A STUDY OF RAPID MITOCHONDRIAL STRUCTURAL CHANGES IN VITRO BY SPRAY-FREEZE-ETCHING

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ABSTRACT

The spray-freeze-etching technique has been used to study energy-linked mitochondrial structural changes in rat liver mitochondria incubated in vitro. The technique involves spraying the suspension of mitochondria into liquid propane at −190°C, and does not require the use of cryoprotectants or chemical fixatives. The results confirmed that freshly isolated mitochondria have a condensed matrix and that this expands at the expense of the outer compartment to give the orthodox configuration when the mitochondria are incubated in a K^+ medium in the presence of substrate and phosphate. Addition of adenosine diphosphate (ADP) caused a rapid shrinkage of the matrix compartment, and the time-course and extent of this shrinkage has been measured quantitatively by coupling a rapid sampling device to the spray-freezing apparatus. These data show that for orthodox mitochondria the onset of phosphorylation is accompanied by a reduction of 30% in the matrix volume in 20 s, and there is no evidence that the decrease in matrical volume affects the phosphorylation efficiency. These results suggest that natural ionophores in the mitochondrial inner membrane make it permeable enough to permit a rapid readjustment of matrix volume after the addition of ADP, and that the associated ion movement does not cause uncoupling of oxidative phosphorylation.

KEY WORDS spray-freeze-etching · mitochondrial structural changes · mitochondria · oxidative phosphorylation · valinomycin effects · mitochondrial ion movements

Energy-linked mitochondrial structural changes occurring in vitro were first examined in the electron microscope by Hackenbrock (7, 8) and Green et al. (6). Following the work of Packer et al. (23), there has been fairly general acceptance that there is a qualitative correlation between changes in the volume of the mitochondrial matrix observed with the electron microscope and changes in light scattering by mitochondrial suspensions. More recently, Hackenbrock (9, 10) and others (28, 29) have used standard freeze-etching techniques to show that the volume of the mitochondrial matrix can vary as a consequence of alterations in the extent to which the mitochondria are energized. However, these results were obtained in the presence of relatively high concentrations of cryoprotectants to prevent ice crystal damage on freezing, and Vail et al. (29) found that glutaraldehyde fixation was necessary to prevent structural damage caused by glycerol when it was used as a cryoprotectant. Hochli and Hackenbrock (11) claim that, if properly used, glycerol will not affect phosphorylation efficiency or damage mitochondrial structure, but the latter point has been disputed by Neidermeyer and Moor (22).

The development of spray-freeze-etching by Bachmann and Schmitt (1) in 1971 suggested that it might be possible to examine changes in mitochondrial structure by spray-freezing without the use of fixatives or cryoprotectants. This new tech-

nique also made it potentially possible to follow the time course of rapid changes in mitochondrial structure after an alteration in incubation conditions. The results we report in this paper confirm that energy-linked changes in the volume of the mitochondrial matrix do occur under appropriate conditions, although the extent of the changes did not influence the efficiency of oxidative phosphorylation. Furthermore, the spray-freezing method has enabled us to show that the addition of adenosine diphosphate (ADP) to mitochondria energized by the presence of substrate and phosphate leads to a shrinkage of the mitochondrial matrix that is much more rapid than is suggested by simultaneous absorbance measurements. The speed and magnitude of this shrinkage have important implications for the permeability properties of the mitochondrial inner membrane.

MATERIALS AND METHODS

Liver mitochondria were isolated from male Wistar rats according to Kielley and Kielley's modification (15) of Schneider's method (25) using unbuffered 250 mM sucrose as the isolation medium. Mitochondrial protein was determined by a biuret method (17).

Incubations (final vol 3 ml) were performed in silica cells of 1-cm path length in a Shimadzu MPS 50 L recording spectrophotometer (Shimadzu Sersakusho Ltd., Kyoto, Japan). Mitochondrial volume changes during incubations were followed by measuring the absorbance at 520 nm. The cuvette chamber included a constant temperature jacket and a magnetic stirrer, and was modified to allow an oxygen electrode (5) to be placed in the top of the cell without affecting the light path. Oxygen consumption rates were followed polarographically (13, 14) by recording changes in oxygen tension during the incubations. The phosphorylation efficiency (ADP/O ratio) was estimated according to the method of Chance and Williams (4). Details of incubation media and additions are given in the legends for the figures and tables.

Samples were withdrawn from the incubation cell and spray-frozen (Bachmann and Schmitt [1], Bachmann and Schmitt-Fumian [2]) using apparatus constructed in this laboratory (Lang et al. [16]).

Spray-frozen specimens were freeze-fractured at -115°C and $1-2 \times 10^{-6}$ torr in an N.G.N. FE600 freeze-etch machine (N. G. N. Limited, Accrington, Lancashire, England) (Robards and Parish (24)). In some cases, fractured specimens were exposed to the vacuum for 1 min at -100°C (etching) before shadowing. Replicas were cleaned in solutions of sodium hypochlorite or chromic acid, washed in distilled water, and mounted on uncoated 400-mesh copper grids. Grids were examined in a Hitachi (Perkin Elmer) HU 12 A electron microscope operated at 75 kV. Images were recorded on Kodak 4489 plates which were developed in D-19 developer. Micrographs have been printed as positives with the direction of shadowing from below so that shadows appear white and point upwards.

Rapid Sampling Method for Measurement of the Time-Course of Mitochondrial Structural Changes

Samples were withdrawn from the incubation cell through Portex tubing (0.65 mm internal diameter) using a proportioning pump, and were delivered directly to the spray gun for immediate spray-freezing. Fig. 1 shows the arrangement of the apparatus.

Air was introduced into the sampling line (*a*) from lines (*b*) and (*c*), and this was mixed with the sample at points (*d*) and (*e*) to prevent it from becoming anaerobic. The minimum time between withdrawing samples from the incubation cell and freezing them was 5 s. The spray gun was held in a clamp above the freezing block containing liquid propane, and the compressed air supply was turned on before the samples were introduced into the spray gun. Thus, samples were sprayed as soon as they entered the spray gun, and there was no accumulation of sample in the spray gun reservoir. Only small samples (<0.1 ml) were sprayed in these experiments so that the time between spraying the first and last parts of each sample as it left line (*a*) was approximately 0.5 s.

