Relationship of Sidedness of Mitochondrial Inner Membrane Vesicles to Their Enzymic Properties†

Lynn Astle‡ and Cecil Cooper*

ABSTRACT: The relationship between the sidedness of sonic and digitonin mitochondrial inner membrane vesicles, the fluorescence changes induced in added probes upon energization with ATP or succinate, and the sensitivity of their oxidative phosphorylation and fluorescence response to atractyloside were studied. It is the current consensus that the membranes of sonic vesicles are inverted while those of digitonin vesicles are not and that the direction of the fluorescence change of an added probe indicates the orientation of the membranes because mitochondria show a fluorescence decrease on energization while sonic particles show an increase. We have found that digitonin particles cause fluorescence increases in probes following energization by succinate or ATP. *Both* digitonin and sonic particles can be fractionated by equilibrium density centrifugation into inverted and noninverted vesicles. The sidedness was characterized by specific labeling of the exposed surface of the membrane with ^{125}I by lactoperoxidase. The outer aspect of the membrane is only slightly labeled while the inner surface is heavily labeled. The inverted and noninverted vesicles respond the same as the unfractioned parent preparation with respect to the direction of their energy-dependent fluorescence changes and the sensitivity of their oxidative phosphorylation to atractyloside. Four conclusions are drawn from their investigation. These are: (1) both sonic and digitonin mitochondrial inner membrane preparations are mixtures of right-side out and inside out vesicles; (2) the direction of the energy-dependent fluorescence responses of probes does not reflect the sidedness of the membrane but rather some state of the membrane; (3) inhibition of oxidative phosphorylation by atractyloside does not correspond to the orientation of the membrane and therefore it must be affecting something other than a nucleotide translocase; and (4) the differences in enzymatic properties of digitonin and sonic particles previously attributed to differences in sidedness are more likely caused by the effects of the preparative procedures used.

The mitochondrial inner membrane is of considerable importance in the regulation of intracellular metabolism because of its selective permeability properties and because it contains transport and energy transducing systems. One of the proposed transport systems, nucleotide translocase, is believed to be intimately related to the operation of the process of oxidative phosphorylation and the observed properties of both are believed to arise from an anisotropic distribution within the membrane of the relevant enzymes (Racker, 1970; van Dam and Meyer, 1971). It has been well established that the properties of isolated inner membrane vesicles depend on the preparative method and the differences observed between sonic and digitonin particles have been used to support theories of membrane organization and distribution of enzymes on one side or the other of the inner membrane (Heldt *et al.*, 1965; Pfaff *et al.*, 1965; Lee and Ernster, 1966; Mitchell, 1966; Slater, 1966; Bygrave and Lehninger, 1967; Malviya *et al.*, 1968; van Dam and Meyer, 1971). The validity of these theories is dependent on the interpretation of the experimental data and it has been our feeling that more than one reasonable interpretation may be made (Hoppel and Cooper, 1969b; Hohnadel and Cooper, 1972). We have therefore used fluorescent probes and other techniques successfully applied to study the erythrocyte membrane to explore further the possible relationship between the enzymatic properties of isolated inner membrane vesicles and their sidedness. Our results suggest that the properties of vesicles made from mitochondrial inner membrane depend on the preparative method employed and *not* on the sidedness of the vesicular membrane.

Materials and Methods

All chemicals employed were reagent grade. Atractyloside, lactoperoxidase, and butylated hydroxytoluene were purchased from Sigma, $Na^{125}I$ from ICN, and the sodium salt of ANS[1] from K & K Laboratories. The ANS was recrystallized three times from water. Ludox, colloidial silica, was a gift from the I. E. DuPont Corp.; *N,N,N′,N′*-tetramethylethylenediamine, acrylamide, and *N,N′*-methylenebisacrylamide were from Eastman Kodak. The acrylamide was recrystallized twice from water. TNS was synthesized and purified according to the method of McClure and Edelman (1966) and was stored at −20° and protected from light. Fluorescence measurements were made on an Eppendorf spectrofluorimeter equipped with a 366-nm band pass excitation filter and a combination of two Wratten gelatin filters (2D plus 45) and a 420–3000-nm emission filter to eliminate light scattering interference.

Rat liver mitochondria were isolated and the outer membrane removed by treatment with digitonin as described by Hoppel and Cooper (1968). The resulting inner membrane–matrix complex was then disrupted either by sonication or by treatment with digitonin to yield the B_{48} preparation described

† From the Department of Biochemistry, Case Western Reserve University, Cleveland, Ohio 44106. *Received August 14, 1973.* This work was supported by a research grant from the Northeast Ohio Chapter of the American Heart Association and a training grant (GM-00035) from the National Institutes of Health.

‡ These data are from the thesis of L. A. in partial fulfillment of the requirements for the Ph.D. degree from Case Western Reserve University.

[1] Abbreviations used are: ANS, 8-anilino-1-naphthalenesulfonate; TNS, 6-*p*-toluidinonaphthalene-2-sulfonate; MOPS, morpholinopropanesulfonic acid.

by Hoppel and Cooper (1969a). The stock preparation containing 30–40 mg of submitochondrial particle protein/ml in 15% dimethyl sulfoxide–1 mM EDTA (pH 7.0) was stored at −80°.

