

# THE SURFACE CHARGE OF RAT LIVER MITOCHONDRIA AND THEIR MEMBRANES

## Clarification of Some Controversies Concerning Mitochondrial Structure

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### ABSTRACT

The surface charge of intact mitochondria and submitochondrial particles was examined by the technique of preparative free flow electrophoresis. When submitochondrial preparations obtained by a swelling-contraction procedure were examined with this technique, two fractions were observed. One of these fractions exhibited the same electrophoretic properties as intact mitochondria, which indicated that it was derived from the outer limiting membrane of the mitochondrion. This fraction was found to contain the enzymes monoamine oxidase and rotenone-insensitive NADH-cytochrome *c* reductase which have been reported to be localized in the outer mitochondrial membrane. The other fraction exhibited an electrophoretic mobility which was different from that of intact mitochondria, and this fraction contained enzymes characteristic of the inner membrane-matrix fraction such as soluble and particulate enzymes of the Krebs cycle. Microsomes exhibited an electrophoretic mobility which was almost identical with that of the outer mitochondrial membrane. In addition to resolving the localization of enzymes in mitochondrial membranes, these data indicate that the outer limiting membrane of the mitochondrion is the sole determinant of the surface charge of mitochondria.

### INTRODUCTION

It is well established that the mitochondria contain two membrane systems. The outer membrane *alone* envelops the organelle and forms the outer surface of the mitochondrion. It is isolated from the inner membrane by an intermembranous space. The inner membrane encloses the hollow of the mitochondrion, the so-called mitochondrial matrix. While the inner membrane serves as an "osmotic barrier" and is able to contract, the outer membrane does not show such properties and its actual function is not yet known. This and related questions can be answered only by studies on isolated, unaltered membranes. Several tech-

niques can be used to fractionate mitochondrial membranes, but there is confusion regarding which membrane should be termed a pure outer or a pure inner membrane and how both membranes can be identified reliably (1). Although the inner membrane can be identified by its morphological appearance (2), the outer membrane is not identified reliably with the electron microscope, and biochemical methods have been used. Even with these techniques, however, it is not clear how an actual separation of the mitochondrial membranes from each other can be realized. Green et al. (3) have stated that enzy-

matic markers should be assigned to the different mitochondrial membranes only after this separation problem is solved. In the past some controversies have arisen between investigators of the mitochondrial structure and function concerning the localization of the enzymes of the citric acid and of the fatty acid oxidation cycles within the mitochondria. There is no doubt that both cycles are located in the mitochondria. The results of Parsons et al. (1, 4), Sottocasa et al. (5), Schnaitman and Greenawalt (6), Levy et al. (7), and Beattie (8) show that the Krebs cycle enzymes are exclusively found in the inner membrane-matrix fraction, whereas the findings of Green et al. (3) and Allmann et al. (9) indicate that these enzymes are present in the outer mitochondrial membranes. A similar situation is discussed for the fatty acid oxidation enzymes found by Beattie (8) in the inner membrane. The localization of enzymes like monoamine oxidase and rotenone-insensitive NADH-cytochrome *c* reductase is another object of controversy. Microsomal impurities which can never be completely excluded when isolating mitochondrial membranes cause additional confusion.

This discussion shows that the main problem is to identify the mitochondrial membranes without using enzymes as markers. A separation technique which uses some of the characteristic properties of the membranes would help identify the membranes. As mentioned above, a separation of the two mitochondrial membranes already has been achieved by several techniques as, for example, water-lysis (10, 11), swelling-shrinking (4, 5, 8, 12), sonification (5, 13), digitonin treatment (6), potassium cholate treatment (14), oleate swelling (9), or phospholipase digestion (9, 15). The membranes thus obtained have been purified mostly by density gradients (4, 5, 7, 10, 13). This purification method is based on the size and density of particles being separated, and these factors are often reliable characteristics but can sometimes be ambiguous.

The present investigation was started in order to clarify some of the confusion described above. A completely new separation technique in combination with commonly used methods was applied to identify the two mitochondrial membranes. It is known that biological particles carry a specific electrical surface charge (16), and it has been shown in several studies, by using free flow electrophoresis techniques developed by Hannig (17), that on the basis of this charge a

separation of microorganisms (18), cells (19, 20), cell organelles (21), and membranes (21) can be achieved. In the present study the surface charge of biological membranes was used to separate and identify the mitochondrial membranes. In order to guarantee a native and unaltered membrane surface, no treatment of the mitochondria with sonication, organic solvents, detergents, chemicals, or enzymes was used in the separation and purification process, but the mitochondria and their membranes were washed several times prior to electrophoresis in order to unmask the electric functional groups from adhesive proteins (20, 21). As a result of this investigation, two mitochondrial membranes were separated by free flow electrophoresis on the basis of their different surface charges, and the thus characterized membrane fractions were studied for their enzyme content.

## MATERIALS AND METHODS

### *Free Flow Electrophoresis*

A FF3 Electrophoresis Apparatus according to Hannig (17) (DESAGA, Heidelberg, Germany, and Brinkmann Instruments Inc., Westbury, N.Y.) was used. The buffer for the separation chamber (electrophoresis buffer) was a 1 + 9 aqueous solution of the isolation medium described below ( $6 \times 10^2$   $\mu$ mhos.). The buffer for the electrode vessel circulation was made of 100 mM triethanolamine and 100 mM acetic acid, pH 7.4 (2 N NaOH). The conditions of the run were as follows: 85 v/cm, 100 mA,  $t = 5^\circ\text{C}$ , and electrophoresis buffer flow 2 ml per hr/fraction. Prior to injection all samples in electrophoresis buffer were spun in a refrigerated centrifuge at 600 *g* for 10 min in order to remove aggregates. The supernatant from this centrifugation was warmed up to  $25^\circ\text{C}$  for about 2 min, cooled down to  $0^\circ\text{C}$ , its protein content was brought to about 15 mg/ml with electrophoresis buffer, and this sample was then injected into the FF3. The injection port was above fraction 70. The sample injection was 0.5 ml/hr. 92 fractions were collected at  $0^\circ\text{C}$ .

### *Preparation of Biological Material*

All experiments were carried out between  $0^\circ$  and  $4^\circ\text{C}$ . An H and an L fraction from a rat liver homogenate were prepared according to Hannig et al. (21) following procedures of de Duve et al. (22). These fractions were either injected into the FF3, either without treatment or after washing with a 0.1 M phosphate buffer, pH 7.2 for 20 min, or exhaustively homogenized in a tight-fitting homogenizer and then used for electrophoresis.

