

Studies on Ultrastructure and Purification of Isolated Plant Mitochondria¹

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Abstract. Sweetpotato mitochondria, that showed respiratory control, were studied with respect to ultrastructure. If fixed in media containing sucrose at 0.4 M, the cristae were dilated and the matrix was highly condensed. A more orthodox ultrastructural form was observed when the mitochondria were fixed in a medium containing sucrose at 0.25 M, *i.e.*, the matrix was more expanded, the cristae were less dilated, and peripherally, the inner membrane element lay adjacent to the outer membrane element. These results are discussed in terms of a sucrose-accessible space (space between outer and inner membrane elements including intracristal space), and a space relatively inaccessible to sucrose (matrix). Ultrastructural shifts were not observed with change in metabolic steady state of the mitochondria. High resolution electron micrographs showed that the ultrastructure of sweetpotato mitochondria is very similar to that of animal mitochondria.

Purity and homogeneity of mitochondrial fractions were followed both by phase-contrast and electron microscopy. Preparations from sweetpotato, using older methods, were relatively homogeneous with respect to particle type and size, whereas avocado preparations contained a high proportion of chloroplasts and cellular debris. A method of purification involving sucrose-density-gradient centrifugation was developed. Purified mitochondria exhibited respiratory control and appeared similar to unpurified mitochondria under the electron microscope.

While investigators have recently attempted to improve plant mitochondrial preparations, using respiratory or acceptor control by ADP as a criterion of functional intactness (13, 35), little attention has been given to the ultrastructure of the mitochondria. Indeed, little attention has been given to the presence of contaminating organelles and cellular debris. The investigation of Parsons, Bonner, and Verboon (23), on the ultrastructure of plant mitochondria showing respiratory control, and the investigation by Chrispeels and Simon (7), on ultrastructure of plant mitochondria and heterogeneity of mitochondrial fractions, are notable exceptions.

In the present investigation, the objectives were: (1) To further examine possible relationships between respiratory control and ultrastructure in plant mitochondria; (2) To study possible ultrastructural shifts depending upon changes in metabolic state of

plant mitochondria or their environment; (3) To develop a method for preparing plant mitochondria which show biochemical and morphological signs of intactness, and which are relatively free of contaminating particles and debris. A preliminary report of this work appeared earlier (5).

Materials and Methods

Plant Materials. Sweetpotato (*Ipomoea batatas*, Poir) roots were of the white variety, Pelican Processor, used in earlier studies (3, 4). The roots were stored at 15° until needed, and were chilled at 0° for 1.5 hr before extraction of mitochondria was begun. Avocado (*Persea gratissima*, Gaertn.) fruit, variety Fuerte, were stored under conditions described by Hobson *et al.* (13), and then chilled for mitochondrial extraction.

Preparation of Mitochondria. (A) *Crude Preparation.* The standard method of mitochondrial preparation used in these studies, was that of Hobson *et al.* (13). Mitochondria were also prepared by the method of Baker and Lieberman (3) for examination by electron microscopy. (B) *Density-Gradient Preparations.* Discontinuous gradients were prepared by layering sucrose solutions into a 30 ml Spinco tube by means of a hypodermic syringe with curved needle. The concentration of sucrose decreased in 0.2 M steps from 1.6 M at the bottom, to 0.4 M at the top of the tube. Other components

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present in each layer were: 0.01 M KCl; 0.01 M tris-0.01 M potassium phosphate buffer, pH 7.2; and 0.75 mg/ml fatty acid-poor bovine serum albumin (BSA). These constituents were included in the gradient, since they were also constituents of the suspending medium for the crude preparation.

Continuous gradients were prepared by the following procedure. A solution containing 1.6 M sucrose was added dropwise to a mixing chamber which contained 10 ml of a solution 0.4 M with respect to sucrose. A small tube led from this chamber to the bottom of a 30 ml centrifuge tube. Both solutions contained KCl, potassium phosphate, tris, and BSA as described for discontinuous gradients. The gradient formed under these conditions was of the convex type (26).

In the early experiments, a crude mitochondrial suspension, prepared by the method of Hobson *et al.* (13) was layered onto the gradient and centrifuged in a Spinco SW-25 rotor at 15,000 rpm for 1 hr at 0°. In order to reduce the total time involved in density-gradient purification, the Hobson method was abbreviated. The brei was strained through muslin, centrifuged at 1500*g* for 15 min, and the supernatant removed and centrifuged at 12,000*g* for 20 min. The pellet from the 12,000*g* centrifugation was taken up in suspending medium, layered onto the gradient, and centrifuged as previously described in the Spinco SW-25 rotor. The mitochondrial fraction was removed carefully with a hypodermic syringe, diluted 5-fold with suspending medium, and centrifuged at 12,000*g* for 15 min. The pellet was taken up in 2 ml of suspending medium. A purified preparation was thus obtained in about the same length of time as required in the Hobson procedure.

Polarographic Measurements. Oxygen consumption was measured polarographically using the apparatus and reaction mixture of Hobson *et al.* (13). The medium used contained 0.25 M sucrose, 0.01 M potassium phosphate buffer, pH 7.2, 10 mM tris-HCl buffer, pH 7.2, 5 mM MgCl₂, 0.5 mM EDTA, and 0.75 mg/ml BSA, in a total volume of 3 ml. The mitochondria experienced a tonicity change with respect to sucrose when introduced into the reaction mixture, since the suspending medium contained 0.4 M sucrose and other components as described for density gradients.

The metabolic steady states defined by Chance and Williams (6), were obtained as follows. Mitochondria were added to the reaction mixture which contained P_i, but not ADP or substrate. These conditions are similar to the State 1 conditions of Chance and Williams, and we shall say that the mitochondria were in State 1. Succinate was then added to the mixture, and a steady state in oxygen consumption was soon reached. At that point ADP was added and the steady state that resulted was State 3. When the ADP was exhausted the rate of oxygen consumption returned to the rate prior to ADP addition, or the State 4 rate. Respiratory control (R.C.) ratios were obtained by dividing the

State 3 rate by the State 4 rate. State 5 was achieved by allowing State 3 to be terminated by oxygen exhaustion.

Electron Microscopy. Sweetpotato mitochondria were pelleted, and suspending medium or sucrose solutions containing 2.5 % glutaraldehyde was added to the pellet, except in the cases where fixation of the mitochondria in States 1, 3, 4, and 5 was attempted. In the latter cases, the reaction mixture was rapidly removed from the reaction vessel and mixed with 25 % glutaraldehyde to a final concentration of 2.5 % glutaraldehyde. The mitochondria were pelleted after 15 min and allowed to stand in the glutaraldehyde solution a total of 2 hr, as in the first case where fixative was added to the pellet. In both cases, pellets were washed briefly with suspending medium and were post-fixed with 1 % OsO₄ dissolved in suspending medium for 10 to 12 hr. The pellets were then washed in suspending medium, and post-fixed for 1 to 2 hr in a 1 % solution of uranyl acetate. They were then washed with distilled water, dehydrated in acetone, and embedded in Vestopal W. In 1 experiment (fig 4) the uranyl-acetate step was omitted, dehydration was accomplished with isopropyl alcohol, and embedding was in Epon 812 (17). Avocado mitochondrial pellets were fixed in the same manner, dehydrated in ethanol, and embedded in Epon 812. A similar procedure was used in preparing sweetpotato tissue for electron microscopy. Thin slices of sweetpotato tissue were cut with a razor blade and rapidly placed in an ice-cold fixing solution containing 0.4 M sucrose and 2.5 % glutaraldehyde. After 1 hr, this solution was removed, and the tissue washed in 0.4 M sucrose, and placed in a solution containing 1 % OsO₄ dissolved in a 0.4 M sucrose solution. The remainder of the procedure was as described for mitochondrial pellets.