Density Gradient Separations. Mixtures of inside out and right-side out inner membrane vesicles were resolved by modifying the colloidal silica method used by Lagercrantz and Pertoft (1972) to separate catecholamine storing synaptosomes. The centrifugation medium contained 28% Ludox (w/v), 0.01 mM dithiothreitol, 1.0 mM EDTA, 12.5% glycerol (v/v), and inner membrane vesicles (up to 20 mg of protein) in a total volume of 5.0 ml. The resolving ability of the gradient is dependent on the osmolarity of the medium (Lagercrantz *et al.*, 1970; Lagercrantz and Pertoft, 1972). The exact characteristics of each preparation of vesicles varied and it was necessary to adjust the osmolarity of the centrifugation medium for each preparation. The ionic strength was adjusted with MOPS buffer (pH 7.2) (0.068–0.080 M). This mixture was centrifuged in a swinging bucket SW-65 rotor at 22,500 rpm (36,000*g*) for 60 min in a Spinco Model L centrifuge. Fractions were collected by careful removal with a Pasteur pipet.

All protein concentrations except those containing Ludox were determined by the Biuret method (Gornall *et al.*, 1949). Ludox precipitated and interfered with the color formation in the Biuret determination; therefore the Lowry *et al.* (1951) method was used for the density gradient samples. The density gradient mixture lowered the optical density readings slightly, probably because of the dithiothreitol present (Geiger and Bessman, 1972), and so an aliquot of the density gradient mixture minus protein, equal in volume to the sample, was added to the standards.

Iodination and Polyacrylamide Disc Gel Electrophoresis of Mitochondrial Inner Membranes. The iodination of the mitochondrial membranes was catalyzed by lactoperoxidase as described for erythrocyte ghosts by Phillips and Morrison (1971). The iodide concentration was monitored by an Orion iodide specific electrode used with a Beckman research pH meter Model 1019. The 5-ml reaction mixture contained 0.05 M MOPS (pH 7.2), 10^{-5} M $Na^{125}I$, 3×10^{-7} M lactoperoxidase, 0.001% butylated hydroxytoluene to prevent lipid peroxidation (Welton and Aust, 1972), and 0.2 mg of protein of the membrane preparation to be iodinated. The reaction mixtures for inner membrane–matrix preparations also contained 0.3 M sucrose to prevent osmotic swelling. The iodination was performed at 0°. The reaction was started by adding enough H_2O_2 to bring the concentration to 8×10^{-6} M, following which the iodide concentration dropped slowly for about 1 min and then remained constant. The iodide concentration was then restored to its original level by adding 10^{-3} M $Na^{125}I$ of the same specific activity. The process was repeated until the iodination was completed (three–five additions). The end point of the iodination of the membrane was indicated by a rapid fall in iodide concentration to near zero upon the addition of hydrogen peroxide. This fall was probably due to the formation of I^{3-} (Morrison *et al.*, 1970). The iodide concentration then spontaneously and slowly returned to the original level in about 15–20 min.

Upon completion of the iodination, the reaction mixture was added to 25 ml of cold water and centrifuged at 48,000*g* for 20 min. The inner membrane–matrix preparations were sonicated to release the matrix proteins (Hoppel and Cooper, 1969a) and centrifuged at 10,000*g* for 10 min to remove any unbroken membranes. The supernatant was then centrifuged at 48,000*g* for 20 min. The inner membrane vesicle pellets or the final sonicated inner membrane–matrix pellets were washed once in 25 ml of water.

The resulting pellets were quantitatively removed and homogenized in 0.2 ml of 3% sodium dodecyl sulfate–1% mercaptoethanol–0.2 M sodium phosphate buffer (pH 7.1) following which 0.1 ml of glycerol plus 0.1 ml of 0.03% Bromophenol Blue tracking dye were added. A 5–20-μl aliquot of the solution was layered onto a 5% polyacrylamide gel (75 × 6 mm) that had been preelectrophoresed at 2 mA/gel for 2 hr (Gabriel, 1971a). Stacking gels were not used. The samples were electrophoresed with 0.05 M sodium phosphate buffer (pH 7.1)–0.5% sodium dodecyl sulfate at 5 mA constant current per tube for 2–2.5 hr at which time the tracking dye had moved two-thirds to three-fourths the length of the gel. The gels were stained in Coomassie Brilliant Blue (Gabriel, 1971b).

Each gel was destained by soaking in 25 ml of 50% methanol–3.5% acetic acid for 2 hr with one change of solution and then in 7% acetic acid to swell them back to their original size. They were then further destained in a Canalco Model 20 electrophoretic destainer with 7% acetic acid for 10–15 min. The destained gels were scanned by a Gilford Model 2000 spectrophotometer equipped with a Gilford Model 2410 linear transport. The gels were then sliced into 1-mm thick slices and counted.