To determine the effects of spraying on mitochondria, samples were sprayed into respiratory medium and then incubated with glutamate as substrate. Table I shows that spraying produced a small decrease in respiration in the presence of ADP, but the sprayed mitochondria retained high acceptor control and ADP/O ratios.

To examine short incubation periods, ADP, uncouplers, or inhibitors could be added through line (*c*). This allowed mitochondria to be incubated in the presence of these reagents for an accurately timed period between 2 and 10 s before freezing. The time of the incubation depended on the length (X) of sampling line which was used. The reagents, dissolved in standard incubation media, were added at concentrations which were adjusted to give the final concentrations reported in the Results section after they had been mixed with the mitochondrial samples in line (*a*). For incubation periods of more than 10 s, all reagents were added to the incubation cell for an appropriate period before switching on the sampler.

Before spray-freezing, the spray gun was placed in a beaker containing double-distilled water in the bath used to maintain the temperature of the incubation cell in the spectrophotometer. This miminized any changes in the temperature of the samples which might have occurred immediately before they were rapidly frozen.

Most of the micrographs in this paper show mitochondria sampled from incubations, and consequently, the spray-frozen droplets contained many areas with a low

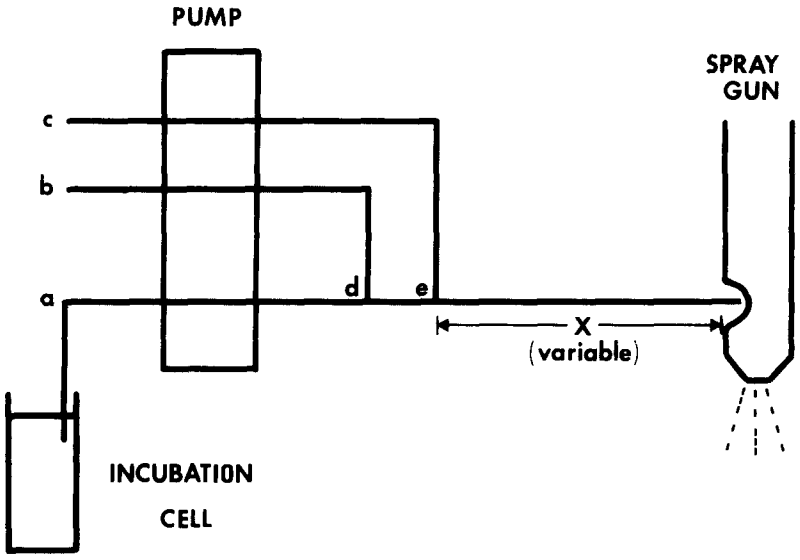

FIGURE 1 Schematic diagram of the rapid sampling apparatus.

density of mitochondria. To show as many profiles as possible, areas containing several mitochondria were chosen for the figures, but the organelles in these areas did not differ in appearance from those which were more widely spaced.

Measurement of Relative Matrical Volumes

Quantitative measurements of changes in mitochondrial matrical volume were made on freeze-etch replicas of samples removed from the incubation cell by the rapid sampling method. Micrographs showing cross-fractured mitochondria were covered with a transparency ruled in 1-mm squares, and the cross-sectional area of the matrix compartments was obtained by counting the number of squares within the matrix of each profile. These data were used to estimate the matrix volume.

The micrographs of spray-freeze-fractured (or etched) mitochondria were printed from a number of negatives so that the final magnification was always × 100,000. Only the largest mitochondrial profiles in any given micrograph were used to estimate the matrical volume because it was assumed that in such cases the fracture plane had passed through or near the center of the mitochondria. The procedure of estimating the matrical volume from the cross-sectional area is reasonably accurate for orthodox mitochondria as the matrix is approximately spherical. However, in condensed mitochondria the shape of the matrix is highly irregular so that the cross-sectional area of any single profile would give an erroneous estimate of the matrical volume. Errors from this cause were minimized by making measurements on at least 40 mitochondrial profiles from each configurational state.

For the purposes of comparison, the estimates of matrical volume were expressed in relation to the matrix volume of fully orthodox mitochondria which was taken to be 100. Thus, the relative matrical volume under any particular conditions is the percentage of the mean orthodox volume. The vertical bars shown in Figs. 6 and 8 give twice the standard error of the mean of at least 40 measurements for each point.

RESULTS

The Structure of Freshly Isolated Rat Liver Mitochondria

Samples of freshly isolated rat liver mitochondria were spray-frozen at −190°C and then fractured at −115°C. Fig.2 shows a typical mitochondrial profile in which the matrix compartment is highly condensed and the outer compartment is greatly enlarged. In samples of freshly isolated mitochondria, all of the profiles revealed by spray-freezing-fracturing show a condensed matrix, and measurements on a large number of profiles gave a mean value for the matrical volume of 53% of the mean value of the matrical volume in orthodox mitochondria.

Mitochondrial Structure After Incubation Under Energizing Conditions

Mitochondria incubated in a medium containing 5 mM $MgCl_2$, 10 mM phosphate (pH 7.0), 18 mM K^+, and 5 mM succinate gradually shift from the condensed configuration shown in Fig. 2 to the type of structure displayed in Fig. 3. At 28°C, this change takes about 15 min to become com-

TABLE I

Effects of Spraying on Mitochondrial Respiration, Phosphorylation Efficiency, and Acceptor Control

Treatment	Rate of respiration: n atoms O min^{-1} $mg\ protein^{-1}$ (a) before ADP	Rate of respiration: n atoms O min^{-1} $mg\ protein^{-1}$ (b) after ADP	Acceptor control ratio b/a	Phosphorylation efficiency ADP/O
Control	14	107	7.6	2.88
Sprayed	14	90	6.4	2.76

The incubation medium contained 10 mM K^+ phosphate, pH 7.0, and 5 mM Tris glutamate, 120 mM sucrose, 5 mM $MgCl_2$, and 1.8 mg mitochondrial protein ml^{-1}, total volume 3 ml, temperature 28°C; 250 μM ADP was added after 5 min.

plete, and measurements on mitochondrial profiles in samples spray-frozen after incubation for different lengths of time indicated that the time-course of the increase in matrix volume is approximately the same as that for the decrease in absorbance shown in Fig. 4. If Na-phosphate or Tris-phosphate buffers were substituted for K-phosphate in the incubation medium, the decrease in absorbance was largely prevented (Table II), and no increase in matrical volume was observed in the samples examined by spray-freeze-etching. Fig. 4 also shows that the slow expansion of the matrix under energizing conditions is accompanied by a slow steady rate of respiration. When the oxygen in the incubation medium is exhausted, the absorbance suddenly increases and this is associated with a rapid reduction in the volume of the mitochondrial matrix. The same result is obtained if respiration is blocked by antimycin A, and the matrix volume changes under these conditions are considered below.