Thin sections of the embedded material were prepared with an LKB Ultratome⁵ and were stained with lead salts and uranyl acetate. A Siemens Elmiskop I and a Philips EM-200 were used in these studies.

Results

Phase-contrast microscopy was used to monitor purity of preparations in studies of ultrastructure and purification by isopycnic separations. When care is used in resuspending sweetpotato mitochondrial pellets, *i.e.*, if the fluffy layer on top of the pellet is removed by gentle agitation with medium, and the starch pellet is left adhering to the tube, a relatively homogeneous preparation is obtained (fig 1A). This is in marked contrast to the preparations obtained from avocado which are complex mixtures

⁵ The mention of specific instruments, trade names, or manufacturers is made for the purpose of identification and does not imply any endorsement by the United States Government.

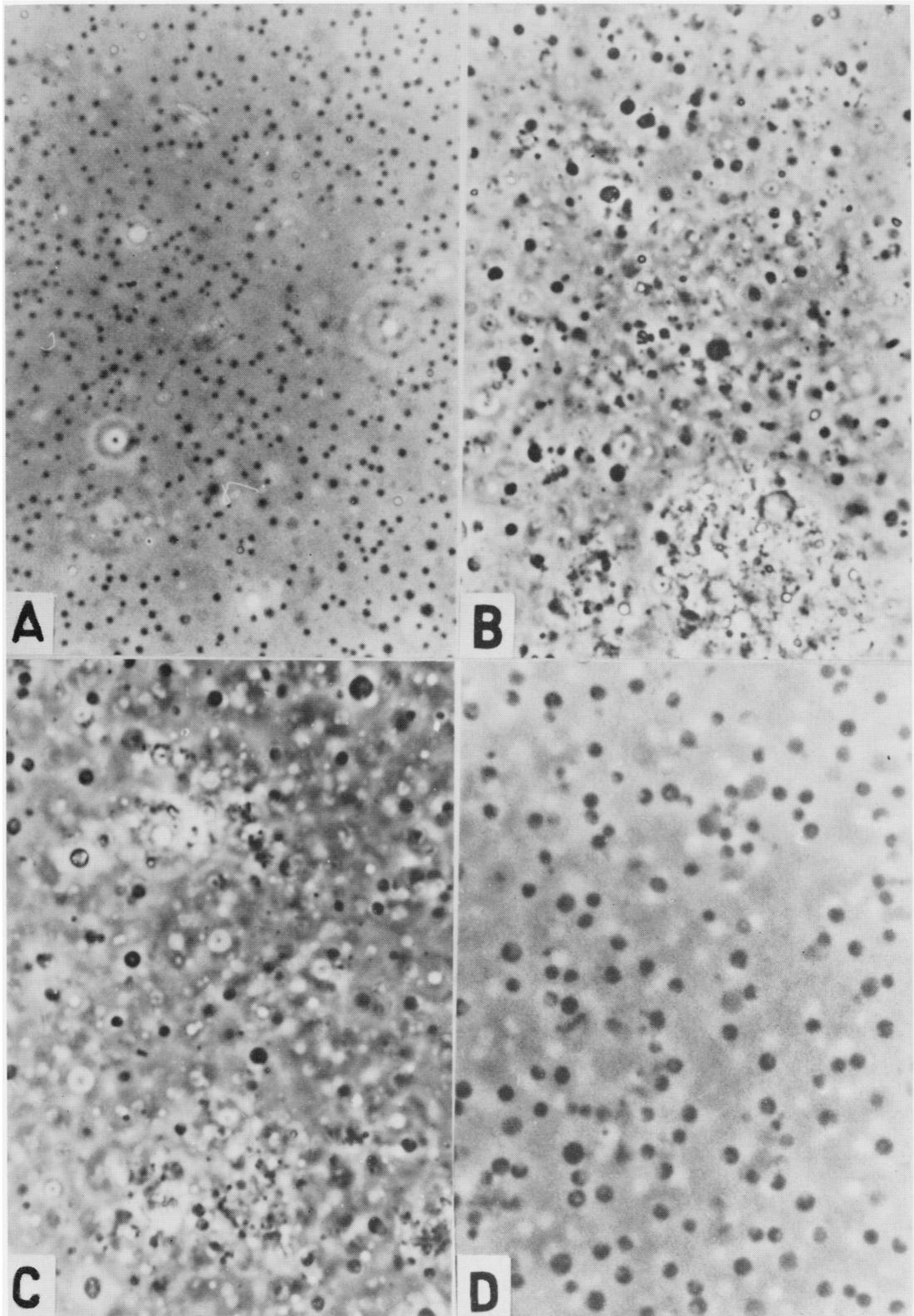


FIG. 1. Phase contrast photomicrographs of particles in plant mitochondrial preparations. All were taken at $1250\times$ magnification on 35 mm film and enlarged $3\times$ in printing. (A) Sweetpotato preparation. (B) & (C) Preparations from climacteric-rise avocado fruit. (D) Avocado mitochondria after density-gradient purification.

