THE SUBMITOCHONDRIAL LOCALIZATION OF MONOAMINE OXIDASE

An Enzymatic Marker for the Outer Membrane of Rat Liver Mitochondria

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ABSTRACT

Controlled osmotic lysis (water-washing) of rat liver mitochondria results in a mixed population of small vesicles derived mainly from the outer mitochondrial membrane and of larger bodies containing a few cristae derived from the inner membrane. These elements have been separated on Ficoll and sucrose gradients. The small vesicles were rich in monoamine oxidase, and the large bodies were rich in cytochrome oxidase. Separation of the inner and outer membranes has also been accomplished by treating mitochondria with digitonin in an isotonic medium and fractionating the treated mitochondria by differential centrifugation. Treatment with low digitonin concentrations released monoamine oxidase activity from low speed mitochondrial pellets, and this release of enzymatic activity was correlated with the loss of the outer membrane as seen in the electron microscope. The low speed mitochondrial pellet contained most of the cytochrome oxidase and malate dehydrogenase activities of the intact mitochondria, while the monoamine oxidase activity could be recovered in the form of small vesicles by high speed centrifugation of the low speed supernatant. The results indicate that monoamine oxidase is found only in the outer mitochondrial membrane and that cytochrome oxidase is found only in the inner membrane. Digitonin treatment released more monoamine oxidase than cytochrome oxidase from sonic particles, thus indicating that digitonin preferentially degrades the outer mitochondrial membrane.

INTRODUCTION

Extensive studies of subcellular fractions from tissue homogenates have shown that many enzymes concerned with respiration and intermediary metabolism are associated with the mitochondria. However, because of the complexity of the mitochondrion itself, which consists of two membrane systems and at least two compartments, the precise intramitochondrial localization of enzymes and chemical components has remained somewhat speculative.

Only recently have attempts been made to separate and characterize the inner and outer mitochondrial membranes. Green et al. (1–3) have examined membrane fractions obtained from beef heart mitochondria by sonication and enzymatic digestion and have concluded that the citric acid cycle enzymes are associated with the outer mitochondrial membrane. Parsons et al. (4, 5) have reported the separation of the inner and outer membranes of rat liver mitochondria follow-

ing induced swelling, and Levy et al. (6) have used digitonin to remove the outer membranes of rat liver mitochondria. Each of these studies has employed the absence of respiratory chain enzymes or morphologically distinct structures such as inner membrane subunits in order to "identify" the outer membrane. In none of these studies has an enzymatic or chemical marker specific for the *outer* membrane been utilized to monitor the enrichment of this membrane in a given submitochondrial fraction. The problem of isolating the outer mitochondrial membrane is compounded by the fact that the inner membrane is extensively folded to form cristae and may comprise 75% or more of the total membrane of the mitochondrion. Therefore, care must be taken to obtain preparations of the outer membrane which are not heavily contaminated with inner membrane. The present paper presents data which indicate that mitochondrial monoamine oxidase is localized in the outer membrane of rat liver mitochondria and that this enzyme provides a suitable biochemical marker for the outer membrane in submitochondrial fractionation procedures.

Monoamine oxidase was selected as a possible outer membrane marker because it is presumably not linked to the respiratory chain or to the major synthetic and degradative pathways of the mitochondrion, e.g. citric acid cycle, fatty acid metabolism, and yet is a membrane-bound enzyme localized primarily in the mitochondria. Cotzias and Dole (7) first reported that rat liver monoamine oxidase activity is predominantly associated with the mitochondrial fraction. Rodriquez de Lores Arnaiz and De Robertis (8) have thoroughly investigated the distribution of monoamine oxidase in rat brain homogenates and concluded that this enzyme is localized exclusively in the mitochondria and is not found in synaptic vesicles or intact nerve endings. Baudhuin et al. (9) studied the distribution of this enzyme in rat liver homogenates by density gradient centrifugation and concluded that it is not associated with lysosomes or microbodies. Oswald and Strittmatter (10) found that the distributions of monoamine oxidase and succinate oxidase are similar in fractions of rat liver homogenates. Gorkin (11) showed that monoamine oxidase is firmly bound to sonic particles derived from rat liver mitochondria.

Vasington and Greenawalt (12) reported that rat liver mitochondria retained the capacity to oxidize succinate and β-hydroxybutyrate and to accumulate Ca^{++} and P_i after controlled osmotic lysis. The morphology and biochemical properties of this "water-washed" preparation have been studied in more detail by Caplan and Greenawalt (13), who showed that the water-washed preparation consists of two populations of particles: large

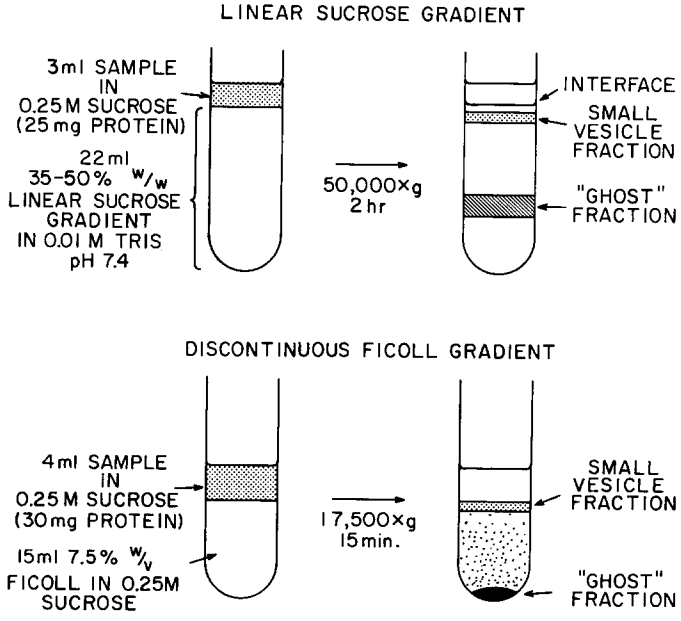

FIGURE 1 Outline of procedures used to fractionate water-washed mitochondrial suspensions on sucrose and Ficoll gradients.

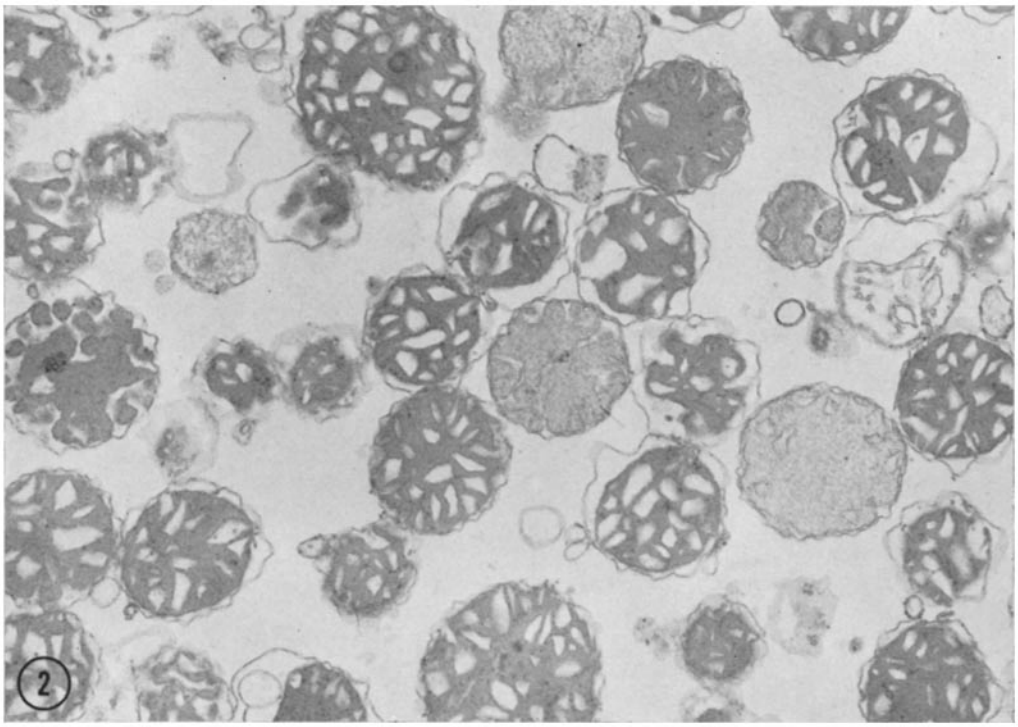

FIGURE 2 Control of freshly isolated rat liver mitochondria. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

membrane-limited bodies termed "ghosts" which have 1.5–2 times the original diameter of the mitochondria, and small empty vesicles less than 0.5 μ in diameter. The ghosts contain relatively few cristae and some granular matrix material. These appear to be partly unfolded inner membrane since they contain cristae and matrix material and since the surface area of the ghosts is much greater than the surface area of the outer membrane. The small vesicles contain no recognizable cristae or matrix material and are presumed to arise from fragmentation of the outer membrane and possibly some fragmentation of the inner membrane during osmotic lysis.