Results

It is a widely held view that the orientation of the inner mitochondrial membrane of intact mitochondria is opposite to that of vesicles prepared by sonication (van Dam and Meyer, 1971). Consistent with this belief is the finding that when these two preparations are energized in the presence of the fluorescent probe ANS they show opposite responses (Azzi, 1969; Jasaitis *et al.*, 1971; Nordenbrand and Ernster, 1971). Intact mitochondria show a decrease in fluorescence (Packer *et al.*, 1969; Azzi and Santato, 1970), whereas sonic vesicles show an increase (Azzi *et al.*, 1969, 1971; Brocklehurst *et al.*, 1970). Although the exact reasons for the fluorescence changes are not clear at present, the opposite *directions* of the fluorescence responses of these two preparations have been reproduced in many laboratories. Since digitonin particles are presumed to have the same inner membrane orientation as intact mitochondria (see Hohnadel and Cooper (1972) for a summary of the evidence) they should give a decrease in fluorescence upon energization with ANS if the direction of the fluorescence response is related to membrane orientation. Because there were no reports in the literature regarding this point, we undertook to test it.

As a preliminary experiment, to familiarize ourselves with the necessary techniques, we repeated the experiments of McClure and Edelman (1967) on the activation of chymotrypsinogen by trypsin and monitored the fluorescence changes with TNS and ANS. We found an increase in fluorescence with TNS as they reported but with ANS there was a decrease in fluorescence under identical conditions. Although McClure and Edelman (1966, 1967) only tested TNS they indicated that ANS should give the same type of response because of the similarity in structure. This unexpected finding made us wonder whether the different responses observed with mitochondria and sonic particles might not arise from factors other than membrane orientation. To rule out any possible effect of the outer mitochondrial membrane we compared the response of ANS and TNS to energization by either succinate or ATP with mitochondria and an inner membrane–

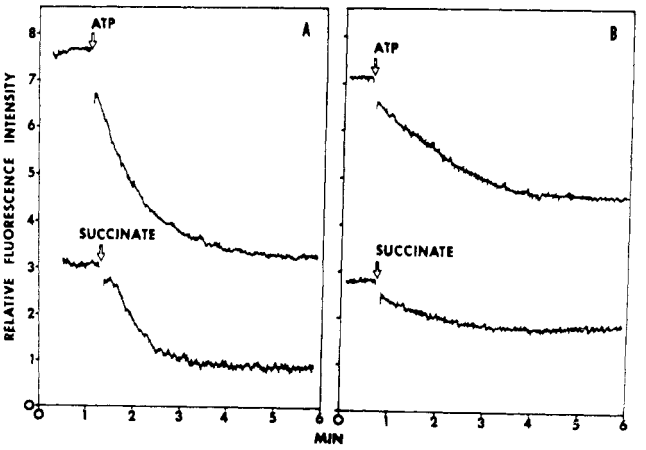

FIGURE 1: The change of relative fluorescence intensity of TNS in the presence of intact mitochondria (A) and inner membrane–matrix preparation (B) upon energization by ATP or succinate. The final concentrations of reactants were: 10^{-5} M TNS, 10 mM $MgCl_2$, 0.2 M MOPS (pH 7.2), and 0.1 mg of mitochondrial or inner membrane–matrix protein in a final volume of 2.0 ml. Changes in fluorescence intensity were induced by the addition of 0.125 mM ATP or 1 mM succinate.

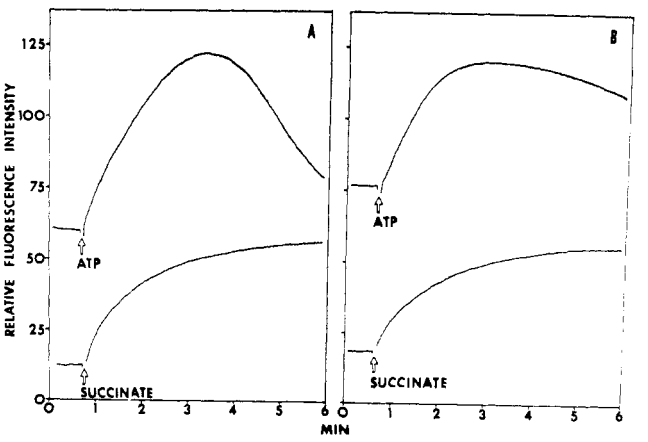

FIGURE 2: The change of relative fluorescence intensity of TNS in the presence of sonicated inner membrane vesicles (A) and digitonin inner membrane vesicles (B). The conditions were the same as Figure 1 except the buffer concentration was 0.1 M.

matrix complex freed of outer membrane (Hoppel and Cooper, 1969a). They both gave a decrease in fluorescence (Figure 1). The results with both probes were qualitatively the same but TNS gave a larger change with all preparations. We then compared the responses of inner membrane vesicles obtained from the inner membrane–matrix preparation by either sonication or treatment with digitonin. These vesicles are free from outer membrane and matrix enzymes and have a higher specific activity for oxidative phosphorylation than do intact mitochondria (Hoppel and Cooper, 1969a) (Table I). Both the digitonin and sonic particles gave an increased fluorescence on energization with either succinate or ATP (Figure 2). If the fluorescence change truly reflects the orientation of the membrane, and if these two preparations have opposite membrane orientations, these two types of particles should have given fluorescence changes that were opposite to each other.

