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A Model Mosaic Membrane: Association of Phospholipids and Cytochrome Oxidase

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Abstract

The structure and physical properties of model membranes formed from lipids and cytochrome *c* oxidase have been examined. The lipid-depleted protein is in the form of 90 Å rods or globules. When phospholipid is added the rods swell and then form sheets and concentric membrane vesicles. The protein is saturated with lipid at 65 g/atoms of phosphorus per mole of heme *a*. Electron microscope examination by negative staining, sectioning, and freeze etching indicates a 50 Å thick unit membrane with 50-60 Å protein globules in the lipid bilayer. Infrared, circular dichroism and fluorescence binding studies are consistent with globular protein units surrounded with lipid. Diolein will substitute for phospholipid but the membrane formed remains as sheets rather than vesicles. Saturated phospholipids will not interact with the oxidase to form membrane. The capacity to form membrane is specific to protein associated with the heme *a*, and other insoluble protein in the original oxidase preparation cannot form membrane.

Introduction

Membrane biochemistry occupies an important position in modern biology in recent years since many cellular functions are associated with biological membranes. Many membrane models have been proposed by investigators. In the Danielli-Davson-Robertson model, every membrane has a continuous lipid bilayer as its core. The membrane is held together by hydrophobic interactions between lipid hydrocarbon chains. Protein forms an outer coating on both sides of the membrane and interacts electrostatically with the lipid polar groups. However, recent biochemical

[1] and biophysical [2, 3, 4] studies indicate that the Danielli-Davson-Robertson model is oversimplified. In order to study organization and lipid protein interaction in membranes, the use of a well-characterized membrane formation protein which also has enzymatic function is desirable.

Cytochrome oxidase (EC 1.9.3.1, ferrocytochrome *c*; oxygen oxidoreductase) constitutes the last carrier in the chain of electron transport and is widely distributed among animals, higher plants and some aerobic microorganisms. Because of its importance in the living system, this enzyme has been extensively investigated. In the present study we would like to propose a model membrane made from interaction of phospholipids and the purified membrane protein, cytochrome oxidase.

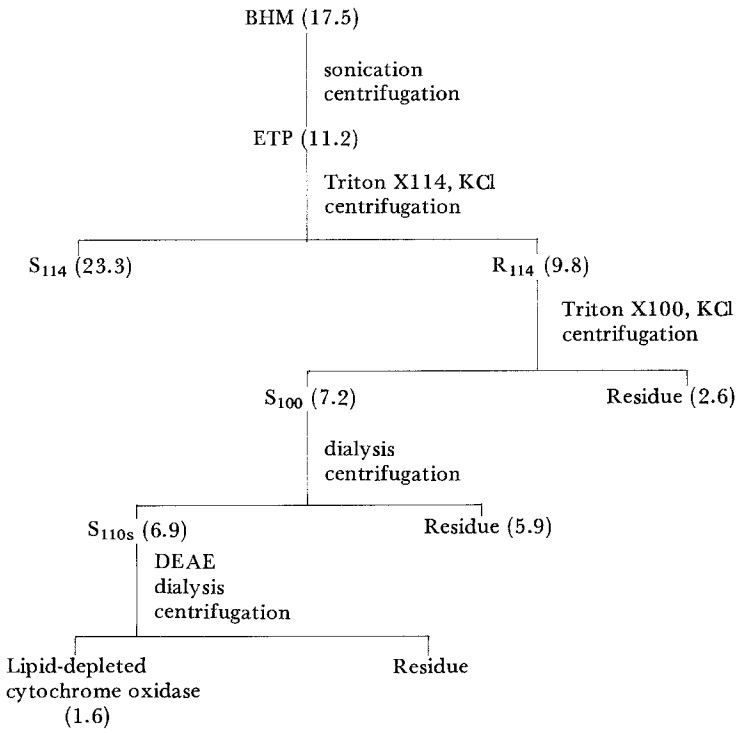
Materials and Methods

Cytochrome Oxidase Preparations

Beef heart mitochondria were prepared by the method of Löw and Vallin [5]. Cytochrome oxidase preparations were prepared as described earlier [6, 7] and diagrammatically shown in a flow chart in Fig. 1, together with phosphorus content at each step. Lipid content decreases as the purification procedure proceeds. After two washes in sucrose-Tris buffer (0.25 M sucrose buffered with 0.02 M Tris HCl, pH 7.4), the dark green residue (R_{114}) obtained after treatment of Triton X114 and KCl was referred to Triton X114 membranous cytochrome oxidase. The final product, the lipid-depleted cytochrome oxidase, contains only 1.3-1.8 μg P/mg protein and has a heme *a* content of 8-9 mμmoles/mg protein* as determined by its differential spectrum, reduced *minus* oxidized, using a millimolar extinction coefficient ΔA at 605 mμ minus 630 mμ as 13.1 [8]. The preparation has two major bands on gel electrophoresis [10]. Figure 2 shows spectra of the lipid-depleted cytochrome oxidase preparation.

Reconstituted membranous cytochrome oxidase: Phospholipid micelles and the lipid-depleted cytochrome oxidase were soluble and could not be centrifuged out separately at 108,000 x g. A typical reconstituted membranous cytochrome oxidase was made as follows. Phospholipid micelles were added to the lipid-depleted cytochrome oxidase at a concentration of 30 μg phosphorus/mg protein. The mixture was sonicated at maximal output with a Branson Sonifer for five 30 sec periods and centrifuged at 108,000 x g for 30 min. The pellet was resuspended in sucrose-Tris buffer and centrifuged at the same speed.

