ULTRASTRUCTURE OF ISOLATED

KIDNEY MITOCHONDRIA TREATED

WITH PHLORIZIN AND ATP

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ABSTRACT

Direct electron microscopic evidence is reported of the ultrastructure of mitochondrial membranes and compartments in mitochondria isolated in 0.5 M sucrose from the rat kidney cortex and the experimental changes they undergo with phlorizin and ATP treatment. A heterogeneous population of mitochondria is recognized under control conditions. The mitochondria appear to be of 3 main types, normal, swollen, and contracted. Under phlorizin treatment, most of the mitochondria swell in less than 15 minutes, apparently at the expense of the matrix. Treatment with ATP, on the other hand, produces, during the same time, a marked contraction of the isolated mitochondria, with many refoldings of the inner membrane and marked increase in the electron opacity of the matrix. It is concluded from these observations that mitochondrial swelling and contraction should be related mainly to the matrix content.

It is well known from the literature (see Lotspeich, 13) that phlorizin blocks the mechanism of sugar transport across the proximal convoluted tubule of the kidney. This mechanism requires the expenditure of energy because glucose is being moved from the tubular lumen to the peritubular capillary blood against chemical and osmotic gradients. The mechanism by which phlorizin inhibits sugar transport, which is not well understood at the present time, has been interpreted as a direct inhibition of the supply of energy for active transport or as an alteration of the cell membranes which affects permeability (13).

Recent studies on the swelling effect of phlorizin upon isolated mitochondria and the prevention of mitochondrial swelling by ATP (8, 12) appear to clarify the manner whereby phlorizin affects energy metabolism in the kidney. According to Keller and Lotspeich (8) the above phenomena may indicate that phlorizin alters primarily the permeability of mitochondrial membranes and that the inhibitions of respiration and oxidative phosphorylation are secondary to a non-specific alteration of mitochondrial structure.

Numerous investigators (see Ernster and Lindberg, 6; Lotspeich, 13; Novikoff, 15, and Lehninger, 11, for reviews of literature) have studied the optical density and chemical properties of mitochondria under normal conditions and during swelling and contraction, but the corresponding morphology of these mitochondria is still very obscure. Therefore, we have undertaken a study of the ultrastructure of isolated mitochondria suspended in 0.5 M sucrose and of the morphological changes produced by adding, to the same medium, ATP and phlorizin. The present results show outstanding modifications in mitochondrial size, density, and membrane characteristics during swelling and contraction.

MATERIALS AND METHODS

Eighteen male rats (Holtzman strain) weighing 190 to 320 gm were used as experimental animals. They were fed ad libitum.

The kidneys were removed by bilateral nephrectomy, rapidly performed under nembutal anesthesia. After as much medullary tissue as possible was removed, the kidney cortex was disintegrated for 3 minutes in a Potter-Elvehjem Teflon-glass homogenizer in 6 volumes of 0.44 M sucrose plus 0.001 M EDTA according to the method of Dounce et al. (4). The homogenate was centrifuged at 0–4°C in a Servall Superspeed angle centrifuge. Nuclei and cell debris were removed by centrifuging 3 ml homogenate with 2 ml of 0.44 M sucrose plus 0.001 M EDTA at 500 g for 10 minutes. The mitochondria were then sedimented from supernatant at 14.000 g for 13 minutes and the opalescent supernatant and fluffy layer were removed. The mitochondrial pellet was then washed twice by resuspending in 4 ml of 0.44 M sucrose alone and centrifuging for 7 minutes at 14.000 g, and finally suspended in 0.44 M sucrose so that 1 ml of final suspension contained mitochondria from 1 gm of fresh kidney.

The mitochondrial suspension was divided into two samples, one for optical densitometry and the other for electron microscopy.

Optical Densitometry

Mitochondrial volume changes were studied at room temperature (23–25°C) by following the optical density of suspensions at 520 mμ with a Beckman spectrophotometer (model B) as described by both Tapley (18) and Keller and Lotspeich (8). The basic test system contained about 2 drops of the mitochondrial stock suspension (sufficient to give an initial optical density of approximately 0.5) in 4.5 ml of 0.5 M sucrose buffered to pH 7.4 with 0.02 M Tris buffer; to this basic test system was added 3 × 10^{-3} M phlorizin or 3 × 10^{-3} M phlorizin plus 5 × 10^{-3} M ATP.

