The rates of specific catalytic events in the mitochondrial inner membrane were determined after enriching the membrane bilayer with exogenous phospholipid. Inner membranes were fused with soybean phospholipid, then separated into buoyant-dense fractions by sucrose density centrifugation. Analysis revealed a progressively increasing phospholipid to protein ratio from the least buoyant (30% phospholipid-enriched) to the most buoyant (70% phospholipid-enriched) membrane fraction. This progressive increase was proportional to an increase in the surface area of the membrane bilayer and a decrease in the lateral density distribution of integral proteins observed as intramembrane particles by freeze-fracture electron microscopy. Thus, the average distance between integral proteins increased as the inner membrane bilayer was enriched with exogenous phospholipid. Electron transfer rates from NADH and succinate to cytochrome c as well as to oxygen decreased in proportion to the phospholipid enrichment and increased surface area of the membrane bilayer. A maximum decrease of 90% to 95% occurred in the most buoyant membranes. NADH- and succinate-ubiquinone reductase activity decreased maximally by 60%, while ubiquinol-cytochrome c reductase activity decreased by 85%. No decrease occurred in the duroquinol oxidase activity of any of the phospholipid-enriched membrane fractions. Activities of the single enzymes, NADH dehydrogenase, cytochrome c oxidase, and ATPase increased with increasing phospholipid enrichment of the bilayer. These data identify a diffusion-limited step in electron transfer between the inner membrane dehydrogenases and cytochromes bc1, and indicate that the dehydrogenases, ubiquinone, and cytochromes bc1, are independent, diffusible membrane oxidation-reduction components.

Lateral diffusion and collision of interacting membrane components such as proteins may be required for eliciting specific catalytic events in a wide variety of cell membranes. In such membranes, the rates of the catalytic events may be determined ultimately by the rates of lateral diffusion as well as by the lengths of the diffusion paths of the interacting membrane components. Furthermore, it is reasonable to assume that in any membrane system where lateral diffusion and collision between interacting components is required for catalytic activity, even small quantitative and qualitative changes in the bilayer lipid will affect the diffusional, collisional, and catalytic activities of such components.

In previous studies from this laboratory, lateral translational diffusion of integral proteins in the mitochondrial energy-transducing membrane was observed to accompany non-destructive liquid crystalline \( \rightarrow \) gel state transitions in the membrane lipid bilayer (1). More specifically, we determined that cytochrome c oxidase, a completely transmembrane protein (2), diffuses laterally in the plane of the membrane and, in addition, that other as yet unidentified smaller integral proteins diffuse independently of the oxidase (3).

Related to these several structural observations, it is of interest to determine whether or not lateral translational diffusion of membrane components has significance in the kinetics of electron transfer and oxidative phosphorylation in the mitochondrial membrane. A number of observations on the rates of catalytic events in the mitochondrial membrane are consistent with the existence of lateral diffusion of interacting membrane oxidation-reduction components. For example, although the oxidation half-time of cytochrome c oxidase may be as rapid as 3 ms, the \( b \) cytochromes require 80 ms and the dehydrogenases require as much as 500 ms for half-oxidation (4). In addition to possible protein diffusion, ubiquinone, a lipid oxidation-reduction membrane component, may subserve a pool function and may diffuse laterally as a mobile carrier to transfer reducing equivalents from multiple dehydrogenases to cytochrome \( b \) (5-7). However, diffusion and collision between NADH-ubiquinone reductase and ubiquinol-cytochrome c reductase has been suggested recently (8, 9). Moreover, it has been proposed that ubiquinone does not act as an independent \( Q^1 \) pool but is the prosthetic group of \( Q \)-binding proteins (10).

Lateral diffusion and collision between the heme proteins is suggested by the finding that the reduction of each cytochrome \( b \) by succinate dehydrogenase results in the reduction of 3 molecules of cytochrome \( c \) and 9 molecules of cytochrome \( c \) oxidase (11). In addition to protein diffusion in this region of the electron transfer sequence, ubiquinone may function as a diffusional oxidation-reduction component between cytochromes \( b \) and \( c \), as part of the proposed \( Q \) cycle of Mitchell (12, 13).

With these observations as a basis for further experimentation, we have developed and report here a novel \textit{in vitro} method for increasing the lipid bilayer surface area of the

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* This investigation was supported by Research Grants PCM77-20689 and PCM79-10968 from the National Science Foundation (to C. R. H.) and research fellowships (to M. H.) from the Swiss National Foundation and The Muscular Dystrophy Association of America. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1. The abbreviations used are: \( Q \), ubiquinone; \( Q-1 \), ubiquinone-1; CCCP, carbonyl cyanide m-chlorophenylhydrazone; PMS, phenazine methosulfate; DCIP, dichloroindophenol; TTFA, thenoyltrifluoroacetone; DBH, ubiquinone having a decyl side chain; IMM, inner membrane-matrix fraction.
mitochondrial energy-transducing membrane which employs fusion of liposomes (small unilamellar vesicles) with the native membrane. Using phospholipid-enriched mitochondrial inner membranes with progressively increased bilayer surface areas, we have examined the diffusion of the electron transfer components and the electron transfer rates in various segments of the electron transfer sequence. Our data reveal that electron transfer is diffusion-limited between the dehydrogenases and cytochromes bc. The data indicate further that the dehydrogenases and cytochrome bc complex as well as ubiquinone are independent diffusible membrane oxidation-reduction components.