* Heme *a* content would be 10-11 mμ moles/mg protein if 16.5 mM^{-1} cm^{-1} was used as the absorbance index for A_{605} (reduced) minus A_{630} mμ (reduced) [9].



0.02 *M* Tris HCl, pH 7.4 was used.
Numbers in parentheses are phosphorus content in μg per mg protein.
BHM, beef heart mitochondria.
ETP, electron transport particles.

Figure 1. Flow chart of cytochrome oxidase preparation procedure and phosphorus content.

After three washes the reconstituted membranous cytochrome oxidase was obtained.

The same procedure was followed to study the binding of phospholipids to cytochrome oxidase with different amounts of phospholipids added to the enzyme.

Detergent-free, lipid-depleted cytochrome oxidase: Soluble lipid-depleted cytochrome oxidase was extensively diluted with 0.02 M Tris HCl, pH 7.4 (10 x dilution) and centrifuged at 270,000 x g for 1 h in a Spinco L2-65. Pellet was collected, homogenized, diluted and centrifuged at the same speed for 30 min. Four or five more washes by centrifugation at 108,000 x g for 20 min were done to remove residual

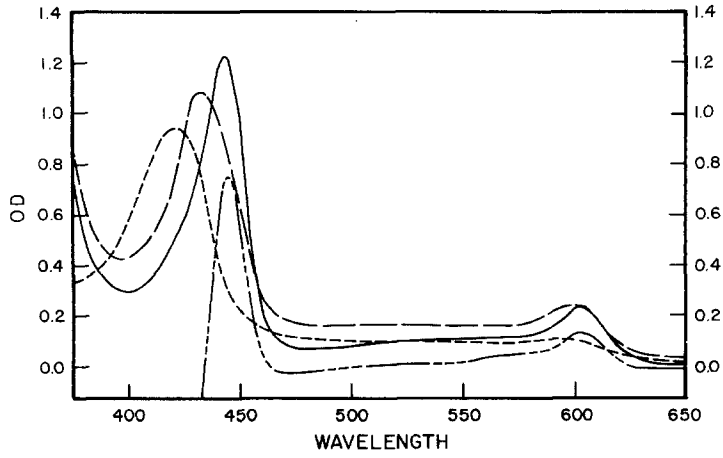


Figure 2. Spectra of lipid-depleted cytochrome oxidase. Spectra taken in 0.5% deoxycholate, protein concentration 1.25 mg per ml. – – – – oxidized, ——— reduced with dithionite, —— —— carbon monoxide plus dithionite reduced, – – – – ———difference spectrum (reduced minus oxidized).

Triton until Triton level decreases to less than 0.03 mg Triton/mg protein.

Enzyme activity was fully recovered after the detergent-free, lipid-depleted cytochrome oxidase resolubilized in Triton-Tris (1% Triton, 0.02 M Tris HCl, pH 7.4) with sonication (five 30 sec periods at maximal power output).

Triton content in the preparations was determined spectrophotometrically by the formation of a blue complex with ammonium cobaltothiocyanate [6].

Protein concentration was determined by the method of double strength biuret [11]. Molecular weight of cytochrome *a* was based on the value of 72,000 [12]. Enzyme activities were assayed polarographically with a Gilson KM oxygraph at 37° [6].

Phospholipids

Mixed total beef heart mitochondrial phospholipids were prepared as described earlier [7]. Purified phospholipids were obtained by applying mixed phospholipids on double thickness silica gel G plates and run in chloroform-methanol-H_2O (100 : 40 : 6, v/v/v). Micelle sols of phospholipids were prepared by sonication method of Fleischer and Fleischer [13]. After centrifugation at 108,000 x g for 1 h, the clear supernatant was kept at 0° under nitrogen and used within ten days.

Phosphorus was determined by the method of Chen *et al.* [14].

Electron microscopy

Negative staining with 2% phosphotungstic acid was carried out as described by Cunningham and Crane [15].

Thin sectioning was accomplished as follows: Specimens were pre-fixed in 4% glutaldehyde, pH 7.4 and post-fixed in 1% OsO_4 buffered in veronal acetate, pH 7.4. Samples were dehydrated in an acetone series and embedded in Epon 812 based on a procedure by Luft [16]. Sectioned specimens were post-stained with 2% aqueous uranyl acetate, followed by lead citrate according to Reynolds [17].

All samples were examined in a Philips EM 300 microscope.

Fluorometry

Fluorescence was measured with an Aminco-Bowman spectrophotofluorometer equipped with a photomultiplier microphotometer. Measurements were carried out at 25° in a temperature-controlled cell holder connected to a circulating water bath.

Infrared Spectroscopy

A Perkin-Elmer model 221 spectrophotometer was used. Solid films were prepared by applying phospholipid micelles or protein in aqueous suspension as a 0.5 x 2.0 cm band in the center of an Irtran disc which was allowed to dry *in vacuo* at room temperature. Extraction of lipids from the film was achieved by immersing the disc in chloroform methanol (2:1, v/v) for 20 min at room temperature. After rinsing with the same solvent, the films were dried in air.