The Relationship Between Mitochondrial Structural Changes and Phosphorylation Efficiency

The absorbance changes given in Table II show how the composition of the incubation medium can influence the extent of the shift in mitochondrial structure under energizing conditions. When Tris- or Na^+-phosphate is substituted for K^+-phosphate, the decrease in absorbance under energizing conditions is cut to one-third. However, the extent to which the matrix swells does not alter the phosphorylation efficiency or the rate of respiration after ADP addition. Even in the K^+ medium, the ADP/O ratio obtained when ADP is added after 1 min is the same as that found when ADP is added after 12 min, despite the fact that the absorbance measurements indicate that the change in matrical volume is more than 10 times as large in the latter case.

Structural Changes During the Phosphorylation of ADP

To examine the structural changes that occur after initiation of oxidative phosphorylation, mitochondria were spray-frozen 30 s after the addition of ADP. Typical results are shown in Fig. 5. The cross-fractured mitochondrial profiles show that the mitochondria are in the condensed configuration, and measurements on a large number of profiles have shown that the average reduction in matrix volume was about 30%. The magnitude of this matrical shrinkage was surprising in view of the relatively small increase in absorbance shown in Fig. 4 after ADP was added.

The Effects of Temperature on the Time-Course of Matrical Shrinkage after ADP Addition

The time-course of the matrical shrinkage was investigated with the rapid sampling device shown in Fig. 1. After spray-freeze-etching of four samples at each time interval, the matrical volumes of at least 40 cross-fractured mitochondrial profiles were estimated as described in Materials and Methods. The mean values of the matrical volume at each time interval studied were then divided by the mean value for the volume of fully orthodox mitochondria to determine the relative matrical volumes which are plotted in Fig. 6. To determine the effects of temperature on the time-course of the structural changes, experiments were carried out at 15° and 5°, as well as at 28°C. At each temperature, mitochondria were incubated in the presence of substrate and phosphate until they became orthodox as judged by both absorbance and spray-freeze-etching. ADP was then added, and samples from the incubation were spray-frozen at the times indicated. The incubation was continued until phosphorylation was complete, as judged by changes in the respiration trace.

The data in Fig. 6 show that at 28°C there is an initial lag period of about 5 s between the addition of ADP and the beginning of the matrical shrinkage. The decrease in matrix volume is complete after a further 15 s at 28°C. Fig. 6 also shows that when the incubation temperature was reduced to

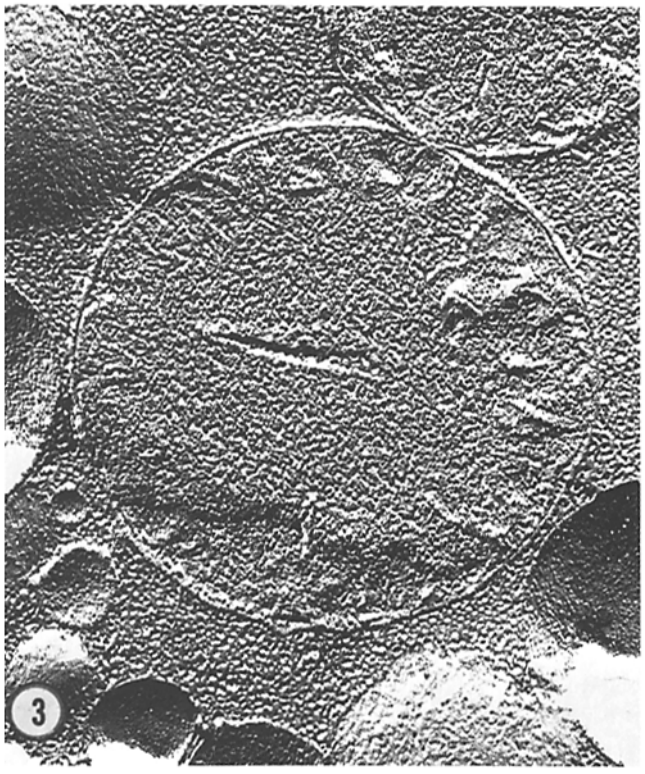

FIGURE 2 Freshly isolated (unincubated) mitochondrion showing the condensed configuration. Mitochondria isolated in 250 mM sucrose spray-freeze-fractured at −115°C and 1.8×10^{-6} torr. × 60,000.

FIGURE 3 Mitochondrion in the fully orthodox configuration after 15-min incubation under energizing conditions in a medium containing 10 mM K^+-phosphate (pH 7.0), 120 mM sucrose, 5 mM $MgCl_2$, 5 mM Tris succinate, and 1.8 mg mitochondrial protein ml^{-1}; total volume 3 ml, temperature 28°C. Spray-frozen and then fractured at −110°C and etched for 1 min at −100°C and 2×100^{-6} torr to emphasize the presence of both inner and outer membranes. × 60,000.