**EXPERIMENTAL PROCEDURES**

**Mitochondrial and Inner Membrane Preparations—**Liver mitochondria were isolated from male Sprague-Dawley rats in 70 mM sucrose, 220 mM mannitol, 2 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes), 9.5 mM of bovine serum albumin/ml, and KOH to pH 7.4. This medium has an osmolarity of about 200 mosmol and is designated H_{2}O medium. Subsequent removal of the outer membrane and purification of the inner membrane-matrix (IMM_{60} or mitoplast) fraction was carried out by controlled detergent incubation (14, 15). The complex topography of IMM_{60} membranes was converted to a simple spherical configuration by washing and resuspending the membranes in 7.5 times diluted bovine serum albumin-free H_{2}O medium (40 mosm) as described earlier (15). This medium is designated H_{0} medium, and the washed inner membrane-matrix fraction is designated IMM_{90} membranes.

**Fusion of Phospholipid Vesicles with IMM_{90}—**Small unilamellar vesicles were prepared from soybean phospholipids (aslofin). Phospholipid, 1.5 g, was suspended in 7.5 ml of H_{2}O medium and sonicated at 0°C using the micropipet probe of a Branson (model W 188) Sonifier set at 40 watts output for three cycles of 10 min each. After each cycle, the pH in the phospholipid vesicle suspension was readjusted to 7.4 with KOH.

Phospholipid vesicles were fused with IMM_{90} as follows. At 30°C with constant stirring, 7.4 ml of IMM_{90} suspension (13 to 14 mg of protein/ml) were placed in a 50-ml glass beaker to which 2.5 ml of phospholipid vesicle suspension were added. The pH was immediately adjusted to pH 6.5 with HCl and kept constant. After 15 min, another 2.5 ml of phospholipid vesicle suspension were added and the pH was readjusted to 6.5. A third and last addition of sonicated phospholipid vesicles (2.5 ml) followed after 15 min. After a total of 45 min, the pH was adjusted to 7.4 with KOH and the IMM_{90}-phospholipid vesicle suspension was placed on ice.

The mixed IMM_{90}-phospholipid vesicle suspension was loaded on a discontinuous sucrose density gradient (0.6, 0.75, 1.0, and 1.25 mM sucrose in H_{2}O medium) in aliquots of 5 ml (16 to 18 mg of protein/100 ml) and centrifuged at 70,000 g for 14 to 16 h at 4°C. The bulk of the phospholipid liposomes remained at the top of the gradient, whereas the IMM_{90} membranes separated into four buoyant-distinct fractions designated Band 1, Band 2, Band 3, and pellet respectively, from least to most dense. To remove the sucrose, each fraction was washed two times with H_{2}O medium.

**Enzyme Assays—**Cytochrome c oxidase, duroquinol oxidase, NADH oxidase, and succinate oxidase activities were measured polarographically with a Clark oxygen electrode (16). Incubation media were as follows. (a) Cytochrome c oxidase: 10 mM potassium phosphate, pH 7.4, 4.5 mM ascorbate, 2.7 mM N,N,N',N'-tetrachloro-p-phenylenediamine dihydrochloride, and 1 mM cytochrome c: (b) duroquinol oxidase: 10 mM potassium phosphate, pH 7.4, 5 mM rotenone, 1 mM CCCP, 1 mM cytochrome c, and 0.6 mM duroquinol ( duroquinone, as 60 mM ethanolic stock solution, was reduced by sodium borohydride(17)); (c) NADH oxidase: 80 mM potassium phosphate, pH 7.4, 2 mM NADH, 1 mM KCN; and 13 mM cytochrome c: (d) succinate oxidase: 80 mM potassium phosphate, pH 7.4, 5 mM sodium succinate, 5 mM rotenone, 1 mM CCCP, and 13 mM cytochrome c. All oxidase assays were started by the addition of enzyme (membrane suspension) except the duroquinol oxidase assay which was initiated by duroquinol. All enzyme-specific activities were expressed as turnover numbers in units of electrons/a/heme, which is equivalent to pairs of reducing equivalents/s/molecule of cytochrome oxidase.

