	0.013
75 μg valinomycin/g protein	0.10	0.036	0.171	1.76	0.05
75 μg valinomycin/g protein	0.40	0.049	0.178	1.74	0.19

[a]Reaction medium contained 115 mM sucrose, 11 mM Tris phosphate (pH 7.0), 2.5 mM Tris succinate, 6 mM $MgCl_2$. Final volume 3 ml; 28°C; 250 μM ADP added after 3 min. The mitochondrial suspension was added to give a final concentration of mitochondrial protein of 2.4 mg/ml and this gave a medium K^+ concentration of 0.10 mM; for the last line of the table 0.3 mM KCl was added.

the K^+ in the medium. Table IV also shows that raising the K^+ concentration in the medium to 0.4 mM gave a further increase in the rate of respiration in the absence of ADP and again quadrupled the absorbance change. Neither the addition of this amount of valinomycin on its own or with 0.4 mM K^+ caused any change in the efficiency of phosphorylation. When this quantity of valinomycin was added to mitochondria incubated with 18 mM K^+ the same total absorbance change was observed but it was complete within 3 sec rather than 3 min (data not shown). This rapid matrical swelling was followed by an equally rapid shrinkage setting up a train of damped volume oscillations similar to those which have been observed by others [34–36].

The Time Course of Changes in the Respiration, K^+ Content, and Structure of Mitochondria Incubated Under Energizing Conditions in the Presence of Valinomycin

Figure 4 shows the time course of the absorbance change in the presence of valinomycin and 0.4 mM K^+, and the electron micrograph in Fig. 5A indicates that mitochondria fixed after the incubation had proceeded for 3 min were orthodox in appearance. The low initial K^+ concentration made it easier to obtain accurate measurements of the K^+ content of the mitochondria by flame photometry. These measurements show that the K^+ content of the mitochondria increased during the 3 min incubation in a manner which reflected the decrease in absorbance. The low medium K^+ concentration also allowed us to follow the changes in medium K^+ with the K^+-sensitive electrode. Although these data are not shown, they confirmed the shape of the K^+ uptake curve given in Fig. 4. The rate of respiration remained nearly constant during the first 3 min of incubation although there was a slight tendency for the rate to decrease with time.

The Time Course of Changes in Mitochondrial Respiration, K^+ Content, and Structure During a Brief Period of Phosphorylation in the Presence of Valinomycin

Figure 4 also shows the effects of adding 250 μM ADP. There was the expected rapid burst of respiration during phosphorylation of the ADP, and this was associated with a sharp but transitory increase in absorbance. Measurements of the K^+ content of the mitochondria indicated that the absorbance increase was associated with a marked decrease in the matrix K^+, and although the data are not shown, this was associated with an increase in medium K^+ as estimated by the K^+-sensitive electrode. The electron

