Title
Methods for mycelial breakage and isolation of mitochondria and vacuoles of neurospora

Journal
Analytical Biochemistry, 128(2)

ISSN
0003-2697

Authors
Cramer, Carole L
Ristow, Janet L
Paulus, Thomas J
et al.

Publication Date
1983-02-01

DOI
10.1016/0003-2697(83)90390-1

ANALYTICAL BIOCHEMISTRY 128, 384–392 (1983)

Methods for Mycelial Breakage and Isolation of Mitochondria and Vacuoles of Neurospora

CAROLE L. CRAMER, JANET L. RISTOW, THOMAS J. PAULUS, AND ROWLAND H. DAVIS

Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, California 92717

Received June 11, 1982

A new and inexpensive glass-bead blender allows rapid, easy, and controlled cell breakage of *Neurospora*, with good organellar survival. The yield of mitochondria and vacuoles is comparable to or better than methods involving sand grinding or snail-gut digestion of cell walls. A method for removing cell wall fragments from a crude homogenate is described. Isolation of mitochondria and vacuoles from the crude homogenate with little cross-contamination is accomplished by density-gradient centrifugation in a fixed-angle rotor (Sorvall).

In our work on mitochondria and vacuoles of *Neurospora crassa* we wished to have a rapid and inexpensive method of preparing both organelles in a reasonably pure state. We report such a method here. It is based in part on the use of a new and inexpensive (approx $3–400) glass-bead blender, the Bead-Beater, recently available from commercial sources. Cell disruption is fast and thorough. The method of organelle isolation described yields as much material as the lengthy, expensive, and variable methods based upon snail-gut digestion of cell walls (1,2). It is less laborious than sand grinding (3). It requires a machine less complex and expensive than some colloid mills or homogenizers described elsewhere (4). Methods for removing cell wall fragments and for separating mitochondria and vacuoles with little cross-contamination are reported. The latter is important, as noted by previous workers (5), if protease-free preparations of mitochondria are to be obtained. Further, *Neurospora* vacuoles, which are rather fragile, have only recently been isolated in a pure state (2); the new method of isolation will facilitate further intensive study. The blender is potentially useful for isolation of any organelle, membrane, or soluble fraction.

MATERIALS AND METHODS

Organisms and Growth

N. crassa wild-type 74A was used. Vogel's medium N with 1.5% sucrose was used for stocks and for all growth media (6). Mycelia were grown at 25°C from heavy conidial inocula (approx 1×10^6/ml, final concentration) for 10 to 20 h, either in low-form culture flasks on a shaker, in round flasks (1000 ml), or in carboys (15 liters) sparged vigorously with hydrated air (6). In all these cases, mycelia of densities exceeding 0.8–1.0 mg dry wt/ml could be collected in a wire basket or a Büchner funnel lined with Miracloth, in which they were rinsed first with a stream of distilled water, and then with the buffer in which they were to be disrupted. For cultures of lower mycelial density, harvests were made in a Büchner funnel with Whatman No. 1 or No. 54 filter paper.

Cell Disruption

The device used to disrupt cells was the "Bead-Beater" (Biospec Products, Bartlesville, Okla.). A baffled chamber (340 ml) is half-filled with glass beads (0.3–0.5 mm di-

0003-2697/83/020384-09$03.00/0

ameter) wetted with the starting buffer. Mycelium, equivalent to 1–10 g dry wt, is added in sufficient buffer to fill the chamber. A Teflon impeller (rotor) is inverted and screwed into the top, leaving no air in the chamber. The assembly is inverted on a blender motor and the mixture is blended for a chosen time. In our experiments, the temperature was maintained at 7°C or less by limiting power to 30- to 60-s bursts, followed by 1-min rests, and by using the ice chamber which surrounds the blender compartment. The buffer used was 10 mM TES[1]–NaOH buffer, pH 7.5, with 1 mM EDTA. For vacuole isolations, the osmoticum was 1 M sorbitol. This reduced the volume of vacuoles (2) and rendered them more resistant to mechanical as well as to osmotic lysis. For mitochondrial isolation, 1 M sorbitol was adequate for many purposes (see below), but the functional integrity of mitochondria may be comprised by the permeation of sorbitol and consequent swelling (7). Therefore, mitochondria were routinely isolated in buffer with 0.33 M sucrose as the osmoticum, with 0.3% bovine serum albumin, if protection during freezing and storage was desired. After disruption of cells, the contents of the flask were poured out through cheesecloth, held in a large Büchner funnel, to remove most of the beads. The homogenate retained by the beads was washed out with a small additional amount of buffer. The beads may be collected, washed, and reused many times.