This preparation was selected as a starting material for the separation of outer and inner membranes of rat liver mitochondria since the preparative procedure involves only mild physical treatment. In addition, a modification of the digitonin fractionation procedure of Levy et al. (6) has been employed to provide an alternative, independent method for separating outer and inner membranes.

Cytochrome oxidase was selected as a possible inner membrane marker because it is an integral part of the mitochondrial respiratory chain, because it is firmly membrane-bound, and because the studies of Brosmer et al. (14) on developing insect mitochondria indicate that this enzyme is associated with the inner membrane. Malate dehydrogenase was chosen as a matrix marker since Klingenberg and Pfaff (15) have shown that mitochondrial malate dehydrogenase is not readily released when the outer membrane is ruptured by phosphate-induced swelling but is released by sonic disruption.

MATERIALS AND METHODS

Mitochondria were isolated from the livers of Carworth Farms albino rats (Sprague-Dawley strain) by the method of Schneider (16). Protein was determined by the method of Lowry et al. (17).

Biochemical Methods

FRACTIONATION OF WATER-WASHED MITOCHONDRIA: Water-washed mitochondria were prepared by the procedure previously described (12, 13). Water-washed mitochondria prepared for

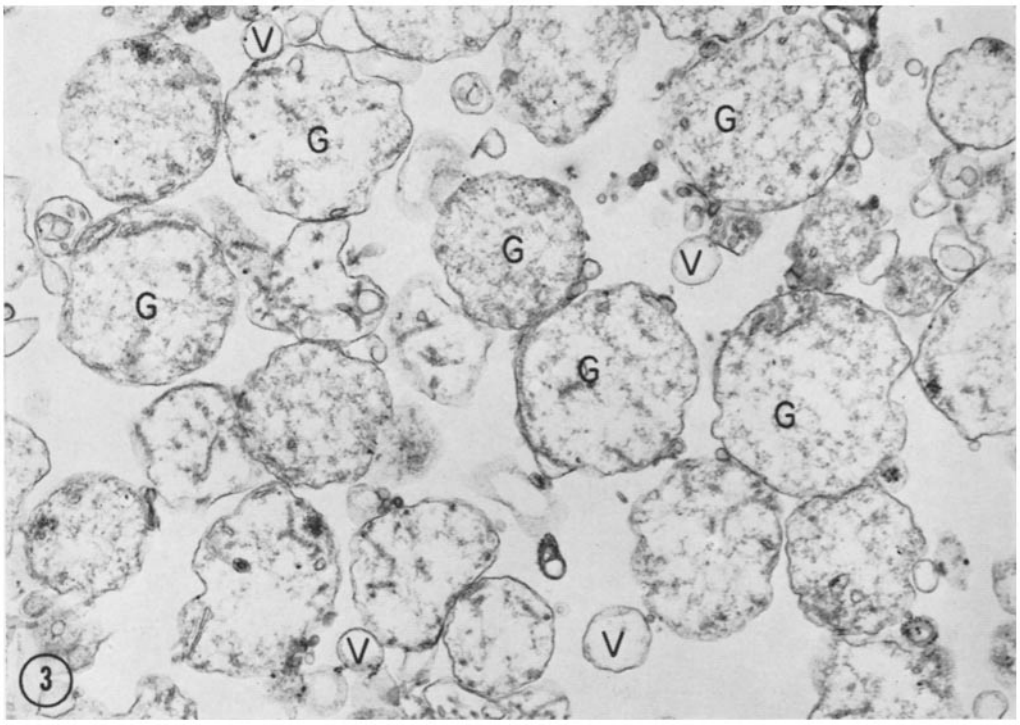

FIGURE 3 Unfractionated water-washed mitochondria. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. Note the presence of ghosts (*G*) and small vesicles (*V*). × 20,000.

loading on Ficoll gradients were given only two washes instead of the normal three. This caused little significant change in the morphology or biochemical properties but reduced aggregation and provided better separation. After the final washing, the pellet was thoroughly suspended in 0.25 M sucrose.

Water-washed mitochondria were fractionated on a sucrose density gradient by layering 3.0 ml quantities of a suspension containing approximately 25 mg of mitochondrial protein on 22 ml linear sucrose gradients containing from 35 to 50% sucrose (w/w) in 0.01 Tris-chloride, pH 7.4. The gradients were centrifuged in a SW 25 swinging-bucket rotor in a Spinco Model L ultracentrifuge at 25,000 rpm (approximately 50,000 *g*) for 2 hr. Two distinct bands resulted, one at a sucrose concentration of about 36% and the other at 46% (Fig. 1).

Water-washed mitochondria were fractionated on discontinuous Ficoll gradients by a modification of the procedure of Kurokawa et al. (18). Four-ml samples of water-washed mitochondria containing about 30 mg of protein suspended in 0.25 M sucrose were layered over 15 ml of 7.5% Ficoll (Pharmacia Fine Chemicals, Inc., Uppsala, Sweden) in 0.25 M sucrose. The gradients were then centrifuged as above at 12,500 rpm (ca. 17,500 *g*) for 13–15 min. This procedure resulted in a sharp band of material at the interface between the Ficoll solution and the solution containing sucrose only, and in a pellet (Fig. 1). A small amount of turbidity was usually noted above the pellet, but in the electron microscope this material appeared identical to that in the pellet.

Bands from the gradients were sampled with J-shaped Pasteur pipettes, and the samples were diluted with 3 volumes of 0.25 M sucrose. Pellets from the Ficoll gradients were suspended in 0.25 M sucrose. Representative samples from three tubes were pooled in order to obtain sufficient material for both electron microscopy and enzyme assays. A portion of each sample was used for electron microscopy, and the remainder was centrifuged at 144,000 *g* for 20 min. The pellets were suspended in cold distilled H_2O by vigorous homogenization and sonically treated for 1–2 min with an MSE ultrasonic probe. The resulting suspension was centrifuged at 144,000 *g* for 1 hr. The pellets were suspended in water or dilute buffer and assayed for enzyme activity and protein. This

procedure removed all of the malate dehydrogenase but did not cause a significant decrease in cytochrome oxidase or monoamine oxidase activities. Unfractionated water-washed mitochondria have been treated with the highest concentrations of sucrose and Ficoll encountered in the gradient procedures and washed and sonicated as described above. This was found to cause no significant change in the total recovery of monoamine oxidase or cytochrome oxidase activities. All procedures were carried out at 0–4°C.

FRACTIONATION OF MITOCHONDRIA BY DIGITONIN TREATMENT: Digitonin (A grade, Calbiochemical Co., Inc., Los Angeles, Cal.) was recrystallized once from hot absolute ethanol, dried, and ground to a fine powder. A 2% stock solution was prepared by adding warm 0.25 M sucrose to the powdered digitonin, mixing briefly, and sonicating for 1–2 min in an ultrasonic bath. The resulting, water-clear solution remained clear for 30–60 min at 0°C. All digitonin solutions were prepared immediately before use.

Aliquots of mitochondrial suspensions in 0.25 M sucrose containing 100–160 mg of protein/ml were placed in an ice bath, and identical aliquots of cold digitonin solutions in 0.25 M sucrose were added with continuous stirring. The digitonin concentration of the final solution was varied over the range 0–1% to give the digitonin-protein ratios described in the Results. The suspensions were incubated at 0° for 20 min after the addition of the digitonin and were then diluted by the addition of 3 volumes of cold 0.25 M sucrose. The diluted suspensions were fractionated by differential centrifugation as described in the Results.