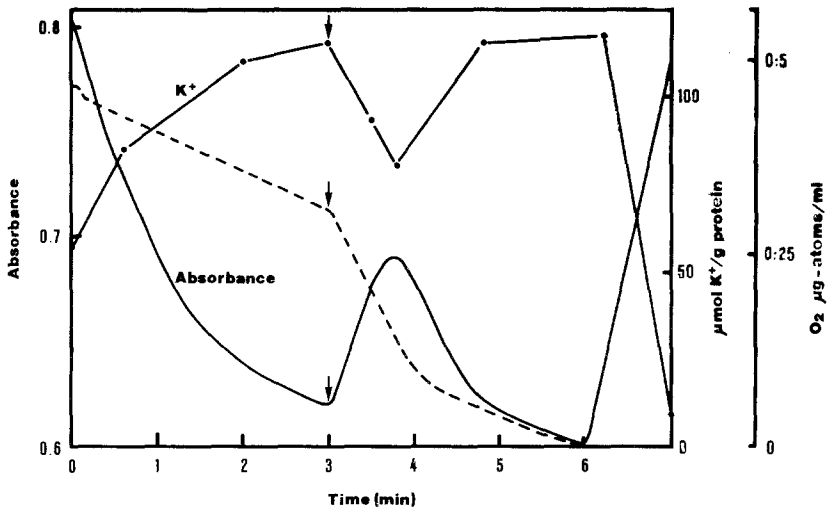


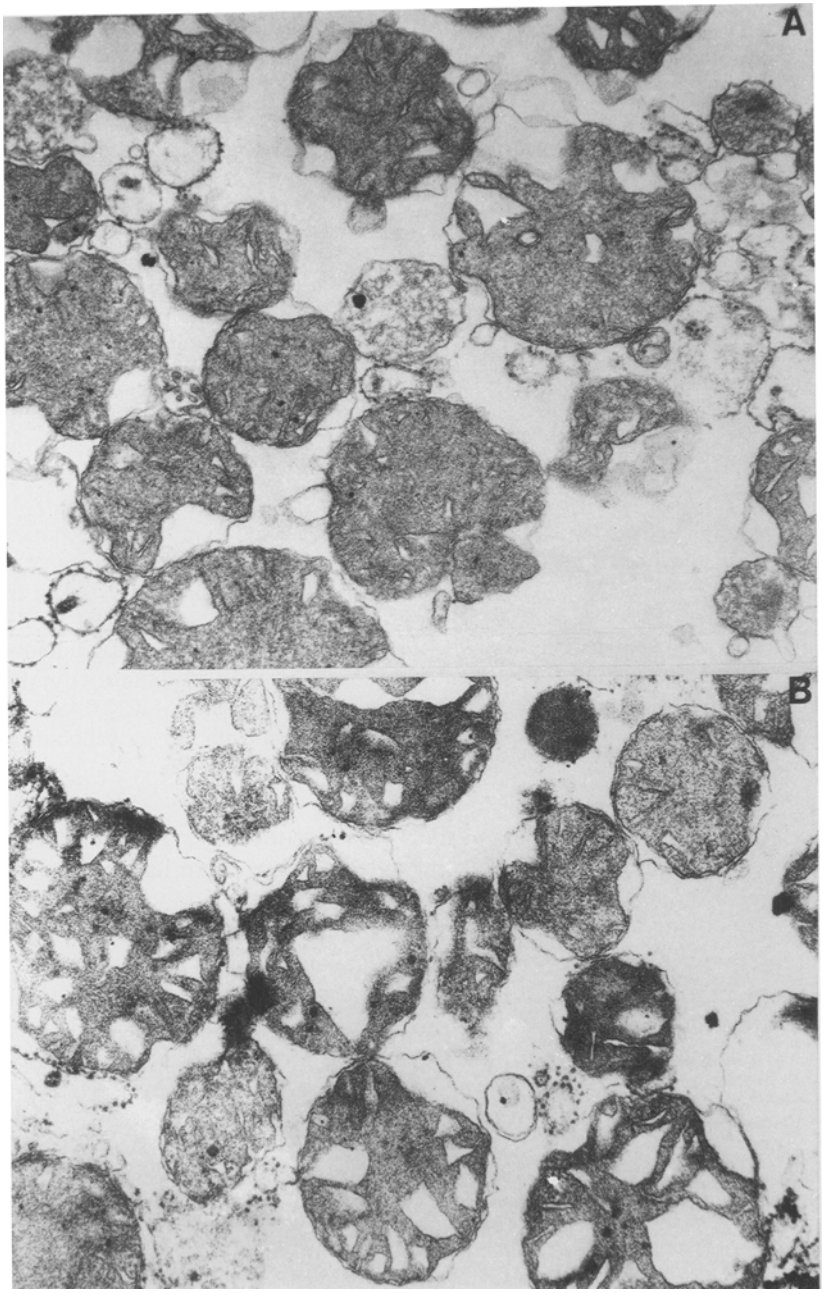
Figure 4. The time course of changes in absorbance, respiration, and K^+ content of mitochondria incubated in the presence of valinomycin. The oxygen content and absorbance of the medium at 510 nm are shown by continuous curves taken from recorder traces. The filled circles show measurements of the K^+ content by flame photometry on 0.5-ml samples of mitochondria taken at the time indicated and separated from the medium by a 20-sec centrifugation. Incubation conditions: 11 mM Tris phosphate (pH 7.0), 115 mM sucrose, 2.5 mM Tris succinate, 6 mM $MgCl_2$, 0.4 mM K^+, 2.4 mg ml^{-1} mitochondrial protein, 28°C, final volume 3 ml; 75 μg valinomycin/g protein was added initially and 250 μM ADP was added after 3 min.