The sensitivity of the fluorescence change with ATP to oligomycin and atractyloside was determined (Table I) and compared with the sensitivity of oxidative phosphorylation with the same preparations. The results with atractyloside appear to be anomalous since the inhibition of phosphate uptake and fluorescence would have been expected to be the same because the fluorescence response is thought to be closely associated with the oxidative phosphorylation system (Azzi *et al.*, 1969). It was found that TNS in the concentrations used does not interfere with oxidative phosphorylation or block the inhibition of same by atractyloside. One possible explanation for the lack of inhibition of fluorescence changes by atractyloside could be that the digitonin preparation contains a mixture of right-side out and a small amount of inside out vesicles. The right-side out vesicles might show a small atractyloside-sensitive decrease in fluorescence typical of intact mitochondria (see Table I), whereas the inside out vesicles might show a large atractyloside insensitive increase in fluorescence typical of sonic particles. The net result could be an atractyloside insensitive increase in fluorescence. If digitonin vesicles are in fact a mixture of inside out and right-side out vesicles, then the sonic particles, that catalyze atractyloside-insensitive oxidative phosphorylation, might also be a mixture. Another possibility is that the probes do not respond to the orientation of the membrane, but rather indicate a difference between altered and unaltered membranes.

In order to answer these questions, we determined whether the preparations are mixtures of inside out the right-side out

TABLE I: Effect of Oligomycin and Atracytyloside on Phosphate Uptake and the Change in Fluorescence Produced by ATP.[a]

Membrane Preparation	P_i Uptake (nmol/min per mg of Protein)	% Inhibition by Oligomycin	% Inhibition by Atractyloside	Fluorescence Response	% Inhibition by Oligomycin	% Inhibition by Atractyloside
Mitochondria	140	99.1	98.7	−15	86	86
Inner membrane–matrix	153	96.3	90.1	−11	85	73
Digitonin vesicles	213	99.5	97.0	+165	100	0
Sonic vesicles	290	98.4	5.0	+108	100	8

[a] The final concentrations of reactants in the [^{32}P]P_i uptake experiments were 20 mM, D,L-3-hydroxybutyrate, 10 mM potassium phosphate containing 7–10 × 10^5 cpm of ^{32}P, 20 mM MOPS (pH 7.2), 2.5 mM ADP, 3 mM $MgCl_2$, 0.25 mM NAD^+, 5 mM glucose, and 20 units of hexokinase (Sigma Type C-300 in a final volume of 200 μl). The reaction was initiated by the addition of 0.2 mg of membrane preparation and the incubations were for 10 min at 30°. The final concentration of atracytloside was 50 μM and 2.5 μg/ml of oligomycin. The formation of glucose 6-phosphate was followed by the extraction procedure previously described by Walters and Cooper (1965). Conditions for the fluorescence measurements were the same as in Figure 1.

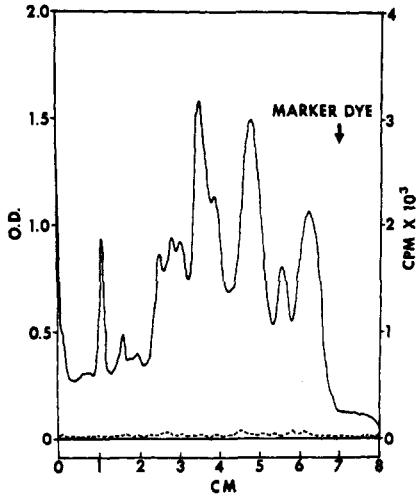

FIGURE 3: Polyacrylamide gel separation of an iodinated inner membrane–matrix preparation. The solid line is the scan of the optical density of the stained gel before slicing. The dotted line is the cpm per 1 mm slice of the gel. The ratio of the areas under the cpm curve to the optical density (OD) curve is 0.03.

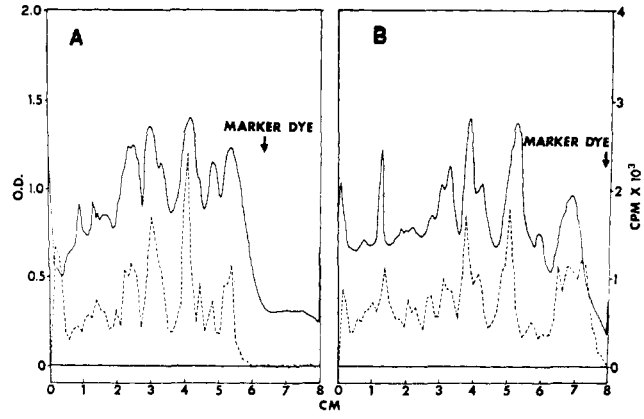

FIGURE 4: Optical density scan (solid line) and radioactivity (dotted line) of a polyacrylamide gel separation of iodinated sonic particles (A) and iodinated digitonin particles (B). The ratios of the areas under the cpm curves to the OD curves are 0.41 for A and 0.44 for B.