The first reading was taken 1 minute after the addition of mitochondria, and subsequent readings 1 minute intervals for the next 30 minutes.

Electron Microscopy

0.5 ml of the mitochondrial stock suspension was incubated for 15 minutes in 3 ml of (a) the basic test system mentioned above (buffered 0.5 M sucrose), (b) same plus phlorizin, and (c) same plus phlorizin-ATP, and then the preparations were fixed in 3 ml of cold 1 per cent OsO$_4$ (Veronal buffer, pH 7.4) plus 0.33 M sucrose (3) for 2 hours at 0°C. After fixation the preparations were centrifuged at 14.000 g for 5 minutes to form pellets.

For the study of mitochondria in situ, as control, kidneys of normal male rats were fixed according to

the method of Latta (9). Fixation of kidney tissue was initiated by continuous dripping of cold 1 per cent OsO$_4$ (Veronal buffer, pH 7.4) plus 0.33 M sucrose upon the encapsulated kidney surface in the living, anesthetized animal for 20 minutes. Subsequently, a cortical slice was rapidly removed from the fixed surface, cut into small pieces, and placed in glass vials containing the same fixative to complete a total fixation time of 2 hours at 0°C.

Both osmium tetroxide–fixed materials were dehydrated in a graded series of cold (0–4°C) ethyl alcohols and embedded in Epoxy resin (14). Thin sections were cut on a Porter-Blum or Huxley ultra-
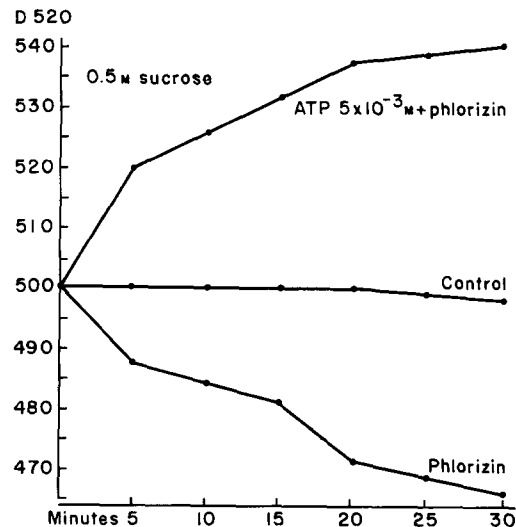
FIGURE 1 Optical densitometry (λ 520) of isolated mitochondria in 0.5 M sucrose, illustrating the swelling of mitochondria effected by phlorizin. ATP is able not only to prevent the mitochondrial swelling but also to bring about a rise in the optical density.

microtome with glass knives and were supported on naked grids. They were stained by floating on a fresh 50 per cent aqueous saturated solution of uranyl acetate for 12 minutes, after which they were rinsed twice with distilled water and stained immediately by lead citrate as described by Reynolds (17). Electron micrographs were taken with an RCA EMU-3D or a Siemens Elmiskop I.

RESULTS

Densitometry

0.25 M and 0.33 M sucrose failed to maintain constant optical densities during the 30 minutes. Molarities of 0.44 and 0.50 proved useful for this purpose. The electron microscopy supported this point by showing that the preservation of mito-

chondrial structure was far better in material suspended in the higher concentrations of sucrose.

As shown in Fig. 1, 0.5 M sucrose consistently maintains the optical density of the mitochondrial suspension at a constant level, whereas phlorizin at a molarity of 1×10^{-3} in 0.5 M sucrose causes a steady fall in the optical density of the mito-