Mitochondria were prepared from the livers of

24-hr starved Sprague-Dawley rats (WIGA, Ottonbrunn, Germany) following methods of Parsons et al. (4), Schnaitman and Greenawalt (6), and Sottocasa et al. (5) which include several differential centrifugation steps. In addition to the washes described in these procedures, the mitochondria were washed twice more at 15,000 *g* for 10 min according to Green et al. (3) in order to remove microsomes as completely as possible. The isolation medium for all these experiments consisted of 10 mM triethanolamine 10 mM acetic acid, 1 mM EDTA, and 0.33 M sucrose, pH 7.4 (2 N NaOH).

A preparation of submitochondrial membranes was obtained using the swelling-shrinking technique described by Parsons et al. (4), Schnaitman and Greenawalt (6), and Neupert et al. (12). In the present experiments swelling of the mitochondria was achieved by suspending the mitochondria from two rat livers in 55 ml of isolation medium from which sucrose and EDTA were omitted. After 10 min of gentle stirring at 0°C, 20 ml of a solution containing 1.8 M sucrose, 8.25 mM ATP, and 2 mM MgSO<sub>4</sub> was added. The suspension was slowly homogenized for 10 min at 0°C and then spun at 35,000 *g* for 30 min. The pellet was homogenized in 100 ml of isolation medium and then spun at 15,000 *g* for 10 min, the obtained pellet again was homogenized in 25 ml isolation medium and then centrifuged at 1000 *g* for 10 min. This pellet was a crude preparation of inner membrane-matrix and was used for purification in the FF3 after being homogenized in electrophoresis buffer. The supernatant from the 15,000 *g* centrifugation contained mostly outer membranes but some small mitochondria, inner membranes, and microsomes also were present. In some experiments this preparation was used directly in the FF3. To obtain high purity outer membranes, the mitochondria and microsomes which could not be removed by electrophoresis were taken out by a centrifugation step (see next paragraph).

Rat liver microsomes were prepared according to Sottocasa et al. (5) in the isolation medium described above.

### *Density Gradient Centrifugation*

To obtain high purity outer membranes, a centrifugation step had to be used prior to electrophoresis. The crude preparation of outer membranes was layered on a 35% sucrose solution (5–6 ml input per 25 ml sucrose solution) in 20 mM phosphate buffer, pH 7.2, and then run in a Spinco SW-25.1 rotor (Beckman Instruments, Inc., Palo Alto, Calif.) at 63,580 *g* for 60 min. The resulting turbid layer at the entrance boundary to the sucrose solution was carefully removed with a bent syringe, washed twice with electrophoresis buffer, and then injected into the FF3. The brownish pellet was discarded.

### *Quantitative Protein and Enzyme Assays*

Protein was determined quantitatively by using a slightly modified Technicon Peptide AutoAnalyzer System (Technicon Corporation, Tarrytown, N.Y.). The samples were automatically collected, totally hydrolyzed by alkaline at 95°C, mixed with ninhydrin reagent, and the color, developed in a 95°C heating bath, was continuously recorded at 570 m $\mu$ . Test solutions with beef serum albumin were used as standards. Controls with the quantitative protein determination according to Lowry et al. (23) were run and found to be in excellent agreement with the results in the Technicon. Special precaution has to be taken with the Lowry test when using triethanolamine buffer (21).

For assaying enzymatic activities, commonly used tests were applied. The spectrophotometric tests were performed in a Beckman DB recording spectrophotometer. Prior to the tests the samples containing cell particles or membranes were either shortly sonicated at 0°C or mixed with Triton-X 100 (final concentration 0.1 mg Triton/10 mg protein) in order to obtain maximal activity. The following abbreviations for enzymes are used in the text: Glucose-6-phosphatase = D-glucose-6-phosphatephosphohydrolase, E.C. 3.1.3.9; NADPH-cytochrome *c* reductase = NADPH<sub>2</sub>-cytochrome *c* oxidoreductase, E.C. 1.6.2.3; NADH-cytochrome *c* reductase = NADH<sub>2</sub>-cytochrome *c* oxidoreductase, E.C. 1.6.2.1; MAO = monoamine:O<sub>2</sub>oxidoreductase (deaminating), E.C. 1.4.3.4; SDH = succinate:(acceptor) oxidoreductase, E.C. 1.3.99.1; cytochrome *c* oxidase = cytochrome *c*:O<sub>2</sub>oxidoreductase, E.C. 1.9.3.1; MDH = L-malate:NAD-oxidoreductase, E.C. 1.1.1.37; GluDH = L-glutamate:NADoxidoreductase, E.C. 1.4.1.2.

NADPH-cytochrome *c* reductase was determined according to Sottocasa et al. (5) and glucose-6-phosphatase following procedures by de Duve et al. (22) and Lowry and Lopez (24). MAO was assayed according to Hannig et al. (21), the assay being based on procedures of Schnaitman (6). Rotenone-insensitive NADH-cytochrome *c* reductase was measured as is described by Sottocasa et al. (5). < 1  $\mu$ M rotenone/3 ml was used. SDH was determined according to Arrigoni and Singer (25) at 550 m $\mu$ . Cytochrome *c* oxidase was tested according to Cooperstein and Lazarow (26). For the quantitation of MDH and GluDH, commercially available test kits were used (Biochimica Test-Combinations, C. F. Boehringer und Soehne, Mannheim, Germany).

### *Electron Microscopy*

Fractions containing cell particles or membranes to be characterized in the electron microscope were spun in 2 ml centrifuge tubes at 37,000 *g* for 10 min in a Spinco L50 preparative ultracentrifuge at 4°C. The

supernatant was discarded and a mixture of a 25% aqueous solution of glutaraldehyde and isolation medium (1 + 1) was placed on the pellet. After 60 min of fixation the solution was renewed. 1 hr later the pellet was washed three times with isolation medium, carefully loosened from the tube wall, and then fixed with OsO<sub>4</sub> (1% in isolation medium) for 1 hr. The dehydration of these samples was carried out with a commonly used ethanol-water passage. All the preceding manipulations were performed at 4°C. The last dehydration was made with absolute isopropanol. Then the pellet was embedded in Epon 812 according to Luft (27). Thin sections were cut in a LKB ultramicrotome (LKB Instruments Inc., Rockville, Md.) and stained with a 2% aqueous uranyl acetate (28) and an alkaline lead citrate solution (29). A Siemens Elmiskop I was used for viewing the sections (80 kv), and photographic plates were taken at magnifications of 20,000 and 40,000.