ENZYMATIC AND CHEMICAL ASSAYS: Malate dehydrogenase (MDH) was determined by the method of Ochoa (19) in an assay medium containing 1×10^{-3} M amytal to inhibit oxidation of NADH. Cytochrome oxidase (cyt ox) was assayed polarographically by measuring the oxygen consumption with a Clark electrode at 23° in an assay system containing 7.5×10^{-2} M phosphate buffer, pH 7.2; 3×10^{-5} M cytochrome *c* (horse heart Type III, Sigma Biochemical Co., St. Louis); 3.75×10^{-3} M sodium ascorbate; and 3×10^{-4} M N,N,N′,N′-tetramethyl - *p* - phenylenediamine dihydrochloride (TMPD). Monoamine oxidase (MAO) was assayed

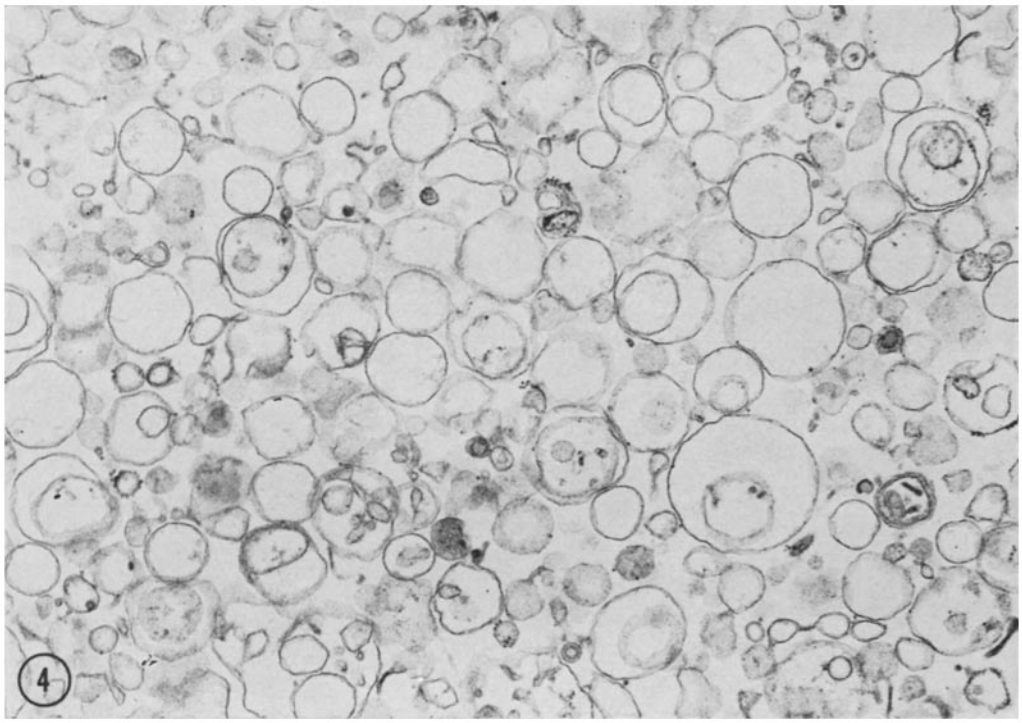

FIGURE 4 The small vesicle fraction obtained by centrifuging water-washed mitochondria in a linear sucrose density gradient. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

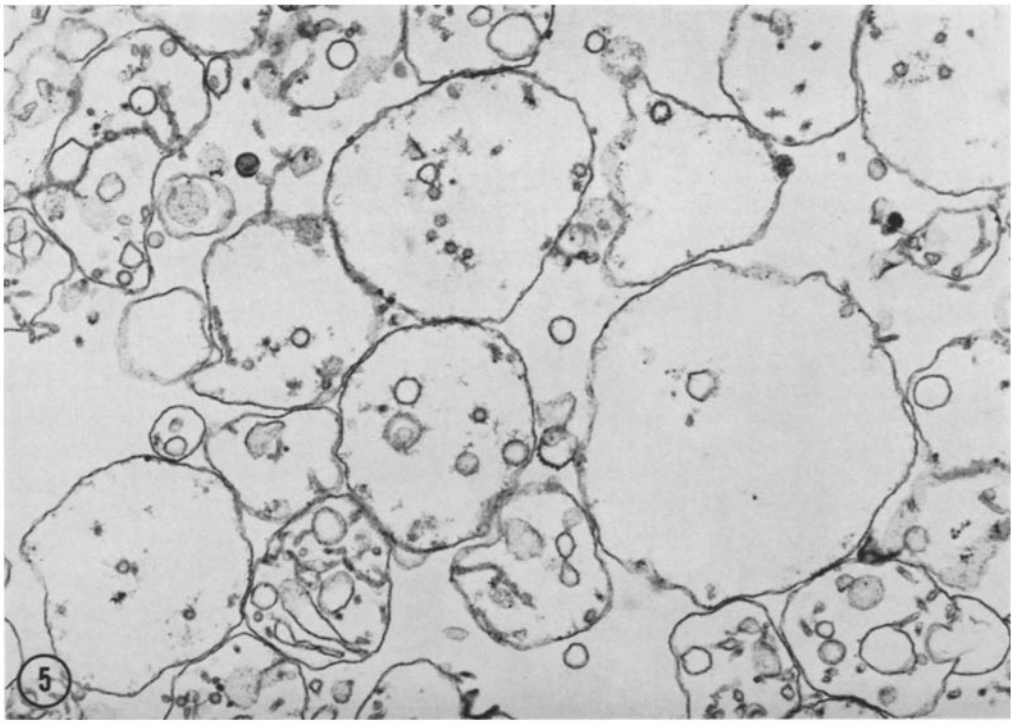

FIGURE 5 The ghost fraction obtained by centrifuging water-washed mitochondria in a linear sucrose density gradient. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

by a modification of the method of Tabor et al. (20) by following the formation of benzaldehyde spectrophotometrically at 250 mμ at 37° in an assay system containing 2.5×10^{-3} benzylamine hydrochloride and 5×10^{-2} M phosphate buffer, pH 7.6.

In order to insure maximal activity, all samples that had not been sonically treated during the course of the preparation were sonicated for about 1 min prior to assaying for malate dehydrogenase and cytochrome oxidase. Sonic disruption was not required for the estimation of monoamine oxidase activity, but caused no loss of activity.

Phospholipids were extracted from aqueous suspensions of the various fractions by the method of Folch et al. (21). The chloroform-methanol extract was washed once with 0.20 volume of 0.9% NaCl and then with 0.40 volume of an aqueous phase prepared by equilibrating 1 volume of 0.9% NaCl with 5 volumes of chloroform-methanol (2:1). The washed extract was dried and the residue was heated to 60–70°C for 5–10 min to denature any remaining protein. The phospholipid was dissolved in chloroform-methanol and aliquots were dried and digested with H_2SO_4 in the presence of H_2O_2. Total phosphate was determined by the method of Gomori (22).

Electron Microscopy

Samples of intact mitochondria, unfractionated water-washed mitochondria, unfractionated digitonin-treated mitochondria, and the resuspended 9,500 *g* pellets obtained after digitonin treatment were fixed by placing a small amount (ca. 0.2 ml) of 6.25% glutaraldehyde (in 0.1 M phosphate buffer, pH 7.2) in the bottom of a Beckman Microfuge tube. A small drop of the suspension (5–20 μl) was placed in the top of the tube. These samples were then centrifuged immediately for 4 min in the Microfuge operated at 120 v. The supernatant was decanted and replaced with fresh glutaraldehyde solution. Resuspended samples of the 40,000 *g* pellet obtained after digitonin treatment and the ghost fractions taken from sucrose and Ficoll gradients were fixed by mixing a sample of the suspensions with an equal volume of 12.5% glutaraldehyde in 0.1 M phosphate buffer. Small aliquots were immediately transferred to the Microfuge tubes and centrifuged for 4 min at 120 v. The supernatant was decanted and replaced with fresh 6.25% glutaraldehyde. A different procedure was adopted in order to sediment completely all of the small vesicles from the Ficoll and sucrose

gradients as well as the resuspended 144,000 *g* pellet from the digitonin fractionation. These samples were fixed by resuspension in a small volume of 0.25 M sucrose to which was added an equal volume of 12.5% glutaraldehyde in 0.1 M phosphate buffer. These suspensions were mixed and allowed to stand for 20 min. Aliquots were then placed in Microfuge tubes and centrifuged for 20 min at 140 v. The supernatant fluid was decanted and replaced with fresh 6.25% glutaraldehyde solution. In all cases, the volume of the sample was adjusted to give Microfuge pellets about 0.5 mm thick. All samples were prefixed for a total of 2 hr in glutaraldehyde.

