Structure of the Outer Mitochondrial Membrane: Ordered Arrays of Porelike Subunits in Outer-membrane Fractions from *Neurospora crassa* Mitochondria

CARMEN A. MANNELLA
Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201

ABSTRACT Light-membrane fractions obtained by hypoosmotic lysis of *Neurospora crassa* mitochondria exhibit buoyant densities and marker-enzyme activities characteristic of outer mitochondrial membranes. SDS PAGE of these membrane fractions indicates that a polypeptide of M_r 31,000 is the main protein component. Under negative-stain electron microscope examination many of the membranes in these fractions appear as large (0.5–1-μm diameter), collapsed vesicles. The surfaces of flattened, open (i.e., ripped) vesicles often exhibit extended two-dimensional arrays of subunits with central, 2–3-nm diameter, stain-accumulating sites. These porelike subunits are arranged into hexagons within each parallelogram unit cell, 12.6 × 11.1 nm (lattice angle = 109°).

The mitochondrion is a double-membrane system: most respiratory chain components are located on, or in the space bounded by, the inner membrane, which is in turn enclosed by an outer limiting membrane. The intact outer mitochondrial membrane is generally considered to be freely permeable to metabolites, excluding only macromolecules such as holocytochrome *c* (1–4; however, cf. reference 5).

Electron microscope observations by Parsons and co-workers (6, 7) suggested a physical basis for the high small-molecule permeability of this membrane. The surfaces of outer membranes of plant mitochondria contain dense (but apparently random) arrays of negative-stain-accumulating sites, each ~3 nm in diameter, which could represent hydrophilic openings of channels through these membranes. These outer membrane subunits could not be correlated at the time with particular components of the plant membrane, nor were they found in the outer membranes from mitochondria of rat or guinea pig liver (7).

Mannella and Bonner (8) and Mannella (9) subsequently found that outer membranes isolated from plant mitochondria display x-ray diffraction patterns consistent with random arrays of subunits having in-plane dimensions like those of the subunits seen in electron micrographs. As in the electron microscope studies, previous x-ray diffraction studies of rat liver mitochondrial outer membranes had given no indication of a prominent in-plane subunit structure (10). Mannella and Bonner (4) noted that mitochondrial outer membranes of various organisms generally contain a more or less prominent class of proteins of M_r ~30,000. In the plant membrane these proteins make up over half the total protein mass (4) and are the only proteins insensitive to trypsin, as is the x-ray diffraction from these membranes (8).

These observations led to a hypothesis (8) that the 30-kdalton polypeptides are responsible for the characteristic porelike subunits detected in the plant mitochondrial outer membrane and that the failure to detect similar pore arrays in animal outer membranes is due to their relatively low content of 30-kdalton polypeptides (11). This hypothesis is consistent with recent reports (12–14) that pore-forming activity of protein-lipid or protein-detergent complexes from mitochondrial outer membranes is associated with polypeptides of M_r 30,000–32,000.

We are interested in the structure-function relations of the intact mitochondrial outer membrane, which probably regulates the entry of large (and perhaps small) molecules into the intermembrane space. For this investigation we set out to isolate such membranes with the highest relative content of 30-kdalton polypeptides. A likely source was the fungus *Neurospora crassa*, from which mitochondrial membranes were first isolated by Cassady (15) and by Neupert and Ludwig (16), whose procedures involved swelling, shrinking, and sonication of the mitochondria. In the latter study outer membrane fractions were found to display a single protein band when electrophoresed on polyacrylamide gels with phenol-formic acid buffer. That the same membranes might also contain prominent porelike subunits was suggested by Stoeckenius's (17)

THE JOURNAL OF CELL BIOLOGY · VOLUME 94 SEPTEMBER 1982 680-687

mention of unpublished electron microscope observations of negative-stain-filled pits in the outer membranes of *Neurospora* mitochondria, like those reported for the plant membrane.

In this report we describe the isolation and electron microscopical appearance of outer membranes isolated from *N. crassa* mitochondria by a procedure which avoids sonication (and detergents) so as to minimize vesiculation and other possible disruption of the membrane structure. Preliminary reports of the composition and ultrastructure of these membranes have been presented earlier (18, 19).

MATERIALS AND METHODS

Neurospora Cultures

For these studies we used a *N. crassa* mutant which lacks a cell wall, commonly referred to as slime (FGSC 326, *fz* [no number], *sg* [no number], *arg-1* [B369], *cr-1* [B123], *aur* [34508], *os-1* [B135] A), a culture of which was kindly provided by R. L. Metzenberg (University of Wisconsin at Madison). Liquid cultures of slime were grown by shaking in Nelson's Medium B at 30°C (20). Long-term stock cultures were grown for several days on 1.5% agar containing Nelson's Medium B, as first suggested by Selitrennikoff (21), and maintained in storage at −70°C.

Isolation of Mitochondria

For mitochondrial membrane isolation we used late-exponential-phase slime protoplasts from 4 l of Nelson's Medium B, inoculated with 7×10^4 cells/ml and shaken for 18 h at 150 strokes/min with a reciprocating shaker or at 210 rev/min with a rotatory shaker. Mitochondria were isolated from the protoplasts by a modification of the procedure developed by Lambowitz et al. (22) for *N. crassa* protoplasts.

Slime cells were harvested by centrifugation at 1,000 *g* for 3–5 min and were resuspended by gentle swirling in 500 ml of a solution containing 0.6 M sucrose, 50 mM NaH_2PO_4, and 5 mM EDTA, pH 6.8 20°C. (This and all subsequent solutions were made with deionized, glass-distilled water.) After 5–10 min, the cells were recentrifuged at 1,000 *g* for 10 min and resuspended in 300 ml of a solution containing 0.25 M sucrose, 10 mM Tris·HCl, 1 mM EDTA, and 0.3% bovine serum albumin (BSA) (fraction V, Sigma Chemical Co., St. Louis, MO) at pH 7.2. (This and all subsequent steps were done at 4°C.) Cell lysis in this medium could be followed by light microscopy. Lysis was generally 30–50% complete after 10–15 min and could be increased to 80% or better by three to six manual strokes with a Potter Elvehjem homogenizer (Wheaton Scientific, Millville, NJ) . Cell lysates were centrifuged at 1,000 *g* for 10 min and the supernatant at 25,000 *g* for 40 min.

