

# Relationship between the Density Distribution of Intramembrane Particles and Electron Transfer in the Mitochondrial Inner Membrane as Revealed by Cholesterol Incorporation

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**ABSTRACT** A low pH method of liposome-membrane fusion (Schneider et al., 1980, *Proc. Natl. Acad. Sci. U. S. A.* 77:442) was used to enrich the mitochondrial inner membrane lipid bilayer 30–700% with exogenous phospholipid and cholesterol. By varying the phospholipid-to-cholesterol ratio of the liposomes it was possible to incorporate specific amounts of cholesterol (up to 44 mol %) into the inner membrane bilayer in a controlled fashion. The membrane surface area increased proportionally to the increase in total membrane bilayer lipid. Inner membrane enriched with phospholipid only, or with phospholipid plus cholesterol up to 20 mol %, showed randomly distributed intramembrane particles (integral proteins) in the membrane plane, and the average distance between intramembrane particles increased proportionally to the amount of newly incorporated lipid. Membranes containing between 20 and 27 mol % cholesterol exhibited small clusters of intramembrane particles while cholesterol contents above 27 mol % resulted in larger aggregations of intramembrane particles. In phospholipid-enriched membranes with randomly dispersed intramembrane particles, electron transfer activities from NADH- and succinate-dehydrogenase to cytochrome *c* decreased proportionally to the increase in distance between the particles. In contrast, these electron-transfer activities increased with decreasing distances between intramembrane particles brought about by cholesterol incorporation. These results indicate that (a) catalytically interacting redox components in the mitochondrial inner membrane such as the dehydrogenase complexes, ubiquinone, and heme proteins are independent, laterally diffusible components; (b) the average distance between these redox components is effected by the available surface area of the membrane lipid bilayer; and (c) the distance over which redox components diffuse before collision and electron transfer mediates the rate of such transfer.

A number of reports have suggested that diffusional and rotational motion of membrane proteins is associated with specific membrane functions (1–6). The extent of lipid motion determines the fluidity of the membrane lipid bilayer which affects the lateral and rotational motion of membrane proteins (7, 8). Thus, a potentially promising approach to investigate diffusion-mediated catalytic activities in membranes is to alter the fluidity or extent of the membrane lipid bilayer through qualitative or quantitative manipulation of the lipid.

We developed a low pH method to fuse liposomes with membranes which permits the incorporation of controlled quantities of exogenous lipid into the bilayer of the mitochondrial inner membrane (9). After enriching the inner membrane with mixed soybean phospholipid, a number of structural, compositional, and functional characteristics were found to be proportionally related. The membrane surface area increased as the phospholipid-to-protein ratio increased. The density distribution of the intramembrane particles (integral proteins)

decreased due to randomization of the particles into the newly expanded bilayer, thus increasing the average distance between integral proteins. Concomitant with the increase in average distance between integral proteins, the rates of electron transfer between specific interacting membrane redox components decreased (9, 10). These data suggested that an increase in the available surface area of the inner membrane bilayer increased the diffusion path length between membrane redox components which normally diffuse independently of one another, thus reducing the number of collisions per unit time between these components, resulting in a decrease in electron-transfer activity.

In the present study, cholesterol was incorporated along with phospholipid into the mitochondrial inner membrane to determine how cholesterol affects structural organization and diffusion-mediated electron transfer in the inner membrane. Cholesterol, virtually absent in the native inner membrane, has been reported to decrease membrane bilayer fluidity above the bilayer lipid transition temperature (11, 12).

We have found that, above 20 mol %, cholesterol causes clustering and aggregation of membrane proteins and concomitant increases in electron-transfer rates from NADH- and succinate-dehydrogenases to cytochrome *c*. The significance of these findings is that they demonstrate the relationship of the diffusion path length between interacting redox components and the rates of electron transfer between such components.

## MATERIALS AND METHODS

Liver mitochondria were isolated from male Sprague-Dawley rats in 70 mM sucrose, 220 mM mannitol, 2 mM HEPES, 0.5 mg bovine serum albumin (BSA)/ml, and KOH to pH 7.4. Removal of the outer membrane and purification of the inner membrane/matrix (mitoplast) fraction was carried out by use of a controlled digitonin incubation (14, 15). Two subsequent washes in 7.5 times diluted isolation medium converted the topographically complex inner membrane into a simple spherical configuration (14).