Differential Centrifugation

The homogenate was centrifuged in tubes or bottles at 600*g* for 5 min. The supernatant (S600) was decanted through a 1- to 12-cm diameter glass-fiber filter (Reeve-Angel Grade 934 AH or Gelman Type A/E) held firmly on a coarse, fritted glass or plastic disk. Suitable surfaces are provided by Pyrex No. 36060 funnels or "fritware" immersion filters or polypropylene "fritware" Büchner funnels from Bel-Art Products, Pequannock, New Jersey. (Standard porcelain Büchner funnels are not suitable because they do not have a uniformly porous surface.) The glass-fiber filter was initially secured by wetting and slight suction to prevent homogenate from passing around it. The filtration step removed virtually all cellular and microscopically identifiable cell wall contamination, without loss of mitochondria or vacuoles. The filtrate was centrifuged for 20 min at 15,000*g*. The supernatant (S15,000) was decanted and saved. The organellar pellet (P15,000) was resuspended very gently but completely with the respective starting buffer (buffered sorbitol or buffered sucrose). This often included very gentle use (e.g., one stroke) of a Teflon–glass homogenizer, with awareness of the fragility of vacuoles.

Organelle Isolation

The organelles were isolated in normal practice in density gradients centrifuged in a fixed-angle, SS-34 Sorvall rotor in the RC-2B refrigerated centrifuge. Somewhat more refined gradients can be achieved in the ultracentrifuge (1,2), but these are unnecessary for routine work.

(a) Mitochondria. Discontinuous gradients were made by layering 10 ml 1.6 M sucrose, 10 ml 1.2 M sucrose, and 10 ml of the P15,000 suspension in a 50-ml clear nylon tube. All gradient material contained 10 mM TES–NaOH, pH 7.5, and 1 mM EDTA. The gradients were centrifuged for 2 h at 43,000*g*. Most vacuoles formed a pellet at the bottom of the tube; mitochondria collected at the 1.6–1.2 M sucrose interface (see Results). Mitochondria were removed with a Pasteur pipet, diluted with an equal amount of starting buffer, and recentrifuged (19,000*g*, 20 min) for a washed mitochondrial preparation. Mitochondria could also be isolated from gradients designed for vacuole isolation (see below).

[1] Abbreviations used: TES, 2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulfonic acid; P, pellet; S, supernatant.

(b) Vacuoles. A gradient was made by layering 4 ml of 1.8 M sucrose and a continuous, 6-ml 1.8 M sucrose–1.0 M sorbitol gradient in a 15-ml Sorvall tube. The gradient materials all contained the standard buffer. The resuspended P15,000 (1–2 ml, in 1 M sorbitol buffer) was added to the top of the continuous gradient. The gradient was centrifuged for 2 h at 43,000*g* in the SS-34 rotor. The vacuolar pellet, after aspiration of the entire gradient, was resuspended in 1.8 M buffered sucrose. (If mitochondria were desired, gradient fractions were collected from the top; mitochondria equilibrated about 1/3 the distance into the gradient.)