vesicles. The idea was to use a method capable of resolving vesicles differing in sidedness and then to use a second method capable of establishing that the separated fractions actually do differ in sidedness. Steck *et al.* (1970a,b) demonstrated that it was possible to resolve erythrocyte membrane vesicles of different orientations on a density gradient of low osmolarity. If the net fixed charges on the two surfaces of the membrane are sufficiently different than the Donnan equilibrium will cause each to swell to a different extent, thus giving different equilibrium densities. Phillips and Morrison (1971) developed a very specific technique for iodinating tyrosine residues exposed on the outer surface of the red blood cell membrane (Morrison *et al.*, 1970; Morrison and Bayse, 1970; on the outer Phillips and Morrison, 1970). Lactoperoxidase, a large enzyme that will not penetrate the membrane, was used to iodinate any exposed protein tyrosine residues on the surface with ^{125}I. The membrane proteins were then resolved by polyacrylamide gel electrophoresis, and the gel sliced and counted to determine which proteins were labeled because they were exposed. A different pattern of labeling was obtained by labeling each side of the red cell membrane.

We first determined the labeling characteristics of the outer surface of the inner mitochondrial membrane. A preparation of mitochondrial inner membrane–matrix was iodinated by lactoperoxidase, and the membranes were dissolved and electrophoresed on a sodium dodecyl sulfate–polyacrylamide gel. After staining with Coomassie Brilliant Blue the distribution of the proteins was determined by an optical density scan (upper trace, Figure 3). The gel was then sliced and the ^{125}I counted (lower trace, Figure 3). It appears that little if any of the proteins of the mitochondrial inner membrane were labeled by lactoperoxidase. Both sonic and digitonin inner membrane vesicles made from this *same* inner membrane–matrix preparation were iodinated and electrophoresed by the same procedure. The resulting protein and radioactivity distributions are shown in Figure 4. Almost every protein band appears to be labeled in both preparations. These data are analogous to those obtained by Phillips and Morrison (1971) who were able to iodinate only one major protein on intact erythrocytes and virtually all of the proteins of disrupted erythrocyte stroma.

The next step was to test whether the pure inner membrane preparations are mixtures of inside out and right-side out vesicles by fractionation on a Ludox gradient and testing the properties of each fraction separately. Two major bands were obtained, with a diffuse dispersion of vesicles in between. The three fractions were collected and the recovery of total protein in each fraction is given in Table II. The two major bands were separated by careful removal with a Pasteur pipet and tested. It was found that both top and bottom bands obtained with either the digitonin or sonic preparations showed oxidative phosphorylation activity and energy-dependent fluorescence changes. When the separated bands were tested for the effect of oligomycin and atractyloside on their oxidative phosphorylation capacity the results shown in Table III were obtained. Both bands from the two preparations showed an increase in fluorescence upon energization with either ATP or succinate and the ATP response was unaffected by atractyloside (not shown). These separated bands therefore give the same types of responses as the parent preparations prior to fractionation. Vesicles from both the top and

TABLE II: Distribution of Protein on Ludox Gradients.[a]

Fraction	% Total Protein Recovered
Sonic Inner Membrane Vesicles [4]	
Top	23 ± 12
Middle	44 ± 13
Bottom	13 ± 4
Digitonin Inner Membrane Vesicles [3]	
Top	18 ± 8
Middle	36 ± 8
Bottom	16 ± 6

[a] The values represent the mean ± the standard deviation of recovery of the total protein applied to the gradient. The figures in brackets are the number of different preparations analyzed. Incomplete recoveries are probably caused by protein being trapped in the Ludox pellet present at the end of each gradient centrifugation.

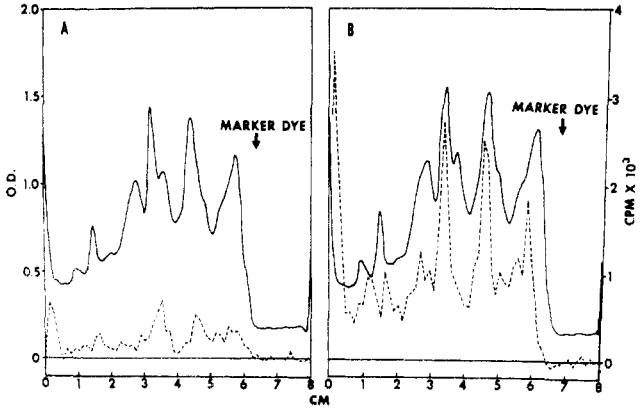

FIGURE 5: Optical density scans (solid line) and radioactivity (dotted line) of polyacrylamide gel separations of the upper (A) and lower (B) bands from a Ludox density gradient fractionation of sonic particles. The ratios of the areas under the cpm curves to the OD curves are 0.15 for A and 0.56 for B.