Circular Dichroism

CD (circular dichroism) measurements were carried out at room temperature with a Cary model 60 spectropolarimeter, using cells with 10 mm path length.

Chemicals

Triton, sodium dodecyl sulfate, diolein, *Clostridium welchii* phospholipase C and DEAE (diethylamino ethyl) cellulose were obtained from Sigma; ANS (8-anilino-1-naphthalene-sulfonic acid) from Eastman. Other chemicals were of reagent grade.

Results

Structure During Enzyme Preparation

Observations of thin sections of the beef heart mitochondria have shown 90 Å thick and the occurrence of both "unit" and "binary" membrane structure [18]. Red green split was obtained upon the action of Triton X114 and KCl. Cytochromes *b, c* and c_1 were present in the red supernatant while green cytochrome oxidase remained in the pellet. The electron micrograph of a negatively stained sample of the washed green pellet, Triton X114 membranous cytochrome oxidase is shown in Fig. 3. 50-60 Å particles are seen over the surface of the membrane vesicles. This section of this membrane shows 50-60 Å thickness with clearly visible "unit" membrane structure. This membranous cytochrome oxidase preparation is essentially the same as the membrane cytochrome oxidase of Sun *et al.* [7], which was prepared using both Triton X114 and Triton X100. Activity of Triton X114 membranous cytochrome oxidase was 10-15 μmoles O_2/min/mg protein while that of mitochondria, used as starting material, was 2-3 μmoles O_2/min/mg protein (Table I).

The lipid-depleted cytochrome oxidase, obtained after DEAE cellulose column, appears as 90 Å diameter globules or assemblies of rod-like structures with the same thickness in the presence of Triton X100 (Fig. 4). One to two molecules of cardiolipin per molecule of cytochrome oxidase or 1.3-1.8 μg phospholipid phosphorus per mg of protein has been reported to be tightly bound to the oxidase [20]. Neither phosphatidyl choline nor phosphatidyl ethanolamine was found in the preparation.

Both preparations of detergent-free, lipid-depleted cytochrome oxidase, prepared by extensive centrifugation or diethyl ether treatment [6], were shown to be aggregates of 90 Å particles or rods by negative staining technique. It is interesting to note that these detergent-free, lipid-depleted preparations appear as small fragments with a "unit" membrane structure in thin section (about 500-1000 Å in length and 75-100 Å in thickness) (Fig. 5). This gives evidence that in such low lipid content, membrane protein can still form the so-called "unit" membrane structures. Formation of these small membranes might be due to both hydrophobic and hydrophilic interactions because they can be broken down to solubilized state by detergents, such as Triton, sodium dodecyl sulfate or deoxycholate with sonication as well as by pH treatment. Triton solubilized more protein in the presence of KCl than in the absence of the salt. The detergent-free enzyme preparation can be solubilized at pH lower than 3 or higher than 12. This enzyme preparation has only one-third of the activity of the regular lipid-

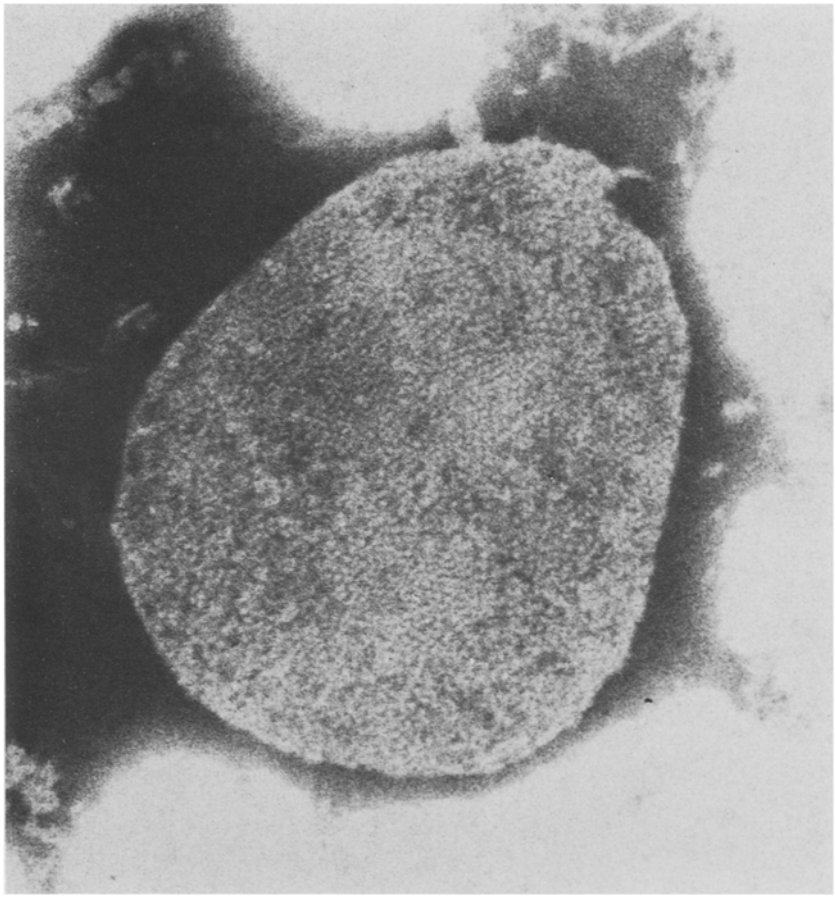


Figure 3. Triton X114 membranous cytochrome oxidase. Negatively stained with phosphotungstic acid. x 160,000.

depleted enzyme (Table I). It can, however, be restored to full activity after resolubilization.