Unilamellar, cholesterol-containing liposomes were prepared as follows: 2.5 g of mixed soybean phospholipid (asolectin) together with either 26 mg, 103 mg, 172 mg, or 240 mg of cholesterol/g asolectin were dissolved in 8 ml of chloroform (Spectral analyzed, Fisher Scientific Company, Pittsburgh, PA). This solution was evaporated to dryness under vacuum for 2 h at 30°C using a rotary evaporator (Rotavapor-R, Büchi AG, Switzerland) in combination with a vacuum pump. 1.5 g of the dry phospholipid/cholesterol mixture were added to 7 ml of 7.5 times diluted isolation medium. After 1-h hydration, the lipid mixture was sonicated at 0°C described previously (9).

Fusion of cholesterol-containing liposomes with inner membranes was carried out as described previously (9, 10). After a fusion period of 45 min the liposome-inner membrane suspension was separated on a sucrose density gradient (9, 10). The resulting four buoyant-distinct inner membrane fractions were designated Band 1, Band 2, Band 3, and Pellet, respectively, from least to most dense.

NADH-cytochrome *c* reductase and succinate-cytochrome *c* reductase activities were determined from cytochrome *c* reduction at 550–540 nm in an Aminco DW-2a spectrophotometer using an extinction coefficient of 19.9 mM<sup>-1</sup> cm<sup>-1</sup> (16). Substrate was either 1 mM NADH or 20 mM sodium succinate plus 5 μM rotenone. The remaining reaction medium was 80 mM potassium phosphate, pH 7.4, 2 mM KCN, and 2 μM CCCP. The reaction was initiated by 50 μM cytochrome *c*, in the case of succinate-cytochrome *c* reductase after a 10-min preincubation of the enzyme (membranes) in the reaction medium at room temperature. NADH-ubiquinone reductase was determined in 20 mM potassium phosphate, pH 7.4 2 mM potassium cyanide, 0.1 mM NADH and 20 mM ubiquinone -1 (17). The lipid-enriched membranes are NADH-permeable (10).

Heme *a* concentrations were obtained from measurements of ΔA 605–630 after dithionite reduction using an extinction coefficient of 13.1 mM<sup>-1</sup> cm<sup>-1</sup> (18). Lipid phosphorus was determined according to Bartlett (19) after acid hydrolysis of the membrane samples with perchloric acid. Cholesterol was determined by colorimetric assay (20) on total lipid extracts obtained by the extraction procedure described for cell fractions (21).

For freeze-fracture electron microscopy, membrane samples were treated with 30% glycerol in 7.5 times diluted isolation medium and frozen in liquid Freon 22, or unglycerinated samples were propane-jet frozen at a cooling rate in excess of 10,000°C/s (22). Freeze-fracturing was carried out in a Balzers BA 360 freeze-

etching apparatus (Balzers, Hudson, NH) (23). Electron micrographs were taken with a JEOL 100 CX electron microscope at 80 kV.

## Materials

Cholesterol and horse heart cytochrome *c* type IV were obtained from Sigma Chemical Co., St. Louis, MO. Asolectin was purchased from Associated Concentrates, Inc., New York, NY and ubiquinone-1 was the generous gift of Hoffman-LaRoche, Basel, Switzerland. All other chemicals were obtained in highest purity available commercially.

## RESULTS

### Incorporation of Controlled Amounts of Cholesterol into the Membrane

Liposomes with four different cholesterol-to-phospholipid ratios were fused with mitochondrial inner membranes. As shown in Fig. 1, the four inner membrane fractions (Pellet, Band 3, Band 2, Band 1), recovered after sucrose density gradient centrifugation, exhibited a different cholesterol-to-phospholipid ratio that was determined by (a) the cholesterol-to-phospholipid ratio of the liposomes used for fusion, and (b) the increase in membrane bilayer lipid due to the fusion. The latter is expressed as a decrease in cytochrome oxidase heme *a* to the total lipid ratio.