Velocity Sedimentation

For comparison of vacuoles isolated from cells broken with glass beads or by homogenization after snail-gut treatment, a double-label velocity-sedimentation experiment was done. Cycloheximide-treated cultures in which the vacuolar arginine pools were labeled with a 10-min pulse of L-[^{3}H]arginine (bead disrupted) or L-[U-^{14}C]arginine (snail gut treated) (2) were disrupted by the methods described above or ones described previously (1,2). The S600 fractions were combined, and a P15,000 fraction was collected in buffered sorbitol. It was applied to a 12-ml continuous gradient which was 1 M sucrose (top) to 1 M sucrose + 20% Ficoll (bottom). Gradients were centrifuged at 100,000*g* for 10, 20, or 30 min at 0 to 4°C in an SW 41 Ti rotor in a Beckman ultracentrifuge. Fractions, collected from a puncture in the bottom of the tube, were counted for ^{3}H and ^{14}C after purification of arginine on columns as described previously (2).

Assays

Arginine was assayed by the method of Van Pilsum *et al.* (8). Protein was measured according to Bradford (9). Ornithine aminotransferase was measured by the method of Weiss and Anterasian (10), ornithine transcarbamylase by the method of Davis (11), succinate dehydrogenase by the method of Pennington (12), and protease by the azocoll method of Lampkin *et al.* (13). Characterization of mitochondrial respiration was done by the methods of Estabrook (14). Cell and cell wall contamination was assessed visually with a Nikon-Orthophot phase-contrast microscope.

Electron Microscopy

Organellar fractions were fixed for 2 h at 4°C in 4% glutaraldehyde solutions (v/v, final concentration) with sucrose or sorbitol, as follows. The P15000 and the recentrifuged, purified mitochondria from cells disrupted in 0.33 M sucrose were fixed in the presence of 0.33 M sucrose. The P15,000 of cells disrupted in 1 M sorbitol was fixed in a 1 M sorbitol solution. The purified mitochondria derived from this P15,000 were taken from the continuous gradient, diluted 1:1 with 1 M sorbitol, and centrifuged at 15,000*g*, and the pellet was resuspended in 1 M sorbitol with 4% glutaraldehyde. The vacuoles from the gradient were fixed in the presence of 50% sucrose. Samples were then washed two to three times in the same buffers lacking glutaraldehyde. Samples fixed in the presence of sorbitol or 50% sucrose were further washed twice with 0.1 M sodium cacodylate buffer, pH 7.2. The samples were postfixed in 1% osmium tetroxide in the cacodylate buffer for 2 h at room temperature. They were dehydrated in a series of increasing ethanol concentrations and embedded in Epon–Araldite. Thin sections were made with a diamond knife on an LKB-3 microtome. Sections were poststained with 10% uranyl acetate in methanol and examined with a Jeol 100C electron microscope at 80 kV.

Materials

Most chemicals were obtained from Sigma Chemical Company. Isotopes were obtained from New England Nuclear Corporation. Miracloth was obtained from Calbiochem.

Glass beads (0.3–0.5 mm) were purchased from Biospec.

RESULTS

Cell Breakage and Differential Centrifugation

Tests of cell breakage with time of blending in buffered, 1 M sorbitol were done on two quantities of wild-type mycelia, 2.8 and 9.5 g dry wt (approx 10 and 40 g wet wt). At each time, samples were removed, centrifuged at 600*g*, filtered, and assayed for protein, ornithine aminotransferase (cytosolic), ornithine transcarbamylase (mitochondrial), and arginine (vacuolar). In addition, the sedimentability of these markers at 15,000*g* was tested to determine the degree of organellar breakage. The results are given in Fig. 1. The arginine was used as a test of cell breakage because dependable values for the arginine content of intact cells could be obtained. As indicated, over 80% cell breakage of the lower weight of mycelium had taken place within 2 min. The same degree of cell breakage was achieved only by 5 min in the case of the higher dry weight. The lower dry weight yielded 30% of the arginine in sedimentable (vacuolar) form (at 2 min), as opposed to 23% in the case of the higher dry weight at 5 min. These figures were representative of vacuolar yield in repeated uses of the apparatus.

The release of protein into the cell-free S600 paralleled that of arginine (Fig. 1). In the case of protein (not shown) and ornithine aminotransferase, less than 10% were sedimentable at 15,000*g*. Over 50% of the ornithine transcarbamylase activity was sedimentable at 15,000*g* in the homogenate derived from the lower dry weight. Much less of the transcarbamylase was sedimentable in the heavier sample, indicating more mitochondrial breakage. This was not due simply to time of blending, since the difference was clear at 5 min for both samples. The regularly higher yield of mitochondria than of vacuoles in the sample of lower weight demonstrates the greater fragility of the vacuoles.