Lipid Concentration and Cytochrome Oxidase Structure

Due to deficiency of lipid in the lipid-depleted cytochrome oxidase, the preparation is very inactive. Figure 6 shows the enhanced activity obtained upon addition of phospholipids or Emasol at different levels. In

TABLE I. Heme *a* and phospholipid contents and enzyme activity of cytochrome oxidase preparations

Preparations	Heme *a* (mμ moles/mg protein)	Phospholipids (μg P/mg protein)	Phospholipids (%, w/w)	Activity (μmoles O_2/min/mg) + None	Activity (μmoles O_2/min/mg) + Phospholipids[a]
BHM	0.8	17.5	30.5	2.6	3.0
ETP	1.0	11.2	21.9	3.2	4.1
Triton X114 memb. oxidase (R_{114})	7.6	9.8	19.7	11.6	11.9
S_{100}	8.0	7.2	15.3	11.8	30.5
S_{100s}	8.3	6.9	14.6	15.9	38.4
Lipid-depleted oxidase	8.6	1.5	3.6	4.0	43.0
Detergent-free oxidase (DF)	8.6	1.5	3.6	1.7	6.2 (37.8[b])
Resolubilized DF[c]	8.6	1.5	3.6	4.1	42.5
Reconstituted memb. oxidase	8.6	20.1	33.4	14.2	15.1
Fowler's prep.[d]	8.1	17.2	30.1	12.1	13.1

[a] Mixed total beef heart mitochondrial phospholipids.

[b] Detergent-free lipid-depleted cytochrome oxidase sonicated with phospholipids. Same level of enzyme activity could be obtained when the enzyme plus phospholipids were incubated at 0° for overnight.

[c] Detergent-free, lipid-depleted cytochrome oxidase was resolubilized in 1% Triton X150, 0.02 *M* Tris HCl, pH 7.4 followed by sonication.

[d] Preparation of Fowler *et al.* [19].

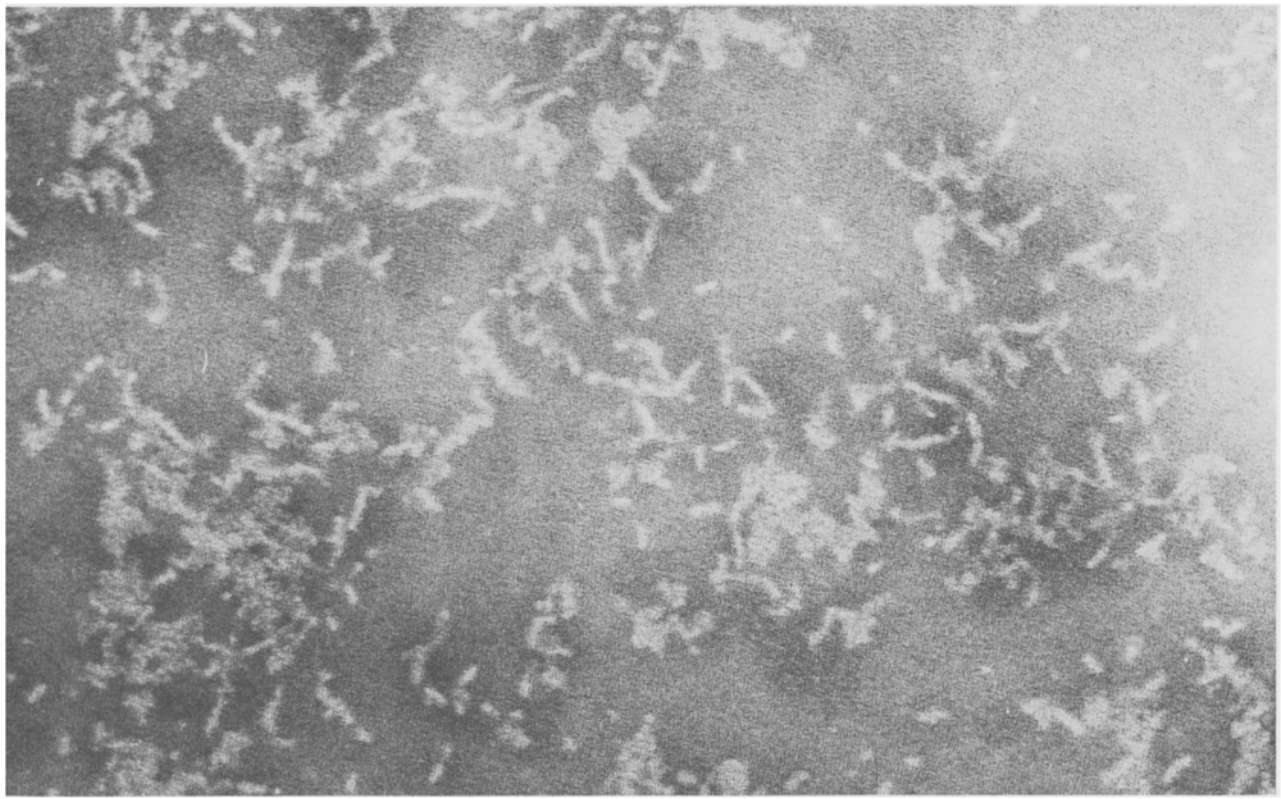


Figure 4. Lipid-depleted cytochrome oxidase. Note 90 Å particles. This preparation contains 0.8 mg Triton X100/mg protein. Negatively stained with phosphotungstic acid. × 160,000.

