Ultrastructural Studies on Mitochondrial Swelling

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Mitochondrial swelling induced by valinomycin, calcium chloride and P_i was studied after potassium permanganate fixation in suspension. Valinomycin induces a rapid K^+ influx, increase of the matrix space and out-folding of the cristae, with good preservation of the matrix material. This swelling is reversible but the cristae do not completely re-form and have a blebbed appearance. On repeated swelling and contraction cycles there is a gradual loss of matrix material. Calcium chloride and P_i produce a slow swelling of the matrix space. Shrinkage induced by ATP was partial and not associated with return to the original structure.

The morphology of fixed isolated rat liver mitochondria has been shown to depend on the method of fixation (Butler & Judah, 1970). Isolated mitochondria resembling those seen in intact liver are only observed after fixation in veronal-buffered potassium permanganate. No conformational changes are seen on varying the respiratory state of the mitochondria, although they seem to appear when glutaraldehyde fixation is used. The method of fixation that was established to retain the conformation *in vivo* of isolated rat liver mitochondria was used to study the process of swelling. In this study the energy-dependent K^+ influx induced by valinomycin (Cockrell, Harris & Pressman, 1966; Harris, Cockrell & Pressman, 1966), and the swelling induced by calcium chloride or P_i are compared. The time-course and the conditions required for these swelling phenomena are well documented (Harris *et al.* 1966; Lehninger, 1959). The ultrastructural changes with procedures based on glutaraldehyde fixation (Packer, Wrigglesworth, Fortes & Pressman, 1968) in suspension or osmium tetroxide fixation of micro-pellets (Hackenbrock & Caplan, 1969; Wlodawer, Parsons, Williams & Wojtczak, 1966) have also been described. However, the conformations of mitochondria in the initial state as seen after glutaraldehyde and potassium permanganate fixation differ. In the present paper the swelling as seen in potassium permanganate-fixed mitochondria is described.

METHODS

Mitochondria were prepared from rat liver in either 0.25 M- or 0.44 M-sucrose as described by Butler & Judah (1970).

The measurements of K^+ movements by cation-sensitive electrodes and of light-scattering were made essentially by the methods of Harris *et al.* (1966). For these the mitochondria (10–20 mg dry wt.) were suspended in a medium containing 40 mM-tris-acetate buffer, pH 7.40, 5 mM-tris-glutamate, 5 mM-tris-malate, 5 mM-KCl and 0.20–0.4 M-sucrose; the final volume was 5 ml. The temperature was 20°C and the gas phase was O_2 . Variations are described in the text and Tables.

Valinomycin where added was at a concentration of 40 μ g/g dry wt. $CaCl_2$ (0.09 μ mol/mg dry wt.)- or P_i (10 mM)-induced swelling was followed by light-scattering measurements in 150 mM-KCl or 250 mM-sucrose.

At appropriate times during the course of the swelling and contraction cycles the contents of the reaction vessel were sampled. The suspensions were poured into approx. 10 ml of 2% (w/v) $KMnO_4$ in veronal-acetate buffer (0.14 M, pH 7.2 at 0°C), left in ice for approx. 10–20 min and centrifuged at 10000 g for 5 min. The tube was then cut down and the pellet was gently removed and diced in buffer in such a way as to preserve the orientation. The blocks were dehydrated through graded alcohols and embedded in Epon 812. The blocks were sectioned on an LKB Ultratome with either diamond or glass knives and were examined in either an RCA EMU3 or an AEI EM6 electron microscope. All specimens shown in the Plates were stained with uranyl acetate.

RESULTS

Valinomycin-induced swelling. During these experiments the appearance of the control mitochondria was as described by Butler & Judah (1970) and as represented in Plate 1. The K^+ uptake induced by valinomycin (40 μ g/g dry wt.) and the concurrent uptake of water were monitored by light-scattering and by K^+ disappearance from the medium. The maximum amplitude of light scattering was reached at 2 min and that of K^+ influx at

about 4 min. At both these times samples were taken and the form of the mitochondria is as seen in Plate 2. The mitochondria are greatly enlarged and spherical. The outer membrane is frequently broken. The cristae have nearly completely evaginated while the matrix is intact. This can be compared with the mitochondria without valinomycin (Plate 1) fixed at the same time as the swollen mitochondria.

Swelling and K^+ uptake were inhibited by dinitrophenol (0.1 mM) or by anoxia, which also brought about shrinkage and loss of K^+ . K^+ concentration and light-scattering returned to the pre-swollen values in 2 min. The cristae had re-formed but in the main were not regular. Frequently bleb-like swellings of the cristae could be seen (Plate 3) and the matrix was evenly granular. This cycle of swelling and contraction could be repeated in experiments where contraction was induced by N_2 . On re-oxygenation there was a K^+ influx and swelling of the mitochondria. A further period of anaerobiosis caused contraction and re-formation of the cristae. However, in each cycle some matrix material appeared to be lost and damaged mitochondria were seen in increasing numbers (Plate 4) with subsequent less active response to oxygenation.