micrograph in Fig. 5B shows that when mitochondria were fixed in suspension at the peak of the absorbance increase there had been a considerable shrinkage of the matrix compartment.

At the conclusion of the period of phosphorylation the respiration decreased to the rate prior to ADP addition. The changes in absorbance and K^+ content indicated that this return to a slow respiratory rate was associated with an increase in matrix volume and the recovery of the K^+ lost to the medium following ADP addition. When these mitochondria were examined in the electron microscope it was clear that they had reverted to the orthodox configuration shown in Fig. 5A.

The Influence of pH on the Changes in Matrix Volume with Valinomycin Present

The decrease in absorbance which occurs under energizing conditions in the presence of valinomycin plus 0.4 mM K^+ and the increase that occurs on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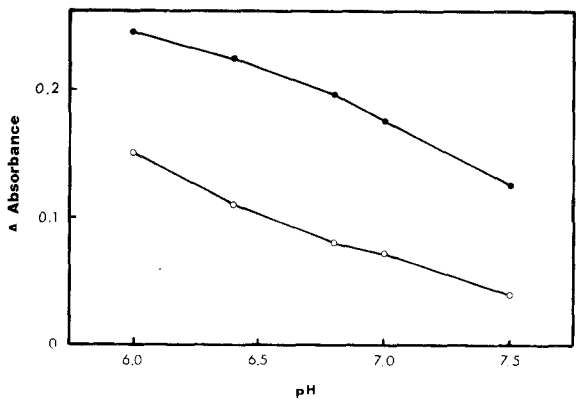

Figure 6. The influence of pH on the absorbance changes that occur during incubation of mitochondria in the presence of valinomycin. Incubation conditions as in Fig. 4 except that the pH of the Tris phosphate was adjusted to the value shown on the horizontal axis; initial $[K^+]$ held constant at 0.4 mM. The filled circles show the decrease in absorbance at 510 nm after incubation for 3 min under energizing conditions; the open circles show the subsequent increase in absorbance in the 1 min following the addition of 250 μM ADP.

subsequent ADP additions are both influenced by the pH of the incubation medium. In Fig. 6 the total absorbance decrease occuring in 3 min under energizing conditions and the total increase occuring during a further 1-min incubation in the presence of ADP are plotted as a function of pH. These results show that over the region from pH 6.0 to 7.5 both volume changes decrease in size as the pH is increased. The phosphorylation efficiency did not change significantly over this pH range.

The Influence of Anaerobiosis on Mitochondrial Structure

The absorbance changes shown in Figs. 2 and 4 suggested that the matrix compartment may shrink when the incubation medium becomes anaerobic. This is confirmed in Fig. 7, which shows an electron micrograph of mitochondria fixed in suspension after incubation for 18 min in the K^+ medium

Figure 5. Mitochondrial structural changes during oxidative phosphorylation in the presence of valinomycin. The incubation conditions were identical with those for Fig. 4. A, mitochondria fixed after 3 min incubation under energizing conditions and before the addition of ADP. B, mitochondria fixed approximately 1 min after the addition of 250 μM ADP at the peak of absorbance increase shown in Fig. 4 ($\times$ 60,000).