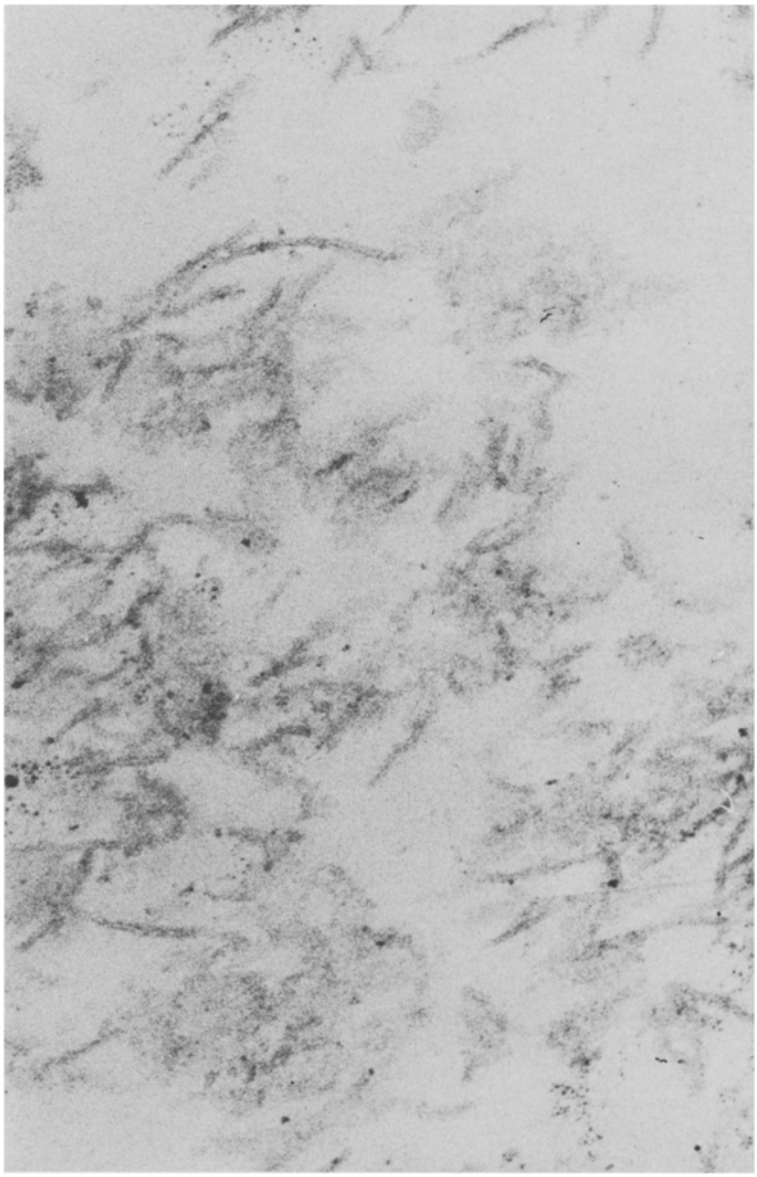


Figure 5. Thin section of detergent-free, lipid-depleted cytochrome oxidase. The preparation was made as indicated in the method. Note the "unit" membrane appearance. x 160,000.

addition to the enhanced enzyme activity upon addition of phospholipids to the lipid-depleted cytochrome oxidase, structural transformation could also be observed depending on the amount of phospholipids added. At low level of phospholipids (10-20 g/Atom P/mole cytochrome *a* or 4-8.5 μg P/mg protein) the 90 Å globules of lipid-depleted cytochrome oxidase (Fig. 4) began to swell to 200 Å in diameter and were gradually transformed to a small sheet of membrane

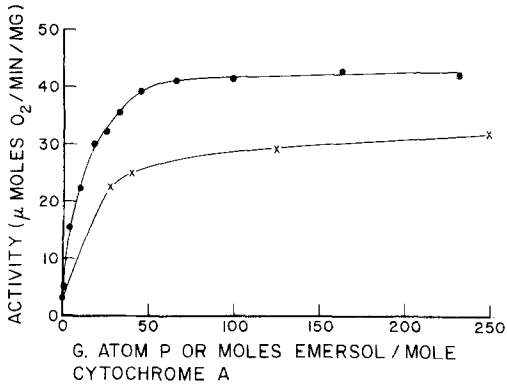


Figure 6. Activation of lipid-depleted cytochrome oxidase by phsopholipids and Emasol. Lipid-depleted cytochrome oxidase was adjusted to 1.5 mg Triton X100/mg protein and incubated with mitochondrial phospholipids or Emasol-1130 for 10 min at 4° before being diluted for assay. x Emasol, 0 phospholipid.

made up of 50-60 Å particles (Fig. 7). With addition of more phospholipids to the enzyme (20-65 g/Atom P/mole cytochrome *a* or 8.5-28 μg P/mg protein), the lipoprotein globules became organized to form a larger sheet of membrane with the same size globules (50 Å) visible in surface views (Fig. 8). At phospholipids concentrations higher than 65 g/Atom P/mole cytochrome *a* or 28 μg P/mg protein, excess phospholipids were observed around the membrane vesicles and the subunits were reduced to about 30-50 Å in diameter (Fig. 9).