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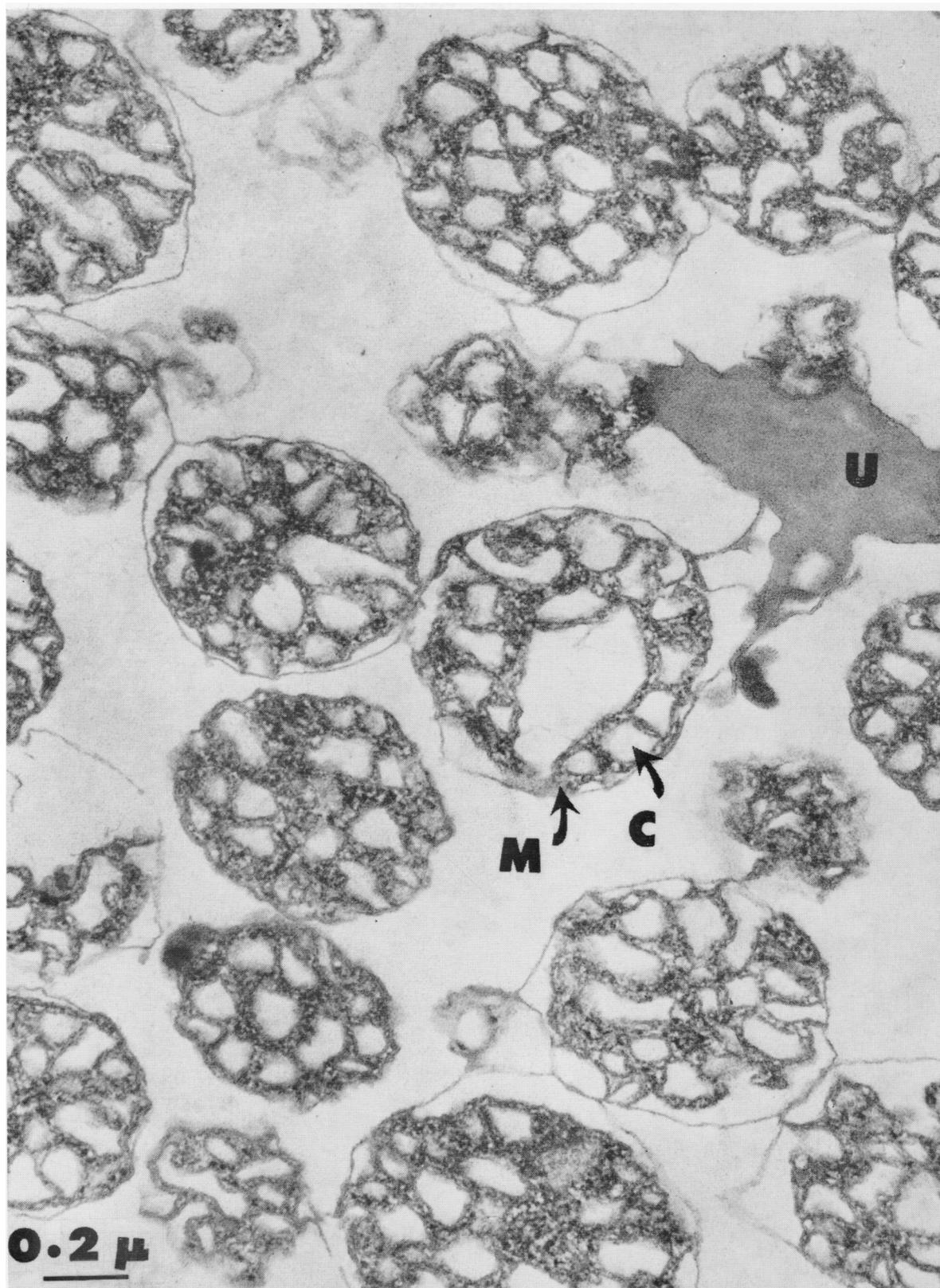


FIG. 2. Sweetpotato mitochondria isolated by the method of Baker and Lieberman (3), and suspended in 0.4 M sucrose. (M) Matrix. (C) Inner membrane invagination or crista. (U) Unidentified material. $\times 70,000$.

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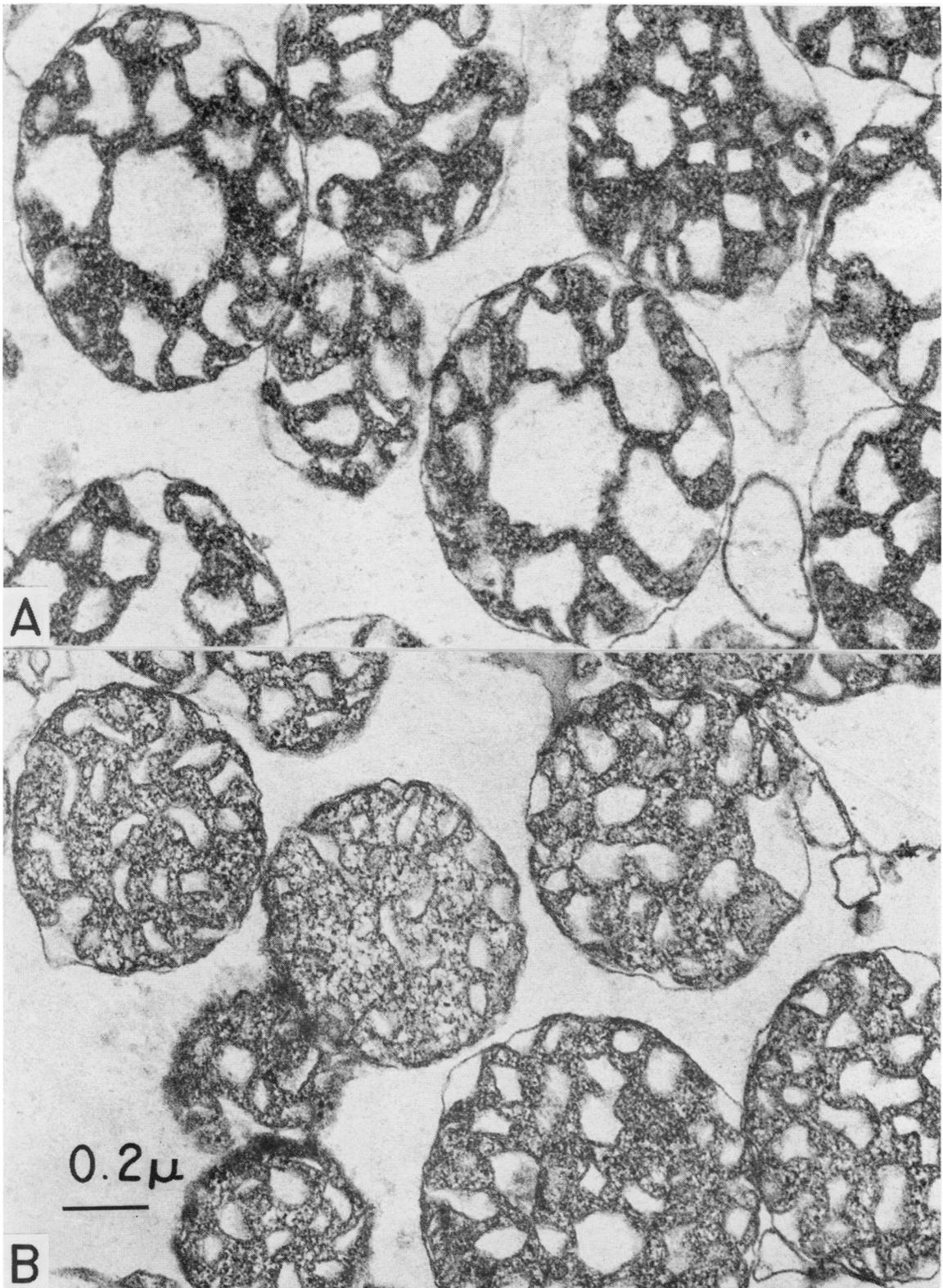


FIG. 3. Sweetpotato mitochondria isolated by the method of Hobson, *et al.* (13). $\times 70,000$. (A) Suspended and fixed in a solution (pH 7.2) containing 0.4 M sucrose, 0.01 M KCl, 0.01 M tris, 0.01 M KH_2PO_4 , and 0.75 mg/ml BSA. (B) Suspended and fixed in the reaction mixture described for polarographic measurements.