NADH-cytochrome c reductase, succinate-cytochrome c reductase, and ubiquinol-cytochrome c reductase activities were determined from cytochrome c reduction measured at the wavelength pair 550-

540 nm in an Amino DW-2a spectrophotometer using an extinction coefficient of 19.8 mM^{-1} cm^{-1} (17). Substrate was either 1 mM NADH,

20 mM sodium succinate plus 5 mM rotenone, or 35 mM reduced DBP, respectively. DBH was reduced as described (18). The remaining reaction medium was 80 mM potassium phosphate, pH 7.4, 2 mM KCN, and 2 mM CCCP. The reaction was initiated by 50 mM cytochrome c: in the case of succinate-cytochrome c reductase, a preincubation for 10 min at room temperature was employed prior to initiation of the reaction. NADH dehydrogenase activity was measured in 20 mM potassium phosphate, pH 7.4, 0.1 mM NADH, and 1 mM potassium ferricyanide (19). Ferricyanide reduction was followed at 420 nm. NADH-ubiquinone reductase was determined in 20 mM potassium phosphate, 2 mM potassium cyanide, 0.1 mM NADH, and 20 mM Q-1 (20). NADH oxidation was followed at 340 nm. Succinate dehydrogenase activity was measured in 80 mM potassium phosphate, pH 7.4, 20 mM sodium succinate, 2 mM potassium cyanide, 0.5 mM TTPA, 2 mM FMS, and 0.1 mM DCIP (21). The reaction was initiated by DCIP and PMS after 10-min preincubation at room temperature, and DCIP reduction was followed at 600 nm.

Succinate-ubiquinone reductase was determined in 50 mM potassium phosphate, pH 7.4, 20 mM sodium succinate, 2 mM potassium cyanide, 0.1 mM DCIP, and 20 μM Q-1. After 10-min preincubulation, the reaction was started by the addition of DCIP and Q-1, and DCIP reduction was measured at 600 nm.

ATPase activity was measured spectrophotometrically at 340 nm by coupling the production of ADP to the oxidation of NADH via the pyruvate kinase and lactic dehydrogenase reactions essentially as described by Pullman et al. (22). The reaction medium tri(bicarbonate and 20 mM potassium phosphate, pH 7.4, 5 mM MgCl_{2}, 1 mM KCN, 0.1 mM NADH, 0.5 mM phosphoenolpyruvate, 2 mM ATP, 15 units/ml of lactate dehydrogenase, and 20 units/ml of pyruvate kinase. Mitochondrial protein was added in final concentrations of 5 to 10 μg/ml. ATP production by oxidative phosphorylation employing succinate as a substrate was measured as previously described (23). Membrane potential (∆ψ) was monitored at the wavelength pair, 511-533 nm, in the presence of 10 μM safranine as described by Akerman and Wikstrom (24).

**Cytochrome b Homed Determinations—**Dithionite-reduced minus air oxidized difference spectra were obtained with an Amino DW-2a spectrophotometer in 0.8% sodium cholate, 65 mM potassium phosphate, pH 7.4, and 0.5 to 2.0 mg/ml of protein. Concentrations of cytochrome bmes, c, b, and a were calculated by simultaneous equations according to Williams (25), employing the extinction coefficients given in Table I. With these coefficients, four simultaneous equations with four unknowns were generated. These were solved to yield:

\[
\text{[cytochrome c]} = 49.3 (ΔA_{550-610}) - 27.9 (ΔA_{604-650}) + 15.8 (ΔA_{635-677})
\]

\[
\text{[cytochrome c]} = -16.8 (ΔA_{560-605}) + 51.1 (ΔA_{605-640})
\]

\[
-12.7 (ΔA_{640-650}) - 3.5 (ΔA_{640-660})
\]

\[
\text{[cytochrome b]} = 3.8 (ΔA_{635-677}) - 4.2 (ΔA_{640-660})
\]

\[
+44.7 (ΔA_{650-677}) + 1.4 (ΔA_{660-680})
\]

\[
\text{[cytochrome a]} = 2.4 (ΔA_{640-645}) - 0.4 (ΔA_{650-677}) + 76.1 (ΔA_{660-680})
\]

where cytochrome concentrations are in micromolar units. Single determinations of heme a were obtained from measurements of ∆A_{605-630} after dithionite reduction, employing an extinction coefficient of 13.1 mM^{-1} cm^{-1} (26).

**Ubiquinone Determination—**The ubiquinone content of the membrane fractions was estimated after solvent extraction with methanol.

<table>
<thead>
<tr>
<th>Table I</th>
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<tbody>
<tr>
<td><strong>Change in millimolar extinction coefficients from dithionite-reduced minus air oxidized difference spectra</strong></td>
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<tr>
<td><strong>Cytochrome</strong></td>
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<tr>
<td></td>
</tr>
<tr>
<td>a</td>
</tr>
<tr>
<td>b</td>
</tr>
<tr>
<td>c</td>
</tr>
<tr>
<td>c</td>
</tr>
</tbody>
</table>

* Major coefficients from Vanneve (26).
* Major coefficients calculated from data of Ereptska et al. (27).
and petrolether according to Krüger and Klingenberg (28). Concentrations of ubiquinone were calculated from the absorbance decrease at 280 - 289 nm after addition of solid sodium borohydride using an extinction coefficient of 8.8 mm-1 cm-1.

**Protein and Phosphorus Determination**—Protein concentration was determined by the method of Lowry et al. (29) and in some cases with the biuret method (30). Lipid phosphorus was measured according to Bartlett (31) after acid hydrolysis with perchloric acid.