The resulting pellets were composed of three layers: a small, clear polysome pellet, above which were the dark-brown mitochondrial layer and a light, somewhat viscous, tan overlay. As much of the overlay as possible was washed off, and the mitochondrial layer was gently resuspended in a few milliliters of isolation medium (0.3 M sucrose, 1 mM EDTA, 0.3% BSA, adjusted to pH 6.8 with NaOH); care was taken to avoid the clear pellet underneath. After all the pellets were resuspended, the total volume was raised to 300 ml with isolation medium, and the suspension was centrifuged at 1,000 *g* for 10 min. This supernatant was centrifuged at 25,000 *g* for 30 min to form the final mitochondrial pellets. Their surfaces were washed gently with a few milliliters of isolation medium to remove any residual light overlay material.

Fractionation of Mitochondrial Membranes

The mitochondrial pellets (generally 0.3–0.8 ml, packed vol) were resuspended by gentle pipetting in a final volume of 1 to 3 ml of isolation medium. The suspension was added rapidly to at least 40 vol of cold lysis medium (0.25 mM EDTA, 0.25 mM EGTA, adjusted to pH 7.0 with NaOH) and centrifuged at 1,000 *g* for 5 min. The supernatant was homogenized gently with three strokes of a Potter Elvehjem homogenizer and stirred on ice for another 25 min. At this point solid mannitol was added to the mitochondrial lysate (final concentration, 5%) to encourage contraction of the inner membrane space.

After 5 min the mitochondrial suspension was layered atop either a 0.9 M sucrose cushion or a 0.55/0.9 M sucrose step gradient and centrifuged at 60,000 *g* for 90 min in a Beckman SW25.2 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA). (Each sucrose layer was 10–15 ml. The sucrose solutions were made in lysis medium containing, in addition, 10 mM potassium phosphate buffer, pH 7.0.) After centrifugation, a white band was visible at the interface above the 0.9 M sucrose layer; this band could be collected with a long-stem Pasteur pipette. The bulk of the mitochondrial material appeared as a dark-brown pellet below the 0.9 M sucrose layer. The rest of the gradient appeared clear.

For assay of enzymatic activity the 0.55/0.9 M sucrose interface fraction was diluted with 2–3 vol of buffer T (1 mM Tris·HCl, pH 7.5) and centrifuged at 60,000 *g* for 60 min. The resulting translucent, slightly yellow pellet was resuspended in a few milliliters of buffer T. The brown pellet from the step-gradient centrifugation was resuspended directly in a few milliliters of buffer T.

For preparation of specimens for gel electrophoresis and negative-stain electron microscopy, a 0.9 M sucrose cushion was generally used for mitochondrial fractionation. Both the interface and pellet fractions were dialyzed for 16–18 h in the cold against 1,000 vol of buffer T. These membrane fractions were then concentrated by centrifugation at 60,000 *g* for 60 min and resuspended in 0.2 to 0.5 ml of buffer T.

Enzyme Assays and Protein Determination

Respiratory activity of the isolated mitochondria was monitored at 25°C by using a Clark oxygen electrode (Yellow Springs Instrument Co., Yellow Springs, OH) and the reaction conditions described by Lambowitz et al. (22).

Antimycin A-sensitive and -insensitive NADH:cytochrome *c* oxidoreductase and succinate:cytochrome *c* oxidoreductase activities of the mitochondrial membrane fractions were monitored as described by Douce et al. (23). L-Kynurenine, NADPH:oxygen oxidoreductase (3-hydroxylating) was measured by the procedure of Nisimoto et al. (24). The reactions were run at room temperature and monitored with a Gilford Instrument Laboratories Inc. (Oberlin, OH) 2400 spectrophotometer. Protein determinations were made by a modified Lowry procedure (25) with crystallized BSA as the standard. Enzyme and protein determination reagents were purchased from Sigma Chemical Co. (St. Louis, MO), except for antimycin A (Boehringer Mannheim, Indianapolis, IN).

Gel Electrophoresis

Mitochondrial membrane specimens were electrophoresed on 1.5-mm-thick polyacrylamide slab gels consisting of a 6% acrylamide stacking gel (2-cm long) and a 12% acrylamide separation gel (20-cm long), prepared and stained (with Coomassie Brilliant Blue G) as described by Chua and Bennoun (26). The buffer systems of Neville (27) or Laemmli (28) were used with 0.1% SDS in the upper reservoir buffer and in the gels themselves. Mitochondrial membrane specimens containing 10–50 μg of protein were prepared in 60-μl mixtures containing 2% SDS, 25% glycerol, 1.5% β-mercaptoethanol, and 0.04% bromophenol blue. These were heated to 100°C for 1 min before they were loaded onto the gels. For molecular weight calibration protein standards of M_r 14,000–94,000 (Bio-Rad Laboratories, Richmond, CA) were electrophoresed on the same gels. The gels were run at 30 mA for 7.5 h (Neville buffers) or at 50 mA for 6 h (Laemmli buffers). Reagents used in preparing the gels, buffers, and specimens were from Sigma Chemical Co., with the exception of acrylamide and *N,N'*methylene-*bis*-acrylamide, which were from Eastman Chemicals (Rochester, NY).

Electron Microscopy

THIN SECTION: *Neurospora* slime cells (in Nelson's Medium B) and isolated mitochondria (in isolation medium or lysis medium) were fixed in suspension with 2.5% glutaraldehyde (EM grade; Polysciences, Inc., Warrington, PA) at 4°C for 0.5 to 1 h. The specimens were pelleted, rinsed with 0.2 M sodium phosphate (pH 7.2), and postfixed for 1 h with 2% OsO_4 in phosphate buffer. After rinsing in phosphate buffer, the pellets were dehydrated in a graded ethanol-water series, with a final rinse in propylene oxide, and embedded in Epon (Polybed 812, Polysciences, Inc.). Grey-to-silver sections were cut from these blocks with either glass or diamond knives (MJO-Diatome, Fort Washington, PA), and the sections were stained for 20 min with 2% uranyl acetate, followed by 20 min with Reynold's lead citrate.

NEGATIVE STAIN: Membrane specimens were negatively stained with either phosphotungstic acid (2% aqueous solution adjusted to pH 7.0 with KOH; Ernest F. Fullam Inc., Schenectady, NY) or uranyl acetate (1% aqueous solution; Ernest F. Fullam Inc.). BSA (crystallized and lyophilized; Sigma Chemical Co.) was generally added to the phosphotungstate solutions at a concentration of 0.01% as a spreading aid (29).