The pellets were removed from the Microfuge tubes by cutting off the tips of the tubes, washed three times with 0.25 M sucrose in 0.1 M phosphate buffer, pH 7.2, and stored overnight in a fresh change of this solution. The pellets were then fixed for 1–1.5 hr in 1% OsO_4 in 0.1 phosphate buffer, pH 7.2. The fixed pellets were dehydrated by passage through an ethanol series and embedded in Epon 812 by the procedure of Luft (23). All fixation and dehydration procedures were carried out at 0–4°.

The pellets were sectioned with a diamond knife on an LKB ultramicrotome, and the sections were collected on unsupported 400-mesh grids. Sections were stained with 1% uranyl acetate for 30 min at 60°C and then with lead citrate by the procedure of Reynolds (24). They were then examined in a Siemens Elmiskop I operated at 80 kv and photographed at plate magnifications of 8,000.

Sections of each specimen were cut from the top, middle, and bottom of the Microfuge pellets, but no qualitative differences were observed in such sections. All micrographs in this paper are of sections cut from the middle of the Microfuge pellets.

RESULTS

Fractionation of Water-Washed Mitochondria

Fig. 2 shows the appearance of freshly isolated rat liver mitochondria. The mitochondria show a distinct outer membrane and most have a condensed, densely staining matrix similar to that described by Hackenbrock (25). Fig. 3 shows the typical appearance of an unfractionated preparation of water-washed mitochondria consisting of

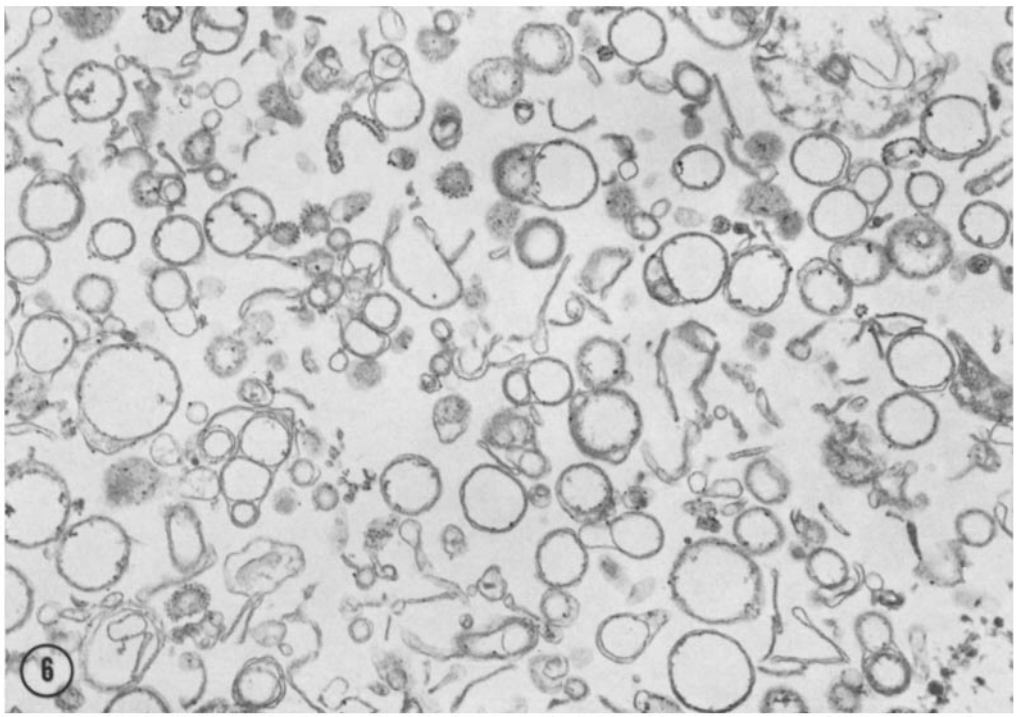

FIGURE 6 The small vesicle fraction obtained by centrifuging water-washed mitochondria in a discontinuous Ficoll gradient. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

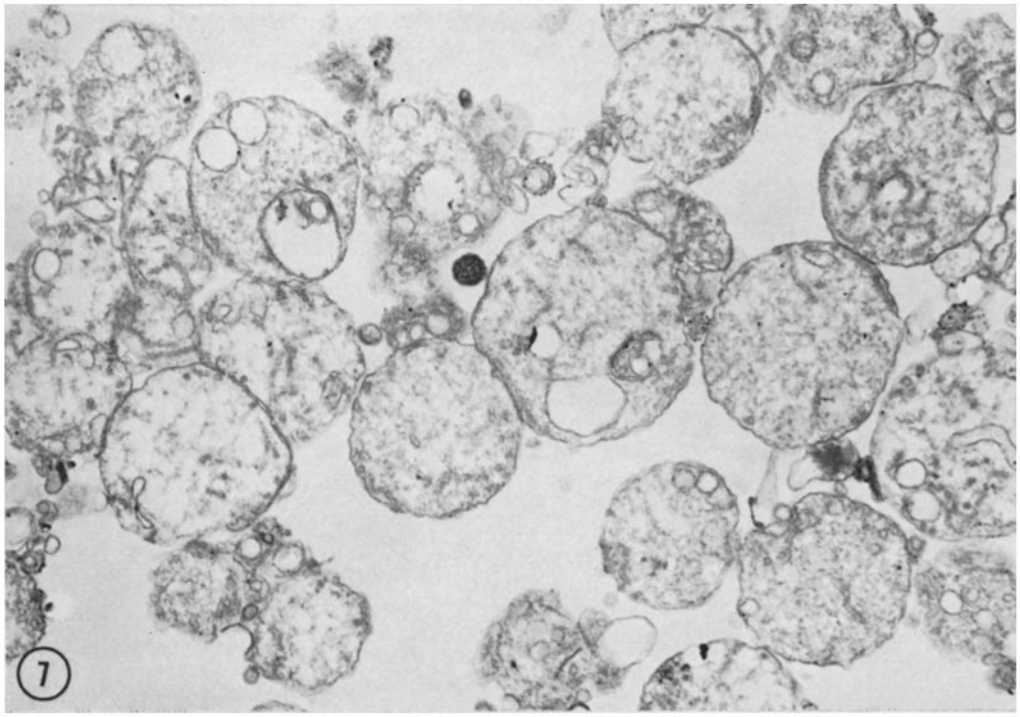

FIGURE 7 The ghost fraction obtained by centrifuging water-washed mitochondria in a discontinuous Ficoll gradient. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

ghosts and small empty vesicles. Despite the loss of 50% of the total protein, this preparation contains all of the cytochrome oxidase and monoamine oxidase activities of the untreated control mitochondria. The concentration of granular matrix material visible in the ghosts appears to be diminished. Fig. 4 depicts the appearance of the small vesicle fraction obtained after fractionation of the water-washed mitochondria on a sucrose density gradient by the procedure outlined in Fig. 1. This fraction consists primarily of small empty vesicles about ¼–½ the diameter of the ghosts. The ghost fraction obtained from the sucrose density gradient appears in Fig. 5. The size of the membranous bodies is about the same as that of the ghosts seen in the unfractionated preparation, and a few cristae are still visible. However, much of the dense-staining matrix material appears to be lost, possibly as a result of the osmotic effects of the high concentration of sucrose in the gradient. The small, round membranous profiles seen within the ghosts may be derived from the cristae. The small vesicle fraction recovered from the interface of the Ficoll gradient (see Fig. 6) is similar in size distribution to the small vesicle fraction from the sucrose gradient although more of the vesicles appear enclosed by multiple layers of membrane. The ghost fraction from the Ficoll gradient is shown in Fig. 7. This fraction is almost identical in appearance to the ghosts seen in the unfractionated water-washed preparation. The good preservation of structure is probably due to the protective effect of the Ficoll and to the isotonic conditions employed in this fractionation procedure. The preparation seems largely free of small vesicles, but serial sections have not been examined to determine to what extent small vesicles may be present.