**Electron Microscopy**—Unfixed membranes were treated with 30% glycerol buffered at pH 7.4 with H2O medium and frozen in liquid Freon 22. Freeze fracturing was carried out in a Balzers BA260 freeze-etching apparatus as described by Moore (32). Electron micrographs were taken with a JEOI 100CX electron microscope operated at 80 kV.

**Materials**—Horse heart cytochrome c type VI was obtained from Sigma. Ubiquinone-1 was the generous gift of Hoffmann-LaRoche, Basel, Switzerland. DBH was kindly supplied by Dr. B. L. Trumpower, Department of Biochemistry, Dartmouth Medical School, Hanover, N. H. Asolectin was purchased from Associated Concentrates, Inc., New York, N. Y. All other chemicals were obtained in highest purity available commercially.

**RESULTS**

**Fusion of Liposomes with Mitochondrial Inner Membranes**—Fusion of liposomes with inner membranes occurred at pH 6.5 and below. Fusion did not occur at pH 7.0 or 7.4 to any significant degree. Four buoyant-distinguish membrane fractions were recovered after the liposome-membrane fusion mixture was centrifuged over a discontinuous sucrose density gradient as described under "Experimental Procedures" demonstrating that fusion did not occur equally in all membranes. The four buoyant-distinguish membrane fractions were designated Bands 1, 2, and 3 and pellet. After incubation for 45 min at 30°C, up to 40% of the total membrane protein was recovered in the third bands, with the remainder recovered in the pellet.

Native (nonfused) inner membranes and the four buoyant-distinguish membrane fractions were quick-frozen from room temperature and examined by freeze-fracture electron microscopy. Native membranes contained closely packed intramembrane particles (integral proteins) in random distribution (Fig. 1a). However, an increase in the average distance between integral proteins occurred progressively from pellet to Band 1 and was proportional to the increased buoyancy of the four buoyant-distinguish membrane fractions (Fig. 1, b to e). Concomitant with this random lateral dilution of membrane proteins was an average increase in membrane surface area as revealed by an increase in the average size of the fracture faces. The increase in membrane surface could also be observed by phase-contrast light microscopy. These observations indicated that the surface area of the membrane bilayer increased by fusion with the liposomes, that the membrane bilayer became highly enriched with exogenous phospholipid, and that the integral membrane proteins diffused laterally to randomize in the newly expanded lipid bilayer.

**Chemical Analysis of the Phospholipid-enriched Inner Membranes**—The four buoyant-distinguish membrane fractions, as well as the native membrane, were analyzed for total protein, cytochrome hemes, ubiquinone, and lipid phosphorus. All four buoyant-distinguish membrane fractions exhibited a nearly identical heme a to protein ratio (Table II). The heme a content of these membranes showed an apparent 2-fold increase over the native membrane due to a loss of mitochondrial matrix protein which occurred equally in all four fractions during the fusion, centrifugation, and washing procedures.

Lipid phosphorus content showed a dramatic increase in the four membrane fractions progressing from pellet to Band 1 (Table II). Using the phosphorus to heme a ratio to determine the ratio of membrane phospholipid to integral membrane protein, it was found that membranes in the pellet fraction became slightly enriched (30%) in phospholipid over control, while membranes in the Band 1 fraction became enriched in phospholipid by more than 7 times over control (Table II).

No significant loss of electron transfer components integral to the membrane occurred during the fusion process. When data were normalized with respect to heme a content, cytochromes b and c, as well as ubiquinone, were recovered quantitatively in all four buoyant-distinguish membrane fractions (Table III). However, cytochrome c, a loosely bound peripheral membrane protein, was lost by 70 to 80% compared to control during the fusion process. These analytical data taken together with the ultrastructural data clearly reveal that the inner membrane bilayer became enriched with phospholipid during the fusion procedure.

**Electron Transfer Activities in the Phospholipid-enriched Inner Membranes**—As noted above, a majority of cytochrome c was lost from the inner membrane during the fusion procedure; therefore, all electron transfer activities were determined in cytochrome c supplemented media as detailed under "Experimental Procedures." Analysis revealed a progressive decrease in both NADH and succinate oxidase activity from pellet to Band 1 membranes proportional to the degree of enrichment of the membrane bilayer with exogenous phospholipid (Table IV). Band 1 membranes, enriched 7-fold, showed only 11 to 13% activity compared to membranes in the pellet fraction. The decrease in these oxidase activities after incorporation of exogenous phospholipid into the membrane bilayer was a striking, reproducible finding. It was of interest, therefore, to determine whether such decreases in activities were related to the increased spacing between integral proteins observed by freeze-fracture electron microscopy or related to a direct inhibitory effect by the newly incorporated phospholipids on the catalytic activities of specific proteins. For this purpose, we examined individual electron transfer components and segments of the electron transfer sequence from NADH and succinate to oxygen.