Visual inspection showed that some cells and cell walls were not removed by centrif-

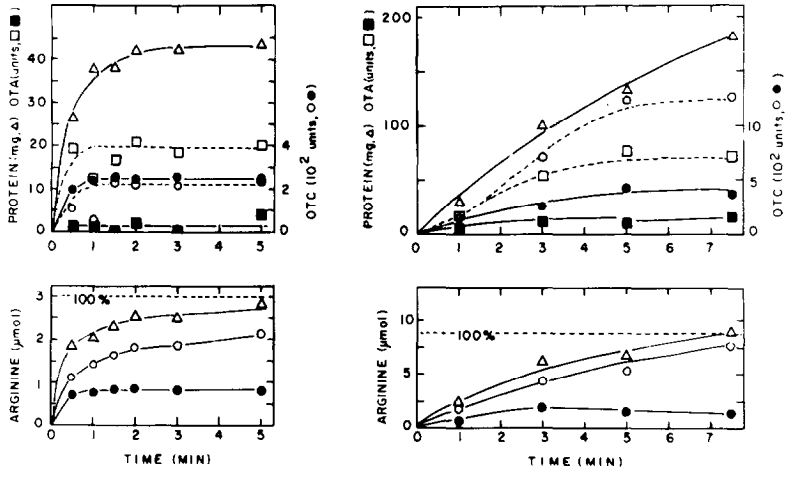

FIG. 1. Kinetics of cell and organelle breakage using the Bead-Beater. Sorbitol (1 M) was used in the suspending medium to disrupt 2.8 g (dry wt equivalent) (left) or 9.5 g (right) of mycelium. Lower graphs: Release of arginine from cells into the S600 (△) and its distribution into sedimentable (●) and nonsedimentable (○) fractions, using 15,000*g* centrifugation. Upper graphs: Release of protein, OTA, and OTC. In the case of protein (△), the fraction shown is the S600, over 95% of which remains nonsedimentable at 15,000*g*. In the case of the enzymes, solid lines and symbols represent sedimentable fractions; dashed lines and open symbols represent nonsedimentable fractions. OTA, ornithine aminotransferase; OTC, ornithine transcarbamylase.

ugation at 600*g*, and thus appeared in the P15,000. While not enough to compromise the results above, it was desirable to remove them from the S600. Glass-fiber filters (see Materials and Methods) removed them definitively from the S600. Matrices which were not completely successful are Whatman No. 1 and 540 filter papers, 6 layers of Miracloth, coarse fritted glass, recentrifugation at 600 or 1000*g* (20 min). Glass-fiber filters allowed passage of mitochondria and vacuoles without detectable loss or breakage, and with little clogging.

Because only the smaller amounts of mycelia gave satisfactory results in the disruption step, large samples were processed in lots: premeasured lots of buffer, beads, and mycelia can be blended in rapid succession. It should be added that the kinetics of breakage were the same for scaled-down samples disrupted in the smaller (30- and 50-ml) chambers available for the machine.

The same kinetics of breakage were seen for 3-g portions of wild-type mycelium in buffered 0.33 M sucrose, with or without 0.3% bovine serum albumin, although some loss of mitochondria at longer times (>3 min) was often seen. Certain cell growth conditions and osmoticums led to different breakage kinetics. Phosphate-starved cells, for instance, are exceedingly fragile and 1 M sucrose is viscous enough to retard breakage seriously. Therefore, a test of each major variation in method is desirable before adopting a routine.

Organelle Isolation

(a) Mitochondria. In this experiment, 1 M sorbitol was used for cell disruption and suspension of the P15,000. This was done to preserve vacuoles so that the vacuolar and mitochondrial distributions could be compared in a gradient. The same pattern was seen in materials processed in 0.33 M sucrose, except that fewer vacuoles survived to the P15,000.