The specific activities of cytochrome oxidase and monoamine oxidase in sonic particles derived from the various fractions of water-washed mitochondria are shown in Table I. The small vesicle fractions from both gradients have a much higher monoamine oxidase activity and a lower cyto-

TABLE I

Gradient-Fractionation of Water-Washed Mitochondria

	Unfractionated		Vesicle fraction		Ghost fraction	
	Cyt. ox.	MAO	Cyt. ox.	MAO	Cyt. ox.	MAO
Sucrose gradient						
A	—	35.8	—	117	—	27.5
B	1.23	35.4	0.60	86.7	1.88	28.3
Ficoll gradient						
A	—	39.6	—	156	—	21.5
B	1.20	30.2	0.36	124	1.50	19.2

A and B are separate experiments.

Cytochrome oxidase is expressed in μatoms O_2 consumed/minute/milligram protein. Monoamine oxidase is expressed in mμmoles benzaldehyde produced/minutes/milligrams protein.

chrome oxidase activity than the unfractionated preparation, while the opposite is observed in the ghost fractions. These results are consistent with the hypothesis that monoamine oxidase is located in the outer membrane while cytochrome oxidase is associated with the inner membrane. A better separation of these enzymes is obtained with Ficoll gradients, possibly because the more favorable osmotic conditions prevent fragmentation of the inner membrane.

Fractionation of Mitochondria after Digitonin Treatment

Initial experiments on the fractionation of mitochondria treated with digitonin were carried out by centrifuging the digitonin-treated suspensions at 9,500 *g* for 10 min. When digitonin-protein ratios were greater than 0.5 mg of digitonin/10 mg of protein, a firm tan pellet, a semiliquid reddish fluffy layer, and a clear yellow supernatant were obtained. In these initial experiments the fluffy layer was decanted and combined with the supernatant as suggested by Levy et al. (6).

The recovery of protein and enzymatic activities in the 9,500 *g* pellet after treatment of the mitochondria with various digitonin concentrations is shown in Fig. 8. The results are expressed as % of total recovered activity since cytochrome oxidase is somewhat activated at the higher digitonin-protein ratios (the activity of cytochrome oxidase at the highest digitonin concentration is about 130% of the activity of untreated mitochondria). The amount of monoamine oxidase in the pellet

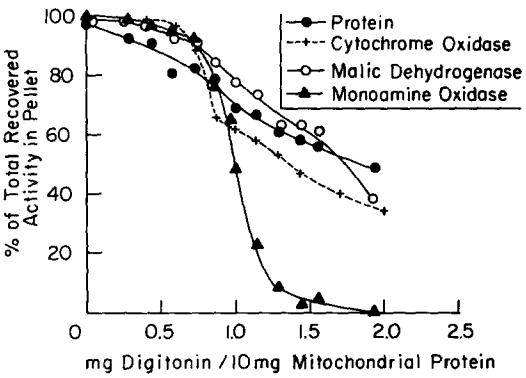

FIGURE 8 The effect of various digitonin concentrations on mitochondria. The percentage of the total recovered protein and enzymatic activities sedimented by centrifugation at 9,500 *g* for 10 min are plotted versus the digitonin concentration.

after treatment with digitonin at concentrations above 1.0 mg of digitonin/10 mg of mitochondrial protein decreases sharply. However, the protein, malate dehydrogenase, and cytochrome oxidase found in the pellet decreased much less. This suggested that the MAO-containing component of the mitochondrion, i.e. the outer membrane, was being removed whereas the inner membrane and matrix were relatively unaffected.

The removal of the outer mitochondrial membrane by the digitonin treatment was confirmed by electron micrographs of the treated mitochondria. Fig. 9 is an electron micrograph of unfractionated mitochondria after treatment with

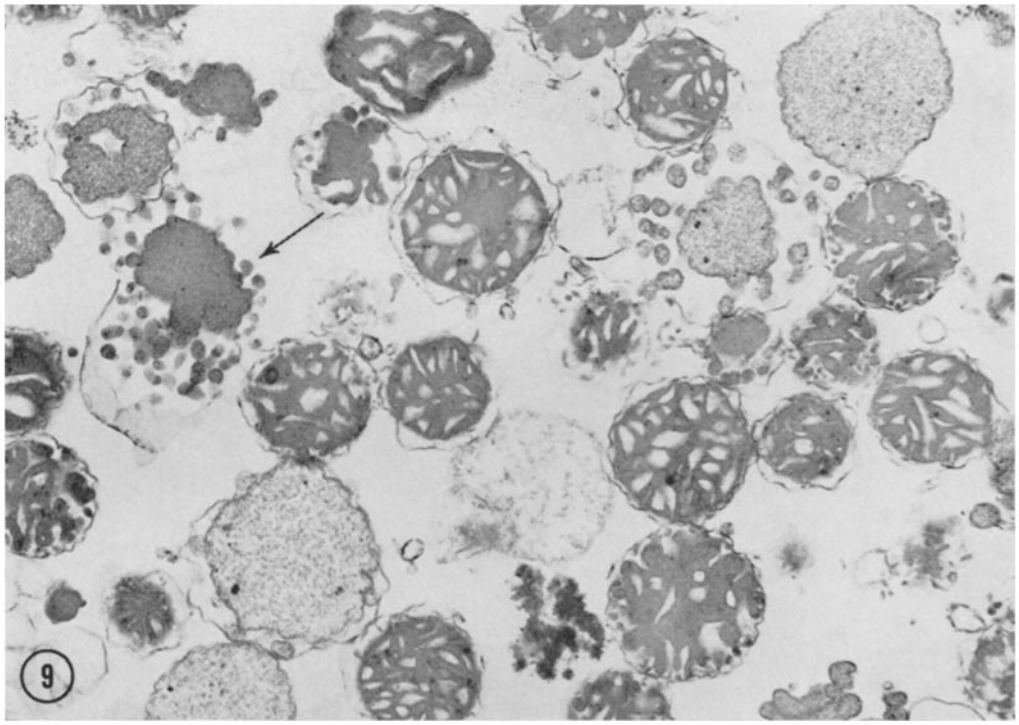

FIGURE 9 Unfractionated mitochondria treated with 0.58 mg of digitonin/10 mg of mitochondrial protein. The arrow indicates a mitochondrion with a partially ruptured outer membrane. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

0.58 mg of digitonin/10 mg of mitochondrial protein. At this digitonin concentration, 95% of the monoamine oxidase and 85% of the mitochondrial protein can be sedimented at 9,500 *g*. The majority of the mitochondria appear intact although the outer membrane is occasionally damaged or ruptured. The cristae of some of the contracted mitochondria unfold to form "fingers" of the inner membrane containing dense-staining matrix (Fig. 9, arrow). After treatment with 1.16 mg of digitonin/10 mg of mitochondrial protein, only 20% of the monoamine oxidase can be sedimented at 9,500 *g* while 60% of the cytochrome oxidase, 73% of the malate dehydrogenase, and 65% of the protein are recovered in the pellet. All of the mitochondria have lost their outer membranes, but many of the mitochondria are still contracted and have retained the densely staining matrix (see Fig. 10). Mitochondria treated with 1.94 mg of digitonin/10 mg of mitochondrial protein are shown in Fig. 11. No monoamine oxidase is sedimented at 9,500 *g* following this treatment, but the pellet still contains a significant portion of the cytochrome oxidase, malate dehydrogenase, and protein. This preparation consists almost entirely of swollen mitochondria with dilute matrices, a few visible cristae, and no outer membranes. Furthermore, small vesicles are almost entirely absent from this preparation.