400 mμ wide and from 2 to 6 μ long. They present numerous cristae piled in planes which are perpendicular to the long mitochondrial axis. The cristae have a 70 A membrane continuous with the inner mitochondrial membrane, and the cristal lumen varies from 100 A to 130 A in width. The matrix has a moderate electron opacity and
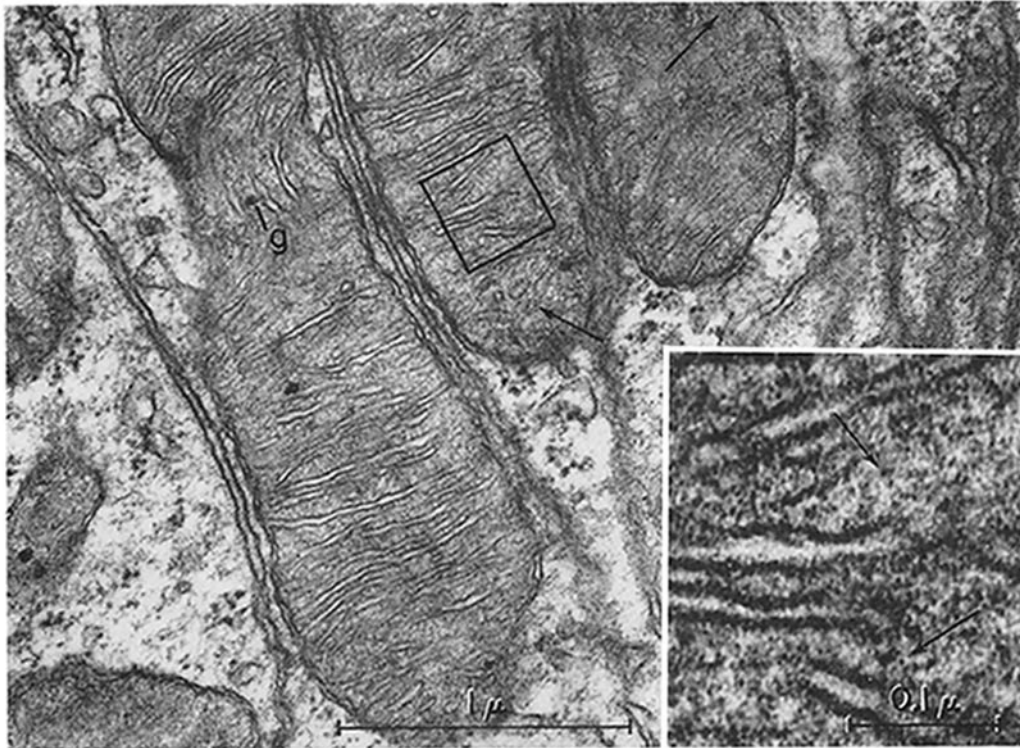

FIGURE 2 Portion of the basal cytoplasm of a cell from the proximal convoluted tubule, showing the elongated mitochondria with transverse cristae and a moderately dense matrix. Shown for comparison with the isolated mitochondria. Granules (grana) (g) and small particulate component (arrows) are seen. Uranyl acetate and lead citrate stained. $\times$ 38,000; inset $\times$ 200,000.

chondrial suspension. This fall is of the order of 10 per cent (average of several experiments) in the first 30 minutes. On the other hand, ATP, when added at a molarity of 5×10^{-3} to the mitochondrial suspension containing phlorizin, is consistently able not only to prevent the decrease in optical density but also to bring about a rise in the optical density of the order of 10 per cent (average of several experiments) in the first 30 minutes.

Mitochondria in Situ

Mitochondria in both the proximal convoluted tubule and distal tubule (Fig. 2) present the following characteristics. They are elongated bodies,

presents dense granules (grana) 200–300 A in diameter scattered within it. The matrix also is stippled with very small granules 40–50 A in diameter, closely packed in some places, scattered in others. This small component is clearly shown in the uranyl acetate–stained sections (Fig. 2).

Most of the data mentioned above are well known from the literature. They are mentioned here to serve as comparison with those of the isolated mitochondria.

Mitochondria Isolated in 0.5 M Sucrose

Most of the mitochondria adopt the spherical or oval shape. Three main types are recognized, in

approximately equivalent amounts, in the heterogeneous population of the washed pellets.

One type is a very dense, zebra-like mitochondrion (M_2, Fig. 3). This is the smallest and most dense type in all the population, and measures approximately 500 mμ in diameter. Some of these dense mitochondria maintain a rod shape. The cristae are numerous with very wide lumens, and narrow strands of dense matrix are interspersed between them. The cristal lumens measure approximately 300 A in width, and the outer mitochondrial compartment is 180 A in width. The matrix granules are difficult to recognize, as seen in the same figure (M_2). The matrices of these mitochondria attain the highest electron opacity.