The P15,000 was applied to a 1.2 M/1.6 M discontinuous sucrose gradient. It was centrifuged for 2 h in a Sorvall SS-34 rotor. Analysis of fractions for arginine, protease activity (both vacuolar), and ornithine transcarbamylase (mitochondrial) are shown in Table 1. Also shown are the results of resuspension and recentrifugation of the mitochondrial material.

Virtually all mitochondria came to lie in the 1.2 M/1.6 M sucrose interface, and only a minor (approx 5–10%) fraction of the arginine was associated with them (Table 1). The same distribution was seen with protease activity: 5% of the recovered protease activity

TABLE 1

DISTRIBUTION OF MARKERS IN A DISCONTINUOUS SUCROSE GRADIENT (1.6–1.2 M SUCROSE) LOADED WITH A RESUSPENDED P15,000

Fraction	Percentage recovery			
	Protein	OTC	Protease	Arginine
Sucrose gradient				
Sample zone	1.4	1.5	4.5	9.3
Interface 1 (sample/1.2 M)	1.4	0.4	4.5	4.0
Interface 2 (1.2 M/1.6 M)	95.5	96.7	4.5	6.7
Pellet	1.6	1.4	86.5	80.0
Mitochondrial wash				
Supernatants	1.3	1.0	39.0	53.0
Pellet	98.7	99.0	61.0	47.0

Note. Figures are percentage of activity recovered from the gradient. Approximately 50 mg protein was used. Distribution of markers in the mitochondrial wash step is also shown.

was found in the mitochondrial zone. Upon diluting mitochondria with an equal amount of fractionation buffer and recentrifuging (20,000*g*, 20 min), about half the arginine and protease activity remained with the mitochondria. The proteases were inhibited by resuspending the mitochondrial pellet in 1 mM PMSF.

In these experiments, vacuoles appeared mainly in the pellet of the discontinuous sucrose gradient. This was indicated by the presence of 80% of the recovered arginine and about 87% of the protease in this fraction. However, the bulk of the protein in the pellet appeared to be contaminating mitochondrial material, as indicated by the protein: ornithine transcarbamylase ratio. Fewer intact vacuoles appeared in the gradient if homogenization and P15,000 resuspension were done in 0.33 M sucrose; the latter is recommended for work involving only mitochondria. Mitochondria isolated in 0.33 M sucrose have ADP:O ratios of 2.5 to 0.3 and respiratory control ratios of 7 to 8, using α-ketoglutarate as substrate. With 0.3% bovine serum albumin in the resuspending medium, mitochondria could be frozen at −70°C with little deleterious effect on their osmotic or respiratory properties.

(b) Vacuoles. For improved yields of the osmotically sensitive vacuoles, breakage and isolation were routinely perfomed using buffer containing 1 M sorbitol. Purified vacuoles were collected as a pellet after centrifugation of the P15,000 on a continuous 1 M sorbitol/1.8 M sucrose gradient with 1.8 M sucrose at the bottom. Over 50% of the arginine applied to the gradient was recovered in the vacuolar pellet. Recovery of intact vacuoles from this pellet required resuspension in osmoticum of at least 1.8 M to avoid lysis. Overall, of the arginine in intact cells, which other evidence showed to be over 95% vacuolar (15), 8–15% was recovered in the sucrose gradient pellet. This is equivalent to vacuolar yields of the more laborious and time-consuming method involving glusulase digestion followed by homogenization of protoplasts (2). The mitochondria band approximately 1/3 the distance into the gradient. Less than 1% of the mitochondria (ornithine transcarbamylase or succinate dehydrogenase activity) in the sample pelleted with the vacuoles as compared with 1 to 4% in the discontinuous gradient described above.