These observations suggested that the high concentrations of digitonin had disrupted the outer membrane vesicles even further so that the MAO-containing structures no longer were sedimented at 9,500 *g*. To investigate this possibility further, the supernatant fluid from the 9,500 *g* pellet was fractionated by centrifugation at 144,000 *g* for 1 hr. The specific activity of monoamine oxidase in the 9,500 *g* pellet, in the unfractionated 9,500 *g* supernatant, and in the 144,000 *g* pellet from the 9,500 *g* supernatant as a function of treatment with various concentrations of digitonin is shown in Fig. 12 The specific activity of monoamine oxidase decreased rapidly in the 9,500 *g* pellet at digitonin concentrations greater than 1

mg/10 mg of mitochondrial protein, and this decrease is accompanied by an increase in the specific activities in the unfractionated supernatant fluid and in the 144,000 *g* pellet. However, the specific activity in the unfractionated supernatant remains high and decreases slightly only at very high digitonin concentrations, while the specific activity in the 144,000 *g* pellet reaches a peak at 1.1 mg of digitonin/10 mg of protein and then declines rapidly. These results suggest not only that digitonin preferentially disrupts the outer mitochondrial membrane into small fragments, but also that the degree of fragmentation is markedly dependent upon the digitonin-protein ratio. In contrast to treatment with about 1.0 mg of digitonin/10 mg of mitochondrial protein, disruption with higher concentrations of digitonin (ca. 1.5 mg of digitonin/10 mg of mitochondrial protein) is so extensive that little monoamine oxidase activity is sedimented by centrifugation at 144,000 *g*. This is reflected by the low monoamine oxidase specific activity (Fig. 12).

It seemed likely, therefore, that a more complete fractionation of the outer mitochondrial membrane from digitonin-treated mitochondria could be obtained by using a digitonin-protein ratio which would give a maximal recovery of monoamine oxidase in the 144,000 *g* pellet. Mitochondria were treated with 1.03 mg of digitonin/10 mg of mitochondrial protein. This suspension was then centrifuged at 9,500 *g* for 10 min and the supernatant fluid and fluffy layer were decanted and combined. The combined supernatant fluid and fluffy layer was then centrifuged at 40,000 *g* for 10 min. This resulted in a clear, yellow supernatant and a reddish semisolid pellet. Centrifugation of the supernatant liquid at 144,000 *g* for 1 hr resulted in a firm red pellet and a clear, pale yellow supernatant fluid. The concentrations and recoveries of protein, monoamine oxidase, cytochrome oxidase, malate dehydrogenase, and phospholipid of these four fractions are given in Table II. The 9,500 *g* pellet contained almost no monoamine oxidase, but did contain most of the original cytochrome

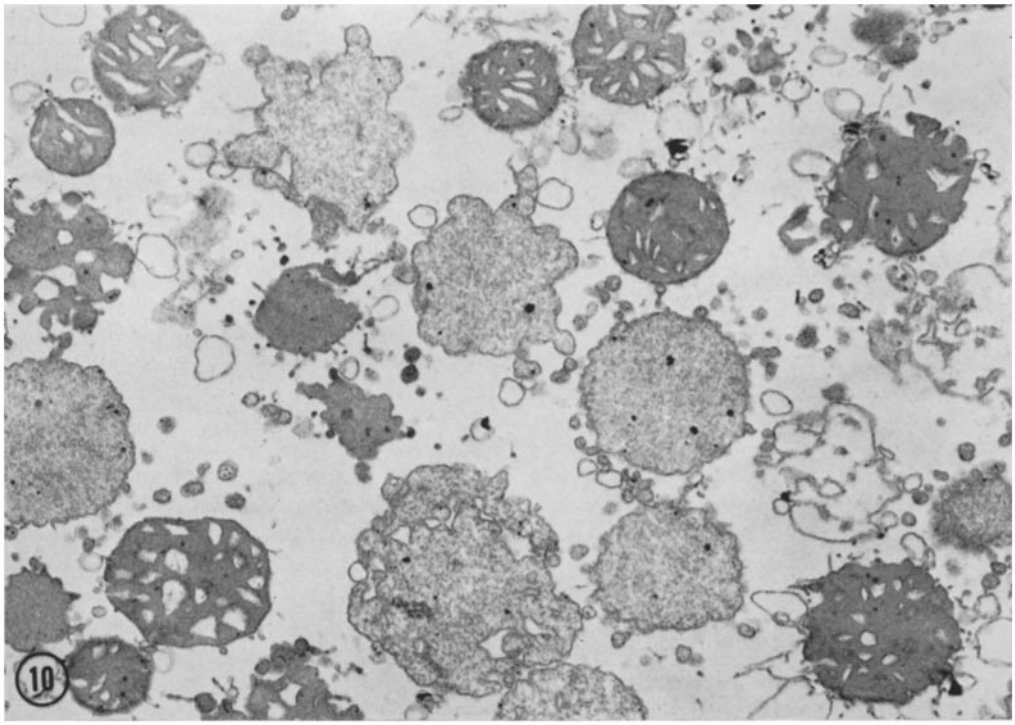

FIGURE 10 Unfractionated mitochondria treated with 1.16 mg of digitonin/10 mg of mitochondrial protein. The outer membrane has been completely removed. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

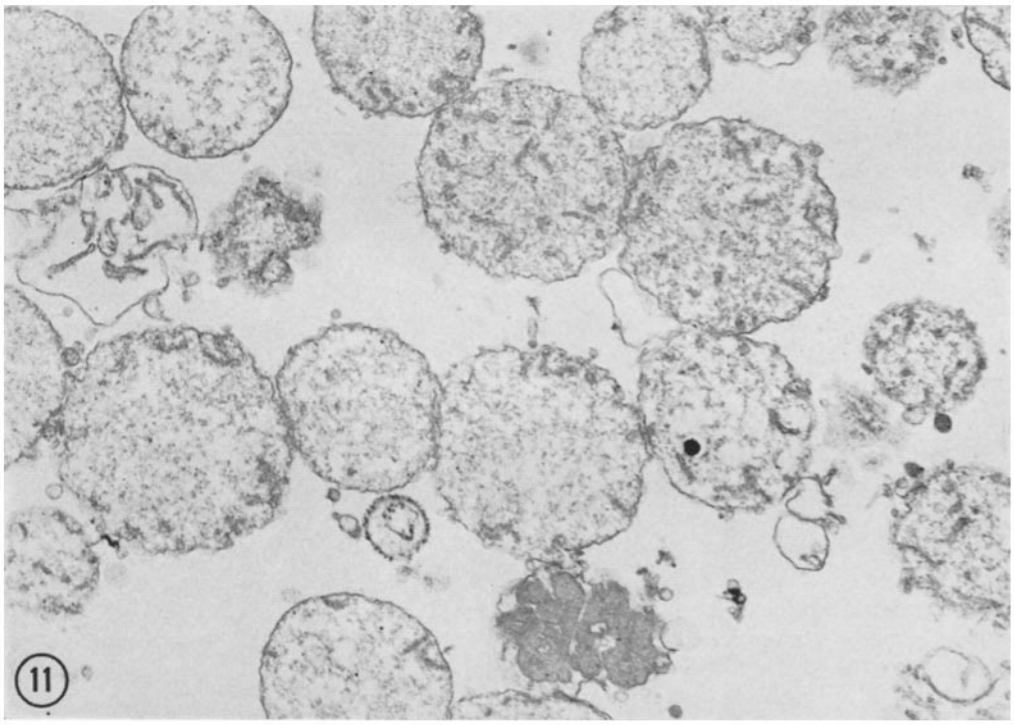

FIGURE 11 Unfractionated mitochondria treated with 1.94 mg of digitonin/10 mg of mitochondrial protein. The outer membranes are absent and most of the mitochondria appear swollen. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

oxidase and much of the original malate dehydrogenase. The phospholipid-protein ratio also indicates that it is rich in membrane material. Most of the monoamine oxidase activity is recovered in the 144,000 *g* pellet and the specific activity of monoamine oxidase in this fraction is 14 times higher than that of intact mitochondria. This fraction is low in cytochrome oxidase and malate dehydrogenase, and the high phospholipid-protein ratio indicates that this fraction is composed largely of membrane. The supernatant fluid contains considerable amounts of malate dehydrogenase and monoamine oxidase, but the phospholipid-protein ratio is very low.

The 9,500 *g* pellet consists of mitochondria essentially devoid of outer membrane; however, many mitochondria remain in the condensed state typical of freshly isolated preparations (Fig. 13). The biochemistry and morphology of this fraction indicate that it consists almost entirely of inner membrane and matrix (cf. Table II). The morphology of the 40,000 *g* pellet (fluffy layer) is shown in Fig. 14. This fraction resembles ghosts isolated on sucrose density gradients (cf. Fig. 5), although it is somewhat more fragmented. However, a few cristae can be recognized. The high cytochrome oxidase activity and the cristae suggest that this fraction consists primarily of fragmented inner membrane. The 144,000 *g* pellet shown in Fig. 15 consists entirely of very small, empty vesicles.