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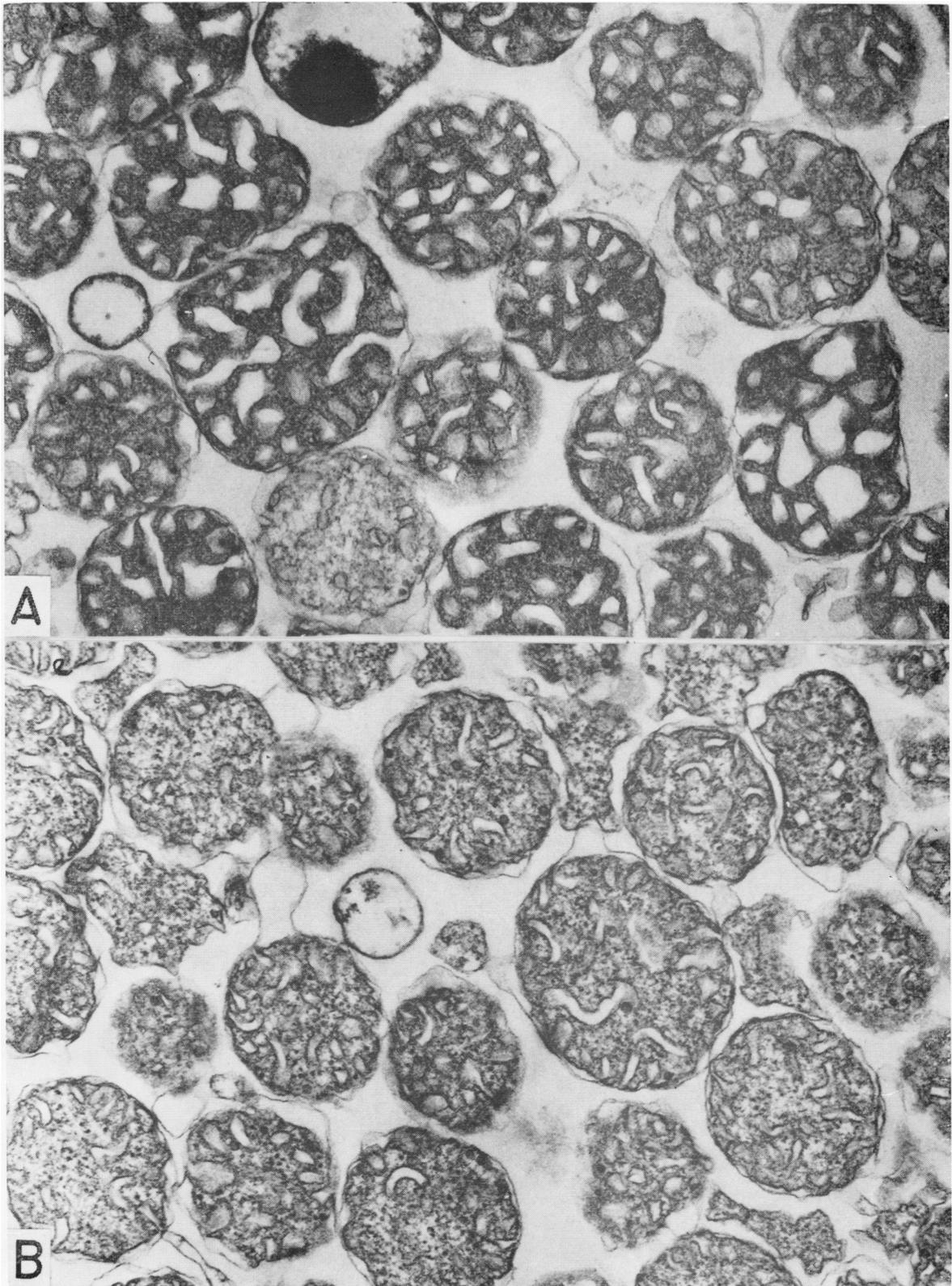


FIG. 4. Sweetpotato mitochondria. $\times 34,100$. (A) Suspended in 0.4 M sucrose, pelleted, and fixed with a solution containing 0.4 M sucrose and 2.5% glutaraldehyde. (B) Suspended in 0.2 M sucrose, pelleted, and fixed with a solution containing 0.2 M sucrose and 2.5% glutaraldehyde.

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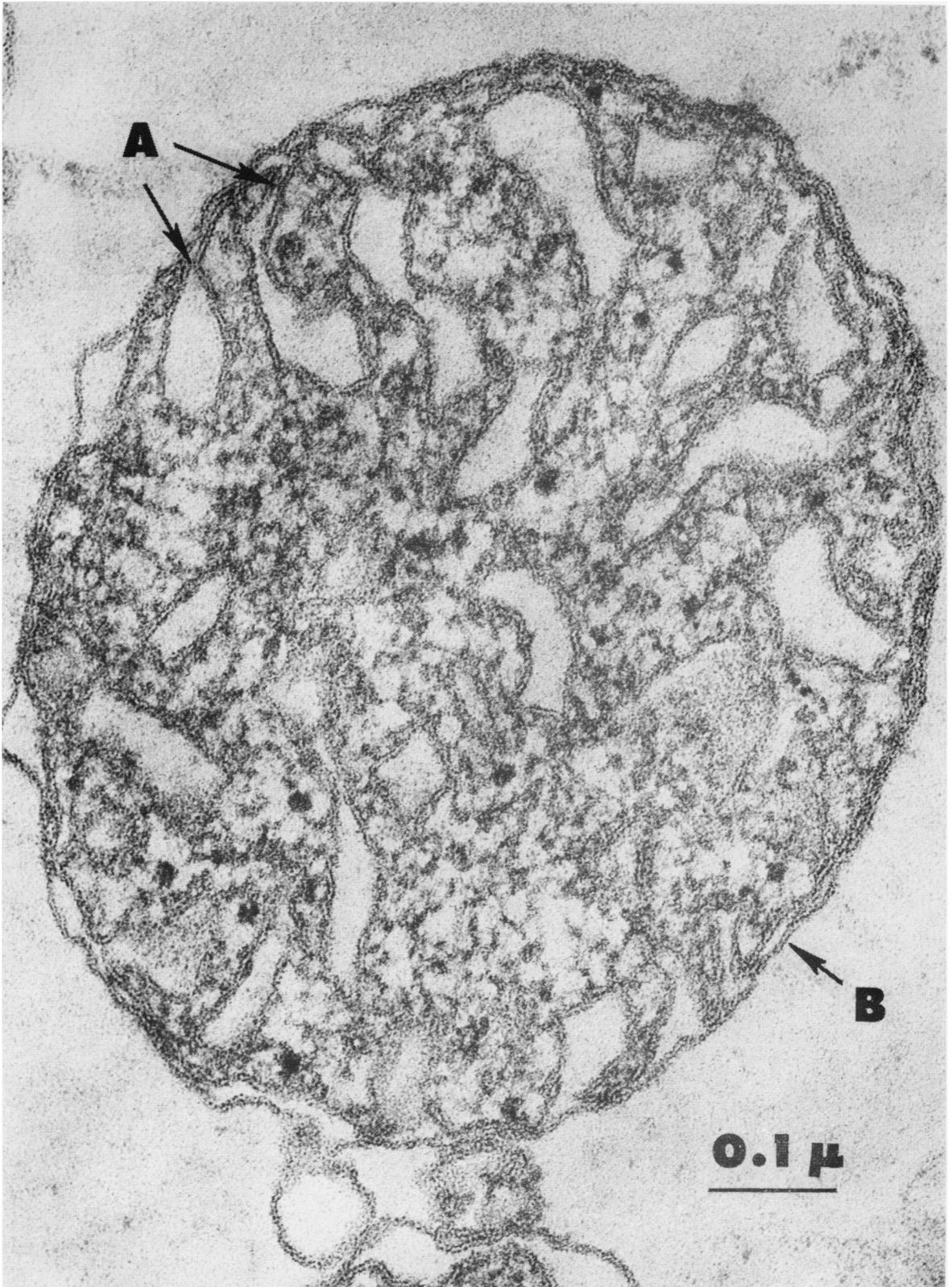