## RESULTS

### *Free Flow Electrophoresis of Swollen Mitochondria<sup>1</sup>*

The experiments of Parsons et al. (4) indicate that suspension in a phosphate buffer results in a swelling and morphologic alteration of mitochondria. In order to study these changes, mitochondria from the L fraction of a rat liver homogenate were suspended in 0.1 M phosphate buffer, pH 7.4, for 20 min and the products were injected into the FF3. Fig. 1 shows the FF3 runs of these mitochondria before and after this treatment. From the figure it is apparent that there is an additional peak in fraction 37 of the run with the phosphate-treated particles. The enzyme pattern in this fraction shows a high activity of SDH and GluDH, whereas MAO is almost absent. The electron micrographs of this additional peak illustrate some lysosomes, but in addition a large amount of particles that appear different from mitochondria (Fig. 2). In these particles only one boundary membrane can be seen and almost no cristae are present. Particles with the same enzyme pattern could be isolated as described before when an H fraction of a rat liver homogenate was homogenized in a tight-fitting homogenizer. Again, the electron micrographs (Fig. 3) show several particles different from mitochondria, but similar to those described by Parsons et al. as inner mito-

<sup>1</sup> The experiments in this paragraph were taken from the Thesis by Roland Stahn, University of Muenchen, 1969.

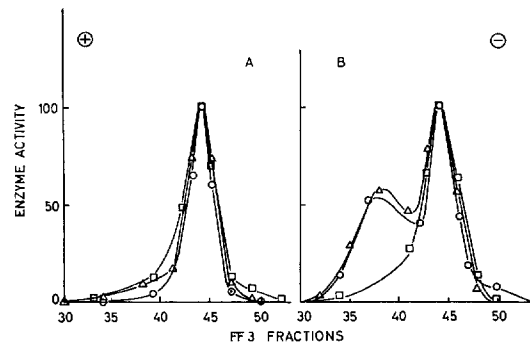
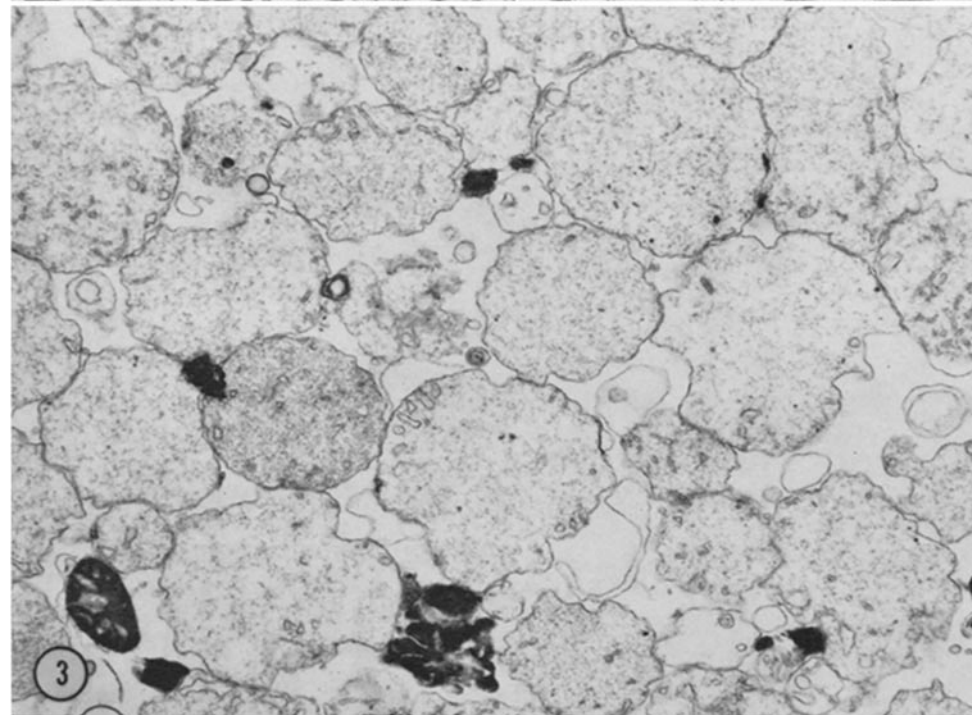
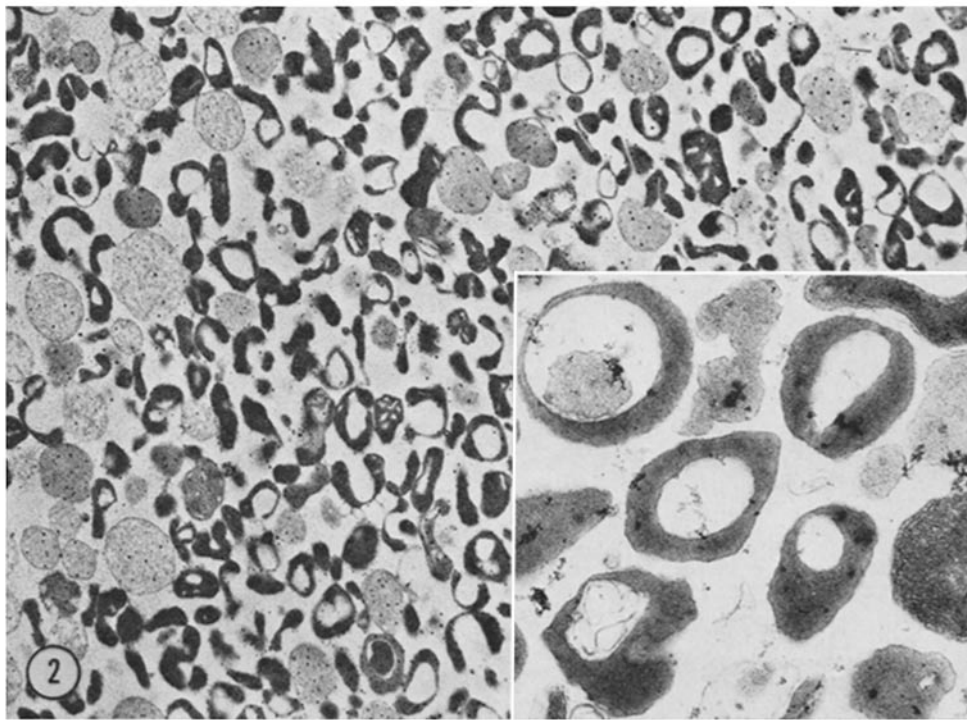


FIGURE 1 Enzyme distribution in a FF3 run of an L fraction from a rat liver homogenate. A, original; B, after a 20 min treatment with a 0.1 M phosphate buffer, pH 7.2, prior to electrophoresis. MAO  $\square$ — $\square$  SDH  $\triangle$ — $\triangle$  GluDH  $\circ$ — $\circ$  Units of enzyme activity, see Hannig et al. (21).

chondrial membranes (1, 4). The ratio SDH:MDH or SDH:GluDH in the particles of fraction 37, however, is the same as in the original mitochondria. These facts indicate that the particles described are the inner mitochondrial membranes still enclosing the matrix, whose electrophoretic mobility is different from that of the original mitochondria (cf. Fig. 1).