It is interesting to note that the supernatant fluid and the 144,000 *g* pellet together contain 89% of the monoamine oxidase and 21% of the total phospholipid, while the 9,500 and 40,000 *g* pellets together contain almost all of the cytochrome oxidase and 77% of the phospholipid. Assuming that all of the cytochrome oxidase is localized in the inner membrane and that all of the monoamine oxidase is in the outer membrane, these values indicate that the inner and outer membranes have been well separated by this procedure.

The results obtained with digitonin-treated mitochondria suggested that digitonin preferentially degrades the outer membrane. To test this

TABLE II

*Fractionation of Digitonin-Treated Mitochondria**

	Protein		Monoamine oxidase		Cytochrome oxidase		Malic dehydrogenase		Phospholipid	
	mg/ml	%‡	*Sp. act.*	%‡	*Sp. act.*	%‡	*Sp. act.*	%‡	Ratio§	%‡
Sample										
9,500 *g* (P)	12.3	63	1.69	7	1.15	82	1.01	66	0.130	53
40,000 *g* (P)	2.6	13	17.4	15	1.88	28	0.37	6.0	0.280	24
144,000 *g* (P)	0.88	4.5	208	62	0.75	3.7	0.24	1.1	0.500	14.7
144,000 *g* (S)	3.8	19.5	20.8	27	0.016	0.3	0.89	19.9	0.054	6.3
Control mito.	19.5	100	15.1	100	0.89	100	0.94	100	0.154	100
Recovery		100		111		114		92		97

Monoamine oxidase is expressed in mμmoles benzaldehyde produced/minute/milligram protein. Cytochrome oxidase is expressed in μatoms O_2 consumed/minute/milligram protein. Malic dehydrogenase is expressed in μmoles $NADH^2$ oxidized/minute/milligram protein.

* 1.03 mg digitonin/10 mg mitochondrial protein.

‡ % as compared to control mitochondria.

§ μmoles phospholipid/mg protein.

(P) indicates pellet. (S) indicates supernatant.

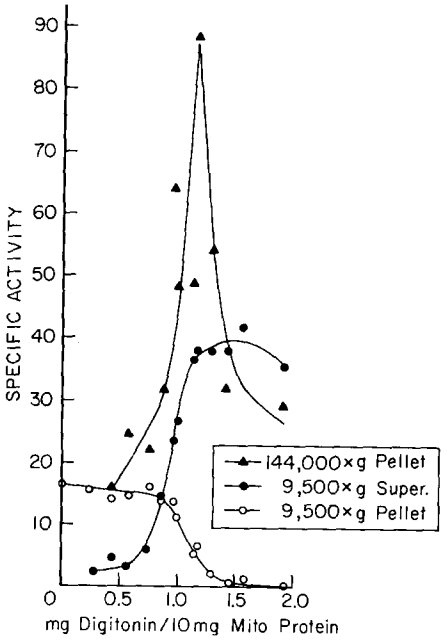

FIGURE 12 The effect of various digitonin concentrations on the specific activity of monoamine oxidase in the 9,500 *g* pellet, 9,500 *g* supernatant, and 144,000 *g* pellet derived from the 9,500 *g* supernatant. The specific activity is expressed in mμmoles benzaldehyde produced/min/mg protein.

possibility, the effect of digitonin on sonic particles derived from whole mitochondria was examined. In such a preparation, inner and outer membranes should be equally exposed to the digitonin. Freshly isolated mitochondria were suspended in distilled H_2O and sonicated for 2 min, and the sonic particles were obtained by centrifugation at 144,000 *g* for 1 hr. The pellet was thoroughly resuspended in 0.25 M sucrose and treated with digitonin as previously described (2.6 mg of digitonin/10 mg of original mitochondrial protein). The digitonin-treated sonic particles were then centrifuged for 1 hr at 144,000 *g* and the pellet and supernatant were assayed for protein, monoamine oxidase, and cytochrome oxidase. The results of this experiment (Table III) indicate that digitonin has preferentially released monoamine oxidase from the sonic particles. Although these data are not conclusive, they support the view that the removal of the outer membrane by digitonin is due, at least in part, to a preferential effect of digitonin on the outer membrane.

DISCUSSION

The correlation between the appearance of monoamine oxidase activity in association with small vesicles and the loss of the outer membrane from the intact mitochondria following digitonin treatment (Figs. 9–14) indicates that this enzyme is localized in the outer membrane. The retention of malate dehydrogenase in the fraction sedimenting at 9,500 *g* suggests that the inner membrane remains well preserved. These results confirm the observation of Levy et al. (6) that digitonin treatment under isotonic conditions strips off the outer membrane.

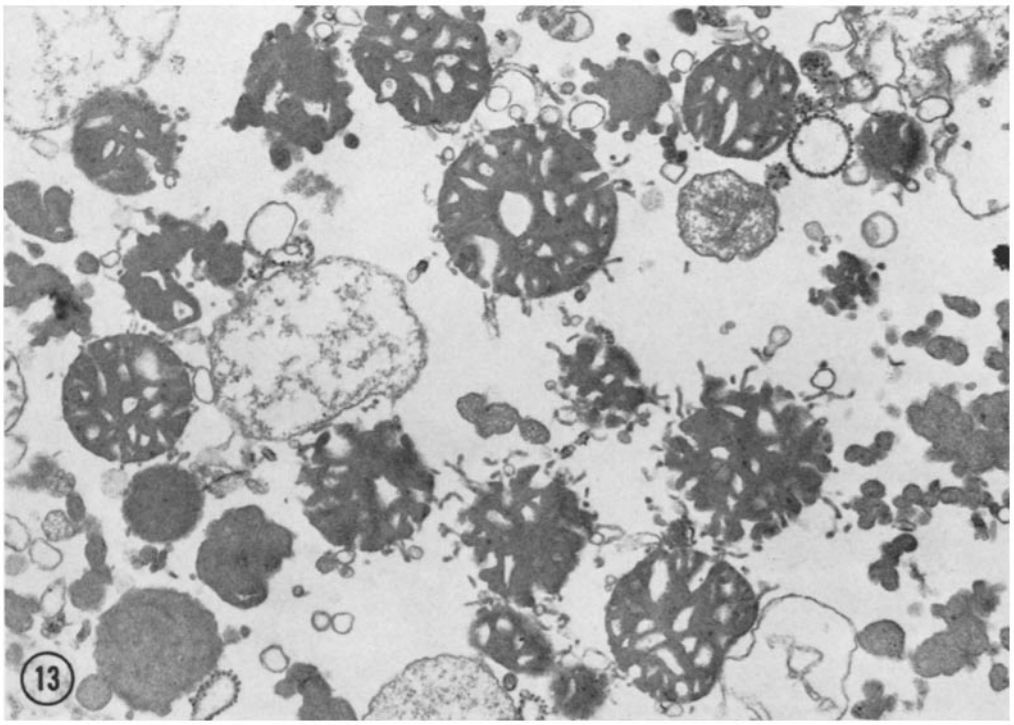

FIGURE 13 The 9,500 *g* pellet obtained from a mitochondrial suspension treated with 1.03 mg of digitonin/10 mg of mitochondrial protein. The outer membrane is absent, but most mitochondria have retained condensed matrices. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

The somewhat selective action of digitonin on the outer membrane is of considerable interest since digitonin is known to combine with cholesterol. The cholesterol-phospholipid ratio of rat liver mitochonria is quite low (26), and it is possible that the outer membrane may have a higher cholesterol content than the inner membrane. The cholesterol content of the submitochondrial fractions described here is currently being investigated

The fractionation of water-washed mitochondria on sucrose and Ficoll gradients provides additional evidence that monoamine oxidase is localized in the outer membrane, since enrichment of monoamine oxidase activity in the small vesicle fraction and a concomitant increase in cytochrome oxidase activity in the ghost fraction was observed with both gradient procedures (Table I). The separations obtained by these procedures are not complete; however, this is probably due in part to aggregation of the particles and to a certain degree of inhomogeneity in the water-washed preparation.