Negatively stained specimens were prepared as follows. A 5-μl drop of membrane suspension (containing ~1 mg of membrane protein/ml) was placed on a Formvar/carbon-coated specimen grid, which had been glow-discharged just before use, and 5 μl of the appropriate stain solution was immediately added to the membrane drop. After 1–5 min (depending on the initial membrane concentration) the drop was removed by touching a piece of filter paper to the edge of the grid, the surface of which was then washed twice with 25-μl drops of the stain solution and allowed to air-dry. These negatively stained specimens were normally examined in the electron microscope within a few hours of preparation.

RESULTS AND DISCUSSION

Structure and Respiratory Activity of the Isolated Mitochondria

In situ the mitochondria of *N. crassa* slime grown in liquid culture display varied cross-sectional shapes. Most are circular or elliptical (and sometimes elongated), but many others are donut- or C-shaped (Fig. 1*A*). The latter types have been previously described for *N. crassa*, both in wild-type mycelia (30) and in the slime mutant grown as hyphlets on agar (31), and may represent sections through cup-shaped mitochondria.

Mitochondria isolated from slime cells in liquid culture (Fig. 1*B*) show predominantly circular cross-sections with diameters ranging from 0.3 to 1.5 μm, the upper limit matching that of the largest mitochondrial cross-sections seen *in situ*. In a few instances adjacent mitochondria appear in thin section to be enclosed by a single outer membrane (arrow, Fig. 1*B*); these might be derived from the dumbbell-shaped mitochondria occasionally seen *in situ* (arrow, Fig. 1*A*). Isolated mitochondria with annular cross-sections are also occasionally detected in these preparations (*insert*, Fig. 1*B*).

When, as in Fig. 1*B*, isolated mitochondria are fixed in media containing 0.25 or 0.3 M sucrose, they show mixed orthodox and condensed inner-membrane configurations with predominantly intact outer membranes. Such mitochondria display state-3 oxidation rates, with NADH as substrate, of 0.9 to 1.2 μatoms oxygen·min^{-1}·(mg protein)$^{-1}$ and respiratory control ratios (i.e. state-3/state-4 respiration rates) of 2.4 to 4.0. Both sets of values are comparable to those of mitochondria isolated from wild-type *Neurospora* mycelia (32).

Mitochondrial Lysis

The procedure used to isolate outer membranes from *Neurospora* mitochondria is based on the osmotic shock/density-gradient centrifugation procedure of Douce et al. (23), devised for higher plant mitochondria, which was in turn derived from the original procedure of Parsons et al. (7) for liver mitochondria. This approach avoids the use of sonication to separate the outer membrane, as used by previous workers with *Neurospora* mitochondria (15, 16) to increase outer membrane yields.

The first step in isolating outer membranes is to dilute the mitochondria in a hypoosmotic medium in order to expand the inner membrane and rupture the outer membrane. In *Neurospora* slime mitochondria suspended for 0.5 h in hypoosmotic lysis medium (Fig. 1*C*), the inner membranes display large-scale swelling like that evidenced with animal mitochondria under similar conditions (33).

The extent of outer membrane lysis can be estimated from the relative activities of reactions involving transfer of electrons between inner-membrane redox sites and exogenous cytochrome *c*, which cannot penetrate the intact outer membrane (3, 4). The unmasking of one such enzyme activity (succinate:cytochrome *c* oxidoreductase) as a function of decreasing osmoticant (sucrose) concentration is illustrated for a typical mitochondrial preparation in Fig. 2. The freshly isolated mitochondria did not display maximum reaction rates after 0.5 h incubation at low osmolarity (presumably corresponding to maximum outer membrane lysis). Instead, maximum cytochrome *c* reduction rates were achieved only after the mitochondrial suspension had stood on ice for 2 h. Most prepara-

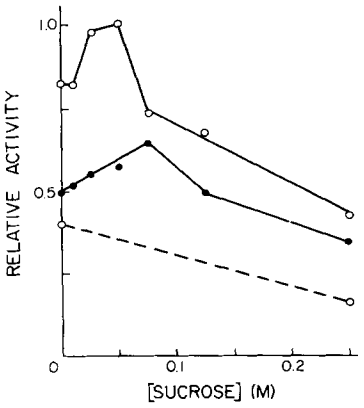

FIGURE 2 Relative activities of succinate:cytochrome *c* oxidoreductase of *N. crassa* mitochondria as a function of osmoticant concentration. The mitochondria (final conc., ~1 mg/ml) were preincubated for 0.5 h at 4°C in solutions containing the specified concentrations of sucrose and either 0.25 mM EDTA plus 0.25 mM EGTA, adjusted to pH 7.0 with NaOH (○), or 0.05 mM $CaCl_2$ (●). Incubations were initiated 0.5 h (---) or 2 h (—) after isolation of the mitochondria.