### *Pure Mitochondria in the FF3*

On the basis of these first results pure mitochondria from rat liver were prepared and run in the FF3 in order to compare their electrophoretic properties with those of the mitochondrial membranes. Fig. 4 shows a section of mitochondria used in the present study. It is evident that contamination by microsomes or other cell particles was very low. The lack of microsomal contaminations is especially significant since Green et al. (3) have claimed recently that the preparations of mitochondria normally used contain a considerable amount of microsomes and are therefore unsuitable for membrane studies or enzyme assignments. As is also evident in Fig. 4, the mitochondria are in excellent morphological condition, the outer and the inner membranes and the cristae being clearly evident. The matrix is densely filled with protein. MAO, rotenone-insensitive NADH-cytochrome *c* reductase, cytochrome *c* oxidase, SDH, MDH, and GluDH could be determined, but no glucose-6-phosphatase and NADPH-cytochrome *c* reductase were found. With some small differences the activities are in good agreement with those found



**FIGURE 2** Inner mitochondrial membrane-matrix isolated from an L fraction of a rat liver homogenate after phosphate buffer treatment and electrophoresis in the FF3 (fraction 38 from Fig. 1 B).  $\times 7000$ ; *inset*,  $\times 12,000$ .

**FIGURE 3** Inner mitochondrial membrane-matrix isolated from a H fraction of a rat liver homogenate in a tight-fitting homogenizer and electrophoresis in the FF3.  $\times 25,000$ .

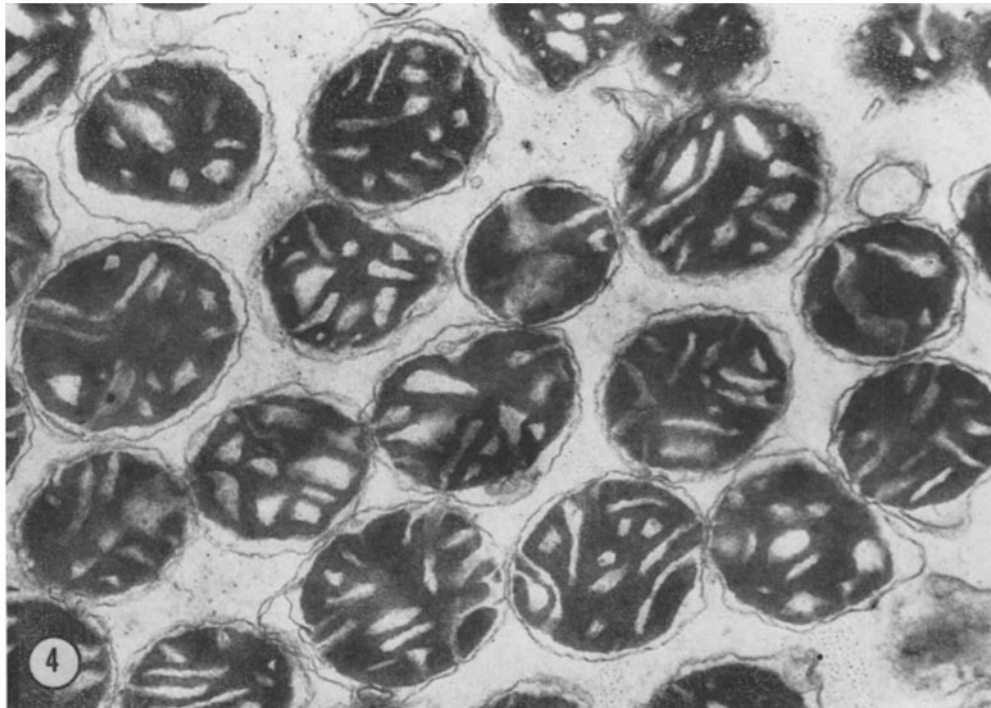


FIGURE 4 Freshly isolated mitochondria of rat liver used in the present experiments.  $\times 20,000$ .

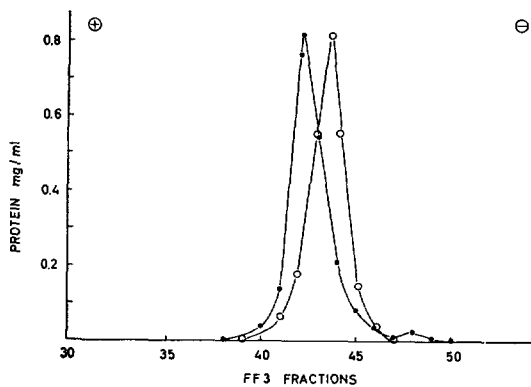


FIGURE 5 Protein distribution in a FF3 run of A, intact rat liver mitochondria  $\circ-\circ$ ; B, rat liver microsomes  $\bullet-\bullet$ .

by other authors (5, 6, 12), indicating that intact, unaltered, and pure mitochondria were used for the present studies.

Fig. 5 A shows the FF3 run of these mitochondria. A sharp peak with its maximum in fraction 44 can be seen. No contaminations with lysosomes and peroxisomes can be detected and no microsomal enzymes are present in this frac-

tion. Examination of thin sections in the electron microscope shows no morphologic alterations or abnormalities in comparison with mitochondria preparations of other authors (3-7). In addition, the mitochondrial enzyme pattern looks exactly the same as that of the original mitochondria.

This is also true for reelectrophoresed mitochondria and for mitochondria which have been washed several times prior to reelectrophoresis. This last result indicates that the surface of the mitochondria used had been "cleaned" as thoroughly as possible and that all the adsorbed protein masking the surface charge groups had been removed.

Consequently, free flow electrophoresis does not alter the content and the surface of mitochondria, and the electrophoretic behavior of these particles (and of their membranes) is actually based on their surface active groups.