In previous experiments it was established that the cytochrome oxidase heme *a* to membrane protein ratio remains constant during phospholipid incorporation (9). The molar ratio of heme *a* to total lipid can thus be used to determine the ratio of membrane lipid to membrane protein. A heme *a* to total lipid (TL) ratio of 0.139–0.144 × 10<sup>-3</sup> as found in Band 1, the most buoyant fraction, corresponds to an increase in bilayer lipid of ~700% over the control, nonenriched inner

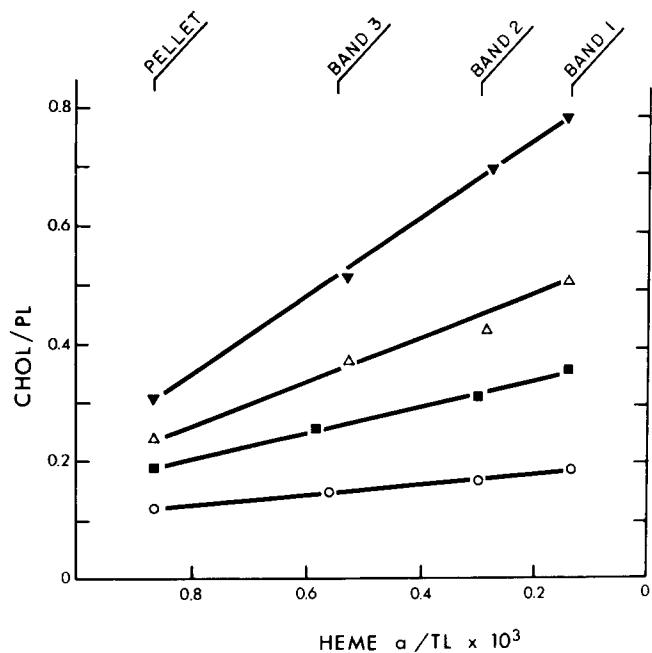


FIGURE 1 Incorporation of cholesterol into the mitochondrial inner membrane. The membrane cholesterol (*CHOL*) to phospholipid (*PL*) ratio is given as a function of the cytochrome oxidase heme *a* to total lipid (*TL*) ratio in the four different membrane fractions (Pellet, Band 3, Band 2, and Band 1) obtained after liposome-inner membrane fusion and sucrose density gradient centrifugation. Liposomes containing 26 mg (○), 103 mg (■), 172 mg (△) or 240 mg (▼) of cholesterol/g phospholipid were fused with inner membranes.