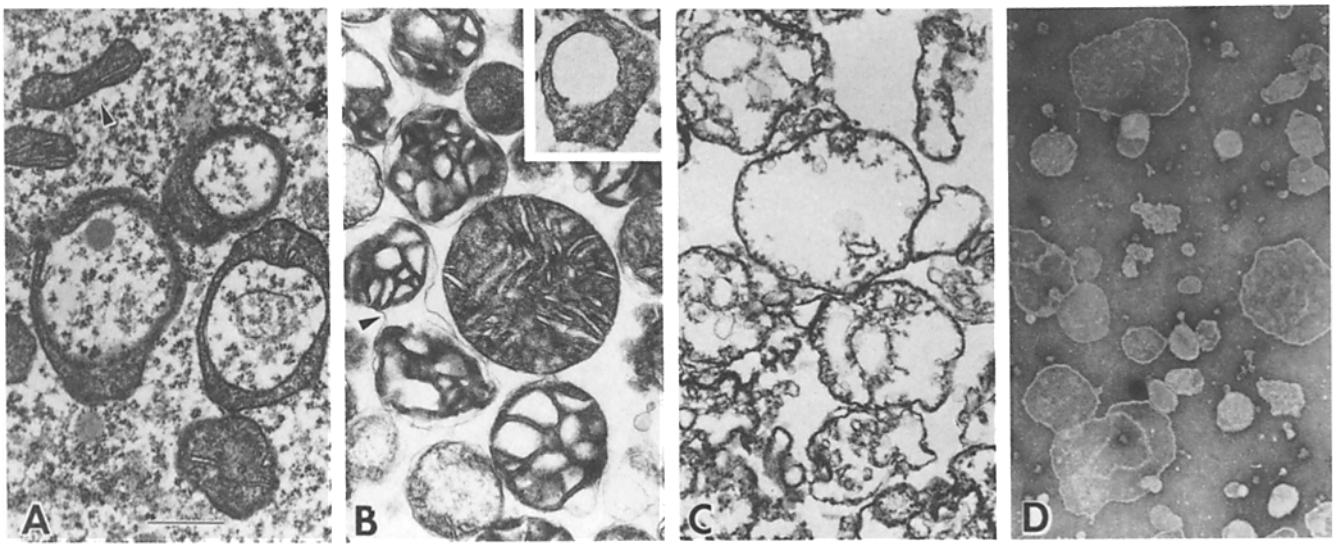

FIGURE 1 (*A–C*) Thin-section electron micrographs of (*A*) cytoplasm of a *N. crassa* slime protoplast, (*B*) isolated *N. crassa* mitochondria in isolation medium, and (*C*) isolated *N. crassa* mitochondria in lysis medium. (*D*) Spread and negatively stained light (outer) mitochondrial membranes. Each micrograph was taken at 80 kV on a Philips EM301 at × 8,700, and all were enlarged to the same final magnification. Bar, 0.5 μm.

tions of *N. crassa* mitochondria displayed this time-dependent susceptibility to hypoosmotic lysis. In some preparations the mitochondria lysed fully soon after isolation. In others the outer-membrane lysis was only 30–50% complete after 0.5 h hypoosmotic incubation, despite several hours preincubation on ice. Electron microscope and light transmission experiments (Mannella, C. A., and S. Rubinstein, work in progress) indicate that the increased susceptibility to osmotic lysis of mitochondria with time after isolation may correlate with slow expansion of the inner membrane space. Preliminary data indicates that treatments which convert freshly isolated *Neurospora* mitochondria to the orthodox conformation (e.g., preincubation with respiratory substrate) can improve outer membrane yields after hypoosmotic lysis. In one such experiment, preincubation of mitochondria with 10 mM NADH for 30 s before swelling approximately doubled the yield of outer membranes relative to that obtained with a control (no NADH) mitochondrial suspension.

Biochemical Characterization of Membrane Fractions

The outer mitochondrial membrane fractions characterized biochemically were obtained by hypoosmotic lysis of mitochondria within 1 h of isolation and were not pretreated in any way to enhance matrix swelling. Metal ion chelators were included in the swelling medium because low concentrations of divalent metal ions can inhibit hypoosmotic lysis of the outer membranes (Fig. 2), as is also true for higher-plant mitochondria (4).

After osmotic swelling, mitochondria from animals and higher and lower plants can be fractionated on sucrose-density gradients into light and heavy membranes, which are found to correspond (by biochemical and ultrastructural criteria) to outer and inner membranes respectively (7, 23, 34). After centrifugation for 2 h at 60,000 *g*, the light membranes of lysed *Neurospora* mitochondria are stopped by 0.7 M but not by 0.6 M sucrose, whereas the heavier membranes in the lysates pellet through 1.0 M sucrose. (The presence or absence of material at particular gradient interfaces in these experiments was determined both by direct visualization and by electrophoresis of interface fractions on SDS polyacrylamide gels; see below). The buoyant density of the light membranes inferred from these experiments, 1.08–1.09, falls within the range reported for mitochondrial outer membranes of higher plants and yeast, 1.07–1.10 (34, 35).

Specific activities and percentage recoveries of outer- and inner-membrane marker enzymes in the light- and heavy-membrane fractions of lysed *Neurospora* mitochondria in a typical experiment are summarized in Table I. That the light-membrane fraction contains predominantly outer mitochondrial membranes is indicated by (*i*) the specific activity of the outer-membrane marker enzyme, kynurenine hydroxylase (15, 16, 35), in this fraction which is sixfold higher than in the starting lysate and (*ii*) its very low specific activity of the inner-membrane marker, antimycin A-sensitive NADH:cytochrome *c* oxidoreductase, essentially all of which was recovered in the heavy-membrane fraction. The outer-membrane fractions also display antimycin A-insensitive NADH:cytochrome *c* oxidoreductase activity but at too low a specific activity to serve as an outer-membrane marker. The activities of this enzyme system for *Neurospora* mitochondrial outer membranes, 10–17 nmol cytochrome *c* reduced $\cdot$ min^{-1} $\cdot$ $(mg\ protein)^{-1}$, is comparable to the 18 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} reported for the corresponding yeast membrane (34) and much smaller than those observed with the higher-plant membrane, 400–700 nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} (23). In fact, the fungal mitochondrial outer-membrane activity is comparable to that which could arise from incomplete (96–98%) inhibition by antimycin A of the inner-membrane NADH:cytochrome *c* oxidoreductase of lysed mitochondria. Thus, the apparent antimycin A-insensitive specific activities of fractions containing either outer or inner membranes of lysed *Neurospora* mitochondria are similar (Table I). However, the ratios of antimycin A-insensitive to antimycin A-sensitive NADH:cytochrome *c* oxidoreductase activities in the light (5.5) and heavy (0.04) membrane fractions indicate that the inhibitor-insensitive activity observed in the light-membrane fractions does not arise simply from contamination by inner membranes.

TABLE I

Specific Activities and Recoveries of Marker Enzymes in Membrane Fractions of Lysed Neurospora Mitochondria

	NADH:cytochrome *c* oxidoreductase		
Fraction*	AA-sensitive‡	AA-insensitive	Kynurenine hydroxylase
	nmol · min $^{-1}$ · (mg protein) $^{-1}$		
Lysate	130	5.3	2.3
Light	2 (0.02%)§	11	14 (5.3%)
Heavy	470 (104%)	12	3.3 (40%)

* Light- and heavy-membrane fractions correspond to material at the 0.55/0.9 M sucrose interface and that which pellets through the 0.9 M sucrose layer, respectively.

‡ *AA*, antimycin A.

§ Percentage recovery of activity in fraction.

Protein recovery in the light-membrane fraction is routinely low, between 0.6% and 2.0% (vs. 2–4% by the procedure of Neupert and Ludwig [16]), as is the percent recovery of the outer-membrane marker enzyme, generally <10%. The bulk of the outer-membrane marker activity is recovered in the heavy-membrane fraction, presumably reflecting incomplete outer-membrane lysis (Fig. 2) or poor separation of the outer and inner membranes. By comparison, recovery estimates for outer-membrane fractions obtained by mitochondrial swelling and step-gradient centrifugation approach 30% for higher-plant mitochondria (23), but only 4.5% for liver mitochondria (7).