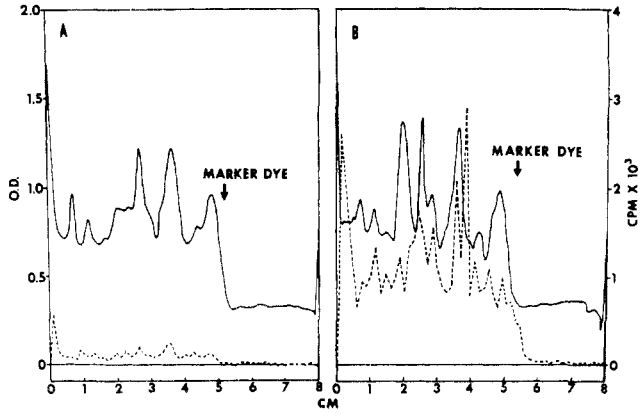

FIGURE 6: Optical density scans (solid line) and radioactivity (dotted line) of polyacrylamide gel separations of the upper (A) and lower (B) bands from a Ludox density gradient fractionation of digitonin particles. The ratios of the areas under the cpm curves to the OD curves are 0.07 for A and 0.68 for B.

TABLE III: Sensitivity Phosphorylation Activity of Fractions from Ludox Gradients to Inhibitors.[a]

Preparation	Gradient Fraction	% Inhibition: Oligomycin	% Inhibition: Atractyloside
Sonic particles	Top band	99	0
	Bottom band	99	0
Digitonin particles	Top band	92	69
	Bottom band	95	75

[a] The incubation conditions were as in Table I except 5×10^6 cpm of $[^{32}P]P_i$ was used and the incubation was for 45 min. The specific activities of the bands isolated from the gradients ranged from 12 to 17 nmol of phosphate uptake/min per mg of protein.

bottom bands were iodinated and electrophoresed. The results are shown in Figures 5 and 6. With digitonin and sonic vesicles the upper band is not readily iodinated, and in this respect appears to behave much the same as the intact inner membrane–matrix preparation. On the other hand, the lower bands are heavily iodinated as could be expected if they were inverted. In keeping with this supposition is the finding that the ratio of the area under the counts per minute (cpm) curve to that under the optical density (OD) curve is greater in the lower bands of Figures 5 and 6 than it is for the parent preparations (Figure 4) that contain a mixture of both bands.

We were concerned that the two bands separated on the Ludox gradient may arise from alterations introduced in a portion of the membrane vesicles by the preparation procedure rather than from differences in sidedness. If the differences in iodination result from a partial alteration of the membrane during the rupture of the intact membrane, than a portion of the resulting pieces might have membrane residues available for iodination and be different enough to separate on a density gradient. If it is assumed that the membrane orientation model currently in vogue is correct than this would mean that the two bands obtained by fractionation of a sonic particle preparation would consist of the following. Both bands would contain inside out vesicles but in the bottom band the inner surface of the membrane would have become partially altered so that it could become iodinated, whereas in the top band the inner surface remained unaltered and could not be iodinated. Placing the iodinating system inside the vesicles should make no difference since we already know that the outer surface does not become iodinated (Figure 3). This may be done by carrying out the iodination during sonication. If, on the other hand, the differences in iodination of the two bands actually result from differences in sidedness then trapping the lactoperoxidase and its substrates inside the right-side out vesicle during the interval between the disruption of the intact inner membrane and the resealing of the pieces to form vesicles should result in the labeling of the upper band. The results of this experiment are shown in Figure 7 and it is clear that the upper band is now also labeled. This supports the idea that the two bands are caused by populations of vesicles differing in sidedness.

Discussion

The purpose of the experiments described above was to relate enzymatic properties and membrane orientation of two different submitochondrial preparations. The concept that the orientation of the inner mitochondrial membrane in digitonin particles is identical with that of intact mitochondria while the sonic particles are inverted has been questioned (Hoppel and Cooper, 1969b; Hohnadel and Cooper, 1972) and the present work raises further questions. The direction of fluorescence change of ANS induced by energization was presumed to be related to membrane orientation (Azzi *et al.*, 1969, 1971; Nordenbrand and Ernster, 1971). However, we found that the direction of the fluorescence change with digitonin particles is opposite to what would have been predicted. We also found that atractyloside does not inhibit the fluorescence changes with digitonin particles but does abolish their oxidative phosphorylation (Table I).

There are at least two explanations for the same fluorescence response of digitonin and sonic particles: (1) both sonic and digitonin inner membrane vesicles may be mixtures of right-side out and inside out vesicles, and (2) the direction of the fluorescence response may be unrelated to membrane orientation. The lack of inhibition by atractyloside could arise from a lack of correspondence of the energization sequence

and the site of action of atractyloside. We evaluated these possibilities by testing the homogeneity of our particulate preparations by density equilibrium centrifugation. Steck *et al.* (1970a) demonstrated that the sidedness of the two membrane fractions they separated on a density gradient were opposite by freeze-etch electron microscopy and by the differential release of sialic acid by neuraminidase. Since the activities we are interested in were completely inactivated by prolonged handling and in gradient materials such as sucrose, dextran, and ficoll, we used Ludox. It has a very low osmotic activity which is an essential requirement for separating vesicles of differing sidedness (Steck *et al.*, 1970b) but it has the disadvantage of partially inhibiting the activities we monitored. This activation may result from the aggregation of Ludox particles on the surface of the vesicle membrane since this is very obvious on examination by electron microscopy. The techniques used by Steck *et al.* (1970a) to evaluate the sidedness of the red cell membrane could not be readily applied to the mitochondrial vesicles. Our attempts to evaluate this by the freeze-etch technique have been unsuccessful to date and the results of Glew *et al.* (1973) suggest there is little carbohydrate associated with the mitochondrial inner membrane. We therefore used the iodination of membrane proteins by lactoperoxidase to evaluate sidedness.