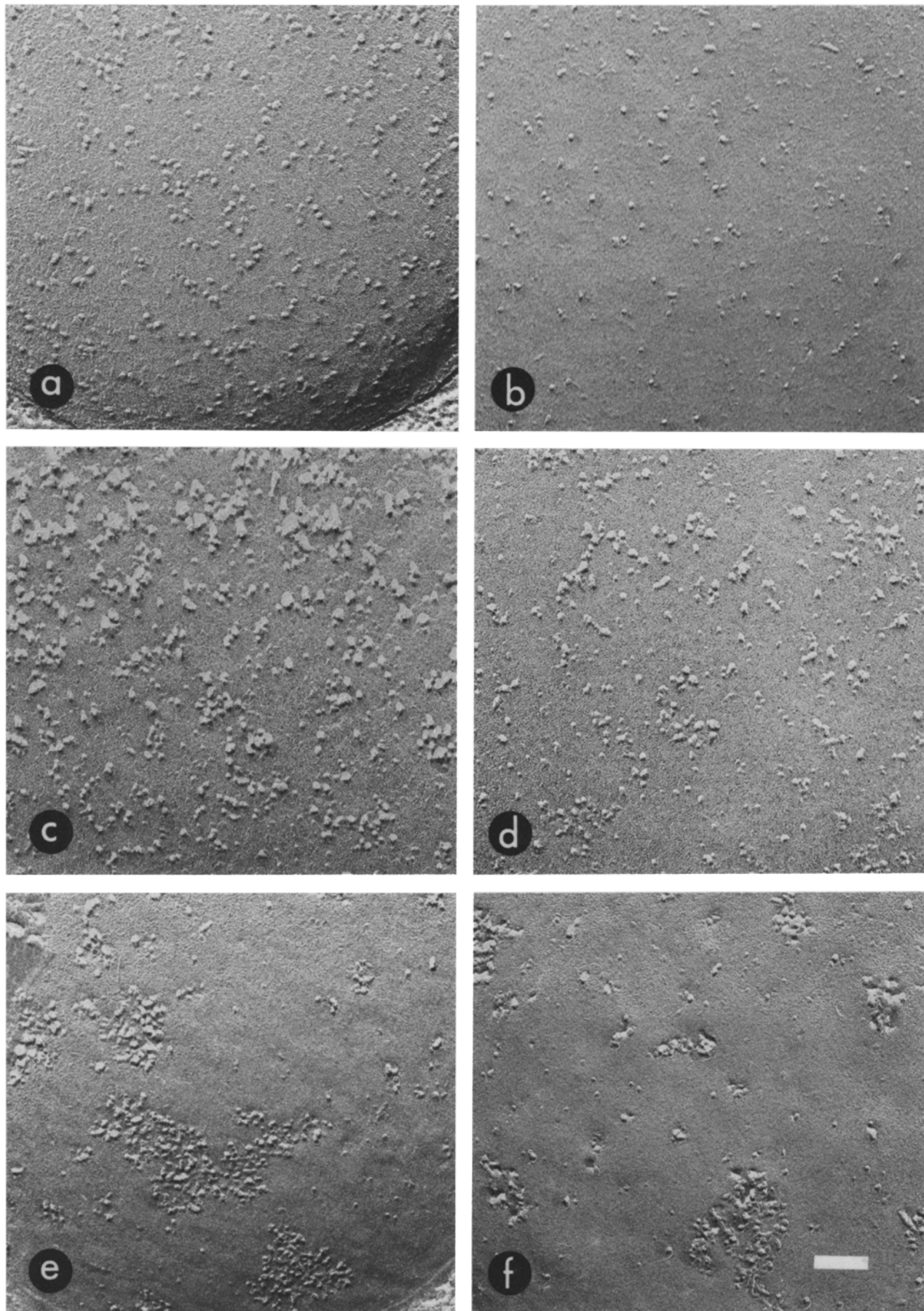


FIGURE 2 Freeze-fracture convex faces of the two most buoyant fractions, Band 2 (*a, c, e*) and Band 1 (*b, d, f*), obtained after fusion of inner membranes with liposomes containing no cholesterol (*a* and *b*), 103 mg cholesterol/g phospholipid (*c* and *d*), and 172 mg cholesterol/g phospholipid (*e* and *f*). The cholesterol concentration in the various membrane fractions is 0 mol % (*a* and *b*), 24 mol % (*c*), 27 mol % (*d*), 30 mol % (*e*), and 34 mol % (*f*). Bar, 100 nm.

membrane fraction (heme *a*/TL ratio of  $1.11 \times 10^{-3}$ ). Membranes in the least buoyant, Pellet fraction (heme *a*/TL ratio of  $0.8-0.85 \times 10^{-3}$ ) are considerably less enriched in exogenous lipid (~30%) (9).

Consistent with true fusion of cholesterol-phospholipid liposomes with inner membranes, the cholesterol-to-phospholipid ratio in the membranes increased proportionally to the decrease in the heme *a* to total lipid ratio (Fig. 1). Thus an increased cholesterol-to-phospholipid ratio in the liposomes used for fusion resulted correspondingly in an increased cholesterol-to-phospholipid ratio from Pellet to Band 1 membranes.

### Effects of Cholesterol on the Structural Organization of the Lipid-enriched Membrane

Freeze-fracture electron microscopy of mitochondrial inner membranes was carried out after phospholipid-cholesterol enrichment. The same results were obtained using glycerinated or nonglycerinated, propane-jet frozen membrane samples. Membranes enriched with phospholipid alone and membranes containing up to 20 mol % cholesterol showed a random lateral distribution of intramembrane particles, regardless of their total lipid content. In addition, the lateral density distribution of the intramembrane particles decreased, resulting in an increase in the average distance between particles (Fig. 2*a* and *b*). However, membrane cholesterol contents of 20–27 mol % resulted in small clusters of particles (Fig. 2*c* and *d*) and cholesterol contents above 27 mol % led to larger aggregations of particles, leaving extensive smooth domains in the bilayer mostly devoid of particles (Fig. 2*e* and *f*). Ultrastructural analysis of all membrane fractions, obtained after fusion with liposomes containing different amounts of cholesterol, is summarized in Fig. 3. Clearly, the pattern of lateral distribution of the intramembrane particles is a function of cholesterol concentration (mol % cholesterol of total lipid) in the various membrane fractions. In general, increasingly higher membrane