SDS PAGE of three different outer mitochondrial membrane preparations is illustrated in Fig. 3. Similar to the finding of Neupert and Ludwig (16) for outer membranes isolated by swelling and sonication of wild-type *Neurospora* mitochondria, these light-membrane specimens (Fig. 3, lanes *b–d*) display a single prominent polypeptide band, in contrast to the numerous bands in the total mitochondrial pattern (Fig. 3, lane *a*). The mobility of the predominant light-membrane protein on 12% polyacrylamide gels is consistent with a molecular weight of 30,500 to 31,000, close to that of the major polypeptides in the outer membranes of higher-plant mitochondria (28,000 and 29,000, measured on 7.5% to 15% polyacrylamide gels [4, 35]).

In addition to the prominent band at M_r 30,000–31,000, minor bands are usually visible in the gel electrophoretic patterns of light-membrane fractions. The strongest and most consistently observed (see Fig. 3) occurs at M_r 38,000. Since the mobility of this band does not coincide with that of a major band in whole mitochondrial patterns, it is probably a minor polypeptide component of the outer mitochondrial membrane. Rarely light-membrane fractions are obtained which display numerous light bands coinciding with major heavy-membrane

bands (indicated by hash marks to the right of Fig. 3). One such fraction was run in lane *d* of the gel in Fig. 3 and was found to contain (by negative-stain electron microscopy, see below) unusually high numbers of tubular, cristae-like membranes.

Electron Microscopy of Light-membrane Fractions

A wide-angle view of a typical negatively stained field of light membranes obtained by step-gradient centrifugation of hypoosmotically swollen *Neurospora* mitochondria is shown in Fig. 1 *D*. The main components of this fraction are membrane vesicles or sheets which vary in width from ~0.1 to >1 μm. When stained with phosphotungstate (as in Fig. 1 *D*), the membranes are usually irregularly shaped with a distinct white (i.e., stain-excluding) border, similar in appearance to unfixed outer membranes isolated by comparable techniques from animal and higher-plant mitochondria (7, 35). The upper limit of the size of outer membranes isolated by this procedure appears to be about twice that of the same membranes isolated by procedures involving sonication (16).

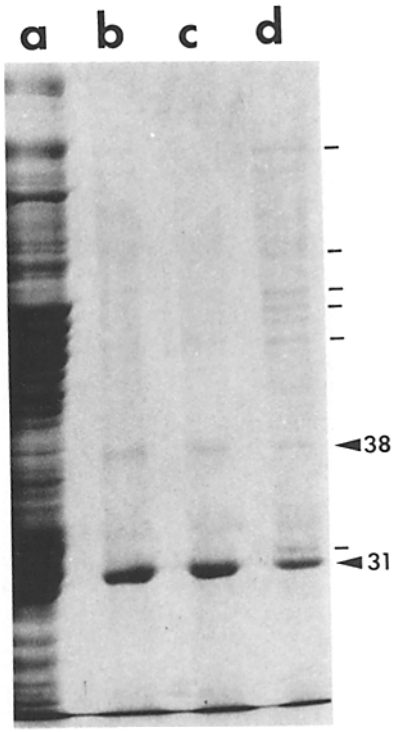

FIGURE 3 SDS PAGE of whole mitochondria (*a*) and light-membrane fractions (*b–d*). The molecular weight range from top to bottom in this gel is 100,000 to 20,000. Numbers to the right of the gel are M_r ($\times 10^{-3}$) of indicated bands.

When large, flat, well-stained membranes in these outer-membrane fractions are viewed at a higher instrument magnification (40,000–60,000), the surfaces of some appear to be covered, entirely or in part, with small close-packed scattering centers, clearly distinguishable (on the phosphor screen and in subsequent micrographs) from the finer granularity in adjacent background fields. These phosphotungstate-accumulating sites are similar in diameter (2.5–3 nm) and center-to-center spacing (4.5–5 nm) to those previously observed by Parsons et al. (7) in images of mitochondrial outer membranes from higher plants. However, unlike the stain centers of the plant membrane, those of the *Neurospora* membrane are organized into two-dimensional crystalline arrays, whenever they are visible in these images.

Three examples of outer membranes bearing ordered arrays of these subunits are presented in Fig. 4. The *insets* in *A–C* of Fig. 4 are the optical diffraction from areas (~150-nm square in each case) at the center of the ordered region of each membrane. The diffraction patterns correspond to two superimposed oblique planar arrays with unit cell parameters (averaged over 23 such lattices) of $\theta = 109 \pm 1°$, $a = 12.6 \pm 0.3$ nm, and $b = 11.1 \pm 0.2$ nm. The two reciprocal lattices in such patterns do not show simple preferred rotational orientations (see *insets* in Fig. 4). Diffraction patterns from obvious single-sheet regions of open membranes consist of one reciprocal lattice, indicating that each of the two reciprocal lattices in the patterns of Fig. 4 originates from the membrane layer on one side of the collapsed vesicles in these micrographs.

The three membranes of Fig. 4 were chosen to illustrate the morphologic classes observed for the crystalline membranes in these outer-membrane fractions. The membrane in Fig. 4 *A* is a large, flattened vesicle, roughly circular in outline, with ordered arrays of subunits visible only in a central area ~200 nm across. In Fig. 4 *B* the large, open membrane vesicle has an