The second type is a more voluminous mitochondrion, 800 mμ in diameter, and less dense (M_1, Fig. 3). The cristal lumens are about 150 A in width, and the outer mitochondrial space measures, as an average, 80 A, like the mitochondria in situ. The matrix presents the same types of granules seen in the in situ mitochondria (inset, Fig. 3).

The largest type is a swollen mitochondrion, 1100 mμ in diameter, with a very pale matrix, and scattered cristae. The cristal lumens are about 100 A, and the outer mitochondrial space is 80 A in width. In some extremely swollen mitochondria the outer mitochondrial membrane is absent or partially interrupted. Granules in the matrix are less evident than in the other types. The large ones (grana) present are apparently like those of the in situ mitochondria, but the small ones conglomerate around the cristae leaving lucid spaces in the matrix.

Intermediate types are also found in this pellet (see Fig. 3).

Mitochondria Isolated in 0.5 M Sucrose Plus 5 ×10⁻³ M Phlorizin

This pellet shows a definite predominance of the swollen type of mitochondria (Fig. 4). They attained the largest volumes (1500 mμ in diameter) and the lowest densities. The width of the outer compartment between the outer and inner mitochondrial membranes and at the cristal lumen

maintains approximately the control values of about 80–100 A. Many mitochondria show cristal lumens that are less in diameter than those of the swollen type of the control ones. Few mitochondria maintain a volume and density similar to those of controls. The matrix has a very low electron opacity. The small particulate component (40–50 A) conglomerates around the cristae. Some of the cristae appear devoid of particles. The granules (grana) of the matrix apparently are seen in the same amount as in controls, sometimes free in the matrix or close to the cristae.

Mitochondria Isolated in 0.5 M Sucrose Plus 5 ×10⁻³ M Phlorizin Plus 3 ×10⁻³ M ATP

As seen in Fig. 5, the dark and small type of mitochondrion predominates. Its diameter is about 500 mμ and its density reaches the highest value. The space between the two mitochondrial membranes maintains almost a normal value in those few places where the outer membrane is present, but at many points the outer membrane is absent, apparently detached from the contracted mitochondria. The diameter of the cristal lumens attains the largest value, which is about 500 A. The matrix, very dense and contracted, shows conglomerates of the small particulate component (40–50 A) and in some places closely packed and irregular membranes of 80 A, apparently related to collapsed cristae. Granules (grana) are difficult to recognize.

DISCUSSION

In this work we report three main observations: first, the heterogeneous population of mitochondria isolated in 0.5 M sucrose which appear in 3 types, contracted, medium, and swollen, in approximately equivalent amounts; secondly, the predominance of the swollen type after phlorizin treatment; and thirdly, the predominance of the contracted mitochondria in the presence of ATP. Data concerning mitochondrial volumes and microdensitometry have been recently published by the present authors (2).

Without ignoring the possibility of structural

FIGURE 3   Control population of isolated mitochondria after 15-minute incubation in 0.5 M sucrose, showing the three main types: M_1 similar to the in situ mitochondria; M_2 the smallest and most dense, and M_3 the largest and most swollen. The arrows point to the small particles of 50 A. Uranyl acetate and lead citrate stained. × 58,000; inset × 200,000.

alterations as a result of the isolation conditions, which applies to all cell fractionation procedures, we suggest that the three main types are normal structural stages of mitochondria. They probably reflect the mitochondrial level of ATP or the ability to hydrolyze ATP, the swollen type having the lower values and the contracted type the higher values. Green (7) and Ziegler and Linnane (21) also describe in isolated beef heart mitochondria a heterogeneous population with differences in their biochemical properties.

The effect of ATP and phlorizin upon isolated mitochondria has been recently reviewed by Lehninger (11) and by Lotspeich (13). They concluded that phlorizin probably exerts its effect by altering the permeability of the mitochondrial membranes, and that a high energy phosphate is required to maintain their normal permeability.