Phospholipid binding studies showed increase of phospholipid content in cytochrome oxidase membranes up to a constant value. Phospholipid micelles and the lipid-depleted cytochrome oxidase were soluble and could not be centrifuged out separately at 108,000 x g for one hour. To study the amount of phospholipids bound to cytochrome oxidase during membrane formation, different amounts of phospholipid micelles were added to 40 mg of the lipid-depleted cytochrome oxidase and the mixture was diluted with sucrose-Tris buffer to 3 mg of protein/ml. The mixture was sonicated at maximal output with a Branson's Sonifer for

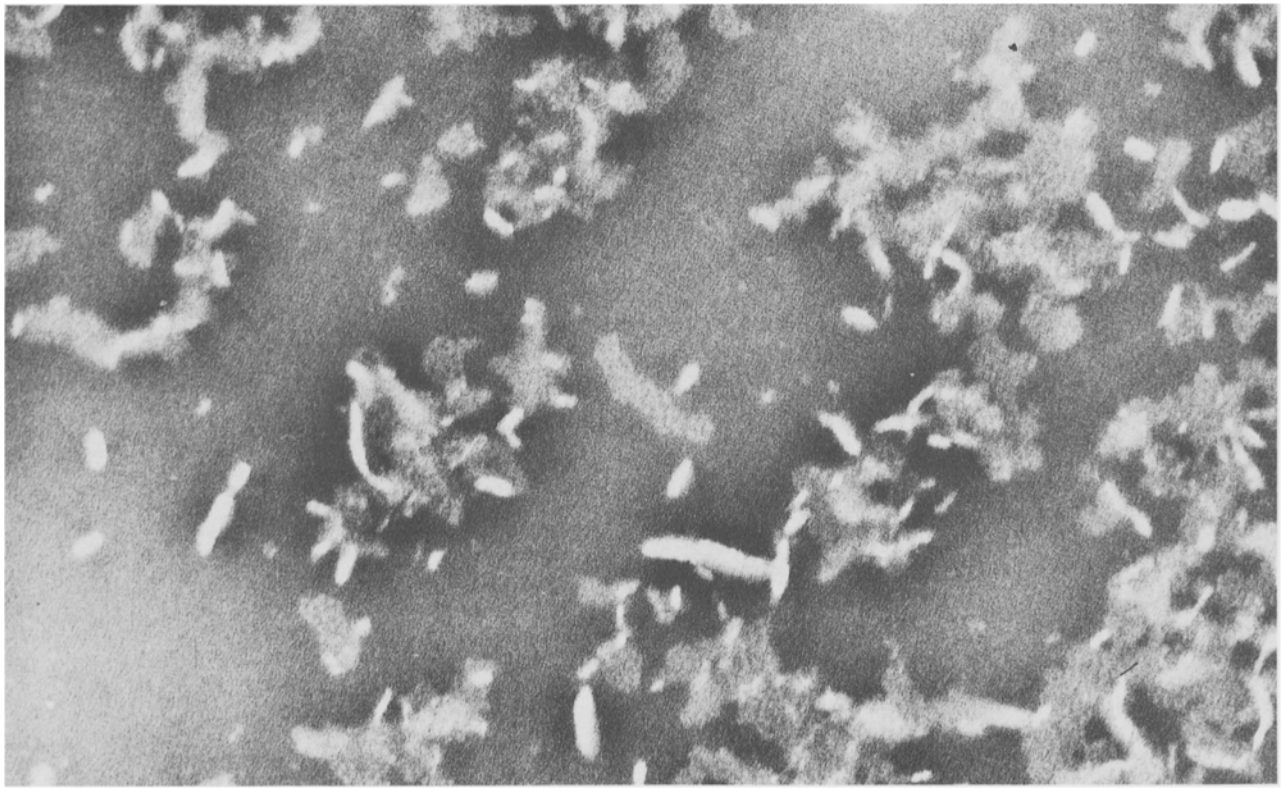


Figure 7. Cytochrome oxidase with 10 g Atom P of phospholipids per mole of cytochrome oxidase (10% phospholipids, w/w). 90Å particles of lipid-depleted enzyme (Figure 4) swell to approximately 200 Å in diameter and organize to form small membrane with 50-60 Å subunits. Negatively stained with phosphotungstic acid. x 160,000.