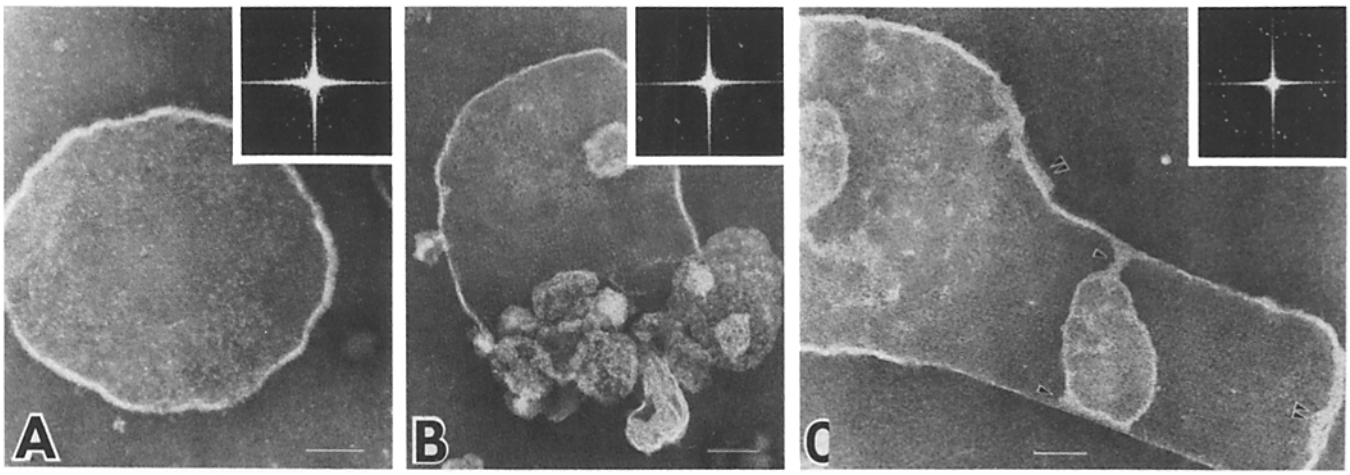

FIGURE 4 Electron micrographs of phosphotungstate-stained outer mitochondrial membranes containing ordered subunit arrays. These micrographs were taken (*A*) at × 63,000 on a Siemens 1 operating at 80 kV or (*B* and *C*) at × 57,000 on a Philips EM301 at 100 kV. *Insets:* optical diffraction from an area, ~1-cm², on the original electron image negatives near the center of the ordered region in each membrane image. A He-Ne laser (Jodon Engineering Assoc., Ann Arbor, MI) was used as the coherent radiation source for these diffraction patterns. Bars, 0.1 μm.

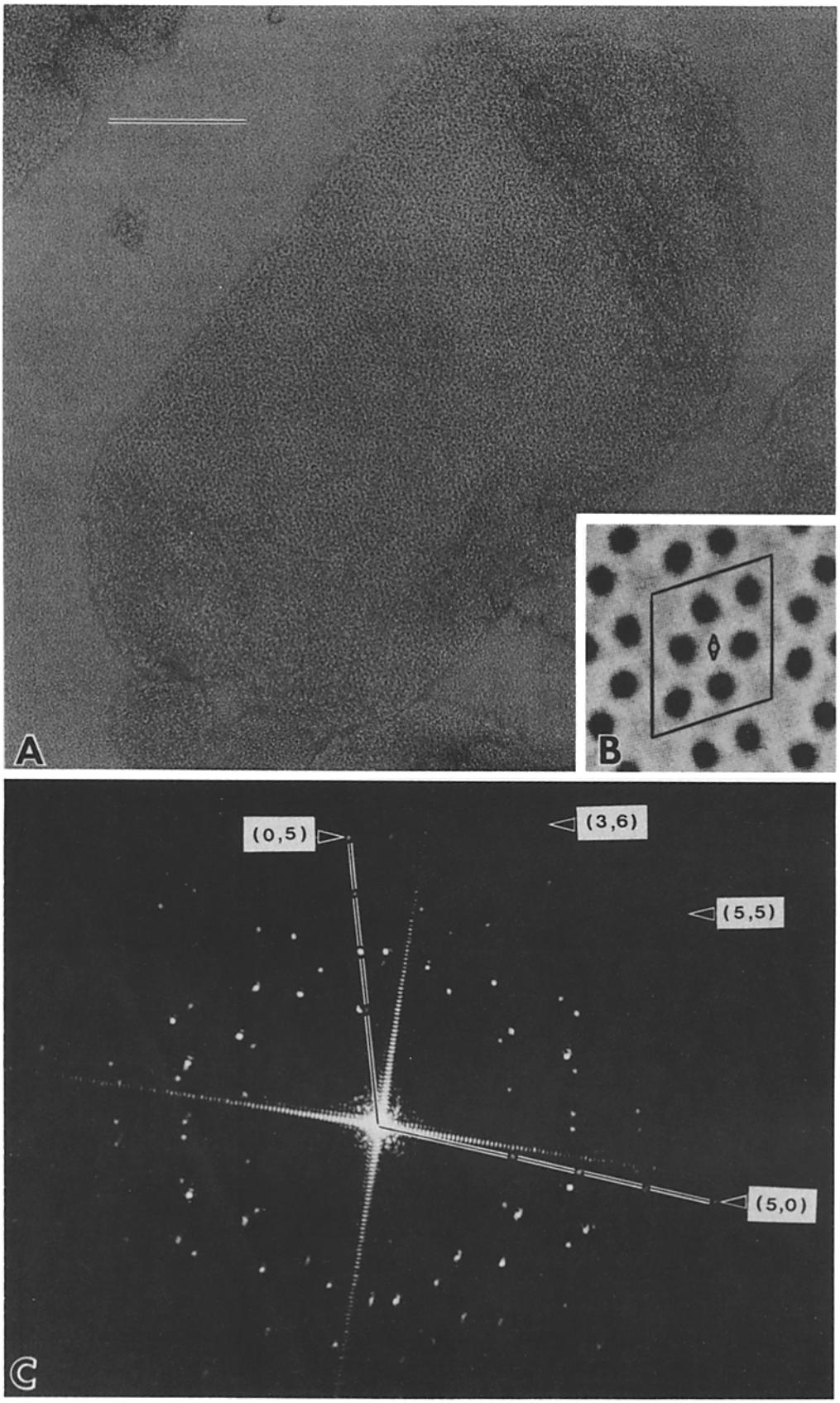

FIGURE 5 (*A*) Electron micrograph of a uranyl acetate-stained outer mitochondrial membrane, obtained at × 57,000 on a Philips EM301 at 100 kV. Bar, 0.1 μm. (*B*) Computer-filtered image of a 23-×-23 nm^2 area of one membrane layer at the center of the collapsed vesicle, reconstructed using reflections out to 1/2.05 nm^{-1} (see text). The unit cell (outlined on this image) contains six stain centers which are arranged in a hexagon with vertices related by a twofold rotation axis, indicated at the center of the hexagon. (*C*) Optical diffraction from a 170-nm square region at the center of the membrane in *A*. The *h,0* and *0,k* axes of one of the two reciprocal lattices in this pattern are indicated, as are several high-order maxima detected in this quandrant of the pattern.

ordered subunit structure across its entire surface. This bag-shaped membrane partially encloses smaller membranes morphologically similar to swollen cristae (7, 35). The apparently single membrane of Fig. 4*C* has two regions with distinctly different shapes and substructures. The left region has a rounded outline with amorphous, somewhat patchy surface staining, very similar to that of the noncrystalline region of the membrane of Fig. 4*A*. In apparent continuity is an elongated region with straight sides and an ordered substructure.