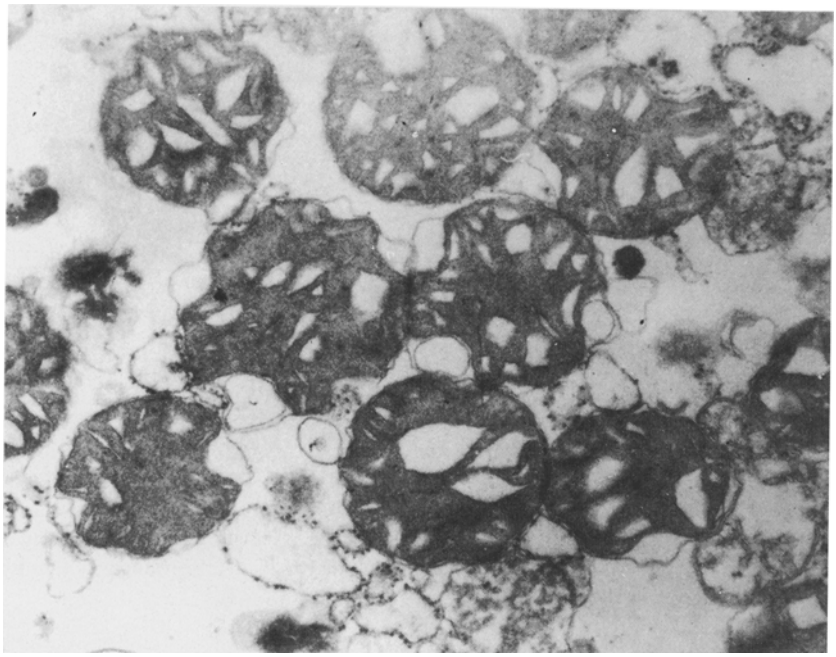

Figure 7. The influence of anaerobiosis on mitochondrial structure. Incubation conditions were the same as those in Fig. 1 except that the incubation was continued for 18 min, or 2 min after the suspension had become anaerobic. The mitochondria were fixed in suspension ($\times$ 45,000).

in the absence of nucleotide addition and without valinomycin present. As indicated in Fig. 2 mitochondria incubated for 18 min under these conditions have been anaerobic for approximately 2 min, and Fig. 7 shows that this causes the mitochondrial matrix to become highly condensed. Identical changes were observed following the addition of Antimycin A to mitochondria after a 15-min incubation in the K^+ medium.

Figure 4 shows that when mitochondria which were incubated with valinomycin and 0.4 mM K^+ became anaerobic after 6 min there was a rapid increase in absorbance which was associated with the loss of most of the K^+ from the matrix. Mitochondria fixed 1 min after the onset of anaerobiosis proved to be even more highly condensed than those shown in Fig. 7, indicating that conversion to the condensed state under anaerobic conditions occured more rapidly in the presence of the ionophore.

Discussion

The data presented in this paper confirm the observations made by Hackenbrock [1, 2] and by Green and his associates [4–7] that freshly isolated mitochondria have a condensed matrix which expands under energizing conditions in the presence of phosphate and contracts again when the mitochondria are deenergized. However, our studies show clearly for the first time that the energized expansion of the matrix of liver mitochondria will not occur in the absence of K^+ and that the increase in volume of the inner compartment is associated with the uptake of K^+. Expansion of the inner compartment is also associated with phosphate uptake, as shown by Green et al. [4, 5], but our data indicate that insufficient extra phosphate is accumulated to balance the K^+ uptake suggesting, as shown by others, that the accumulation of other divalent anions such as succinate also takes place [14].

We have shown that the conversion from the orthodox state with its expanded matrix back to the condensed configuration does not occur instantaneously and a change to the fully condensed state requires the complete deenergization of the mitochondria as occurs under anaerobic conditions. The partial deenergization following ADP addition produces a smaller shrinkage of the matrix unless fixation is delayed until the mitochondria have been centrifuged from the incubation medium. It seems likely that the more complete condensation that occurs in the latter case is due to the fact that the mitochondria become anaerobic before fixation when this procedure is used.