#### *Electrophoresis of Mitochondrial Membranes*

The main point of interest is the electrophoretic behavior of the mitochondrial membranes. In the present study, all manipulations which could create alterations of the electrophoretic properties

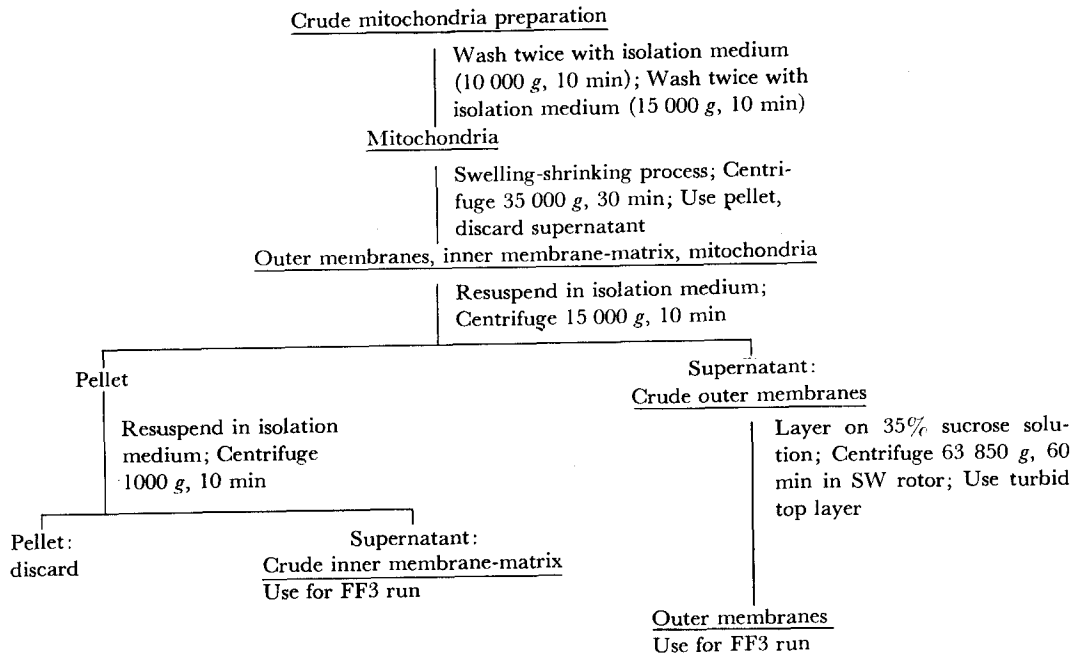


FIGURE 6 Isolation scheme for mitochondrial membranes from rat liver.

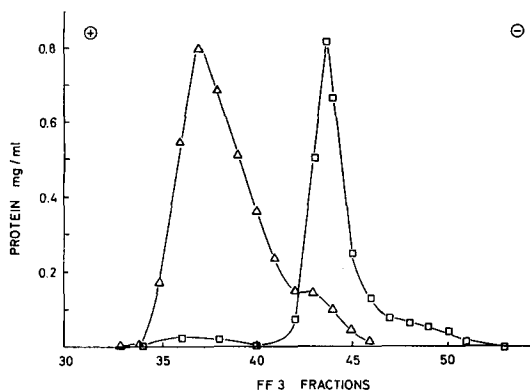


FIGURE 7 Protein distribution in a FF3 run of mitochondrial A, inner membrane-matrix  $\triangle-\triangle$ ; B, crude outer membranes  $\square-\square$ .

of the membrane surfaces were carefully avoided. Even a density gradient centrifugation was omitted. The separation of the two mitochondrial membranes was achieved by the swelling-contraction technique that is based on the different osmotic and contractile behavior of the outer and the inner mitochondrial membranes. The crude membrane preparations obtained by the isolation scheme of Fig. 6 were injected into the FF3.

Fig. 7 illustrates the protein distribution in the

electrophoresis fractions. Fig. 7 A shows the electrophoresis of the crude inner membrane fraction after the swelling-contraction procedure. As can be clearly seen from the graphs, the protein maximum is found exactly at the same position as the additional peak found in the phosphate buffer-treated L fraction or H fraction of the rat liver homogenate (Fig. 1, fraction 37). This particle fraction was suspected to be the inner membrane-matrix fraction from mitochondria on the basis of the electron micrographs and enzyme determinations. Fig. 8 shows a thin section of material from fraction 37 of Fig. 7 A. Regular particles with only one membrane can be seen. These particles look similar to those shown in Fig. 3 and identical to those described by other investigators as the inner membrane-matrix fraction (1, 4, 7). The protein content appears to be less dense than that of mitochondria, but the particles are larger than mitochondria because of swelling. In Fig. 7 A, a shoulder can be seen at the position where mitochondria are expected (fraction 44). A section of this fraction shows unaltered or somewhat damaged mitochondria and some distinct and considerably smaller vesicles which are presumably microsomes that

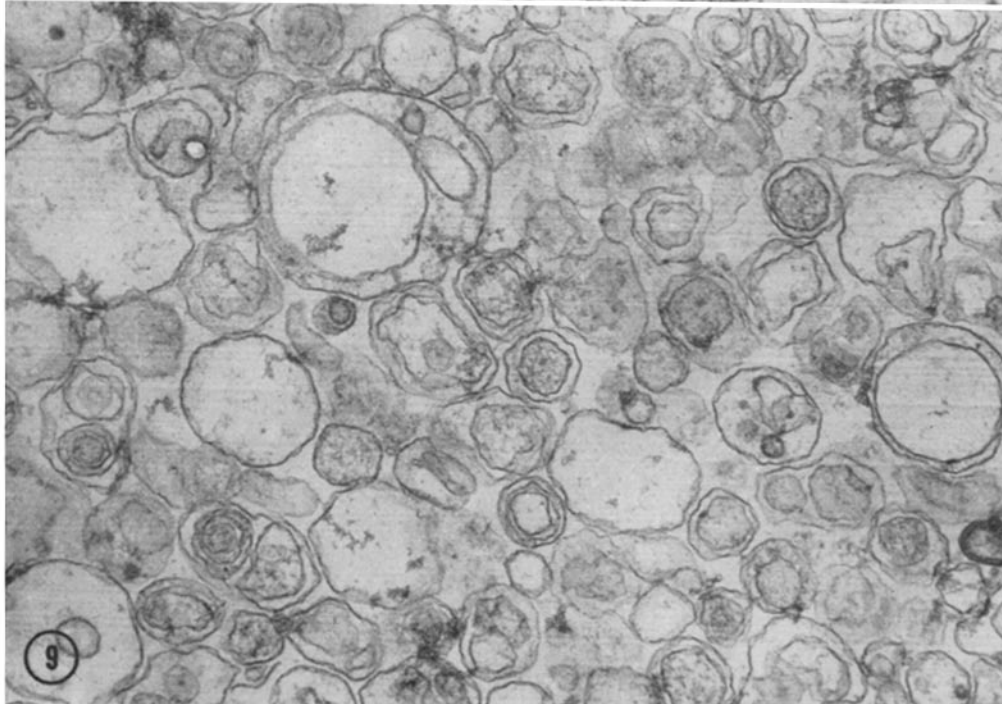
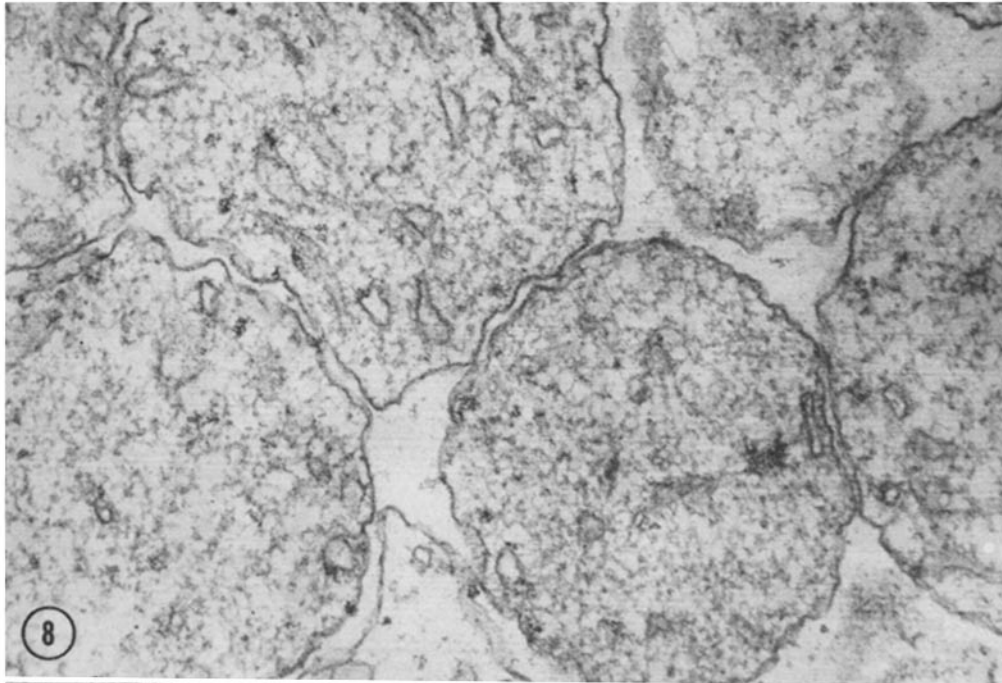