Cytochrome c oxidase and duroquinol oxidase activity were measured in order to evaluate electron transfer through the cytochrome aa3 and bc segments, respectively, of the electron transfer sequence (Table V). In the pellet membrane fraction, cytochrome c oxidase activity was 168 e-/s/heme a, in good agreement with Tritos-solubilized rat liver mitochondria (33). As the phospholipid content of the membrane bilayer increased, there was a slight increase of the turnover number up to 212 in Bands 1 and 2. Related to this finding, other workers have reported that the activity of phospholipid-depleted cytochrome c oxidase is restored to a greater extent by asolectin than by mixed phospholipids isolated from mitochondria or isolated from the oxidase (34). In any case, it was clear in our studies that enrichment of the membrane bilayer by fusion with asolectin liposomes did not specifically inhibit the catalytic activity of cytochrome c oxidase.

Within experimental error, the specific activity of duroquinol oxidase was the same in all four lipid-enriched membrane fractions (Table V). Antimycin inhibition was 90% in all fractions, and total inhibition with cyanide was used to correct for the auto-oxidation of duroquinol. Since duroquinol oxidation involves the electron transfer segment from cytochrome b to cytochrome c oxidase and since duroquinol reacts specifically with cytochrome b (35), it is clear that phospholipid enrichment of the membrane bilayer neither decreased electron transfer between the various heme proteins nor specifically inhibited the catalytic activity of any of these proteins.

Unlike electron transfer from duroquinol or cytochrome c...
**Fig. 1.** Freeze-fracture convex faces of mitochondrial inner membranes from inner membrane-matrix fraction (control) and four buoyant-distinct fractions after fusion with liposomes. *a,* inner membrane-matrix fraction (control); *b,* pellet fraction; *c,* Band 3 fraction; *d,* Band 2 fraction; *e,* Band 1 fraction. Average distance between intramembrane particles (integral proteins) increases progressively from pellet to Band 1. × 200,000.
Diffusion of Electron Transfer Components

### TABLE II

<table>
<thead>
<tr>
<th>Molar ratio of lipid phosphorus to heme a</th>
<th>c/a</th>
<th>b/a</th>
<th>Q/a</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMM&lt;sub&gt;α&lt;/sub&gt;</td>
<td>0.69</td>
<td>0.31</td>
<td>0.63</td>
</tr>
<tr>
<td>Pellet</td>
<td>0.18</td>
<td>0.31</td>
<td>0.63</td>
</tr>
<tr>
<td>Band 3</td>
<td>0.13</td>
<td>0.31</td>
<td>0.63</td>
</tr>
<tr>
<td>Band 2</td>
<td>0.12</td>
<td>0.31</td>
<td>0.58</td>
</tr>
<tr>
<td>Band 1</td>
<td>0.21</td>
<td>0.31</td>
<td>0.58</td>
</tr>
</tbody>
</table>

*Nonfused inner membrane-matrix fraction (control).*

### TABLE III

<table>
<thead>
<tr>
<th>Specific activities of NADH and succinate oxidase in mitochondrial inner membranes after liposome fusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>NADH oxidase</td>
</tr>
<tr>
<td>--------------</td>
</tr>
<tr>
<td>e⁻/s/heme a</td>
</tr>
<tr>
<td>Pellet</td>
</tr>
<tr>
<td>Band 3</td>
</tr>
<tr>
<td>Band 2</td>
</tr>
<tr>
<td>Band 1</td>
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</table>

### TABLE IV

<table>
<thead>
<tr>
<th>Specific activities of cytochrome oxidase and duroquinol oxidase in mitochondrial inner membranes after liposome fusion</th>
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<tbody>
<tr>
<td>Cytochrome oxidase</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>e⁻/s/heme a</td>
</tr>
<tr>
<td>Pellet</td>
</tr>
<tr>
<td>Band 3</td>
</tr>
<tr>
<td>Band 2</td>
</tr>
<tr>
<td>Band 1</td>
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### TABLE V

<table>
<thead>
<tr>
<th>Specific activities of NADH dehydrogenase and NADH-ubiquinone reductase in mitochondrial inner membranes after liposome fusion</th>
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<tbody>
<tr>
<td>NADH dehydrogenase</td>
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<td>--------------------</td>
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<td>e⁻/s/heme a</td>
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<tr>
<td>Pellet</td>
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<tr>
<td>Band 3</td>
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<tr>
<td>Band 2</td>
</tr>
<tr>
<td>Band 1</td>
</tr>
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</table>

### FIG. 2

**Relationship of the relative activities of NADH-linked electron transfer reactions, ubiquinol-cytochrome c reductase, duroquinol oxidase, and cytochrome c oxidase to the lipid phosphorus (P) to heme a ratio.**

Relative activities of NADH dehydrogenase (NADH → Fe(CN)₆), NADH-ubiquinone reductase (NADH → Q⁻), NADH-cytochrome c reductase (NADH → cyto c), NADH oxidase (NADH → O₂), ubiquinol-cytochrome c reductase (DBH → cyto c), duroquinol oxidase (DQH₂ → O₂), and cytochrome c oxidase (cyto c → O₂) are given as average values of three experiments.