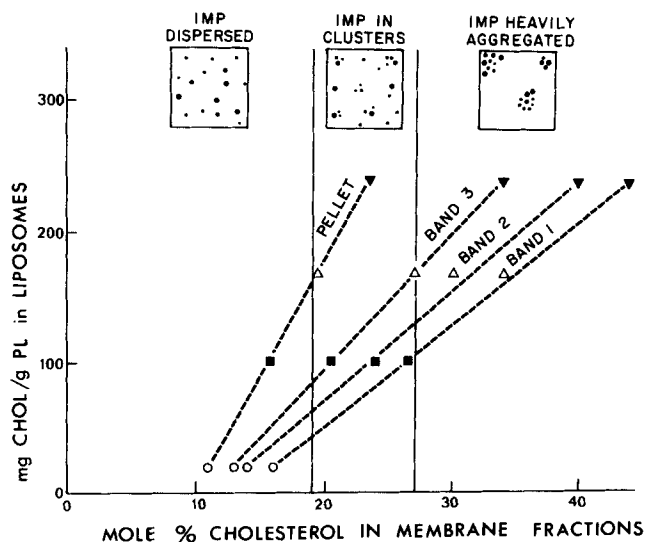


FIGURE 3 Summarized ultrastructural analysis. The distribution of intramembrane particles is shown as a function of the cholesterol concentration (MOL %) in the lipid-enriched inner membrane fractions (Pellet, Band 3, Band 2, and Band 1) and the cholesterol content (mg CHOL/g PL) in the liposomes used for the fusion with inner membranes. Electron micrographs derived from membrane fractions after fusion with liposomes containing 26 mg (○), 103 mg (■), 172 mg (△), or 240 mg (▼) of cholesterol/g phospholipid.

cholesterol was required to cluster or aggregate particles in membranes with higher total lipid to protein ratios.

These results can be interpreted in terms of the formation of cholesterol-rich bilayer domains in cholesterol-enriched membranes, the size of the domains depending on the cholesterol-to-phospholipid ratios in the membranes. It appears that the intramembrane particles are excluded from such cholesterol-rich domains, leading to particle clustering and, as the domain size increases, to large aggregations of particles. It should be noted, however, that some solitary particles appear trapped within the cholesterol-rich domains.

### Effects of Cholesterol-induced Intramembrane Particle Aggregation on Electron Transfer

As previously reported, the increase in the average distance between intramembrane particles or integral proteins upon enrichment of the inner membrane lipid bilayer with exogenous phospholipid was accompanied by a proportional decrease in electron-transfer rates from either NADH or succinate to  $O_2$  (9). A more detailed study revealed a diffusion-mediated electron transfer between the inner membrane NADH- and succinate-dehydrogenases and the  $bc_1$ -cytochrome complex, indicating that these redox components normally diffuse independently of one another (10). Thus, it was of interest to determine how the cholesterol-induced particle aggregation affected the diffusion-mediated electron transfer from the dehydrogenases to the  $bc_1$ -cytochrome complex.

When membrane cholesterol reached concentrations which caused distinct particle clustering or aggregation (20–27 mol %), electron-transfer activity from NADH- and succinate-dehydrogenase to cytochrome *c* increased distinctly in comparison to control membranes enriched with phospholipid alone or membranes containing <20 mol % cholesterol (Figs. 4 and 5). Generally, increasingly higher membrane cholesterol was required to increase electron transfer activity in membranes of higher total lipid-to-protein ratios.