FIG. 5. High resolution electron micrograph of a sweetpotato mitochondrion from the preparation shown in figure 4, showing triple-layered membrane elements. (A) Space between outer and inner membranes communicates with the intra-cristal space *via* constricted cannulla. (B) Arrow points to triple-layered membrane element. $\times 210,000$.

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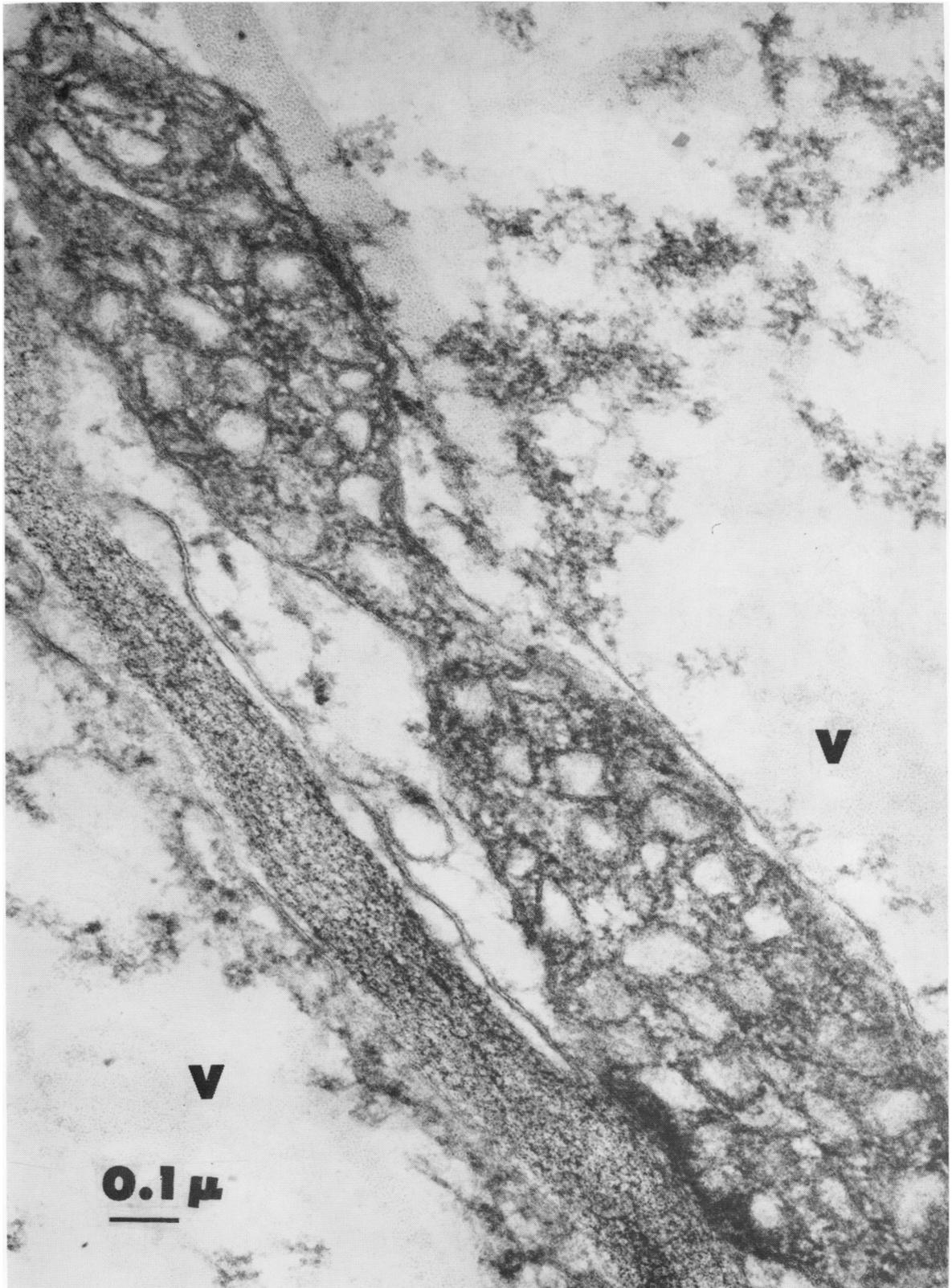


FIG. 6. Sweetpotato mitochondria *in situ*. Cytoplasm forms a thin layer along the cell wall and surrounds a large vacuole (V). $\times 114,000$.