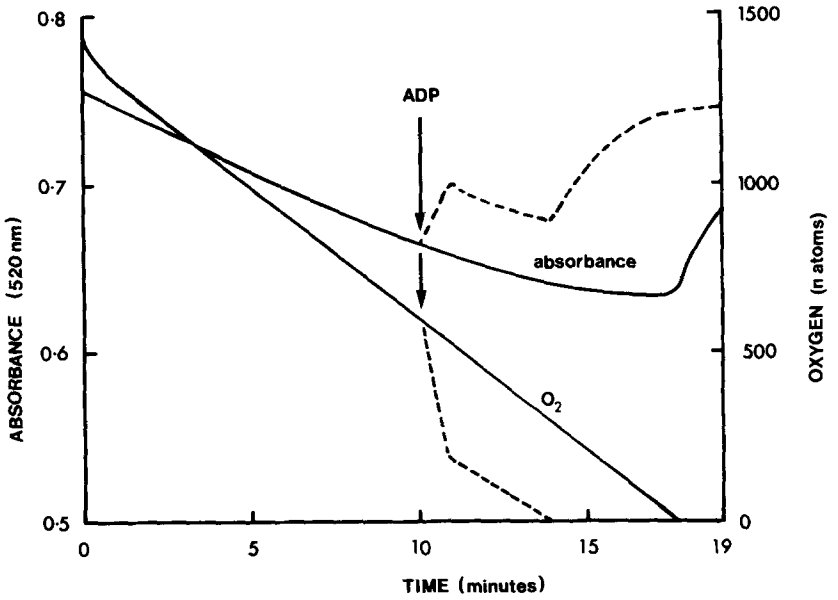

FIGURE 4 Respiration and absorbance changes of mitochondria incubated under energizing conditions (substrate + phosphate) and during phosphorylation (substrate + phosphate + ADP) in a K^+ medium. The curves are taken from continuous recorder traces of absorbance and oxygen tension. The broken lines indicate changes occurring after the addition of 250 μM ADP at the point indicated by the arrows; for incubation conditions, see Fig. 3. In both the presence and absence of ADP, the incubation eventually became anaerobic leading to an increase in absorbance; this occurred after about 14 and 18 min, respectively.

TABLE II

The Influence of Cation Content and Incubation Time on Mitochondrial Swelling, Respiration, and Phosphorylation Efficiency

Principal cation	Time before ADP addition	Change in absorbance at 520 nm before ADP addition	Rate of respiration (n atoms 0 min^{-1} $mg\ protein^{-1}$)		ADP/0
			before ADP	after ADP	
	(*min*)				
K^+	1	0.010	23	130	1.82
	12	0.104	23	133	1.82
Tris	1	0.004	20	122	1.74
	12	0.034	21	134	1.75
Na^+	1	0.006	22	132	1.81
	12	0.037	21	143	1.83

The incubation medium contained 10 mM K^+ phosphate, 10 mM Tris phosphate or 10 mM Na^+ phosphate, and 5 mM Tris succinate; 250 μM ADP was added at the time indicated. For other incubation conditions, see Fig. 3.

15° or 5°C, the lag period lengthened to 15 or 20 s, respectively, although there was relatively little change in the extent of the matrical shrinkage. At 28°C, the maximum rate of shrinkage resulted in a reduction in the relative matrical volume of 2.5%/s. The equivalent rates of change at 15° and 5°C were 1.5%/s and 1.0%/s, respectively. At the lowest temperature studied, the full change from orthodox to the condensed structure typical of phosphorylating mitochondria takes nearly 1 min, or three times as long as it does at 28°C. However, we would like to emphasize that at all temperatures studied the structural change which follows ADP addition is complete in less than half the time required for the phosphorylation of all the ADP which was added.

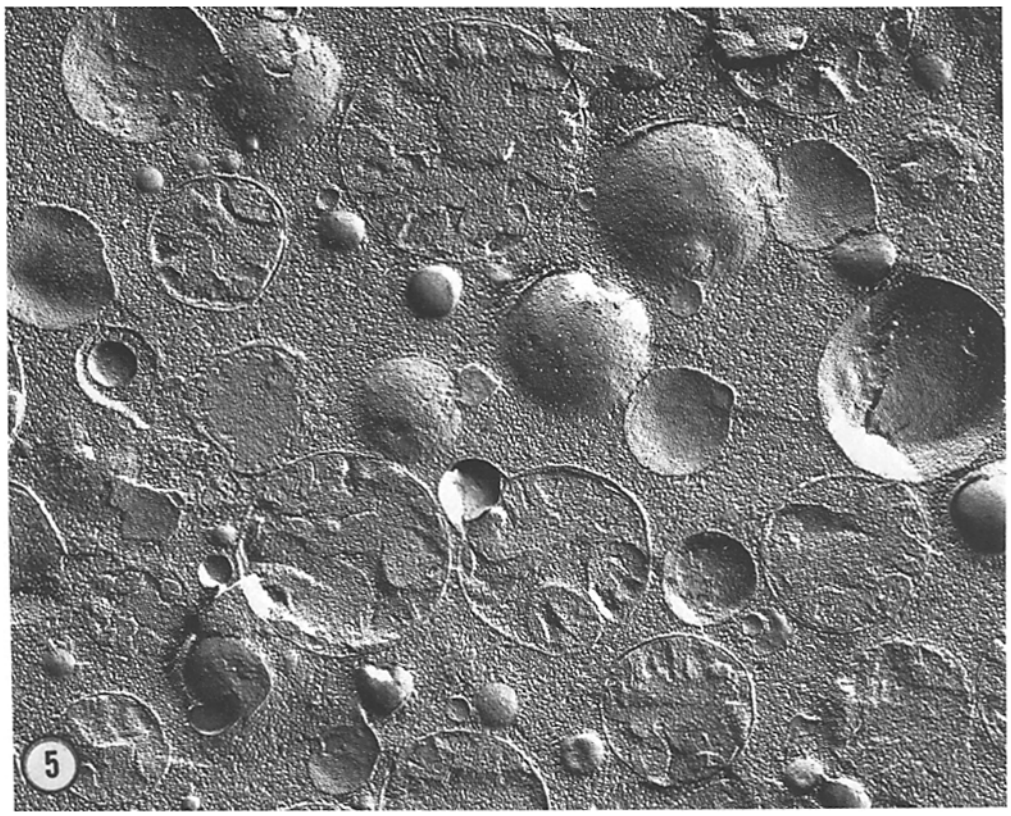

FIGURE 5 Mitochondria spray-frozen 30 s after the addition of 250 μM ADP showing a partially condensed configuration. Before ADP addition, the mitochondria had been incubated for 15 min under energizing conditions in a medium containing 5 mM Tris succinate and 10 mM K^+-phosphate so that they were fully orthodox; for other conditions, see Fig. 3. Spray-freeze-etched. × 29,000.