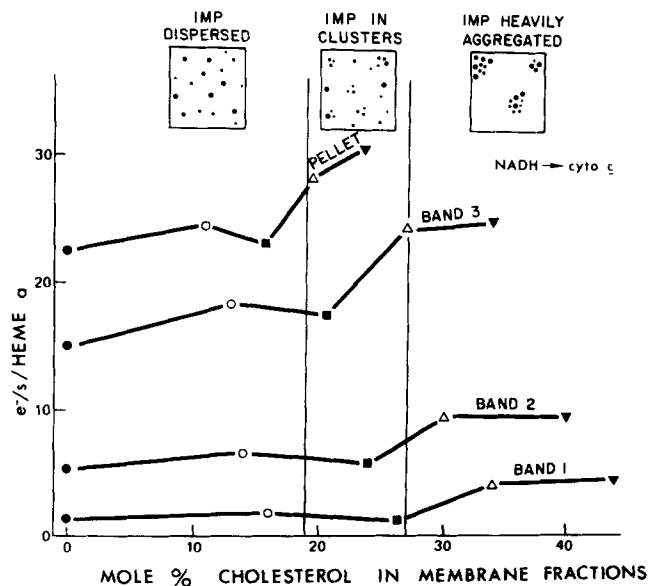


FIGURE 4 Specific activity of NADH-cytochrome *c* reductase (NADH → cyto *c*) as a function of the cholesterol concentration (MOL %) in the lipid-enriched inner membrane fractions. The liposomes used for fusion contained no cholesterol (●), 26 mg (○), 103 mg (■), 172 mg (△), or 240 mg (▼) of cholesterol/g phospholipid.

When the activities of the four density-distinct cholesterol-enriched membrane fractions are compared, it is clear that for any one membrane fraction there is an increased activity at cholesterol contents which result in particle clustering and aggregation (Figs. 6 and 7). It is also clear, however, that a general decrease in activity occurs from Pellet to Band 1 proportional to the amount of newly incorporated lipid and independent of the membrane cholesterol concentration (Figs. 6 and 7). This observation indicates that the dilution of ubi-

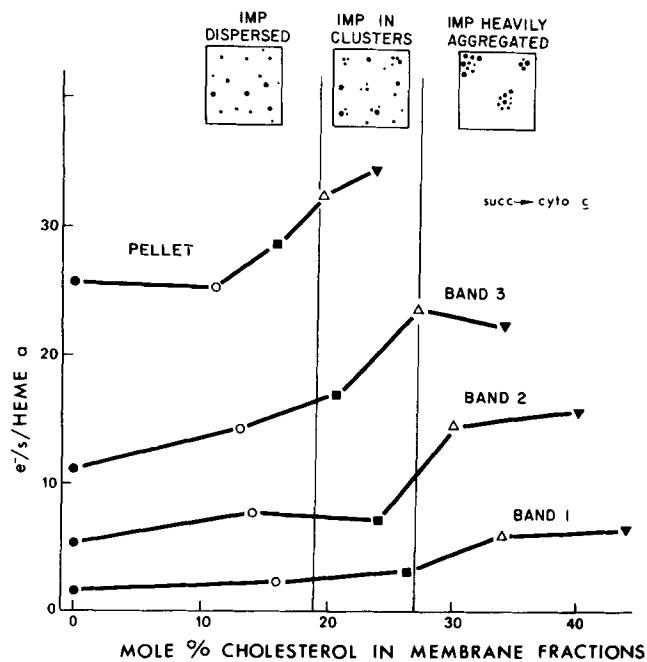


FIGURE 5 Specific activity of succinate - cytochrome c reductase (succ  $\rightarrow$  cyto c) as a function of the cholesterol concentration (MOLE %) in the lipid-enriched inner membrane fractions. The liposomes used for fusion contained no cholesterol (●), 26 mg (○), 103 mg (■), 172 mg (△), or 250 mg (▽) of cholesterol/g phospholipid.