---

*Nonfused inner membrane-matrix fraction (control).*

---

NADH-ubiquinone reductase activity decreased to only 40% in Band 1 membranes, while NADH-cytochrome c reductase activity decreased to 6% in Band 1 membranes (Fig. 2). NADH dehydrogenase activity was not responsible for the decreased activities in NADH-cytochrome c reductase and NADH-ubiquinone reductase. NADH dehydrogenase measured as NADH-ferricyanide reductase activity revealed an increase rather than decrease in activity as the phospholipid content of the membrane bilayer increased (Table VII).

Succinate dehydrogenase and succinate-ubiquinone reduc-
tase activities were measured employing DCIP as terminal electron acceptor. PMS in the presence of TTFA was the electron mediator for succinate dehydrogenase measurements, and Q-1 was the mediator for succinate-ubiquinone reductase measurements. The latter measurements were inhibited 100% by TTFA in all membrane fractions. Both succinate dehydrogenase and succinate-ubiquinone reductase activities decreased to a similar extent in proportion to the enrichment of the membrane bilayer with exogenous phospholipid (Table VIII). However, these decreases were not as great as the decreases which occurred in succinate-cytochrome c reductase and succinate oxidase (Fig. 3). The decreases in the activities of succinate dehydrogenase, succinate-ubiquinone reductase as well as succinate-cytochrome c reductase, were not the result of differential activation of these enzymes in the four different membrane fractions. Preincubation with succinate, a known activator, for 10 min produced the same relative activation in all four fractions (data not shown). Reduced ubiquinone is also an activator of succinate-linked electron transfer activity. However, addition of TTFA (which inhibits ubiquinone reduction) during preincubation with succinate did not alter the relative activation of the succinate-linked activities.

Ubiquinol-cytochrome c reductase activity, measured with reduced DBH as the electron donor, decreased in proportion to the increase in membrane phospholipid (Table IX). It is significant that the decrease in activity of 85% for Band 1 was more pronounced than that for NADH- and succinate-ubiquinone reductase and less pronounced than that for NADH- and succinate-cytochrome c reductase (Figs. 2 and 3). Exogenously added quinones, such as DBH and Q-1, have been reported to interact with endogenous ubiquinone (36). Thus, the relative decreases in NADH-, succinate-, and ubiquinol-linked electron transfer activities which occurred as additional lipid was incorporated into the membrane bilayer indicate that there are two distinct diffusion-limited steps which occur on the two sides of ubiquinone between the dehydrogenases and cytochrome b.

### Table VIII

<table>
<thead>
<tr>
<th>Succinate dehydrogenase</th>
<th>Succinate-ubiquinone reductase</th>
</tr>
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<tbody>
<tr>
<td><strong>Activity</strong></td>
<td><strong>Activity</strong></td>
</tr>
<tr>
<td>e-/s/heme a</td>
<td>%</td>
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<tr>
<td>Pellet</td>
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<td>Band 3</td>
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<td>Band 2</td>
<td>112.8</td>
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<tr>
<td>Band 1</td>
<td>112.8</td>
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### Table IX

<table>
<thead>
<tr>
<th>Ubiquinol-cytochrome c reductase</th>
<th>Activity</th>
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<tbody>
<tr>
<td>e-/s/heme a</td>
<td>%</td>
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<tr>
<td>Pellet</td>
<td>100</td>
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<tr>
<td>Band 3</td>
<td>89.8</td>
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<tr>
<td>Band 2</td>
<td>71.8</td>
</tr>
<tr>
<td>Band 1</td>
<td>19</td>
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</table>

### Table X

<table>
<thead>
<tr>
<th>ATPase specific activities in mitochondrial inner membranes after liposome fusion</th>
</tr>
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<tbody>
<tr>
<td>mol ATP hydrolyzed/s/ mol heme a</td>
</tr>
<tr>
<td>Pellet</td>
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<tr>
<td>Band 3</td>
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<tr>
<td>Band 2</td>
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<tr>
<td>Band 1</td>
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### Discussion

The mitochondrial inner membrane supports a number of rapid macromolecular interactions which elicit specific catalytic events required in the processes of electron transfer and oxidative phosphorylation. Such rapid interactions may require freedom of lateral motion or diffusion in the plane of the membrane by the various interacting membrane components involved. For lateral diffusion to occur, adequate space as well as fluidity is required in the membrane lipid bilayer.

With regard to adequate space, it is commonly recognized that the mitochondrial inner membrane is composed of 75% protein (37, 38); however, it is seldom recognized that only
one-half of this protein is integral to the membrane bilayer (39, 40). Consistent with the latter are our recent ultrastructural observations which determined that the proteins of the inner membrane occupy only one-third to one-half of the total surface area of the membrane lipid bilayer (41). These and a number of other observations reviewed recently reveal that there is a large, potential lateral space in the inner membrane bilayer in which the diffusion of integral proteins may occur (42).