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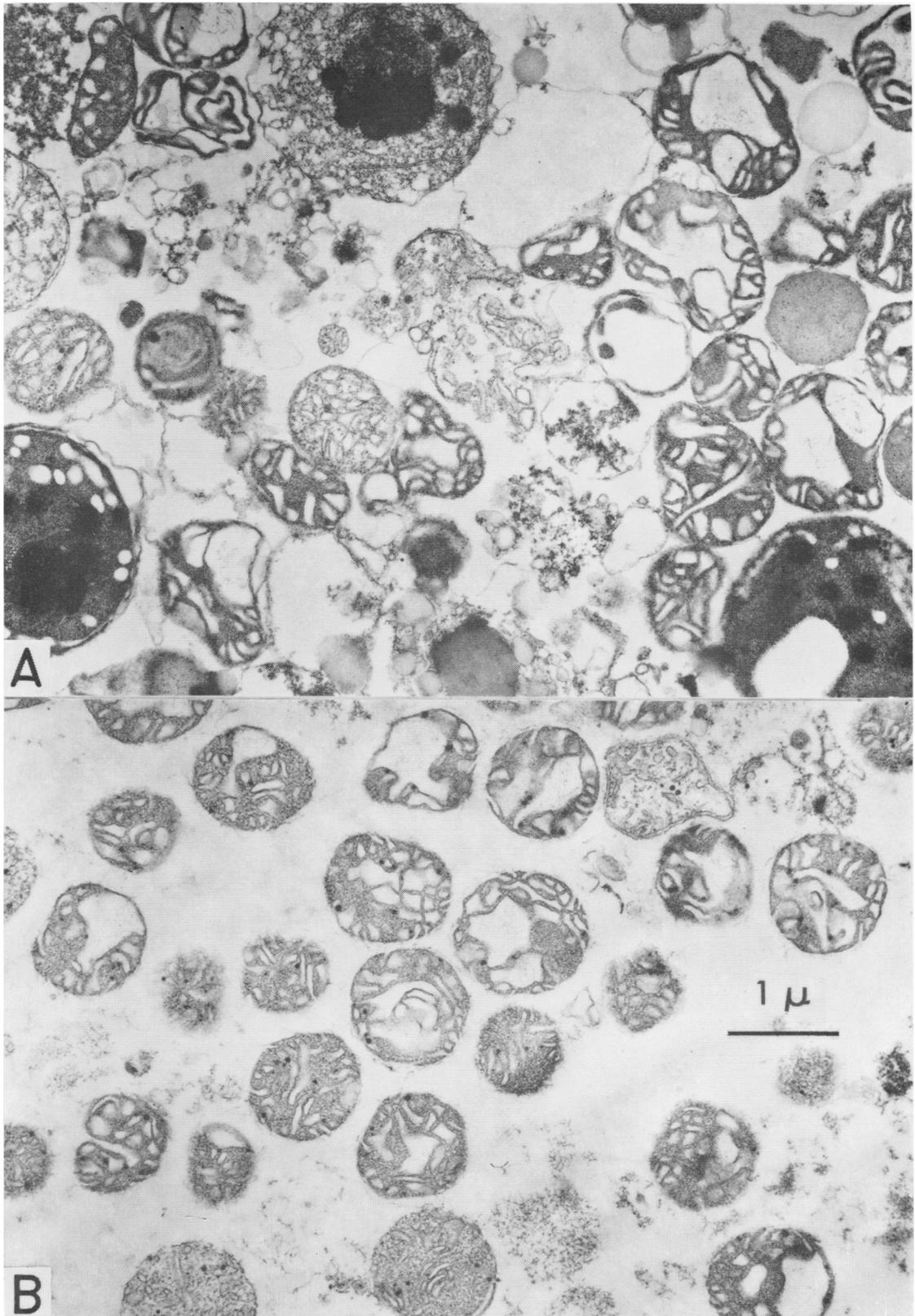


FIG. 7. Mitochondria from climacteric-rise avocado fruit, suspended and fixed in the medium described for figure 3A. $\times 18,000$. (A) Fraction obtained by the method of Hobson, *et al.* (13). (B) Fraction obtained by density-gradient centrifugation.

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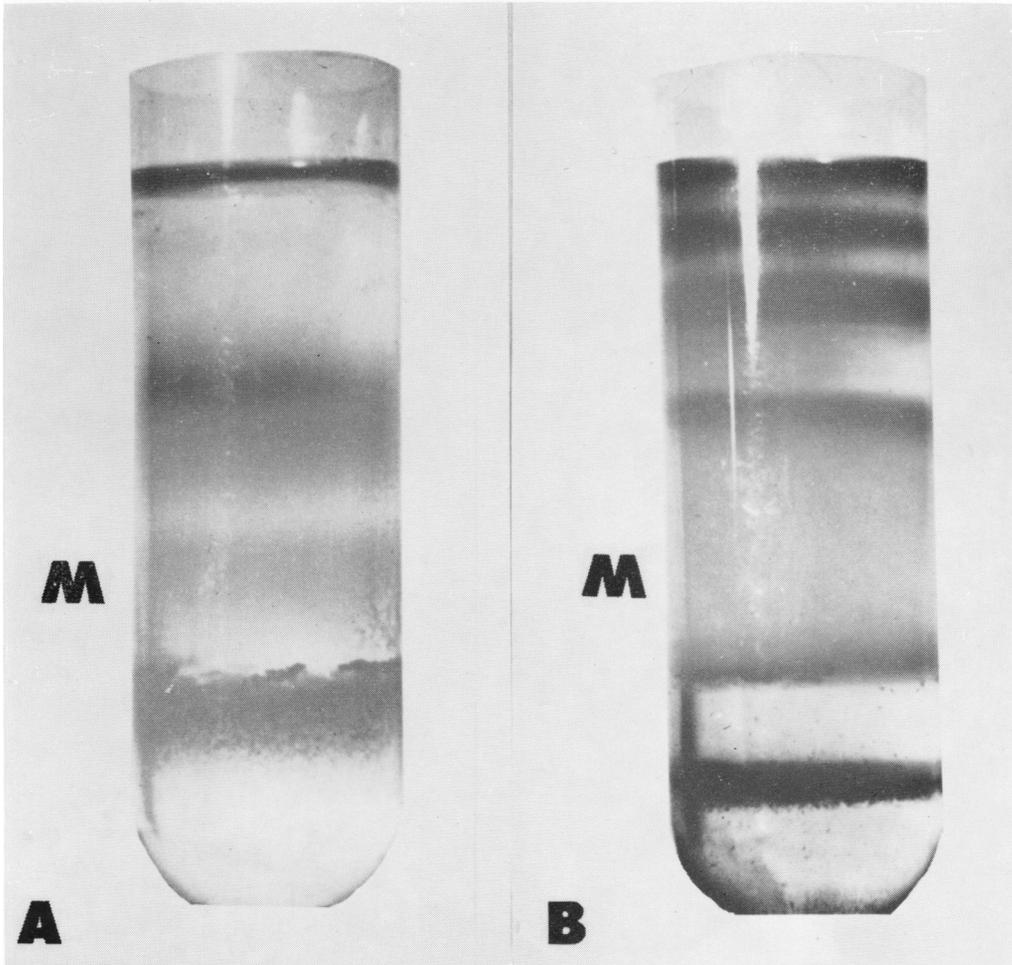


FIG. 8. (A) Avocado mitochondrial preparation centrifuged in a continuous sucrose gradient. (B) Avocado mitochondrial preparation centrifuged in a discontinuous sucrose gradient. (M) Mitochondrial fraction.

of chloroplasts, chloroplast fragments, oil droplets, and other cellular debris (fig 1, B and C).

Electron micrographs, of a sweetpotato preparation made by a method used in earlier studies (3, 4), show a population of mitochondria rather homogeneous with respect to size, and containing only occasional unidentified bodies (fig 2). The mitochondria show a relatively well-preserved ultrastructure in contrast to the appearance of isolated plant mitochondria in many electron micrographs previously presented in the literature. By "well-preserved," it is meant that the mitochondria contain a rather dense, continuous matrix, triple-layered inner and outer membranes, and otherwise resemble mitochondria in fixed tissue. The mitochondria in this preparation show a regular pattern of dilated cristae, and a condensed matrix consisting mainly of dense granules about 60 Å in diameter. Close inspection reveals that in some cases, parts of the outer membrane are missing, and occasionally the entire outer membrane may be absent. These mitochondria were suspended in a 0.4 M sucrose solution, and they were also fixed in a solution containing 0.4 M sucrose.

Mitochondria prepared according to the method of Hobson *et al.* (13) were very similar in appearance to the mitochondria described above (fig 3A). The suspending medium in this case also contained 0.4 M sucrose, but in addition, tris, KH_2PO_4 , KCl, and BSA. The outer membrane was very seldom missing from these mitochondria, but some empty vesicles were evident in the preparation.