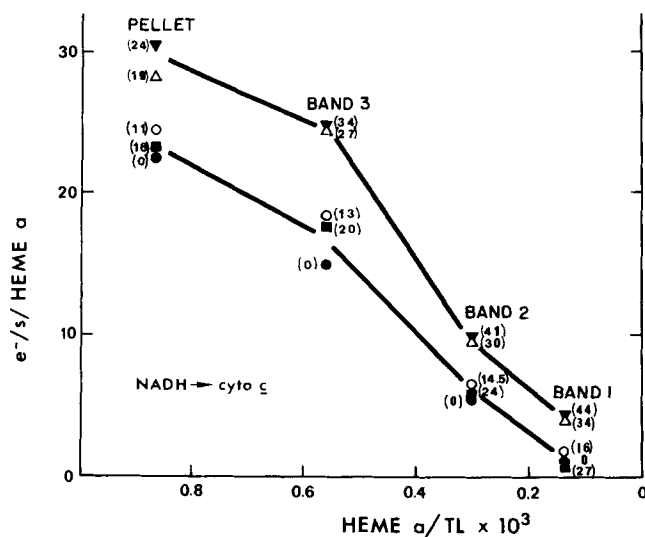


FIGURE 6 Specific activity of NADH - cytochrome c reductase (NADH  $\rightarrow$  cyto c) in lipid-enriched membranes as a function of the heme a to total lipid (TL) ratio. Liposomes used for fusion contained no cholesterol (●), 26 mg (○), 103 mg (■), 172 mg (△), or 240 mg (▽) of cholesterol/g phospholipid. The mol % cholesterol in the membranes is given in brackets.

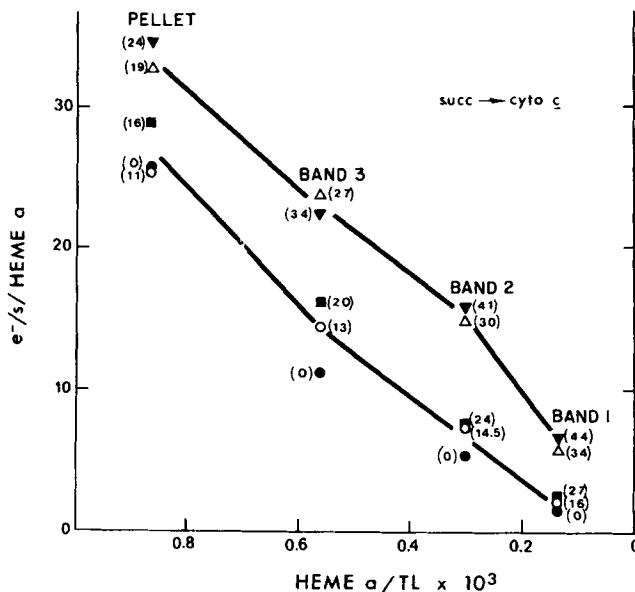


FIGURE 7 Specific activity of succinate-cytochrome c reductase (succ  $\rightarrow$  cyto c) in lipid-enriched membranes as a function of the heme a to total lipid (TL) ratio. Liposomes used for fusion contained no cholesterol (●), 26 mg (○), 103 mg (■), 172 mg (△) or 240 mg (▽) of cholesterol/g phospholipid. The mol % cholesterol in the membranes is given in brackets.

quinone (by the newly incorporated lipid) that is in part responsible for the observed decreases in electron transfer as the membrane phospholipid content increases (6) also occurs in cholesterol-enriched membranes. In addition, solitary intramembrane particles which are trapped within cholesterol-rich domains are removed from the catalytically active pool of aggregated intramembrane particles.

## DISCUSSION

Liposome-membrane fusion is an excellent tool to use to investigate relationships between structure and composition of a membrane with its functions. The low pH liposome-membrane fusion method (6, 9, 10) not only permits qualitative and quantitative alterations of the lipid component of membranes but also can be used to insert components that are not native to the membrane. In the present study, we used liposomal fusion to insert controlled amounts of cholesterol into the mitochondrial inner membrane which is virtually free of cholesterol in its native form (29, 30). The interest to incorporate cholesterol into the mitochondrial inner membrane lipid bilayer grew out of two observations. First, mitochondrial electron transfer may be a diffusion-mediated process (6, 9, 10) and, second, it is reported that cholesterol reduces the fluidity of the lipid bilayer thus modulating the diffusional and rotational mobility of membrane lipids and proteins (28, 31, 32).

We have determined that incorporation of cholesterol into the mitochondrial inner membrane produced protein aggregation similar to that observed by Cherry et al. (13) upon inserting cholesterol into phospholipid vesicles containing bacteriorhodopsin. As in the latter system, protein aggregation in mitochondrial inner membranes was dependent on the concentration of cholesterol compared to phospholipid in the bilayer. Random, lateral distribution of integral proteins was observed at cholesterol concentrations below 20 mol % while protein clustering occurred at concentrations from 20 to 27 mol % cholesterol and protein aggregation occurred above 27 mol %.