In addition to adequate space for diffusion, the inner membrane lipid bilayer is highly fluid. This is consistent with the membrane's virtual lack of cholesterol and its low saturated to unsaturated phospholipid ratio (38), as well as its subzero liquid crystalline to gel state lipid phase transition (43). Further characteristics supportive of a highly fluid inner membrane bilayer are the high mobility of phospholipid hydrocarbon chains (44) and intrinsically low microviscosities, in the range of 0.1 to 0.9 poise at 30°C (45, 46), reported for the mitochondrial membrane lipid component.

Finally, lateral diffusion of some integral proteins has been shown by ultrastructural studies to occur in the inner membrane (1, 3). The rate and catalytic significance of such diffusion has not been determined.

Lateral diffusion of inner membrane components may relate directly to the rates and mechanisms of electron transfer and oxidative phosphorylation. To explore the possible existence of such a relationship, we developed an in vitro method for increasing the surface area of the membrane bilayer with exogenous phospholipid to potentially increase the diffusion distance between the various interacting membrane components which catalyze electron transfer and oxidative phosphorylation.

Enrichment of the inner membrane lipid bilayer was achieved by fusion of sonicated, small unilamellar phospholipid vesicles with the purified inner membrane at pH 6.5. Subsequent sucrose density gradient centrifugation yielded four buoyant-distinct membrane fractions. Exogenous phospholipid was incorporated into the inner membrane bilayer. It was not simply adsorbed to the membrane surface since the density distribution of intramembrane particles (integral proteins) decreased as the surface area of the membrane bilayer increased. This finding was not due to a loss of integral proteins since the ratio of heme a to total protein remained constant as the lipid phosphorus to heme a ratio increased. Moreover, cytochromes b and c, as well as ubiquinone were recovered quantitatively in all four buoyant-distinct membrane fractions. By contrast, cytochrome c, a loosely bound peripheral protein, was largely lost during the fusion process.

It should be emphasized that the decrease in density distribution of intramembrane particles during the membrane-liposome fusion process was proportional to the decrease in the ratio of heme a to lipid phosphorus. Moreover, the particles or integral proteins remained randomly distributed, i.e. they diffused laterally in the membrane as fusion progressed.

Having established the occurrence of free lateral diffusion of integral proteins in the inner membrane enriched with exogenous phospholipid, we proceeded to explore the relationship of diffusion to the activity of specific membrane components known to interact catalytically during electron transfer and oxidative phosphorylation. We incorporated exogenous phospholipid into the bilayer to potentially increase the distance between catalytically interacting integral membrane components. Under such conditions, alterations in the catalytic activity might be expected to occur by at least two mechanisms: 1) a decrease in activity caused by an increase of diffusion distance and, consequently, of diffusion time between the catalytically interacting components; and 2) an increase or decrease in activity caused by the influence of chemical and physical properties of the newly incorporated phospholipid directly on the specific membrane components themselves.

After the fusion process, electron transfer from NADH and succinate to cytochrome c and to oxygen decreased in proportion to the increase in bilayer surface area and, therefore, as well to the increase in average lateral distance between integral proteins observed as intramembrane particles. Significant decreases occurred in NADH- and succinate-cytochrome c reductase activities, whereas no decreases could be detected in any segments of the electron transfer sequence between cytochromes bc1 and oxygen using duroquinol as the electron donor. These results suggest that the average lateral distance between the dehydrogenases and cytochromes bc1 increased as the membrane surface area expanded during enrichment with exogenous phospholipid. From these data, we conclude that a diffusion-limited step is involved in the transfer of reducing equivalents from the dehydrogenases to cytochromes bc1 in the native membrane.

Several models could account for the diffusion-limited step between the dehydrogenases and cytochromes bc1, as illustrated in Fig. 4. Ubiquinone has been characterized as a mobile carrier, shuttling reducing equivalents between the various dehydrogenases and cytochrome b (5). Further, ubiquinone appears to subserve a pool function, i.e. it occurs in the membrane in a homogeneous pool rather than, in separate functional compartments, interacting completely with the various dehydrogenases (6, 7). Therefore, no direct physical interactions between the dehydrogenases and cytochrome b may be required in this segment of the electron transfer sequence. Such a random structural arrangement in our studies could account for the decreases in NADH- and succinate-ubiquinone reductase activities using Q-1 as electron acceptor, as described by Lewis and Chance (8). However, two additional models are also consistent with our data. One is the compartmentation model postulated by Chance (9, 10) in which the dehydrogenases are compartmented in the bilayer, while cytochrome b is located in the aqueous phase to oxidize ubiquinone. The second model is the existent membrane model proposed by Chance (11) in which the dehydrogenases are located within the membrane bilayer while cytochromes bcl and oxygen are located in the aqueous phase. These models will be discussed further below.