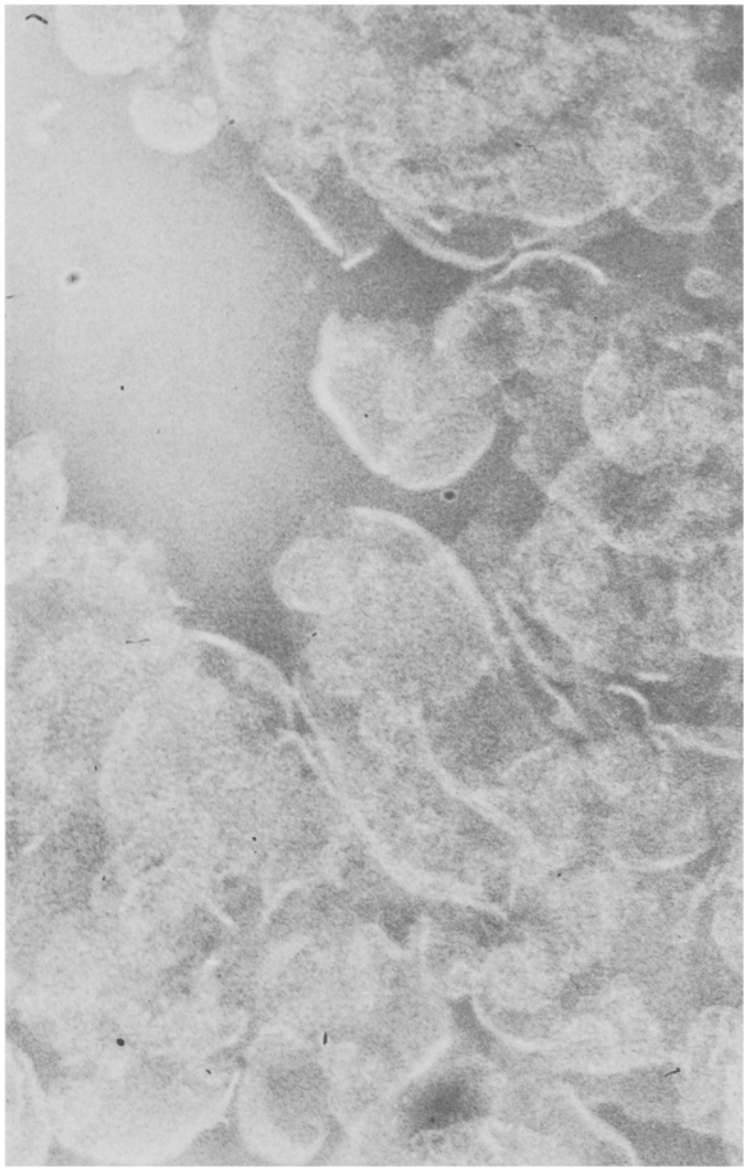


Figure 8. Cytochrome oxidase with 31 g Atom P of phospholipids per mole of cytochrome oxidase (25% phospholipids, w/w). Negatively stained with phosphotungstic acid. x 160,000.

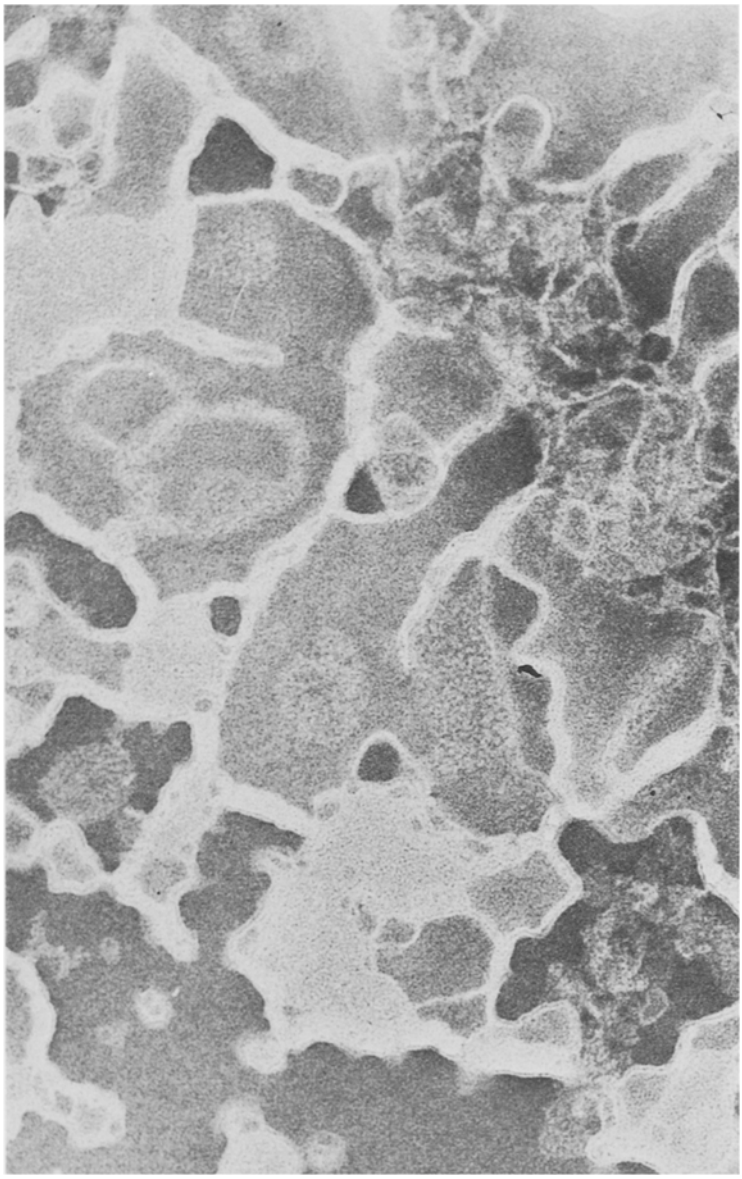


Figure 9. Cytochrome oxidase with 235 g Atom P of phospholipids per mole of cytochrome oxidase (70% phospholipids, w/w). Negatively stained with phosphotungstic acid. x 160,000.