Four or five orders of maxima are usually detected in optical diffraction patterns from images of phosphotungstate-stained membranes, extending on average out to 1/2.5 nm^{-1} in reciprocal space. Up to six orders of diffraction maxima are obtained with images of uranyl acetate-stained membranes (Fig. 5), corresponding to a resolution of 1/2.0 nm^{-1}. Images of individual membrane layers have been reconstructed, using the SPIDER image-processing system (37), by inverse transformation of the Fourier spectra corresponding to single reciprocal lattices (38). (For details of this procedure see references 18, 19; also C. A. Mannella and J. Frank, manuscript in preparation.)

The filtered image of a unit cell near the center of the uranyl-stained outer membrane of Fig. 5*A* shows six approximately circular, negative-stain centers arranged in a hexagon with twofold rotational symmetry (Fig. 5*B*). The diameters of the negative-stain-accumulating sites in filtered images like Fig. 5*B* appear somewhat smaller with uranyl (2 to 2.5 nm) than with phosphotungstate (2.5 to 3 nm) as negative stain. Assuming that these negative-stain centers represent the projections of transmembrane channels, an inner pore diameter of 2 to 3 nm would be consistent with the sieving behavior of outer membranes of animal and plant mitochondrial (1, 3, 4).

Frequency of Occurrence of Crystalline Membranes

The proportion of large (≥0.5 μm wide), well-spread, evenly stained membranes with visible crystalline substructure in these outer mitochondrial membrane fractions was found to correlate with the morphology and intactness of the membranes. Circular vesicles with unbroken borders and no obvious rips or cracks seldom show visible subunits. (The membrane in Fig. 4*A* is an exception.) Freshly isolated outer membranes (such as those in Fig. 1*D*) generally fall into this category, and the frequency of detection of crystalline membranes in such fractions is <5%. After overnight dialysis against low-salt buffer the fraction of open vesicles (i.e. baglike membranes, like that in Fig. 4*B*, or tubular membranes, like that in Fig. 4*C* but open at one or both ends) generally increases to 10–25% of the total membrane population, and most of these show ordered arrays of stain centers.

The proportion of outer mitochondrial membranes containing crystalline regions before isolation cannot be inferred from these negative-stain electron microscope observations. The apparent inverse correlation between membrane intactness and detection of subunit arrays can have at least two explanations: (*i*) Visualization of subunits may require that the negative stain have access to both the inner and outer surfaces of these membranes. Thus, all the membranes in these fractions might contain crystalline regions, but this substructure might not be visible in intact membranes due to poor stain penetration. (*ii*) Crystalline membranes might be initially rare and derive with time from the amorphous membranes in the outer-membrane fractions. The observed association between crystallinity and nonintactness in outer-membrane fractions might then be due to a physical difference between crystalline and amorphous membranes: membranes consisting of ordered subunit arrays may be more prone to ripping or less able to fuse again after rupture.

The morphology of the membrane in Fig. 4*C* is suggestive of the latter possibility, i.e., that ordered membranes evolve in vitro from nonordered membrane regions. However, it is also possible that this membrane enclosed in situ one of the complex mitochondria common in *Neurospora*, e.g., an annular or dumbbell-shaped mitochondrion with one or more large, round regions connected to relatively narrow, tubular sections.

Nature of Crystalline Membrane Arrays

Assuming that the porelike subunits which form the crystalline arrays in these membranes are lipid-protein complexes, it is likely that the major protein component of the subunits is the predominant 31-kdalton polypeptide, since (*i*) no correlation was found between the occurrence of minor bands in gel electrophoretic patterns and the frequency of crystalline membranes in various outer-membrane preparations and (*ii*) the minor outer-membrane protein components together never comprised >10% of the total Coomassie-Blue-stained intensity in these gels, while the frequency of crystalline membranes can be as high as 25%.

That lipid-protein phase separations may occur in the isolated outer membranes (and that they may be involved in formation of crystalline membranes) is suggested by the granular, white (nonstaining) material sometimes seen to connect the borders of crystalline membranes to smaller, amorphous membranes (Fig. 4*C*, arrows). These bridgelike structures might represent transfer or segregation of material between, for example, protein-rich (ordered) and lipid-rich (amorphous) membrane regions. Note that the same peripheral, granular, nonstaining material is also present at each end of the ordered region of the membrane in Fig. 4*C* (double arrows). Experiments are in progress to determine whether the crystalline and amorphous membranes in these outer mitochondrial membrane fractions are compositionally distinct.

The author thanks Drs. Donald F. Parsons, Joachim Frank, and Donald S. Berns for helpful discussions in the course of this research and for critically reviewing this manuscript. The technical advice of Drs. William Goldfarb and William Samsonoff and Ms. Adriana Verschoor is also gratefully acknowledged, as well as the expert technical assistance of Messrs. Kevin Christian, Kurt Asmus, and Bernard Cognon.

This material is based upon work supported by grant PCM-8021789 from the National Science Foundation and grant 37005-80 from Health Research, Inc.

Received for publication 15 March 1982, and in revised form 28 May 1982.

REFERENCES

1. Werkheiser, W. C., and W. Bartley. 1957. The study of steady-state concentrations of internal solutes of mitochondria by rapid centrifugal transfer to a fixation medium. *Biochem. J.* 66:79–91.
2. Pfaff, E., M. Klingenberg, E. Ritt, and W. Vogell. 1968. Korrelation des unspezifisch permeablen mitochondrialen Raumes mit dem "Intermembrane-Raum." *Eur. J. Biochem.* 5:222–232.
3. Wojtczak, L., and H. Zaluska. 1969. On the impermeability of the outer mitochondrial membrane to cytochrome *c*. I. Studies on whole mitochondria. *Biochim. Biophys. Acta.* 193:64–72.
4. Mannella, C. A., and W. D. Bonner, Jr. 1975. Biochemical characteristics of the outer membranes of plant mitochondria. *Biochim. Biophys. Acta.* 413:213–225.
5. Brdiczka, D. 1978. Impermeability of the outer mitochondrial membrane for small polar molecules. *Hoppe-Seyler's Z. Physiol. Chem.* 359:1063 (Abstr.).