Micrographs of Hobson-type mitochondria suspended and fixed in a reaction medium which was 0.25 M with respect to sucrose, show less dilated cristae and a more expanded matrix (fig 3B). Mitochondria of this type were fixed in States 1, 3, 4, and 5 described by Chance and Williams (6). Neither ultrastructural shifts of the type reported by Hackenbrock (12) for rat-liver mitochondria, nor the smaller shifts of the type reported by Penniston *et al.* (25) for heart mitochondria were observed. *i.e.* sweetpotato mitochondria in States 1, 3, 4, and 5 appeared identical to those shown in figure 4. Micrographs of sweetpotato mitochondria which were fixed in solutions containing only sucrose and glutaraldehyde are shown in figure 4. Mitochondria fixed in 0.4 M sucrose show a highly condensed matrix and dilated cristae, while mitochondria fixed in 0.2 M sucrose show a more expanded matrix, and cristae which are much less dilated.

Higher resolution micrographs of sweetpotato mitochondria from the 0.25 M sucrose, reaction medium (fig 5), reveal that both the outer and inner membranes are triple-layered units with a thickness of 50 to 60 Å, as Sjostrand demonstrated in osmium-fixed rat-kidney mitochondria (30). It may also be observed that the intracristal space is a continuation of the space between outer and inner membranes. In some cases it appears that the cristae have separated from the peripheral inner membrane and that the intracristal space forms a separate

compartment. This is perhaps due to the fact that the crista is constricted where it joins the peripheral inner membrane, and this part would frequently lie out of the plane of the section.

An electron micrograph of sweetpotato mitochondria *in situ* is shown in figure 6. These mitochondria were similar in appearance to isolated mitochondria, but elongate as well as spherical forms were observed. The cristae are somewhat dilated perhaps reflecting hypertonicity of sucrose in the fixation medium. Because sucrose at 0.4 M is commonly used in the preparation of plant mitochondria, it was included in the fixation medium for tissue. The results presented indicate that 0.4 M sucrose is hypertonic. In the case of the elongate type shown in figure 6, it would appear that a mitochondrion was fixed in the act of dividing, or that 2 mitochondria were fixed in the act of coalescing. Both phenomena are observed by phase-contrast microscopy in the living cell where mitochondria are dynamically pleomorphic (14, 15). However, an alternative likely explanation is that the section is cut through a mitochondrion with a curved or bent form.

Electron micrographs of avocado mitochondria show an ultrastructure similar to that of the isolated sweetpotato mitochondrion (fig 7A). The cristae in places are somewhat more swollen or bulbous and the matrix more condensed than in sweetpotato mitochondria. As expected from observations by phase-microscopy, the preparation is heavily contaminated with altered chloroplasts, as well as vesicular and granular structures of unidentified nature. While the ultrastructure of the mitochondria may be described as unorthodox, it is important to note that animal mitochondria often show similar ultrastructural features. Bovine-brain mitochondria (28) and rat-kidney mitochondria in 0.5 M sucrose (24), show morphological features very similar to those of the avocado mitochondria in figure 7.

Density-Gradient Centrifugation. Avocado mitochondria, from preclimacteric fruit, accumulated in the 1.2 M sucrose layer of a discontinuous gradient (fig 8B). After resuspension and equilibration in the original medium containing sucrose at 0.4 M, respiratory activity and acceptor control were measured using succinate as substrate (fig 9A). This measurement was made 3 hr after the activity of the unpurified mitochondria was measured. Respiratory control (R.C.) ratios were as high in the gradient preparations, as in the original preparation.

Continuous gradients, covering the same range in sucrose concentration, proved more satisfactory for purifying mitochondria from fruit at all stages of development. Particles accumulated in 3 zones: the middle zone contained the mitochondria (fig 8A). As with mitochondria from the discontinuous gradients, these showed R.C. ratios similar to the original preparation, and specific activity (O_2 uptake/ x) was greater than in the original (fig 9B). In this experiment, the final suspension from the Hobson method was centrifuged in the gradient. The modi-

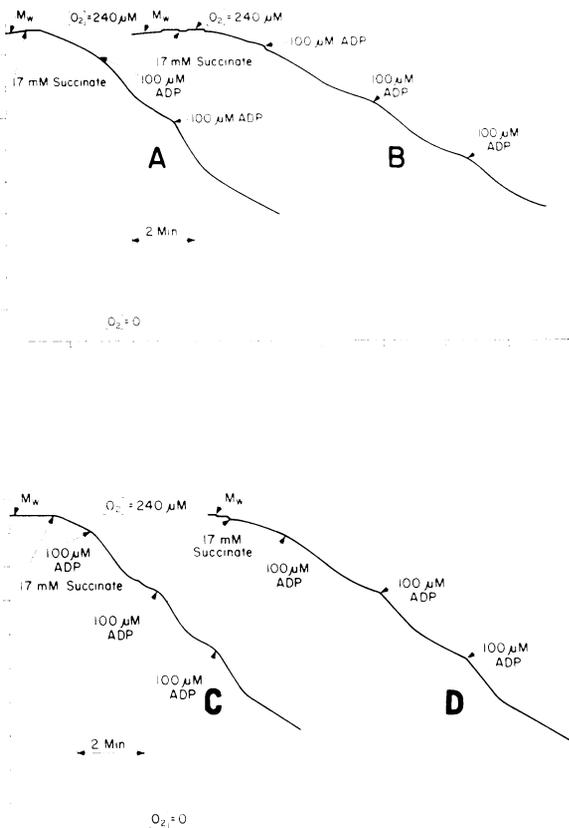


FIG. 9. Polarographic traces showing respiratory activity of mitochondria from avocado fruit. (A) Crude preparation ($60 \mu\text{g N}$) from preclimacteric fruit. (B) Fraction ($50 \mu\text{g N}$) obtained by centrifuging preparation (A) in a discontinuous sucrose gradient. (C) Crude preparation ($130 \mu\text{g N}$) from climacteric rise avocado fruit. (D) Fraction ($85 \mu\text{g N}$) obtained after centrifuging preparation C in a continuous sucrose gradient.

fied abbreviated method produced similar results in a much shorter time.

Avocado mitochondria after gradient purification and resuspension in the original medium, were examined by electron microscopy. The mitochondria (fig 7B) appeared quite similar to untreated mitochondria. Phase-contrast microscopy showed a relatively homogeneous population of particles in the density-gradient fractions (fig 1D).