Our data demonstrate that at constant osmolarity the structure of the isolated mitochondria is influenced by whether or not the mitochondria are energized. However the expansion of the matrix is dependent on the presence of cations and anions that can be accumulated in the inner compartment by an energy-dependent process. It is also clear that the phosphorylation efficiency of the mitochondria is not significantly affected by whether or not the structural changes occur. For example, the addition of 5 mM ATP is sufficient to prevent the structural change even in the K^+ medium although it does not alter the ADP/O ratio. In fact we found that when the mitochondria were incubated for extended periods (more than 30 minutes) in the absence of added ADP or ATP, the coupling (as reflected by respiratory control and phosphorylation efficiency) deteriorated, whereas in the presence of 5 mM ATP tight coupling was maintained and at the same time the respiration and matrix swelling under energizing conditions (State 4) were inhibited (data not included).

Valinomycin acts solely to increase the permeability of the mito-

chondrial inner membrane to K^+ [37–39] and in this paper we show that one result of this change is to increase the rate at which mitochondria can be converted from their initial condensed state to the orthodox state under energizing conditions. The time required for the full condensed to orthodox transition depends on the amounts of valinomycin and K^+ added, and in the presence of 75 μg valinomycin/g of mitochondrial protein and 0.4 mM K^+, we find that the transition is complete in 3 min. The use of low medium concentrations of K^+ in the presence of valinomycin have enabled us to show that the decrease in absorbance, which represents an increase in the volume of the matrix compartment, is quantitatively associated with the accumulation of K^+. Direct measurement on a large number of mitochondrial profiles has shown that in our condensed mitochondria the matrix compartment occupies about 60% of the total mitochondrial volume. When the mitochondria are converted to the orthodox configuration the matrix compartment expands to occupy 96% of the total volume without any increase in mitochondrial size. Values for the water content of the inner compartment of mitochondria vary considerably [40–42] but the highest generally accepted figure would appear to be that obtained by Harris and van Dam [41] who found an average value of 0.7 g water/g mitochondrial protein by measuring the sucrose-inaccessible space. If we assume an internal osmolarity of 0.25 [23], then the 60% increase in matrix volume associated with the complete condensed to orthodox conversion would require the uptake of 35 μmol of the K^+ salt of a divalent anion (i.e., 70 μg ions of K^+) per g mitochondrial protein. From the data in Fig. 4, the K^+ uptake during the 3-min period of energization was 64 μg ions/g mitochondrial protein. Thus, in the presence of valinomycin, we can account fully for the condensed to orthodox transition in terms of the uptake of K^+ and an equivalent amount of divalent anion. Although the relatively high medium K^+ levels necessary to observe structural changes in the absence of valinomycin made estimates of K^+ uptake under these conditions less reliable, it is interesting to note from the data in Table III that comparable amounts of K^+ were taken up during 15 min energization in the presence of succinate and 18 mM K^+ phosphate. The similarity of K^+ uptake in the presence and absence of valinomycin indicates that the ionophore only alters the rate at which the structural changes occur under energizing conditions but does not alter the nature of cation movement involved.

However, the presence of valinomycin does alter the mitochondrial structural changes that follow the initiation of a rapid period of phosphorylation with ADP since there is a partial reversion of the matrix structure to the condensed form and the release of some K^+. At the completion of the rapid phase of respiration the matrix again expands to the fully orthodox condition

and the lost K^+ is reaccumulated. From the data in Fig. 4 it can be calculated that the ratio of K^+ efflux to ADP phosphorylated is about 0.4. Assuming from the previous calculation that the total water in the mitochondrial matrix had increased to 1.1 g H_2O/g mitochondrial protein in changing to the orthodox form, the measured loss of K^+ plus an equivalent amount of divalent anion should give a reduction in matrix volume of 24%. This value corresponds fairly closely to the 20% decrease in absorbance. The data in Fig. 4 for K^+ efflux following ADP addition may be an overestimate since the mitochondria had to be centrifuged from the medium, and we have shown that in the presence of ADP this procedure results in an increase in matrical shrinkage. However the general pattern of K^+ efflux on ADP addition was verified by the increase in medium K^+ estimated with the K^+-sensitive electrode.