Our freeze-fracture observations suggest the formation of cholesterol-rich bilayer domains which exclude the proteins laterally into cholesterol-poor phospholipid domains. Such domain formation has been described to occur in cholesterol-containing phospholipid bilayers as a result of phospholipid-cholesterol complex formation at cholesterol concentrations above 20 mol % (24–27, 33, 34).

This structural effect of cholesterol raised the question: How does cholesterol-induced domain formation and protein aggregation affect mitochondrial electron transfer? From previous experiments we learned that increasing the average distance between the membrane integral proteins by increasing the surface area of the membrane lipid bilayer with exogenous phospholipid resulted in decreases in electron transfer from the membrane dehydrogenases to the  $bc_1$  cytochrome complex proportional to the amount of newly incorporated phospholipid (9, 10). Thus, it would be reasonable to assume that decreasing the average distance between membrane proteins through aggregation, brought about by incorporation of sufficient amounts of cholesterol, should increase the electron-transfer rates between dehydrogenases and the  $bc_1$  cytochrome complex. Indeed, electron-transfer activity from NADH- and succinate-dehydrogenase to cytochrome  $c$  increased as the cholesterol concentration in the membranes reached a level where protein clustering occurred, i.e., 20 mol % cholesterol, and increased even further upon aggregation of the proteins at levels above 27 mol % of cholesterol. No further activity increases occurred after protein aggregation had been maximized in the various membrane fractions, even when the cholesterol level was raised to 44 mol %.

Since the rate of electron transfer from the dehydrogenase to the cytochrome  $bc_1$  protein complexes increases rather than decreases with increasing cholesterol, it is more likely that the incorporation of cholesterol causes a decrease in the average distance between the dehydrogenases and cytochrome complexes rather than a decrease in the bilayer fluidity and protein mobility. This conclusion is also in agreement with our previous findings, which revealed that ubiquinone mediates electron transfer between spatially separated dehydrogenases and cytochrome  $bc_1$  complexes through rapid diffusion (6). Therefore, the average distance between the independently diffusible dehydrogenase and cytochrome  $bc_1$  complexes, i.e., the average path length over which ubiquinone has to diffuse, is an important factor that determines the rate of electron transfer.

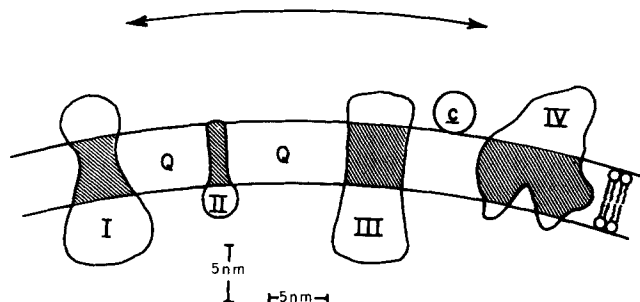


FIGURE 8 Four integral protein complexes (I–IV), ubiquinone (Q) and cytochrome  $c$  (c) comprise the “respiratory chain” of the mitochondrial inner membrane. The sequence of electron transfer is from Complex I (containing NADH dehydrogenase) and Complex II (containing succinate dehydrogenase), to ubiquinone, to Complex III (containing  $b$  and  $c_1$  cytochromes), to cytochrome  $c$ , to Complex IV (cytochrome oxidase). The arrow indicates independent, lateral diffusion of these components in the membrane plane.

The observations reported here, taken together with other previously reported observations (3, 35, 36), support the concept that mitochondrial electron transfer is mediated by energetically favorable collisional interactions between specific, rapidly diffusing, small redox components (ubiquinone, cytochrome  $c$ ) with slower diffusing large redox protein complexes (dehydrogenases,  $bc_1$  cytochromes, cytochrome oxidase) (Fig. 8). Our observations currently do not support the occurrence of a physically continuous chain of redox components, a “respiratory chain,” to account for the rate of electron transfer in the mitochondrial inner membrane.

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