FIGURE 8 Inner mitochondrial membrane-matrix isolated by the swelling-contraction procedure from rat liver mitochondria and purification in the FF3 (fraction 37 from Fig. 7 A).  $\times 40,000$ .

FIGURE 9 Crude outer mitochondrial membranes isolated by the swelling-shrinking technique of rat liver mitochondria after running in the FF3 (fraction 44 from Fig. 7 B).  $\times 20,000$ .



had been removed from the inner membranes by electrophoresis.

When a crude outer membrane fraction was injected, a completely different picture was obtained (Fig. 7 B). The protein maximum was in fraction 44, the same position where mitochondria had been found in preceding experiments. A small hump can be seen in the inner membrane region (fractions 34–39). In fact, the particles in this hump can be identified with the electron microscope as inner membranes. But the particles in the protein maximum look completely different (Fig. 9). For the most part, “empty” vesicles, which are similar in size to mitochondria, can be seen showing only one membrane and apparently very little protein content. Parsons et al. (4), Schnaitman and Greenawalt (6), and Sottocasa et al. (5) have described similar particles and have designated them as outer mitochondrial membranes. Some unaltered small mitochondria and considerably smaller vesicles with distinct boundary membranes, very similar to smooth microsomes, can be detected in the electron micrographs of fraction 44. Note that the maximum of this outer membrane fraction coincides *exactly* with the maximum of the mitochondria.

Both kinds of particles have the same electrophoretic mobility.

#### *Microsomes in the FF3*

As Green et al. (3) have stated, microsomal contaminations in mitochondria and mitochondrial membrane preparations may lead to false conclusions concerning the enzyme patterns of these particles. Therefore, microsomes of rat liver were run in the FF3 in order to test their electrophoretic behavior. Hannig et al. (21) have found previously that microsomes are deflected slightly more towards the anode than mitochondria. A separation of microsomes from mitochondria in the FF3 was observed but was not satisfactory for the preparation of pure fractions. This fact was confirmed in the present studies. The protein maximum of the microsomal run was in fraction 42 (Fig. 5 B), but the overlap with the mitochondrial peak was so large that no complete separation of these two organelles can be expected. Small amounts of microsomal impurities, however, can be partly removed from mitochondria. The electron micrographs from fraction 42 illustrate the typical appearance of microsomes (Fig. 10). Rough and smooth membranes

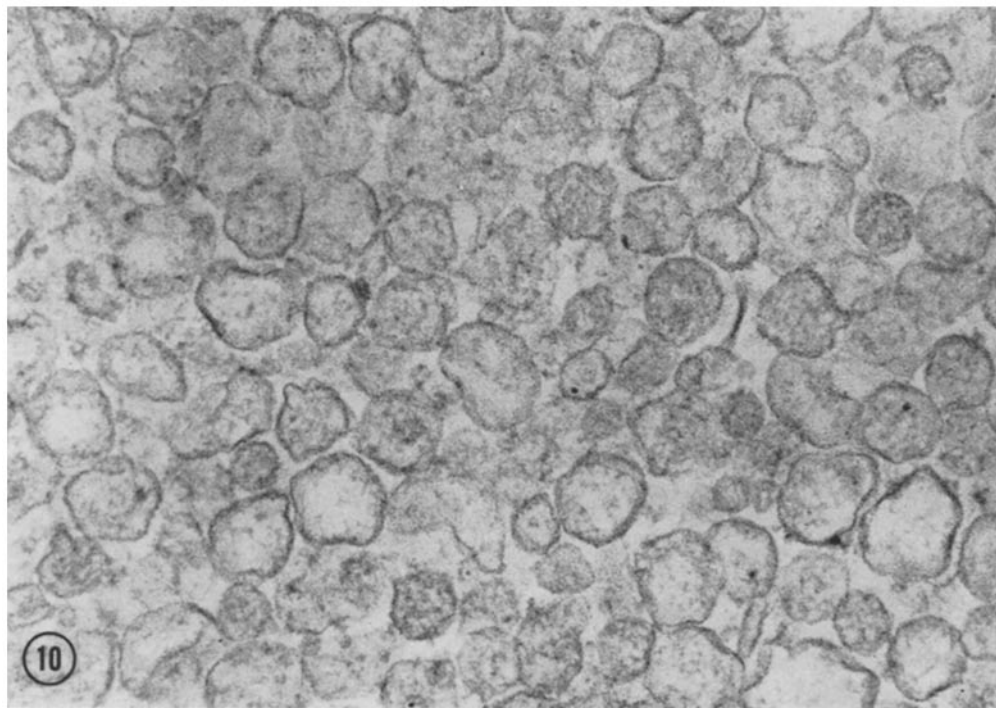


FIGURE 10 Microsomes from rat liver after a FF3 run (fraction 42 from Fig. 5 B).  $\times 40,000$ .

can be recognized. Some of these same vesicles were also seen in fraction 44 of the crude outer membrane run in Fig. 9.