FIG. 4. Diffusional models of electron transfer between dehydrogenases and cytochromes bc1. a, dehydrogenases (DH), cytochrome bc1 complex (bc1), and ubiquinone (Q and QH2) are independently diffusing electron transfer components. b, same as a, but ubiquinone (Q) is bound to a diffusible quinone binding protein (x) as a prosthetic group. c, electron transfer through direct interaction between diffusible dehydrogenases (DH) and cytochrome bc1 complex (bc1) with ubiquinone (Q and QH2) as a coenzyme.
Diffusion of Electron Transfer Components

tron acceptor and ubiquinol-cytochrome c reductase using DBH as electron donor as the surface area of the inner membrane bilayer is increased. It is reported that DBH as well as Q-1 interacts with endogenous ubiquinone (36). Thus, our data reveal a decrease in activity on both sides of ubiquinone-enriched membranes is increased. It is reported that DBH as well as endogenous ubiquinone present in mammalian membranes, result in a relatively high rigidity, compared to a fatty acyl chain of a phospholipid, which may decrease its mobility as well as the fluidity of its local membrane environment. An additional limitation in mobility is introduced by the length of the isoprenoid side chain (48 Å), since the lateral mobility of a lipid molecule is inversely proportional to the square of its length (53).

Decreases in activity in the electron transfer segments from cytochromes b or c to oxygen were not observed as the membrane bilayer was expanded by the incorporation of exogenous phospholipid. Although we have previously demonstrated that cytochrome c oxidase diffuses in the native membrane independently of other as yet unidentified integral proteins (3), the present data suggest that the electron transfer segment from cytochrome b to cytochrome c oxidase diffuses as one structural entity, as shown in Fig. 5a. It is important to point out, however, that we cannot exclude the possibility that cytochrome c, a peripheral membrane protein, may diffuse in the low viscosity aqueous phase along the membrane surface at a rate too rapid to be kinetically limiting (Fig. 5b). Others have reported data which indicate that cytochrome c is a highly diffusible membrane component in the process of electron transfer (11, 54, 55).

Since no decrease in duroquinol oxidase occurred as the membrane bilayer was expanded, it is most likely that cytochromes b and c1 occur as a complex in the native membrane. This finding is consistent with the stoichiometric heme b and c1 composition of the isolated Complex III of Haitzi et al. (56).

Since some membrane-bound enzymes require phospholipids with specific head groups or with fatty acyl chains of specific length and saturation (57), the chemical and physical properties of the newly incorporated phospholipid may have a direct influence on the specific activity of various components in the inner membrane. Such a direct effect probably explains the observed increases and decreases in the specific activity of NADH dehydrogenase, cytochrome c oxidase, succinate dehydrogenase, and ATPase, since none of these integral membrane proteins require collisional interaction with other integral membrane proteins for their activity. The low fact, certain properties of the ubiquinone molecule might limit rather than enhance the capacity for lateral diffusion. The 19 truns double bonds in the isoprenoid side chain of ubiquinone-10, the most common ubiquinone present in mammalian membranes, result in a relatively high rigidity, compared to a fatty acyl chain of a phospholipid, which may decrease its mobility as well as the fluidity of its local membrane environment. An additional limitation in mobility is introduced by the length of the isoprenoid side chain (48 Å), since the lateral mobility of a lipid molecule is inversely proportional to the square of its length (53).

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Although we have determined that electron transfer between the dehydrogenases and cytochrome b is diffusion-limited, further studies will be required to determine which of these three models best describes the diffusion mechanism. Presently, the lateral diffusion coefficients of the dehydrogenases, cytochrome b (or bc1), and ubiquinone in the native and lipid-enriched membranes are unknown. For integral proteins presumably unrestricted by interaction with proteins peripheral to the membrane bilayer, lateral diffusion coefficients of 1 to 6 x 10^-13 cm^2 s^-1 have been reported (50, 51). Using the reported low viscosity (0.1 to 0.9 poise at 30°C) of the cholesterol-free mitochondrial membrane (45, 46), this diffusion coefficient translates into a root mean square displacement of approximately 50 nm in 1 ms. Thus, even long range random lateral diffusion by the dehydrogenases and cytochrome b (or bc1) can occur within a time frame equal to their known turnover times of electron transfer (4).

Ubiquinone represents a neutral lipid which is generally presumed to have a high lateral diffusion coefficient. However, recent data suggest that ubiquinones with long side chains (Q-8) are not highly mobile within membrane bilayers (52). In

![Fig. 5. Models of electron transfer between cytochromes bc and cytochrome c oxidase. a, the electron transfer segment from cytochromes bc1 (bc1) to cytochrome c oxidase (c ox) diffuses as one structural entity. b, cytochrome c (c) shuttles between independently diffusing cytochromes bc1 and cytochrome c oxidase in the aqueous phase along the membrane surface.](image)
pH fusion procedure has the potential of becoming a valuable technique for determining the specific phospholipid requirements of integral membrane enzymes, since harsh and potentially denaturing extraction steps and reconstitution are not required and since preliminary experiments have demonstrated that lipid mixtures of specific and well-defined composition can be fused with the mitochondrial inner membrane.

In conclusion, these data identify a diffusion-limited step in the electron transfer sequence between the membrane dehydrogenases and cytochromes bc, and indicate that the dehydrogenases, ubiquinone, and cytochromes bc are independent, diffusible membrane oxidation-reduction components.

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REFERENCES
42. Hackenbrock, C. R. (1976) Nobel Symp. 34, 199-234