The total K^+ uptake during the initial swelling phase was some $400 \mu\text{mol}$ of K^+/g dry wt. of mitochondria. The time-course of K^+ movements and light scattering is shown in Fig. 1, which also shows the changes following addition of dinitrophenol (0.1 mM).

As can be seen from Plate 2 not all the mitochondria respond to valinomycin. Occasional mitochondria retain the conformation seen in the control

pellet. The mitochondria have regular cristae and do not appear to be mitochondria that have swollen and contracted. Similarly there is a variation in the phase of contraction. Most mitochondria show re-formed cristae whereas others appear to be still swollen.

Ca^{2+} - and P_i -induced swelling. We examined these conditions to contrast them with the rapid valinomycin-induced change. The swelling induced by either Ca^{2+} or P_i is considerably slower than that induced by valinomycin. In these experiments the swelling was monitored by light-scattering. The swelling induced by P_i (10 mM) was examined with mitochondria prepared and suspended in 0.44 M-sucrose. The swelling phase lasted from 6 to 20 min with the appearance of mitochondria as seen in Plate 5. The outer membrane is frequently lost or only partially associated with the inner membrane. The inner membrane is usually continuous and the cristae are very sparse and when seen appear to be near the junction of the outer and inner membranes. The matrix is very scanty.

The swelling induced by calcium chloride ($0.09 \mu\text{mol/g}$ dry wt.) is essentially similar to that induced by P_i . The swelling seen in sucrose medium is slow. The appearance of the mitochondria shows spherical forms with loss of matrix material. The outer membrane is frequently intact and the ballooning seen in the P_i -swollen mitochondria is seldom observed. The cristae are sparse but usually

EXPLANATION OF PLATES 1-4

1. Mitochondria prepared in 0.44 M-sucrose and fixed in suspension from the reaction vessel as described in the Methods section. The cristae are regular and the matrix is finely granular. The outer membrane shows some breaks. This appearance of the mitochondria is constant when samples are taken at point A of Fig. 1 or from the control mitochondria throughout the swelling cycle. (The bars represent $1 \mu\text{m}$.)

2. Mitochondria swollen by the addition of valinomycin ($40 \mu\text{g/g}$ dry wt.) and 5 mM-potassium acetate. The mitochondria have an expanded matrix compartment with only a few peripheral cristae. The outer membrane is usually broken. Note the mitochondrion that has not swollen. The appearance of the mitochondria is found at either the lowest point of the light-scattering record or at the peak of K^+ uptake (Fig. 1).

3. Mitochondria swollen by the addition of valinomycin and 5 mM-potassium acetate and contracted by the addition of dinitrophenol at the peak of K^+ uptake. The sample was taken when the light scattering recording was returned to its original value. The mitochondria have partially contracted showing cristae with uneven dilations.

4. Mitochondria swollen by the addition of valinomycin and potassium acetate. The sample was taken after two periods of anaerobiosis with further swelling induced by re-oxygenation. The matrix is partially lost.

Fig. 1. Diagrammatic representation of records of light-scattering and K^+ uptake induced by valinomycin and potassium acetate. Valinomycin ($40 \mu\text{g/g}$ dry wt.) was added at point A and dinitrophenol was added at the peak of K^+ uptake. Samples for electron microscopy were taken at point A (Plate 1), the initial trough of light-scattering (Plate 2), the peak of K^+ uptake (Plate 2) and on the return to initial values of K^+ on light-scattering (Plate 3). L. S., light-scattering; K^+ , cationic electrode.

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regular (Plate 6). The swelling seen on suspension in potassium chloride (150 mM) was more rapid than when sucrose was the suspending medium. In many respects the mitochondria were similar in that the membranes were frequently intact and the cristae remained. In the most swollen mitochondria the cristae appeared as a crescent. The main difference seen in the calcium chloride-swollen mitochondria in potassium chloride was that the matrix material appeared somewhat better preserved (Plate 7). This swelling was partially reversible by the method of Lehninger (1959) but the original structure was not restored. As can be seen in both Plates 5 and 6 not all the mitochondria swell.

The calcium chloride-induced swelling can be prevented at the outset by addition of dinitrophenol to the reaction mixture (Plate 8). In this case the membranes are intact and the matrix is of similar density to the controls.

When Plates 7 and 8 are compared with Plate 1, it is seen that inner membranes appear to be better preserved in the presence of Ca^{2+} . This appearance may be due to a better contrast between membranes and matrix space which in turn is due to loss of electron-dense material from the matrix. There may also be some direct effect of Ca^{2+} on the membranes. If one examines the contaminating microsomes, it is seen that their membranes stand out very sharply in the presence of Ca^{2+} (Plates 1 & 7).