Vacuoles released from isotopically labeled, bead-homogenized (^{3}H), and glusulase-treated (^{14}C) cultures were compared on velocity sedimentation gradients. Vacuoles of both types formed complex but indistinguishable profiles. The cosedimentation was not due to equilibration of label between the vacuole populations, because label added to crude cell homogenates does not equilibrate during this procedure ((15) and Cramer, unpublished). Moreover, similar methods have previously been used to demonstrate differences in profiles of vacuoles derived from sand-ground and glusulase-treated cultures (2), as well as profiles of vacuoles from cultures in different nutritional states (Cramer, unpublished). We conclude that vacuoles isolated from normal cells disrupted by the Bead-Beater have the same size distribution as those derived from glusulase-treated cells.

(c) Electron microscopy. Electron microscopy was performed on relevant preparations from purification steps optimal for each organelle. The P15,000 from cells disrupted in 0.33 M sucrose (Fig. 2A) reveals the heterogeneity of organelles, including many mitochondria, electron-dense vacuoles, and other materials. The discontinuous gradient fraction containing mitochondria (Fig. 2B) shows no vacuoles and a predominantly mitochondrial population accompanied by some membranes and other organelles. In both fractions, the mitochondrial matrix is condensed, in keeping with the osmotic strength of the medium. The P15,000 from cells disrupted in 1 M sorbitol is also heterogeneous (Fig. 2C), but the mitochondrial matrix is distended because sorbitol does not serve as an osmoticum (it is permeant to mitochondria in swelling assays; Paulus and Davis, unpublished results). The mitochondrial band from the sorbitol–sucrose gradient (Fig. 2D) is quite ho-

mogeneous, and the contraction of the matrix due to sucrose in the gradient and carried over in the large pellet is apparent. Vacuoles from the bottom of this gradient (Fig. 2E) are quite pure; no recognizable mitochondrial material was seen. Cell wall fragments were absent from all organellar preparations.

DISCUSSION

The methods described here allow one quickly to disrupt substantial amounts of *Neurospora*, to prepare organellar pellets, and to separate vacuoles and mitochondria by gradient centrifugation. The process can be accomplished in about 3 h, most of which is spent in the 2-h gradient centrifugation step. The yield of vacuoles per gram of cells is as good as in the best alternative method (2); mitochondria can be prepared in bulk with little protease activity, and more easily than in prior methods requiring ultracentrifugation (5). Both mitochondria and vacuoles can be prepared from a single homogenate. The method avoids disadvantages of the snail-gut enzyme method, which is slow, expensive, and accompanied by metabolic changes and addition of extraneous catabolic enzymes. The method also avoids the labor of sand grinding and the associated fine sand and porcelain particles which sediment with vacuoles. No cell walls were found in preparations of either organelle. Finally, the small chambers available allow determination, in scaled-down samples, of changes in organelles as cultures grow.

Some cautions are appropriate. First, cells in different metabolic states may vary in their susceptibility to breakage. For instance, mycelia which have been starved for phosphate for 3 h can be broken in 1/5 to 1/6 the time it takes to break normally grown cells (Cramer, unpublished). It is therefore advisable to do controls for cell and organelle breakage before adopting a routine procedure. Another point is that the "mitochondrial" fractions described contain cell structures other than those specifically tested for; it is known that glyoxysomes are present on biochemical grounds (2), and the electron micrographs