#### *Pure Outer Mitochondrial Membranes*

The electron micrographs of the crude outer membrane preparation and the results of the FF3

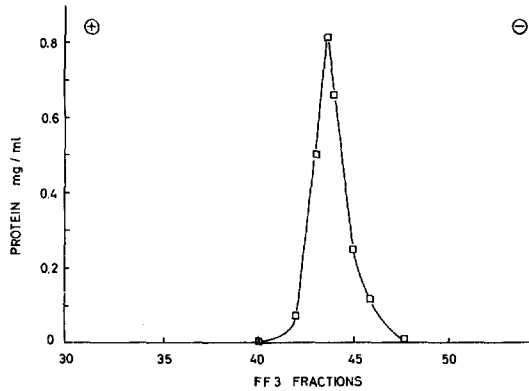


FIGURE 11 Protein distribution in a FF3 run of outer mitochondrial membranes after purification on a 35% sucrose solution prior to electrophoresis.

run of microsomes indicate that pure outer membranes cannot be produced by the free flow electrophoresis technique alone. An additional centrifugation step must be applied. In the present study, a 35% sucrose solution was chosen for separating aggregates, small mitochondria, and some of the microsomes from the outer membranes. After the centrifugation the turbid layer at the entrance boundary of the sucrose solution contained mostly outer membranes. When this preparation was injected into the FF3, a sharp peak was obtained in fraction 44 (Fig. 11). Since outer membranes and mitochondria have the same electrophoretic mobilities, and since microsomes cannot be separated from mitochondria satisfactorily by free flow electrophoresis, this figure alone does not provide evidence for a pure outer membrane preparation. The electron microscope, however, suggests the outer membranes to be pure (Fig. 12). No mitochondria and no noticeable amounts of other particles can be detected. Better evidence for the purity of the outer membranes is obtained from the following enzyme determinations.

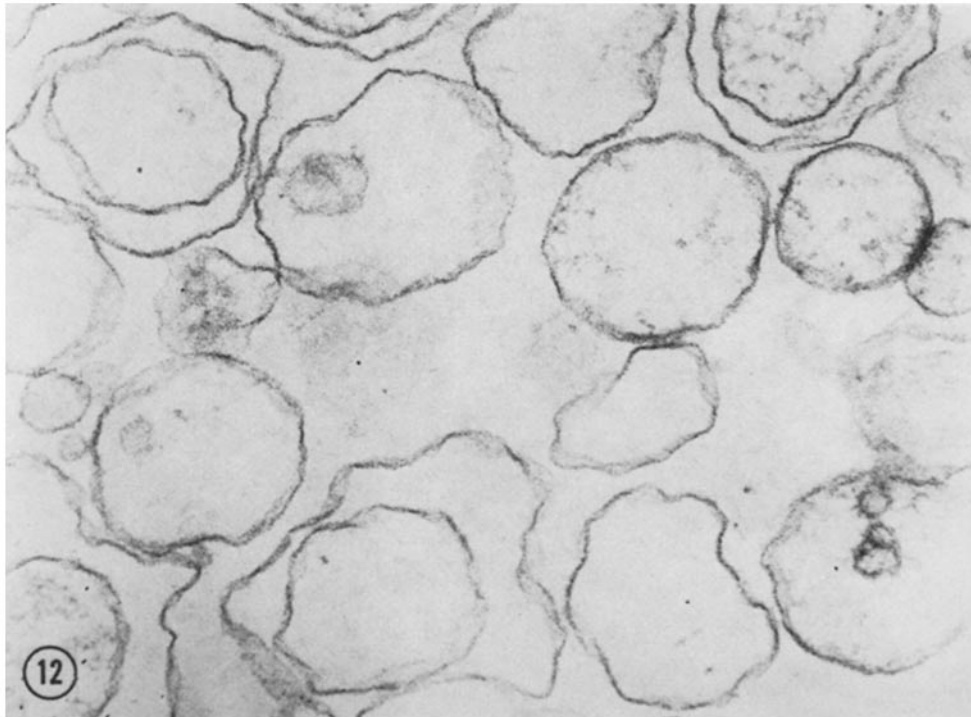


FIGURE 12 Pure mitochondrial outer membranes after purification on a 35% sucrose solution and FF3 electrophoresis (fraction 44 from Fig. 11).  $\times 40,000$ .

*Determination of the Purity of the Membranes and Assignment of Enzymes to the Mitochondrial Membranes*

The membrane fractions obtained with the FF3 and the microsomal preparation were tested for their enzyme content in order to assess the purity of the membranes and to assign some enzymes to the mitochondrial membranes. Note that this assignment was attempted after the membranes were isolated and identified by electrophoresis. As was shown above, the mitochondria that were used for the membrane separation here possessed the normal mitochondrial enzyme pattern. All of these enzymes should also be found in the membranes if they are bound to them. In addition, it was necessary to prove that no microsomal contamination was present in the membranes. Fig. 13 shows the enzyme distribution in the electrophoresis runs of the outer membranes, the inner membrane-matrix, and the microsomes in the FF3. For each membrane, only one representative enzyme is shown in the plot. In addition, glucose-6-phosphatase, rotenone-insensitive NADH-cytochrome *c* reductase, cytochrome *c* oxidase, MDH, and GluDH have been tested. On the basis of the enzyme content, the graphs clearly show that there are three different particles or membranes present. This conclusion already was made from the electron micrographs.

Fig. 13 A, demonstrating the enzyme distribution in the outer membranes, illustrates MAO as the marker enzyme (11, 30). This enzyme has no comparable maximum in the inner membrane-matrix (Fig. 13 B), and only a small one in the microsomes (Fig. 13 C). On the other hand, the main enzymes of the inner membrane-matrix and of the microsomes are either absent, or present only in negligible amounts in the outer membrane. This confirms the observations with the electron microscope in which the outer membranes looked extremely pure (Fig. 12).