The matrical shrinkage during phosphorylation in the presence of valinomycin probably reflects the decreased membrane potential [43] which has been shown to occur under these conditions by Mitchell and Moyle [44]. One explanation for the K^+ efflux is that it could partially compensate for the electrogenic exchange of external ADP^{3-} for internal ATP^{4-} [45]. This compensatory movement of K^+ could lead to a decrease in matrix volume if there were an equivalent loss of matrical phosphate and this could occur if the matrix phosphate required to form ATP from ADP was not completely balanced by extra phosphate uptake. In the absence of valinomycin there is evidence that the exchange will be largely compensated by H^+ cotransport with ATP^{4-} [40].

The pH dependence of the changes in matrical volume in the presence of valinomycin (Fig. 6) indicates that the changes are enhanced by increasing the Δ pH across the mitochondrial inner membrane. It is widely accepted that anion transport is controlled by the Δ pH [46–48] and our data therefore suggest that with valinomycin present the K^+ permeability of the inner membrane is no longer limiting so that the entry and exit of phosphate govern the extent of the shift in matrix volume.

Our data are consistent with previous work on mitochondrial structural changes with the exception of the study by Hackenbrock et al. [23] in which it was claimed that the structural changes were not associated with ion movement. However, the data presented by these authors shows that expansion of the matrix under energizing conditions is associated with some ion uptake after an initial rapid loss. They point out that the addition of ADP caused a reversion to the condensed state without any compensating loss of ions but our results make it likely that the structural change after ADP addition was exaggerated as a result of fixation after the mitochondria were spun out of the incubation medium.

Our results support previous observations that mitochondrial structural changes can follow the energization–deenergization cycle in isolated mitochondria but add the essential qualification that appropriate ions such as K^+ and phosphate must be present. Under some conditions other cations should be able to substitute for, or accompany, K^+ uptake in the energized expansion of the matrix [49–51]. However, our results indicate that 6 mM Mg^{2+} was unable to support significant matrix swelling in the absence of K^+. This is in agreement with other reports indicating that Mg^{2+} can be accumulated and released in energy-dependent reactions by heart mitochondria, although it is not accumulated to any great extent by liver mitochondria [36–40]. It should be stressed that our observations indicate that ultrastructural changes visible in the electron microscope are dependent on appropriate ion movements and do not play a direct role in the energy-coupling process, but this conclusion does not in itself provide a basis for favoring either the chemiosmotic or conformational model of energy coupling.

The results reported in this paper indicate that the mitochondrial inner membrane is normally poorly permeable to K^+ salts. Increasing this permeability within limits by the addition of valinomycin does not reduce the efficiency of coupled ATP synthesis, although it is important to recognize that the increases in the respiration rate in the absence of ADP means that the respiratory control ratios will be reduced.

There have been a number of reports of rapid structural changes in the mitochondria within intact cells [28, 56, 57] and this work suggests the existence of natural ionophores which could increase the K^+ permeability of mitochondria in situ. Furthermore, the high intracellular K^+ concentration could give rise to sizeable K^+ movement on energization or deenergization of mitochondria even if the mitochondrial inner membrane remains relatively impermeable to K^+ in vivo.

Note Added in Proof

Since this paper was submitted, a paper has been published from this laboratory (R. D. A. Lang and J. R. Bronk, *J. Cell Biol.* **77**, 134–147, 1978) describing a spray-freeze-etching study of the time course of the shrinkage of the mitochondria matrix following ADP addition. These results indicate that matrical shrinkage occurs more rapidly than the associated decrease in absorbance, although they confirm the observations made in this paper that valinomycin increases both the amount of shrinkage and the rate at which it occurs.

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