Separation of the small vesicles and ghosts on the Ficoll gradient is probably caused by differences in size and mass of these particles and indicates a "sieve-like" action at the Ficoll-sucrose interface resulting from the large size of the polymer molecules. However, the separation of these particles on a sucrose density gradient indicates a difference in densities of the membranes of the ghosts and the small vesicles. It appears unlikely that this difference in density may be attributed to the presence of matrix proteins in the ghosts, since the gradient procedure causes a loss of much of the matrix material (Fig. 5). Therefore, the possibility remains that the difference in density of the ghosts and small vesicles is due to inherent differences in the composition of the inner and outer mitochondrial membranes. The inner membranes, especially the cristae, of the ghosts often appear somewhat thickened and "fuzzy" in profile. This may reflect the presence of additional protein adsorbed or bound to the inner membrane resulting in increased density.

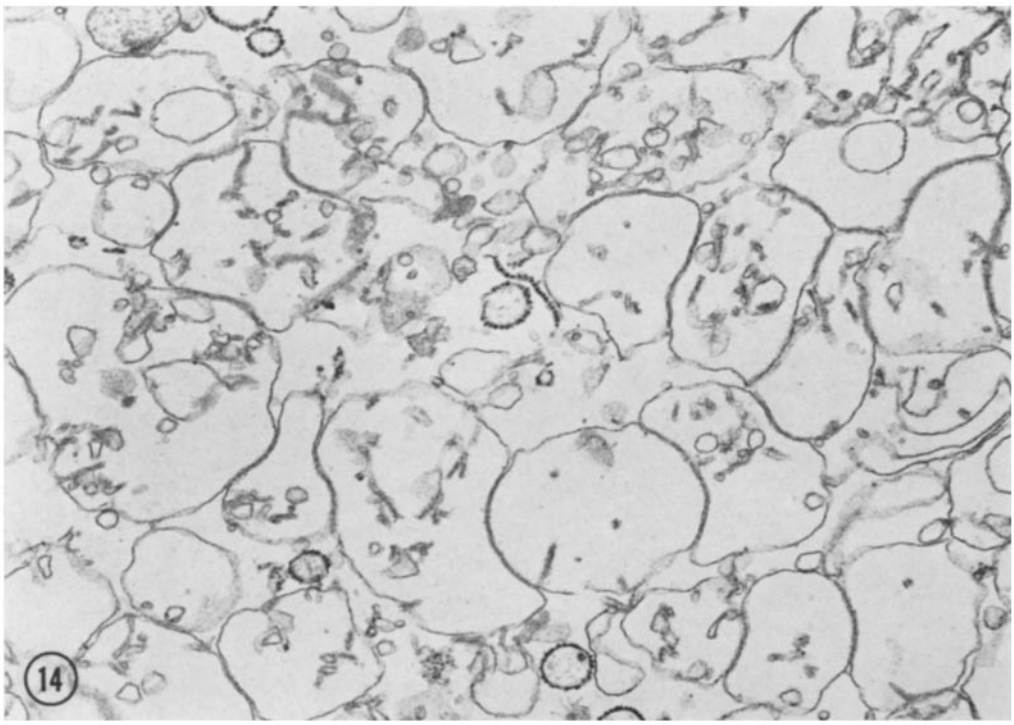

FIGURE 14 The 40,000 *g* pellet obtained from a mitochondrial suspension treated with 1.03 mg of digitonin/10 mg of mitochondrial protein. This fraction contains the fluffy layer, and appears to consist primarily of disrupted inner membrane. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

However, this does not preclude the possibility that the difference in density may be the result of differences in lipid composition of the membranes as well.

Some vesiculation of the outer membrane similar to that observed with both the water-washing and digitonin procedures probably occurs during the preparation of liver mitochondria. This may explain why small amounts of monoamine oxidase activity have been found in the microsomal fraction of liver homogenates (9, 10). Monoamine oxidase has not been found in the microsomal fraction of brain homogenates (27), and it is known that brain mitochondria are more resistant to physical damage and swelling than are liver mitochondria (28).

The evidence presented here suggests that monoamine oxidase is a suitable enzymatic marker for the outer membrane of rat liver mitochondria. The enzyme is fairly stable and can be stored for several days at 4° without significant loss of activity. It is easily assayed and, when benzylamine is used as an assay substrate, the activity is unaffected by extended anaerobic sonic treatment (11), nonionic detergents, or other disruptive procedures which may be required for mitochondrial subfractionation.

The 90 A inner membrane subunits associated with the mitochondrial membranes in negatively stained preparations (29) have been used by other workers as a means of identifying the inner membrane (1–5). Although present evidence (30, 31) supports the view that these subunits are found only on the inner membrane, the events leading to the visualization of these subunits during negative staining are not fully understood. In addition, the visualization of these particles by negative staining depends on factors such as the spreading of the phosphotungstate on the microscope grids and the disruption of the mitochondrial structures by the phosphotungstate which cannot be strictly controlled. Therefore, only fixed, sectioned prepara-

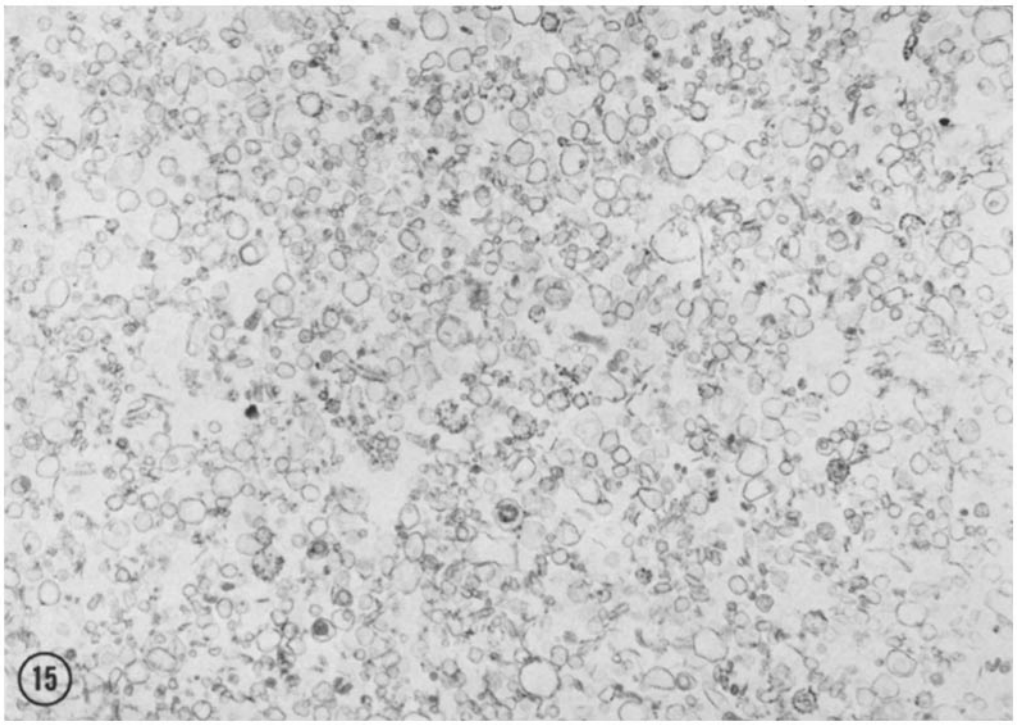

FIGURE 15 The 144,000 *g* pellet obtained from a mitochondrial suspension treated with 1.03 mg of digitonin/10 mg of mitochondrial protein. This fraction contains most of the monoamine oxidase activity of the mitochondria. Fixed with glutaraldehyde and OsO_4 and stained with uranyl acetate and lead citrate. × 20,000.

TABLE III

Effect of Digitonin on Sonic Particles*

	Protein	Cyt. Ox.‡	MAO‡	Ratio of specific activities Cyt. ox./MAO
	%	%	%	
Untreated sonic particles	100	100	100	2.8×10^{-2}
Digitonin-treated* 144,000 *g* pellet	65	85	46	5.3×10^{-2}
144,000 *g* Supernatant	27	34	61	1.74×10^{-2}

* 2.6 mg digitonin/10 mg total mitochondrial protein.

‡ % as compared to untreated sonic particles.

tions have been used in this study, and the presence or absence of the characteristic double membrane structure of the mitochondria and the presence of clearly recognizable structures such as cristae and granular matrix material have been used as a means of identifying the membrane fractions obtained. The distribution of these morphological characteristics in fractions obtained by mild physical and chemical treatments has been correlated with the distribution of specific enzymatic activities in order to obtain enzymatic markers for the identification of the inner and outer mitochondrial membranes.

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