Fig. 7 shows a typical result obtained when a sample of mitochondria was incubated at 5°C and spray-frozen 20 s after the addition of ADP. All the profiles which are cross-fractured show mitochondria that are still in the orthodox state as shown in Fig. 6, indicating that at 5°C the contraction of the matrix does not begin until after a 20-s lag period.

Inspection of the respiration traces at the various temperatures indicates that the lag period between the addition of ADP and the start of matrical contraction coincided with a similar lag in the attainment of maximal respiratory rates. Some of the lag period is due to the dead time of mixing, but independent estimates indicate that mixing is complete in 1 s.

Changes in Matrical Structure in the Presence of Valinomycin

The absorbance data reported in Table II suggest that the energized matrical swelling which occurs in the presence of substrate and phosphate also requires the presence of K^+ ions. Further evidence for this view was provided by adding low concentrations of valinomycin to the incubation medium. In the presence of 50 ng of the K^+-ionophore/mg mitochondrial protein, the full condensed to orthodox conversion under energizing conditions occurred in 3 min, even when the K^+ content of the incubation medium was restricted to 0.4 mM. Other work in this laboratory has shown that this amount of K^+ is just sufficient to allow the complete conversion of mitochondria from the condensed to the orthodox state under energizing conditions.[1] We have now used the spray-freezing technique to investigate the time-course of the matrical shrinkage which follows ADP addition to orthodox mitochondria previ-

[1] Webster, K. A., and J. R. Bronk. 1978. Ion movements during energy-linked mitochondrial structural changes. *J. Bioenerg. Biomem.* In press.

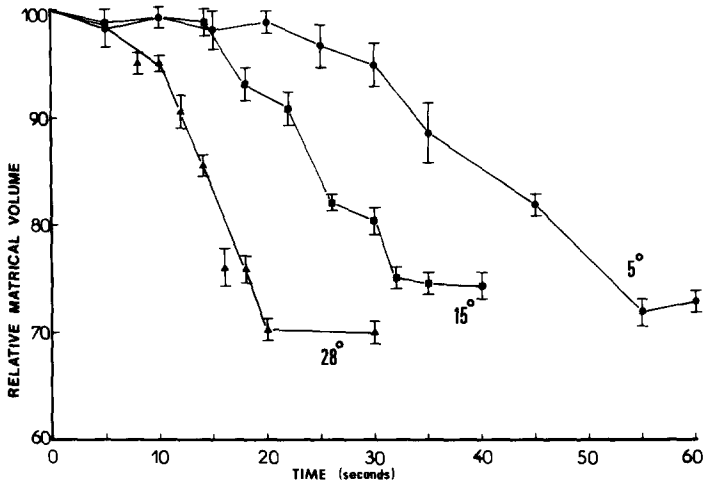

FIGURE 6 Effects of temperature on the time-course of ADP-induced shrinkage of orthodox mitochondria. The curves show the decrease in the relative matrical volume of mitochondria after the addition of 250 μM ADP at time zero. Before ADP addition, the mitochondria were incubated under energizing conditions until they were fully orthodox; ▲, 28°C; ■, 15°C; ●, 5°C. The reaction system was as described in Fig. 3; each point represents the mean of measurements on at least 40 profiles, and the vertical bars are twice the standard error of that mean. The time-course at 28°C is significantly ($P < 0.001$) different from those at the other two temperatures from 10 to 30 s inclusive; the time-course at 15°C is significantly ($P < 0.005$) different from the other two time-courses from 18 to 40 s, inclusive.

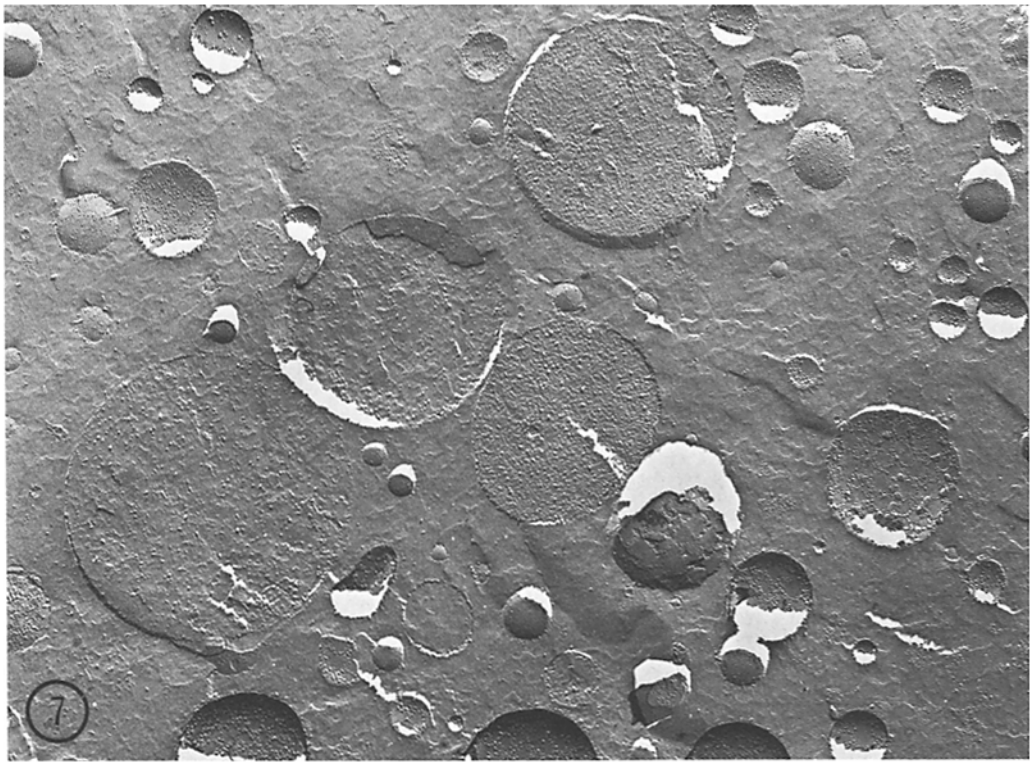

FIGURE 7 Mitochondria spray-frozen 20 s after the addition of 250 μM ADP. The mitochondria were incubated at 5°C, and before ADP addition they had been incubated for 63 min under energizing conditions in the K^+-phosphate medium described in Fig. 3 so that they were fully orthodox when the ADP was added. Spray-freeze-fractured. × 38,000.