6. Parsons, D. F., W. D. Bonner, Jr., and J. G. Verboon. 1965. Electron microscopy of isolated plant mitochondria and plastids using both the thin-section and negative-staining techniques. *Can. J. Bot.* 43:647–655.
7. Parsons, D. F., G. R. Williams, and B. Chance. 1966. Characteristics of isolated and purified preparations of the outer and inner membraneous of mitochondria. *Ann. N. Y. Acad. Sci.* 137:643–666.
8. Mannella, C. A., and W. D. Bonner, Jr. 1975. X-ray diffraction from oriented outer mitochondrial membranes. Detection of in-plane subunit structure. *Biochim. Biophys. Acta* 413:226–233.
9. Mannella, C. A. 1981. Structure of the outer mitochondrial membrane. Analysis of x-ray diffraction from the plant membrane. *Biochim. Biophys. Acta.* 645:33–40.
10. Thompson, J. E., R. Coleman, and J. B. Finean. 1968. Comparative x-ray diffraction and electron microscope studies of isolated mitochondrial membranes. *Biochim. Biophys. Acta.* 150:405–415.
11. Melnick, R. L., H. M. Tinberg, J. Maguire, and L. Packer. 1973. Studies on mitochondrial proteins. I. Separation and characterization by polyacrylamide gel electrophoresis. *Biochim. Biophys. Acta.* 311:230–241.
12. Colombini, M. 1980. Structure and mode of action of a voltage dependent anion-selective channel (VDAC) located in the outer mitochondrial membrane. *Ann. N. Y. Acad. Sci.* 341:552–562.
13. Zalman, L. S., H. Nikaido, and Y. Kagawa. 1980. Mitochondrial outer membrane contains a protein producing nonspecific diffusion channels. *J. Biol. Chem.* 255:1771–1774.
14. Roos, N., R. Benz, and D. Brdiczka. 1982. Identification and characterization of the pore forming protein in the outer membrane of rat liver mitochondria. *Biochim. Biophys. Acta.* In press.
15. Cassady, W. E. 1969. A study of the localization of enzymes in *Neurospora* mitochondria. PhD Thesis. University Texas, Austin.
16. Neupert, W., and G. D. Ludwig. 1971. Site of biosynthesis of outer and inner membrane proteins of *Neurospora crassa* mitochondria. *Eur. J. Biochem.* 19:523–532.
17. Stoeckenius, W. 1970. Electron microscopy of mitochondrial and model membranes. *In* Membranes of mitochondria and chloroplasts. E. Racker, editor. Van Nostrand Reinhold, New York. 53–90.
18. Mannella, C. A., and J. Frank. 1980. Lattice structure in the outer membrane of *Neurospora* mitochondria. *In* Electron Microscopy 1980. P. Brederoo and W. DePriester, editors. 7th European Congress on Electron Microscopy Foundation. Leiden (The Netherlands). 2:618–619.
19. Mannella, C. A., J. Frank, and A. J. Ratkowski. 1980. Structure of the outer mitochondrial membranes of plants and the fungus, *Neurospora crassa. J. Cell Biol.* 87(2, Pt. 2):210*a* (Abstr.).
20. Nelson, R. E., B. Littlewood, and R. L. Metzenberg. 1975. Toward the domestication of slime. *Neurospora Newsl.* 22:15–16.
21. Selitrennikoff, C. P. 1978. Storage of slime strains. *Neurospora Newsl.* 25:16.
22. Lambowitz, A. M., E. W. Smith, and C. W. Slayman. 1972. Electron transport in *Neurospora* mitochondria. Studies on wild-type and *poky. J. Biol. Chem.* 247:4850–4858.
23. Douce, R., C. A. Mannella, and W. D. Bonner, Jr. 1973. The external NADH dehydrogenases of intact plant mitochondria. *Biochim. Biophys. Acta.* 292:105–116.
24. Nisimoto, Y., F. Takeuchi, and Y. Shibata. 1975. Isolation of L-kynurenine 3-hydroxylase from the mitochondria outer membrane of liver. *J. Biochem. (Tokyo).* 78:573–581.
25. Miller, G. L. 1959. Protein determinations for large numbers of samples. *Anal. Chem.* 31:964.
26. Chua, N.-H., and P. Bennoun. 1975. Thylakoid membrane polypeptides of Chlamydomonas reinhardtii: wild-type and mutant strains deficient in photosystem II reaction center. *Proc. Natl. Acad. Sci. U. S. A.* 72:2175–2179.
27. Neville, D. M., Jr. 1971. Molecular weight determinations of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. *J. Biol. Chem.* 246:6328–6334.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.
29. Parsons, D. F. 1963. Negative staining of thinly spread cells and associated virus. *J. Cell Biol.* 16:620–626.
30. Weiss, B. 1963. An electron microscope and biochemical study of *Neurospora crassa* during development. *J. Gen. Microbiol.* 39:85–94.
31. Van Winkle, W. B. 1970. Ultrastructural aspects of development in the slime mutant (S1-327) of *Neurospora crassa.* PhD Thesis. University Texas, Austin.
32. Lambowitz, A. M., E. W. Smith, and C. W. Slayman. 1972. Oxidative phosphorylation in *Neurospora* mitochondria. Studies on wild-type, *poky* and chloramphenicol induced wild-type. *J. Biol. Chem.* 247:4859–4865.
33. Wlodawer, P., D. F. Parsons, G. R. Williams, and L. Wojtczak. 1966. Morphological changes in isolated rat-liver mitochondria during swelling and contraction. *Biochim. Biophys. Acta.* 128:34–47.
34. Bandlow, W., and P. Bauer. 1975. Separation and some properties of the inner and outer membranes of yeast mitochondria. *Methods Cell Biol.* 12:311–333.
35. Mannella, C. A. 1974. Composition and structure of the outer membranes of plant mitochondria. PhD Thesis. University of Pennsylvania.
36. Mannella, C. A., and A. J. Ratkowski. 1979. Structure of the pore complex in the outer membrane of mitochondria. *J. Cell Biol.* 83(2, Pt. 2):270*a* (Abstr.).
37. Frank, J., B. Shimkin, and H. Dowse. 1981. SPIDER- a modular software system for electron image processing. *Ultramicroscopy.* 6:343–358.
38. Klug, A., and D. J. DeRosier. 1966. Optical filtering of electron micrographs: reconstitution of one-sided images. *Nature (Lond.).* 212:29–32.
39. Mannella, C. A., and J. Frank. 1982. Effects of divalent metal ions and chelators on the structure of outer mitochondrial membranes from *Neurospora crassa. Biophys. J.* 37:3–4.