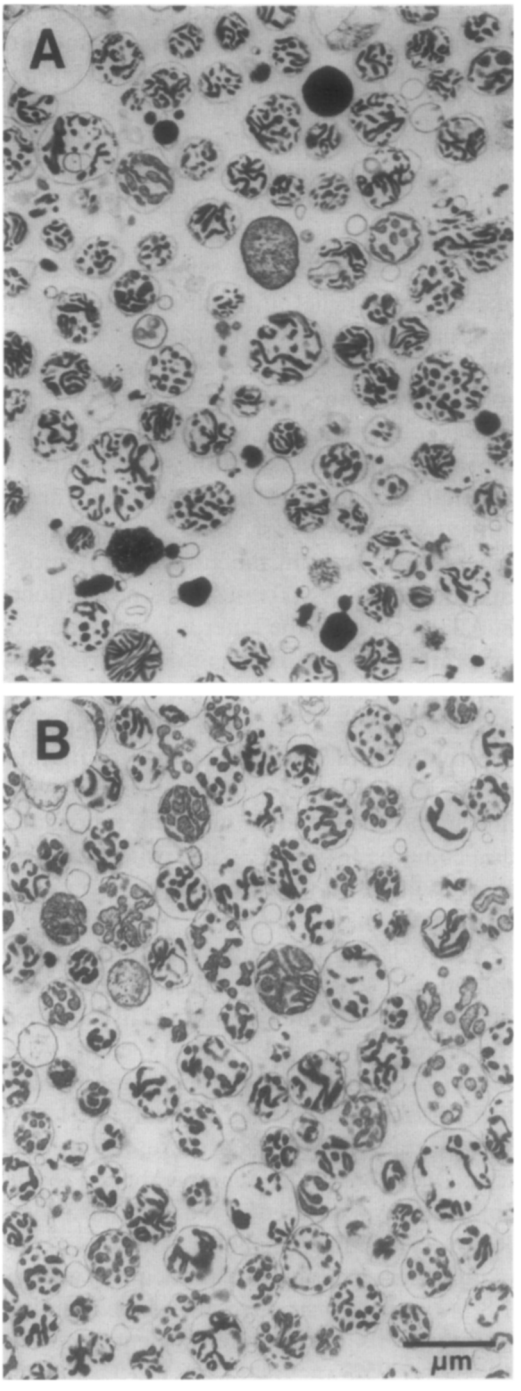

FIG. 2. Electron microscopy of P15,000 and purified fractions. (A) P15,000 from cells disrupted in 0.33 M sucrose. (B) Purified mitochondria (from discontinuous gradient) derived from cells disrupted in 0.33 M sucrose. (C) P15,000 from cells broken in 1 M sorbitol. (D) and (E) Mitochondrial band and vacuolar pellet, respectively, derived from cells disrupted in 1 M sorbitol and taken from continuous sorbitol–sucrose gradient.