ously incubated under energizing conditions with valinomycin and 0.4 mM K^+. The results, which were obtained at 28°C and are shown in the upper curve in Fig. 8, suggest that the presence of the K^+-ionophore largely eliminated the lag period and also increased the extent of the shrinkage to 40% of the orthodox matrical volume. The presence of valinomycin also increased the maximum rate of matrical shrinkage to about 5%/s, although the time required for the full change was only decreased slightly to 13 s. Fig. 9 shows a sample of mitochondria from an incubation at 28°C in the presence of valinomycin which were spray-frozen 10 s after addition of ADP. All of the cross-fractured profiles show condensed mitochondria.

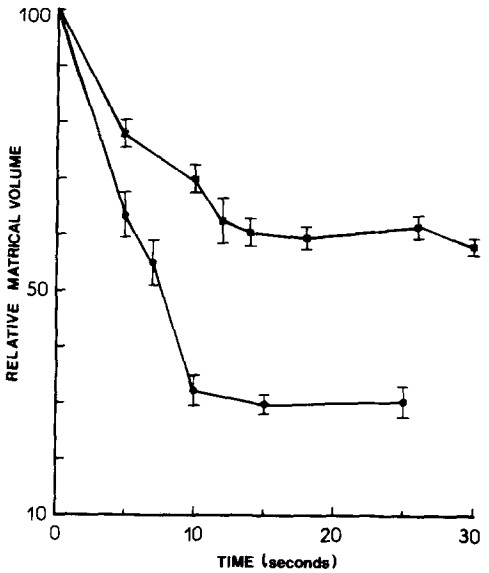

FIGURE 8 Effects of valinomycin and antimycin A on mitochondrial shrinkage. ■, time-course of mitochondrial shrinkage induced by the addition of 250 μM ADP. Before ADP addition, the mitochondria had been incubating at 28°C under energizing conditions in the presence of 50 ng valinomycin/mg mitochondrial protein and 0.4 mM KCl, until they became orthodox (3 min). The incubation medium also contained 120 mM sucrose, 5 mM Tris succinate, 5 mM $MgCl_2$, and 10 mM Tris phosphate (pH 7.0) and 1.9 mg ml^{-1} mitochondrial protein. ●, time-course of shrinkage of orthodox mitochondria at 20°C after the addition of antimycin A (2 μg mg^{-1} protein) at zero time. Before antimycin A addition, the mitochondria had been incubated under energizing conditions at 20°C in the medium described in Fig. 3. Each point shows the mean of measurements on at least 40 profiles, and the vertical bars are twice the standard error of the mean. The two time-courses are significantly different ($P < 0.01$) at 5 s and all subsequent times.

The Time-Course of Structural Changes After Antimycin A Addition

When respiration was inhibited by anaerobiosis or the addition of antimycin A, there was a rapid shrinkage of the matrix. With the spray-freezing method, we were able to investigate the time-course of this change after the addition of antimycin A to mitochondria which had previously been incubated under energizing conditions (K^+-phosphate medium plus substrate) until the mitochondria became orthodox. The results form the lower curve in Fig. 8 and suggest that the shrinkage occurs without any lag period. Both the maximum rate of shrinkage (7%/s) and the extent of the change (70% shrinkage) are much larger than those observed on the addition of ADP, even in the presence of valinomycin. Fig. 10 illustrates the highly condensed form of the mitochondrial matrix 10 s after the addition of antimycin A. This sample was spray-frozen from a sample that had previously been incubated under energizing conditions until the mitochondria were all in the orthodox condition.

A Comparison of the Time-Course of Changes in Mitochondrial Structure as Observed by Spray-Freeze-Etching and Absorbance Measurements

The results reported above with the spray-freezing technique show that the mitochondrial matrix shrinks rapidly when ADP is added to mitochondria previously converted to the orthodox form by energization in the presence of substrate and K^+-phosphate. Because our results seemed somewhat at variance with absorbance changes measured under similar conditions, we have made a direct comparison of the time-course of matrical shrinkage followed by these two techniques, and the results are shown in Fig. 11. Fig. 11*a* shows the time-courses of matrical shrinkage at 28°C expressed as a percentage of the maximum change for each method of measurement. According to the spray-freeze-etching technique, the changes were complete in 20 s, whereas the absorbance changes took 60 s. Fig. 11*b* shows the time-course of changes in matrix volume at 28°C in the presence of 50 ng valinomycin/mg mitochondrial protein and 0.4 mM K^+. As shown in Fig. 8, the valinomycin increases the rate at which the changes occur, and this is true for both methods of measurement. Nevertheless, the spray-freeze-etching technique shows that the matrical volume