We found virtually no label incorporated into the membranes of intact inner membrane–matrix preparations, whereas almost every major protein band from the membranes of both digitonin and sonic vesicles was labeled. When the sonic or digitonin preparations were first fractionated on Ludox gradients and then iodinated the upper bands of both preparations were only slightly iodinated while the lower bands of both preparations were extensively labeled. These results indicate that the upper bands of both preparations are very similar to those obtained with preparations having an intact unaltered inner membrane in terms of their inability to serve as substrate for the iodination reaction. The labeling of the lower band is consistent with the hypothesis that they are inverted and are now exposed and available for iodination.

Alternate explanations are that during the preparative procedures a fraction of the vesicle membranes had their surface altered in some way, such as the loss of some protective component like mucopolysaccharides, or underwent a change in permeability (Kant and Steck, 1972). Such changes could possibly alter the density and iodination characteristics of the vesicles and account for the formation of two bands on a Ludox gradient. To test this possibility we iodinated the membranes during the sonication procedure when the membranes probably exist momentarily as open sheets and then close to form vesicles containing trapped lactoperoxidase and its substrates. This procedure ensures that all proteins with available residues will be iodinated whether they are exposed on the outside or protected on the inside of the vesicles. The facts that these vesicles are still able to be resolved on a Ludox gradient and that both upper and lower bands are now labeled strongly suggest that the upper band is right-side out while the lower band is inside out and that these bands are resolvable because of their sidedness and not because of alterations in membrane structure.

Further investigation of the properties of the bands resolved on a Ludox gradient showed that both bands from sonic and digitonin preparations responded identically with respect to the direction of fluorescence change on energization with succinate or ATP and the latter response was not affected by atractyloside. In addition, both bands obtained from digitonin preparation, but neither band obtained from the sonic preparation, show an inhibition of oxidative phosphorylation by atractyloside. These characteristics are identical with those of the unfractionated preparations. Although the residual activities in these assays are low because of inhibition by Ludox it is reasonable to assume that the remaining activity is respresentative of the activity of the initial preparations.

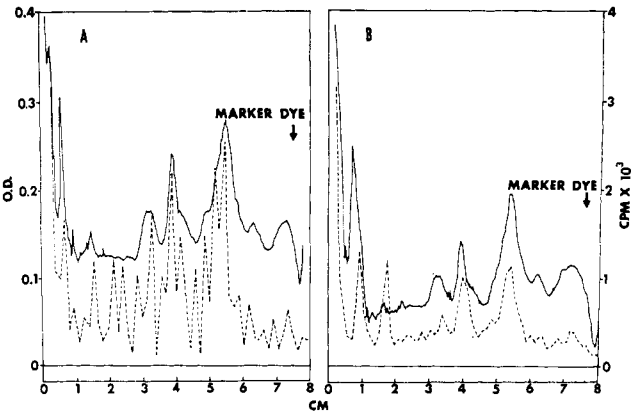

FIGURE 7. Optical density scans (solid line) and radioactivity (dotted line) of polyacrylamide gel separations of the upper (A) and lower (B) bands from a Ludox density gradient fractionation of vesicles that were iodinated during sonication of the inner membrane–matrix preparation and then separated on the gradient. Lactoperoxidase (3×10^{-7} M), $Na^{125}I$ (10^{-5} M), and butylated hydroxytoluene (0.001%) were in the 10-ml sonication medium. During the 60 sec of sonication 10-μl aliquots of 8×10^{-3} M H_2O_2 were added at 10-sec intervals. The ratios of the areas under the cpm curves to the OD curves are 0.49 for A and B.

It is apparent that two previously held theories need revision. Since both inverted and noninverted vesicles respond alike it is clear that fluorescent probes do not indicate the orientation of the membranes by the direction of the change in fluorescence induced by energization. It is more likely that the direction of the response is related to the "intactness" of the membrane structure and how this is affected by the preparative procedures. Secondly, the proposed mechanism of inhibition by atractyloside must be questioned. We have shown that both digitonin and sonic inner membrane preparations are mixtures of inverted and noninverted vesicles. The oxidative phosphorylation catalyzed by the noninverted ones of both preparations would be expected to be sensitive to atractyloside if the atractyloside-sensitive nucleotide translocase hypothesis is correct. In actual fact, both the noninverted and inverted vesicles obtained by digitonin treatment are atractyloside sensitive while the inverted and noninverted fractions obtained by sonication are not. These findings are consistent with the idea that the inability of atractyloside to inhibit oxidative phosphorylation in sonic particles depends on changes produced in the membranes by the preparative procedures and not the sidedness of the membrane and the ability of atractyloside to inhibit oxidative phosphorylation in digitonin particles does not depend on inhibition of a nucleotide translocase. In addition the idea that mitochondrial inner membrane vesicles are pure in the sense that they are completely homogeneous with regard to sidedness appears to be untenable.