DISCUSSION

From studies of valinomycin-induced swelling it can readily be seen that the swelling can be trapped by our fixation procedure. Further, there appears to be little increase in the space between the outer and inner membranes. There is a considerable increase in the cross-sectional area of the matrix compartment of the mitochondria and also of the overall cross-sectional area of the mitochondria. This appearance is different from that described by Packer *et al.* (1968) where the volume enclosed by the outer membrane remains constant. Only the matrix space increases. Packer *et al.* (1968) also describe the appearance of the valinomycin-swollen mitochondria as resembling mitochondria *in vivo*. In the experiments described here the starting point is mitochondria resembling the condition *in vivo* and on induction of swelling by valinomycin the appearance is modified. There is a considerable increase in both cross-sectional area of the mitochondria and in the size of the matrix space. This can be interpreted as swelling of mitochondria. The appearance of mitochondria fixed in glutaraldehyde indicates that water is moved to the matrix space from the intermembrane space with no change in total mitochondrial volume. This

difference possibly reflects the differences of mechanism of fixation.

Harris & van Dam (1968) demonstrated a decrease in the sucrose-accessible space with an increase in the total water content with K^+ uptakes similar to those described in these experiments; at lower values of K^+ uptake there is less modification of the sucrose-accessible space but an increase in the total water. The decrease in sucrose space with an increase in total water is consistent with the findings here of an increase in matrix space and evagination of cristal membranes.

On shrinkage, induced by either N_2 or dinitrophenol, at which stage the mitochondria are depleted of energy, they do not return to their original conformation. The overall cross-sectional area is less than that of the swollen mitochondria but the cristae have not re-formed to the pattern of the controls. The cristae are frequently irregular with the formation of blebs (Plate 3). Under these conditions contracted mitochondria as described by Packer *et al.* (1968) are not seen. The capacity of the mitochondria to transport K^+ is still intact since on re-oxygenation a further K^+ uptake can be induced. The morphological characteristics of subsequent swelling cycles are similar to those seen in the first, except that there is a gradual loss of matrix material and a diminution of the K^+ influx.

This damage is probably due to Ca^{2+} shifts. E. J. Harris (personal communication) has informed us of experiments in which mitochondria subject to anoxia lose Ca^{2+} to the medium. On oxygenation, they regain it rapidly. The sudden access of this Ca^{2+} then causes the mitochondria to swell. The increase in sucrose space can be related

EXPLANATION OF PLATES 5-8

5. Mitochondria prepared and suspended in 0.44 M-sucrose and swollen in P_i for 20 min. There is detachment of the outer membrane and nearly complete loss of matrix. A few remaining cristae can be seen usually in association with surviving outer membrane. Note mitochondria that have not swollen.

6. Mitochondria prepared and suspended in 0.44 M-sucrose and swollen in CaCl_2 for 20 min. The outer membrane is broken but the ballooning seen in Plate 5 is not apparent. What cristae remain are regular in form and there is considerable loss of matrix.

7. Mitochondria prepared in 0.44 M-sucrose, resuspended in 0.15 M-KCl and swollen in CaCl_2 . The appearance is similar to that of Plate 6 except that somewhat more matrix material is preserved.

8. Mitochondria prepared in 0.44 M-sucrose and resuspended in 0.15 M-KCl, CaCl_2 and dinitrophenol added together. Note that mitochondria have not swollen, except for a single profile, the cristae are regular and the matrix is intact.

to the loss of matrix enabling the matrix compartment to be partially permeable to sucrose.

The swelling induced by either Ca^{2+} or P_i differs from that caused by valinomycin in that under the present experimental condition it was not readily reversible. Morphologically the swelling occurs by increase of the matrix space. However, there is considerable loss of matrix material. This loss resembles that seen after repeated episodes of anoxia after the addition of valinomycin to the mitochondria (cf. Plates 4 and 6) and can be related to the increase in sucrose space (Harris *et al.* 1966). The appearance of mitochondria swollen in Ca^{2+} or P_i is similar to that reported by Wlodawer *et al.* (1966) but the swelling is much more extensive than that reported by Hackenbrock & Caplan (1969). The main differences between these studies reflects the observations of Butler & Judah (1970) that pellet fixation distorts the morphology of isolated rat liver mitochondria.

In all the swelling experiments it is obvious that not all the mitochondria do react to the same extent. This heterogeneity of response is similar to that reported by other workers.

In conclusion, it would appear that when rat liver mitochondria swell there is an increase in the matrix compartment while the intermembrane space is comparatively unaltered. The valinomycin-

induced swelling is both rapid and reversible, initially the matrix being well preserved. The Ca^{2+} - and P_i -induced swellings are both slow and only partially reversible. This may be related to the loss of matrix material.

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