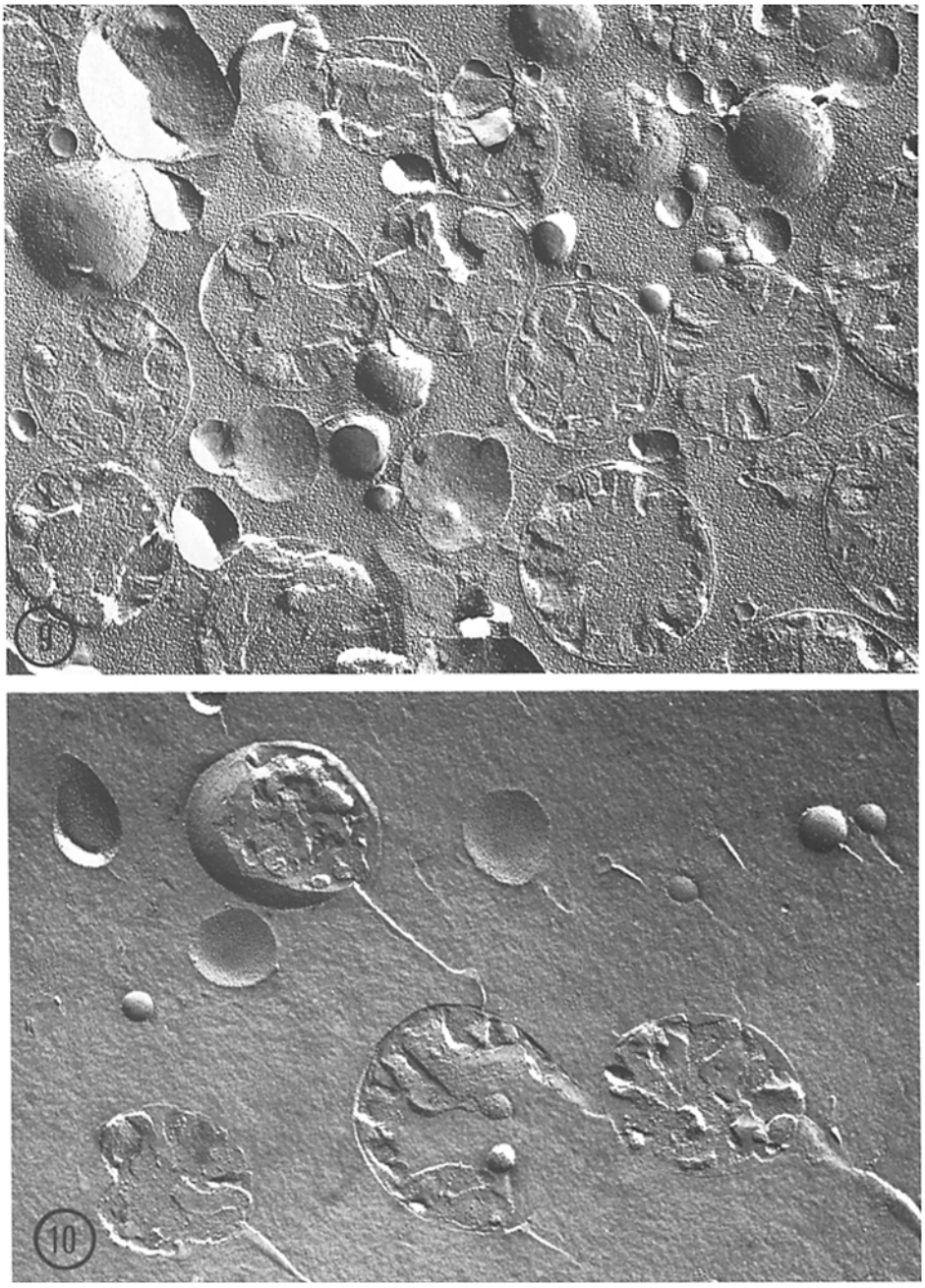

FIGURE 9 Mitochondria spray-frozen 10 s after addition of 250 μM ADP. Before ADP addition, the mitochondria had been incubated for 3 min under energizing conditions at 28°C in the presence of 0.4 mM K^+ and 50 ng valinomycin/mg mitochondrial protein so that the mitochondria were fully orthodox. The incubation medium contained 120 mM sucrose; 5 mM $MgCl_2$; 10 mM Tris phosphate (pH 7.0); 5 mM Tris-succinate; 0.4 mM KCl, 1.9 mg mitochondrial protein ml^{-1} and valinomycin. Spray-freeze-etched. × 29,000.

FIGURE 10 Mitochondria spray-frozen 10 s after addition of 2 μg of antimycin A/mg mitochondrial protein. Before this addition, the mitochondria were incubated at 20°C for 19 min under energizing conditions so that they were in the orthodox configuration. The incubation medium is given in Fig. 3. Spray-freeze-fractured. × 29,000.

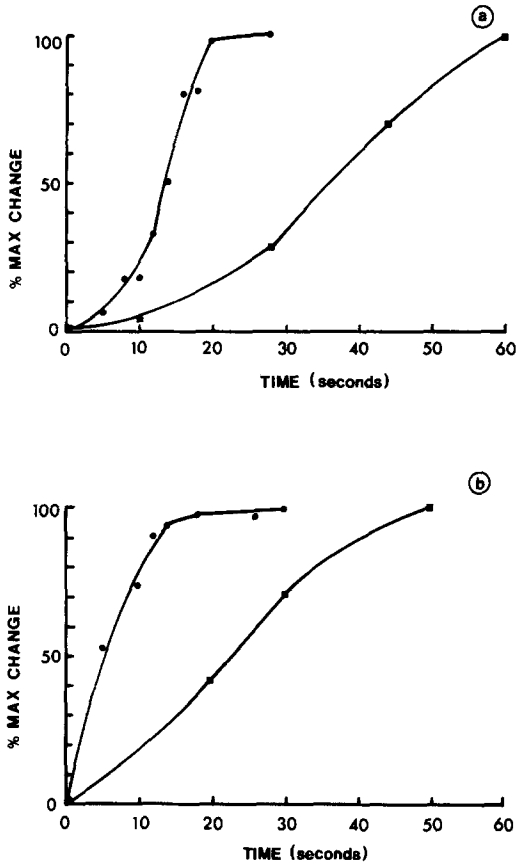

FIGURE 11 Comparison of rate of ADP-induced shrinkage of the inner compartment of orthodox mitochondria with and without valinomycin as indicated by measurements of relative matrical volume (●) and by absorbance changes (■). (*a*) In the absence of valinomycin, temperature 28°C; 250 μM ADP added at zero time to mitochondria which had been incubated for 15 min under energizing conditions in the medium given in Fig. 3. (*b*) In the presence of 50 ng valinomycin/mg mitochondrial protein temperature 28°C; 250 μM ADP added at zero time to mitochondria incubated for 3 min under energizing conditions as described in Fig. 9.

changes occur in one-third the time indicated by the absorbance changes.