In our hands the study of isolated mitochondria treated with phlorizin shows that mitochondrial membranes do not change in thickness, no matter how much the outer membrane stretches to accommodate the four-fold increase in volume of the swollen mitochondria, and the inner membrane apparently unfolds or unpleats in many of the cristae. The volume of the outer mitochondrial compartment and cristal lumens maintains approximately the same (or lower) values as those of the controls; but the volume of the inner compartment increases, on average, four times, concomitantly with a marked decrease in the optical and electron opacity of matrix.

Mitochondria treated with phlorizin plus ATP contract, on average, to half the control volume. This remarkable contraction is accompanied by an increase in the optical density and electron density of all the mitochondria present in the pellet. This contraction apparently takes place in the mitochondrial matrix, leaving enlarged cristal lumens and a very dense and reduced inner compartment. A similar effect has been obtained with ATP alone (1). In our hands ATP not only prevents phlorizin-induced swelling but causes a marked mitochondrial contraction.

In a recent paper Weinbach and coworkers (20) present evidence of reversible changes in the morphology of liver mitochondria suspended in

0.25 M sucrose concomitant with swelling and uncoupling, but they were unable to restore, with ATP alone, the normal conditions. In our opinion, this discrepancy can be ascribed to the lower molarity of the sucrose and to the different source of mitochondria.

The small particulate component is shown only in those sections stained with uranyl acetate (1) and appears in the vicinity of the cristae and internal mitochondrial membrane. These particles accompany such membranes during swelling and appear closely packed and very dark during contraction with ATP. Probably they correspond to the granulations described by Watson (19) in the matrix of uranyl acetate-stained liver mitochondria.

Another interesting observation is the finding of membranes in the cristal lumens and apparently also in the inner mitochondrial compartment which have dimensions corresponding roughly with those of the mitochondrial membrane. However, we believe that these membranes correspond to the inner mitochondrial membrane proper which, after the mitochondrial contraction, conglomerates by puckering or refolding as the volume of the mitochondrion decreases (1).

From the morphological point of view, the mitochondrial compartment that undergoes the most important changes during swelling and contraction of mitochondria is the inner one. It appears from this observation that the mitochondrial matrix contains a colloidal gel component with properties similar to those of the actomyosin complex, able to change its physical state in the presence of ATP. Ernster (5), Price, Fonnesu, and Davies (16), and Lehninger (10) advance a similar suggestion based on biochemical studies. In recent studies, we have observed with negative staining, the contraction of the inner compartment in ATP-treated mitochondria (1); at the same time, we have obtained new evidence for a contractile component in the mitochondrial matrix and evidence for the non-contractility of the mitochondrial membranes (Aoki, Burgos and Iñón, unpublished results).

In contrast, the mitochondrial outer compartment does not appear to participate actively in

FIGURE 4   Phlorizin-treated mitochondria, showing the predominance of the swollen type. They attained the largest volume and lowest densities at the expense of the internal compartment. g, grana; arrow points to conglomerations of the small particulate component around the cristae. Uranyl acetate and lead citrate stained. X 58,000.

any important structural change, except for the elongation and contraction of the outer membrane during swelling and contraction, respectively. The variations in size of isolated mitochondria reported here and in the literature appear to be produced by changes in volume of the inner compartment during swelling and contraction. In all cases the mitochondrial membranes, at the level of resolution we obtained, maintain the same thickness and characteristics, except for the outer membrane which appears frequently detached from the mitochondrial body in the ATP-treated pellets. This point is the subject of further investigations (1).

Note: After the present paper was completed, we learned that Ohnishi and Ohnishi had succeeded in extracting a contractile protein from intact liver mitochondria with many properties similar to those of actomyosin (*J. Biochem., Japan,* 1962, 51, 380).

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FIGURE 5 Phlorizin-ATP-treated mitochondria showing predominance of contracted mitochondria. Cristal lumens (c) attain the largest diameter, and the matrix shows conglomerates of particles of high density (arrow). Closely packed membranes at m, and detachment of the outer membrane at om. Uranyl acetate and lead citrate stained. Inset: arrows point to the small particles of 50 A. Uranyl acetate and lead citrate stained. X 58,000; inset, X 200,000.