Discussion

Ultrastructure of Plant Mitochondria. One of the objectives at the outset of this investigation was to determine, if possible, whether or not relationships between respiratory control and morphological features existed in plant mitochondria. Plant mitochondria showing respiratory control oxidize external DPNH (35). On the other hand, appreciable rates of DPNH oxidation by animal mitochondria are

observed only after their exposure to conditions which increase permeability (16). Apparently ADP control of respiration is not a good criterion of structural intactness or the permeability characteristics of plant mitochondrial membranes differ basically from those of their animal counterparts. Sweetpotato mitochondria prepared by an older method of Baker and Lieberman (3), which utilizes the Waring Blendor and 0.15 M tris buffer in the isolation medium were first examined. Respiratory control ratios in such mitochondria are usually low. The analysis shows that the mitochondria are relatively well-preserved, as defined by the presence of outer and inner membranes, and a matrix resembling those of *in situ* mitochondria (fig 6). Mitochondria prepared by the newer method of Hobson *et al.* (13), and which consistently show good R.C. ratios, were then examined. These mitochondria were similar in appearance to mitochondria prepared by the older method. Perhaps the outer membranes were more intact in the Hobson preparation than in the Baker-Lieberman preparation. Examination of the latter-type mitochondria indicates that parts of the outer membranes are missing, and in some cases may be completely absent. Electron micrographs of mitochondria from Hobson-type preparations rarely indicated missing outer membranes, but did show the presence of empty vesicles (fig 3). On occasion, mitochondria in these preparations showed low R.C. ratios, and electron micrographs (not presented) showed a high incidence of vesicles in the preparation. The presence of damaged mitochondria in all preparations hampers conclusions, but our results indicate that low R.C. ratios may reflect either damaged outer membranes of plant mitochondria, or a high incidence of empty vesicles in the preparation. However, the results do not indicate to what extent isolated mitochondria were altered from their *in situ* condition.

Hackenbrock (12) found that rat-liver mitochondria isolated in 0.25 M sucrose showed a condensed matrix and increased intracristal and outer-compartmental space compared to mitochondria in fixed tissue. Short-term incubation (15 min) of these isolated mitochondria in States 1 or 4 resulted in a shift to orthodox (as in fixed tissue) ultrastructure. During transition from State 4 to State 3, a shift to the condensed unorthodox form was observed. The data presented in the present paper suggest that the plant mitochondria studied do not undergo obligatory ultrastructural shifts with change in metabolic state. Only an initial shift to a form with less condensed matrix was observed, when the sweetpotato mitochondria were placed under State 1 conditions (fig 3B). This shift is evidently in response to a change in osmolarity of sucrose, since the suspending medium contained sucrose at 0.4 M, and the reaction mixture contained sucrose at 0.25 M, and since no subsequent metabolic-dependent changes were observed. This assumption was confirmed in another experiment in which mitochondria were fixed in

solutions containing only sucrose at various concentrations. Electron micrographs (fig 4) showed that a condensed matrix, and dilated, bulbous cristae were characteristic of mitochondria fixed in 0.40 M sucrose. Whittaker (34) obtained somewhat similar results with rat-liver mitochondria. While it is possible that the methods used in this study might have obliterated ultrastructural shifts, it seems probable that an unorthodox form with condensed matrix such as that observed in rat-liver mitochondria in State 3, would have been detected, since we could detect different forms with respect to sucrose concentration. Investigations on this subject are continuing. One problem that exists is the possibility that the fixative agent, glutaraldehyde, contributes to the osmolarity of the sucrose-accessible space and causes changes before the structures are fixed. However, Deamer *et al.* (8), on the basis of light scattering data, concluded that glutaraldehyde rapidly fixed ultrastructural states in rat-liver mitochondria.

Changes in mitochondrial morphology due to change in sucrose concentration, as discussed above, may be explained on the basis of 2 compartments in mitochondria, one readily accessible, and the other relatively inaccessible, to sucrose. If the space between membranes, including the intra-cristal space is accessible to sucrose, and the matrix is not, then hydration of the matrix will vary depending upon the concentration of sucrose in the medium. That 2 such spaces are present in mitochondria is indicated by the work of Amoore and Bartley (1), and Malamed and Rechnagel (19). In fact, Malamed and Rechnagel measured the volume of these 2 spaces in isolated rat-liver mitochondria, and showed that the space inaccessible to sucrose was the space behaving as an osmometer, *i.e.*, obeying the Boyle-van't Hoff law. Tedeschi and Harris (33) showed that mitochondria obey this law and, until the work of Malamed and Rechnagel (19), it seemed an apparent contradiction that mitochondria acted as osmometers, yet were permeable to sucrose. Tedeschi (32) has recently concluded that 2 spaces need not be postulated. Most of the evidence, however, indicates that mitochondria contain 1 compartment, which is readily accessible to sucrose and other solutes, and another space, probably the matrix, which is not readily accessible to these solutes (21, 24). Recent studies, *e.g.*, that of Bachmann *et al.* (2), show that the outer and inner membranes of mitochondria are of different composition. It is therefore reasonable to assume that they have different permeability characteristics.

Considering the fine structure of sweetpotato mitochondria *per se*, we observed that high-resolution micrographs revealed triple-layered membrane elements in the outer as well as inner membranes. The matrix showed mainly dense granules *ca.* 60 Å in diameter, but also some larger granules, possibly ribosomes, in general agreement with the observations of Parsons *et al.* (23). The morphology and fine structure of sweetpotato mitochondria, are essen-

tially identical to that of rat-kidney mitochondria after osmium fixation (30). The cristae are somewhat ballooned in the sweetpotato mitochondria even in 0.25 M sucrose. Parsons, Bonner, and Verboon (23) also concluded that the ultrastructure of plant mitochondria is basically similar to that of animal mitochondria, but that cristae are more randomly oriented in plant mitochondria than in animal mitochondria, and the cristae of some plant mitochondria form "closed loops" in some instances. None of the electron micrographs of sweetpotato or avocado mitochondria observed in the present study showed closed-loop mitochondrial membranes. The inner membrane structures first demonstrated in animal mitochondria by negative staining techniques (10, 22), were not studied in this investigation. The work of Sjostrand *et al.* (31) indicated that the knob-like structures observed in negatively-stained mitochondria were artifacts. In view of the recent work of Stiles and Crane (29), and Fleischer *et al.* (11), the possibility that inner membrane structures are present in mitochondria, must again be considered. These structures have been demonstrated in negatively-stained plant mitochondria by Nadakavukaren (20), and by Parsons, Bonner, and Verboon (23).

Density-Gradient Purification of Mitochondria. The technique for purification presented herein, is considered a prototype. For certain purposes it may prove useful as described, *e.g.*, in determining the contribution of contaminating organelles such as chloroplasts in studies of chemical composition in mitochondrial preparations. We used the most primitive technique and equipment for producing gradients, yet achieved considerable purification of avocado mitochondria. To achieve better purification, the zonal centrifuge is necessary in order to take advantage of the sedimentation coefficient as well as the equilibrium density of a given organelle, as Price and Hirvonen (27) have done in purifying plastids.

In view of the reports of deleterious effects of high sucrose concentration on mitochondria (9, 18), the stability of avocado mitochondria to sucrose density-gradients observed in the present study, was unexpected. The presence of BSA and salts throughout the gradient may have contributed to the observed stability. The criteria used to determine whether mitochondria were altered in the gradient centrifugation were R.C. ratio, with succinate as substrate, and morphology of the mitochondria in electron micrographs. As far as the studies went, no significant alteration of the mitochondria was indicated. Further work on loss of soluble protein and other activities of the mitochondria is necessary to reveal possible alterations.

Acknowledgments

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