DISCUSSION

The results presented in this paper show that in freshly isolated rat liver mitochondria the matrix compartment is reduced to about half the volume that it would occupy in orthodox mitochondria. If these condensed mitochondria are incubated in the presence of substrate and K^+-phosphate, the matrix slowly expands to give the orthodox configuration. This aspect of our work confirms the earlier ultrastructural data obtained by Hackenbrock (7–9), and Green et al. (6) with chemical fixation. However, our results are noteworthy because they are the first to show structural changes in suspensions of mitochondria to which no additions of fixatives or cryoprotectants have been made. The freeze-etching study by Hackenbrock (9, 10) indicated that mitochondrial structural changes observed by freezing mitochondria did not differ from those revealed by chemical fixation, but in his work there was still a danger of artifacts because of the need to employ 25% glycerol as a cryoprotectant. The negative staining technique has been used by Muscatello and coworkers (18–21) to follow mitochondrial structural changes in the absence of chemical fixation. Their work with the negative stain ammonium molybdate also confirmed Hackenbrock's earlier observations (7, 8); however, the isotonic ammonium molybdate partially inhibited respiration and reduced phosphorylation efficiency so that the technique may have introduced artifacts. By contrast, the spray-freezing technique requires no special additions to the mitochondrial suspension. Because our results confirm that mitochondria isolated in a sucrose medium have a condensed matrix which gradually expands to the orthodox configuration when the mitochondria are energized, it now seems reasonable to conclude that there is no support for the view that these structural changes are due to the type of fixative used as suggested by Butler & Judah (3), or that they resulted from osmotic changes after the fixative was added as suggested by Stoner & Sirak (26, 27) and Hunter & Brierley (12) from their work with heart mitochondria.

In addition to avoiding the need for cryoprotectants, the very rapid fixation of the spray-freezing technique has made it possible for us to obtain an accurate measurement of the time-course of the structural changes after the initiation of phosphorylation by the addition of ADP. Hackenbrock's studies with conventional electron microscopy and chemical fixation (7, 8) indicated a dramatic reduction in matrix volume for mitochondria fixed 30 s after ADP addition. However, he was not able to measure the time-course of the change he observed, and the data obtained by Hunter & Brierley (12) led them to question the validity of ultrastructural studies on mitochondria fixed chemically during a period when their volume is changing rapidly. It was also evident that Hack-

enbrock's observations showed a larger and more rapid structural change on ADP addition than was indicated by absorbance measurements. Our results confirm Hackenbrock's observation that the initiation of phosphorylation is associated with a substantial decrease in matrix volume within 30 s, although the time-course studies indicate that the shift in structure is gradual rather than instantaneous. The fact that the rate of change in matrix volume is dependent on temperature as shown in Fig. 6 is consistent with the suggestion that it is a consequence of the movement of solute out of the matrix.

The spray-freeze-etching time-course studies also show that the changes in matrix volume after the addition of ADP occur more rapidly than is indicated by the rate at which the absorbance changes. Our results suggest that absorbance changes are not a good indication of alterations in matrix volume when rapid changes are occurring, although for the slow changes in volume under energizing conditions no discrepancy was apparent. We do not know of any evidence that will explain why the absorbance changes lag behind the shift in matrix volume, but it could be an indication of the time required for the loss from the outer compartment of the extra solutes displaced from the matrix when it shrinks.

In the incubation medium we used, which included $MgCl_2$, both the increase in matrix volume after energization of the mitochondria, and the decrease on ADP addition appear to be largely due to the movement of K^+ and associated anions into and out of the mitochondrial matrix, respectively. Our evidence for this is that when Tris- or Na^+-phosphate is substituted for K^+-phosphate, the expansion of the matrix on energization is reduced by nearly 70% (Table II), whereas when the permeability of mitochondrial inner membrane to K^+ is increased by the addition of valinomycin, the rate at which the matrical volume shrinks after ADP addition was doubled (Fig. 8).

Our data also suggest that the extent to which the volume of the mitochondrial matrix decreases under particular conditions is dependent on the degree to which orthodox mitochondria are de-energized. This is apparent from a comparison of Fig. 8 with Fig. 6, as the addition of antimycin A completely de-energizes the mitochondria, whereas the addition of ADP produced only a partial de-energization. In the former case, the matrix volume decreased by 70%, but the addition of ADP only causes a 30% shrinkage of the matrix. These results suggest that energization of the mitochondria is essential to maintain the mitochondria in the orthodox configuration with the matrix expanded to the limit imposed by the outer membrane. Other experiments performed in this laboratory to be reported elsewhere[1] show that the different reductions in matrical volume caused by the addition of antimycin A and ADP are accompanied by comparable differences in the efflux of K^+ and anions from the matrix. Thus, the decrease in matrix volume on de-energization reflects the establishment of a new steady state at a lower energy level which results in a lower matrical ion content.

Our data show that the initiation of phosphorylation in orthodox mitochondria at 28°C is associated with a 30% decrease in the matrix volume in 20 s. Although the addition of valinomycin doubles the rate at which shrinkage of the matrix occurs, the rapidity of the change in the absence of the K^+-ionophore indicates that the mitochondrial inner membrane contains enough natural ionophores to permit the rapid exit of K^+ and associated anions that is necessary to allow the matrix volume to decrease. It seems likely that the fall in the matrical K^+ content compensates partially for the electrogenic component of the exchange of ADP^{3-} for ATP^{4-}. Some of the associated anion loss could be due to the utilization of matrix phosphate for the synthesis of ATP from the incoming ADP. A new steady state is established within 20 s after ADP addition, and this probably occurs when the rate of respiration has increased sufficiently to balance the depolarizing effects of the ADP/ATP exchange and phosphorylation.

The large decrease in matrix volume that follows the addition of antimycin A to mitochondria requires an efflux of K^+ and associated anions which is even greater than that evoked by ADP addition. The rate at which this ion movement occurs suggests that blocking of the respiratory chain may increase the ion permeability of the mitochondrial inner membrane in some way.

The data reported in this paper generally confirm the view that mitochondrial structural changes are an osmotic consequence of changes in the ionic content of the mitochondrial matrix. However, our time-course measurements and quantitative data show that the inner membrane must be sufficiently permeable to the predominant internal ions (K^+ and phosphate) to allow 30–70% changes in matrix volume within 10–20 s.

This conclusion suggests that the slow rate of increase in matrix volume on energization in absence of ADP is not due to any restraint imposed by the cation permeability of the mitochondrial inner membrane.

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