The inner membrane-matrix fraction (Fig. 13 B) is characterized by a high SDH and MDH activity. In fraction 37, a maximum trails down to a shoulder in fraction 44. No MAO can be found in the main peak but it is present in the shoulder. The particles in this shoulder had been identified earlier in the electron microscope as mitochondrial contamination which had been removed by electrophoresis from the inner membrane-matrix. This is confirmed by the enzyme

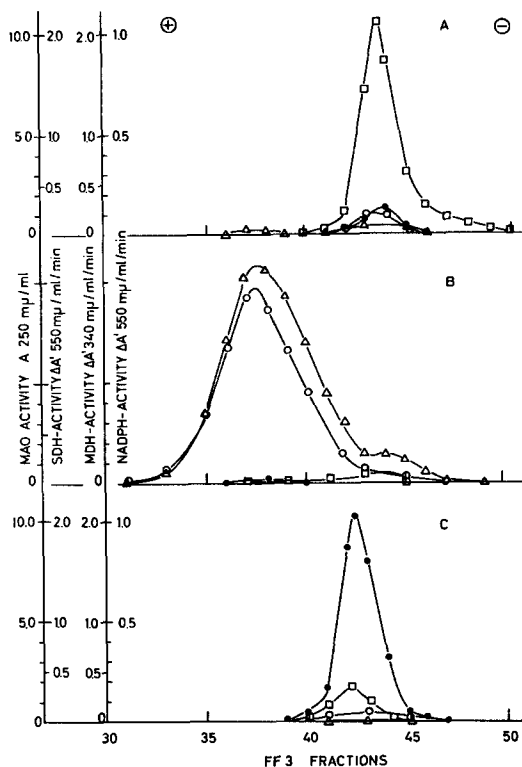


FIGURE 13 Enzyme distribution in FF3 runs of A, outer membranes (from Fig. 12); B, inner membrane-matrix (from Fig. 7 A); C, rat liver microsomes (from Fig. 5 B). MAO  $\square$ - $\square$  SDH  $\triangle$ - $\triangle$  MDH  $\circ$ - $\circ$  NADPH-cytochrome *c* reductase  $\bullet$ - $\bullet$ .

assays which show that enzymes of both outer and inner membranes are found in this shoulder.

The microsomes are distinguished by a high NADPH-cytochrome *c* reductase activity, an enzyme which cannot be found in the two mitochondrial membranes (Fig. 13 C).

The other enzymes tested but not shown in the graphs can now be assigned to the various membranes. The rotenone-insensitive NADH-cytochrome *c* reductase distribution was parallel to that of MAO, a typical outer membrane enzyme (5, 8). The inner membrane-matrix fraction was characterized not only by the citric acid cycle enzyme SDH, but also by MDH and GluDH which belong to the same cycle. After treatment with detergents or sonification, however, these latter enzymes are released from the membranes and only SDH is retained in the particles. In addition to NADPH-cytochrome *c* reductase, the microsomal fraction contained glucose-6-phos-

phatase. This latter enzyme was definitely not present in the inner membrane-matrix, and only a small amount was found in the outer membrane fraction.

#### DISCUSSION

The data obtained from these studies indicate that suspension in a phosphate buffer or mechanical mistreatment can alter mitochondria: the outer membrane is partly stripped off. With the technique of swelling-shrinking, two different membranous systems can be produced from mitochondria and characterized by free flow electrophoresis. One membrane has the same electric charge as the mitochondria themselves. The other possesses completely different electrophoretic properties. In addition, the electron microscope and enzyme assays characterize the membranes as being different from each other. Therefore, there can be no doubt that the relatively mild technique of swelling-shrinking disintegrates the mitochondria only into two pieces: the outer and the inner membranes. Only stronger treatments or reagents can further disintegrate the membranes. The production of only two membranous constituents from mitochondria and their separation into two fractions in the FF3 makes it relatively easy to identify these systems. The two membrane fractions differ from the original mitochondria, from each other, and from microsomes in their morphologic and enzymatic properties, and from each other in their electrophoretic behavior. In addition, it has been demonstrated with the FF3 technique which of the two membranes produced after swelling-shrinking of the mitochondria is the outer and which is the inner membrane. The main criterion for designating one of the membranes as the outer mitochondrial membrane was the identical surface charge of the intact mitochondria and this membrane.

This criterion must be considered to be very significant and specific (16-21). Recently, Hannig et al. (20) have demonstrated that during the generation cycle of immune-competent cells, the surface charge of the cell membrane must undergo a significant change.<sup>1</sup> These changes can be used for the separation of lymphocytes, blast cells, and plasma cells via the FF3. The same investigators have shown that the surfaces of these cells must be washed carefully in order to remove adhesive proteins from the charged functional groups. The same observation was made during studies of the isolation of rat liver lysosomes in the FF3

(21). For this reason, the particles and membranes to be separated in the present investigation had been washed carefully until the electrophoretic mobility remained constant, thus indicating that the surface was free of masking proteins. This is sufficient evidence to indicate that the separation of the mitochondrial membranes had been achieved exclusively on the basis of their surface active groups. The nature of these groups is completely unknown yet, but is under investigation. A possible relationship with surfaces of other biological membranes would be only speculative. It should, however, be pointed out that the electrophoretic behavior of mitochondrial outer membranes and microsomal membranes are very similar. This fact might support the speculations of Parsons et al. (1) and Schnaitman and Greenawalt (6) about the possible origin of the outer mitochondrial membrane. Little is also known about the number of these groups and about their absolute charges. Investigations concerning these problems are under way.

Another question has been finally resolved in the present study. This is the location of several enzymes in the mitochondrial membranes, a problem which is continually discussed by several authors (1, 3-9, 11, 30, 31). The discrepancies between all these results apparently originate in the hitherto unsolved crucial problem of the choice of criteria for the recognition of inner and outer membranes in submitochondrial fractions. Once these fractions of mitochondria were separated in the FF3 and identified on the basis of their surface charges, it was possible to associate reliable enzymatic markers with specific membranes. The localization of the enzymatic markers and the morphology of the membrane fractions supported the conclusion made from the electrophoresis data. It was found that the enzymes of the citric acid cycle which were assayed were exclusively associated with the membrane which has a different surface charge than intact mitochondria. This membrane must be the inner membrane. These enzymes of the citric acid cycle are not present in the membrane having the same electric properties as intact mitochondria which must be the surface-forming mitochondrial membrane, i.e., the outer membrane. NADH-cytochrome *c* reductase and MAO were found only in this membrane fraction. Microsomes, which had only a slightly different electrophoretic mobility than outer mitochondrial membranes, did not possess noticeable rotenone-insensitive

NADH-cytochrome *c* reductase activity, contrary to some reports in the literature.

The clearness of these results was made possible by using the mild swelling-shrinking technique for the preparation of the mitochondrial membranes. It will be of interest to determine whether other separation techniques take the mitochondria apart into more pieces which can be separated by free flow electrophoresis. Racker (32) has been successful in taking apart parts of the inner mitochondrial membrane and then reconstituting them. Similar experiments in combination with investigations of the membrane surface might be helpful in studying the origin and function of the outer mitochondrial membrane. The relatively unknown technique of free flow electrophoresis has been able, in the present

investigation, to provide evidence which cannot be obtained by other techniques but which can effectively support other results. This technique will be of future interest especially in the field of cell, cell particle, and membrane analysis.

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