Acknowledgment

We greatly appreciate the technical assistance of Ms. Ksenija Dimitrov and Ms. Judy Drumm.

References

Azzi, A. (1969), *Biochem. Biophys. Res. Commun. 37*, 254.

Azzi, A., Chance, B., Radda, G. K., and Lee, C. P. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 612.

Azzi, A., Gherardini, P., and Santato, M. (1971), *J. Biol. Chem. 246*, 2035.

Azzi, A., and Santato, M. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 135.

Brocklehurst, J. R., Freedman, R. B., Hancock, D. J., and Radda, G. K. (1970), *Biochem. J. 116*, 721.

Bygrave, F. L., and Lehninger, A. L. (1967), *Proc. Nat. Acad. Sci. U. S. 57*, 1409.

Gabriel, O. (1971a), *Methods Enzymol. 22*, 565.

Gabriel, O. (1971b), *Methods Enzymol. 22*, 578.

Geiger, P. J., and Bessman, S. P. (1972), *Anal. Biochem. 49*, 467.

Glew, R. H., Kayman, S. C., and Kuhlenschmidt, M. S. (1973), *J. Biol. Chem. 248*, 3137.

Gornall, A. G., Bardawill, C. J., and David, M. M. (1949), *J. Biol. Chem. 177*, 751.

Heldt, H. W., Jacobs, H., and Klingenberg, M. (1965), *Biochem. Biophys. Res. Commun. 18*, 174.

Hohnadel, D. C., and Cooper, C. (1972), *Biochemistry 11*, 1138.

Hoppel, C., and Cooper, C. (1968), *Biochem. J. 107*, 367.

Hoppel, C., and Cooper, C. (1969a), *Arch. Biochem. Biophys. 135*, 173.

Hoppel, C., and Cooper, C. (1969b), *Arch. Biochem. Biophys. 135*, 184.

Jasaitis, A. A., Kuliene, V. V., and Skulachev, V. P. (1971), *Biochim. Biophys. Acta 234*, 177.

Kant, J. A., and Steck, T. L. (1972), *Nature (London), New Biol. 240*, 26.

Lagercrantz, H., and Pertoft, H. (1972), *J. Neurochem. 19*, 811.

Lagercrantz, H., Pertoft, H., and Stjärne, L. (1970), *Acta Physiol. Scand. 78*, 561.

Lee, C. P., and Ernster, L. (1966), *Biochim. Biophys. Acta Lib. 7*, 218.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.

Malviya, A. N., Parsa, B., Yodaiken, R. E., and Elliot, W. B. (1968), *Biochim. Biophys. Acta 162*, 195.

McClure, W. O., and Edelman, G. M. (1966), *Biochemistry 5*, 1908.

McClure, W. O., and Edelman, G. M. (1967), *Biochemistry 6*, 567.

Mitchell, P. (1966), *Biol. Rev. 41*, 445.

Morrison, M., and Bayse, G. (1970), *Biochemistry 9*, 2995.

Morrison, M., Bayse, G., and Danner, D. J. (1970), *in* Biochemistry of the Phagocytic Process, Schultz, J., Ed., Amsterdam, North-Holland Publishing Co., p 51.

Nordenbrand, K., and Ernster, L. (1971), *Eur. J. Biochem. 18*, 258.

Packer, L., Donovan, M. P., and Wriggelsworth, J. M. (1969), *Biochem. Biophys. Res. Commun. 35*, 832.

Pfaff, E., Klingenberg, M., and Heldt, H. W. (1965), *Biochim. Biophys. Acta 104*, 312.

Phillips, D. R., and Morrison, M. (1970), *Biochem. Biophys. Res. Commun. 40*, 284.

Phillips, D. R., and Morrison, M. (1971), *Biochemistry 10*, 1766.

Racker, E. (1970), *in* Membranes of Mitochondria and Chloroplasts, Racker, E., Ed., Washington, D. C., American Chemical Society, ACS Monograph, 165, p 164.

Slater, E. C. (1966), *Compr. Biochem. 14*, 327.

Steck, T. L., Straus, J. H., and Wallach, D. F. H. (1970a), *Biochim. Biophys. Acta 203*, 385.

Steck, T. L., Weinstein, R. S., Straus, H. J., and Wallach, D. F. H. (1970b), *Science 168*, 255.

van Dam, K., and Meyer, A. J. (1971), *Annu. Rev. Biochem. 40*, 119.

Walters, E., and Cooper, C. (1965), *Anal. Biochem. 10*, 370.

Welton, A. F., and Aust, S. D. (1972), *Biochem. Biophys. Res. Commun. 49*, 661.