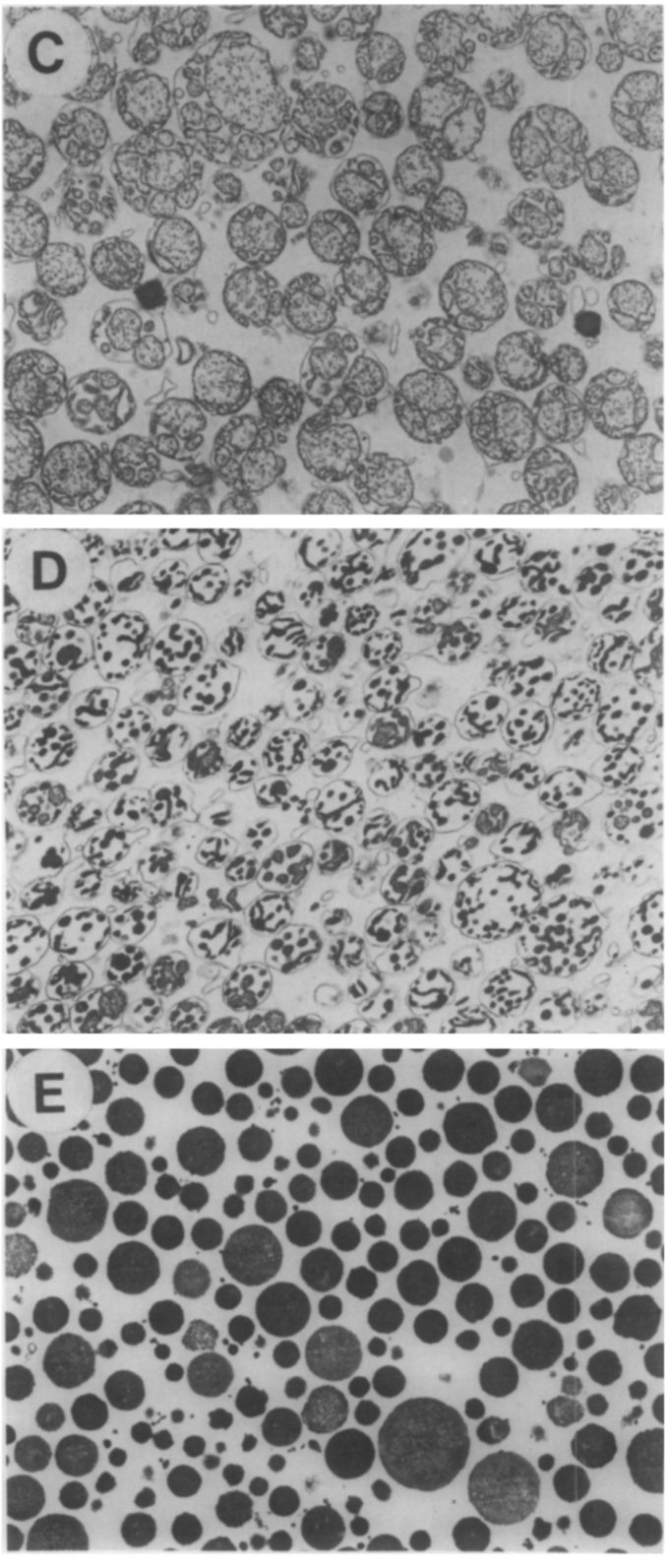

FIG. 2—*Continued.*

demonstrate some heterogeneity. It is likely that glycogen and surviving nuclei are sedimented in the 600*g* centrifugation. Most cellular membranes are left in the S15,000; thus, endoplasmic reticulum, plasma membrane, and broken organelle membranes do not heavily contaminate the mitochondria or vacuoles and can probably be isolated from each other by appropriate methods.

ACKNOWLEDGMENTS

This study was supported by Research Grant AM 20083 from the National Institute of Arthritis, Metabolic, Digestive, and Kidney Diseases. C. L. C. was a predoctoral trainee supported by Public Health Service Training Grant GM-0734. We thank Ms. Juta Kiethe for the electron microscopy and Dr. E. J. Bowman, Dr. B. J. Bowman, and Dr. R. L. Weiss for discussion of methods and critical review of the manuscript.

REFERENCES

1. Cramer, C. L., Vaughn, L. E., and Davis, R. H. (1980) *J. Bacteriol.* **142,** 945–952.
2. Vaughn, L. E., and Davis, R. H. (1981) *Mol. Cell. Biol.* **1,** 797–806.
3. Davis, R. H., Ristow, J. L., and Hanson, B. A. (1980) *J. Bacteriol.* **141,** 144–155.
4. Weiss, H., v. Jagow, G., Klingenberg, M., and Bucher, T. (1970) *Eur. J. Biochem.* **14,** 75–82.
5. Michel, R., Liebl, A., Hartmann, A., and Neupert, W. (1976) *Hoppe-Seyler's Z. Physiol. Chem.* **357,** 415–426.
6. Davis, R. H., and de Serres, F. J. (1970) *in* Methods in Enzymology (Tabor, H., and Tabor, C. W., eds.), Vol. 17, Part A, pp. 79–143, Academic Press, New York.
7. Paulus, T. J., unpublished observations.
8. Van Pilsum, J. F., Martin, R. P., Kito, E., and Hess, J. (1956) *J. Biol. Chem.* **222,** 225–236.
9. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254.
10. Weiss, R. L., and Anterasian, G. P. (1977) *J. Biol. Chem.* **252,** 6974–6980.
11. Davis, R. H., (1962) *Arch. Biochem. Biophys.* **97,** 185–191.
12. Pennington, R. J. (1961) *Biochem. J.* **80,** 649–654.
13. Lampkin, S. L. IV, Cole, K. W., Vitto, A., and Gaertner, F. H. (1976) *Arch. Biochem. Biophys.* **177,** 561–565.
14. Estabrook, R. W. (1976) *in* Methods in Enzymology (Estabrook, R. W., and Pullman, M. E., ed.), Vol. 10, pp. 41–47, Academic Press, New York.
15. Weiss, R. L. (1973) *J. Biol. Chem.* **248,** 5409–5413.