five 30-sec periods and centrifuged at 108,000 x g for 30 min. The pellet was resuspended in sucrose-Tris buffer and centrifuged at the same speed. After three washes all free lipid and free cytochrome oxidase which were not in membranes were washed out. Figure 10 shows the amount of phospholipid phosphorus bound to cytochrome oxidase. A

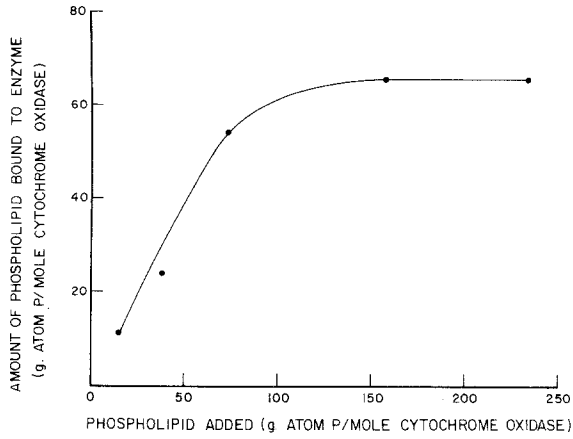


Figure 10. Amount of phospholipids bound to cytochrome oxidase. Various amounts of phospholipids were added to the lipid-depleted enzyme preparation. The mixture was then sonicated, centrifuged and washed to remove unbound phospholipids and free cytochrome oxidase.

saturation value of 65 g/Atom P/mole cytochrome oxidase or 28 μg P/mg protein, which corresponds to 41% (w/w), was found in the membrane. No layered micelle structure was observed around the edges of these washed membrane vesicles (Fig. 11). Subunits of the membrane consistently showed a diameter of 50-60 Å. Thin sectioning and freeze-etched micrographs of this reconstituted membranous cytochrome oxidase are shown in Figs 12 and 13. The dimension of the membrane is 50 to 60 Å.

Lipid Specificity in Membrane Formation

Mixed total mitochondrial phospholipids could reactivate the lipid-depleted cytochrome oxidase and reconstitute it to a membrane form as indicated above. Purified mitochondrial phospholipids, e.g. phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin, have been shown to have the same capability as mixed total phospholipids to

form membrane vesicles [21]. Figure 14 shows the membrane structure of phosphatidyl choline-cytochrome oxidase membrane. It is indistinguishable from the mixed total phospholipids reconstituted cytochrome oxidase membranes. Experiments were designed to test whether hydrophobic or hydrophilic groups of phospholipids are mainly involved in the lipid-protein interaction in membrane formation.

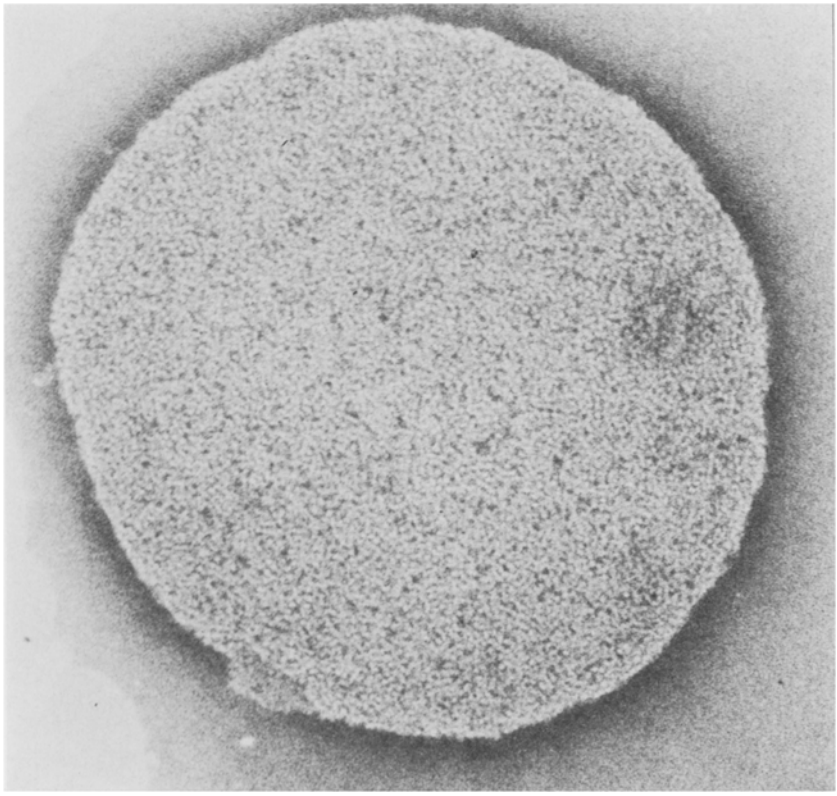


Figure 11. Reconstituted membranous cytochrome oxidase. Negatively stained with phosphotungstic acid. x 160,000.

Phosphatidyl choline of the reconstituted membranous phosphatidyl choline-cytochrome oxidase was digested by *Clostridium welchii* phospholipase C to release the ionic group of the phospholipid, phosphoryl choline. In spite of the removal of 80% of the lipid phosphorus, it is indistinguishable from the original undigested membrane in negatively stained electron micrographs. The digested

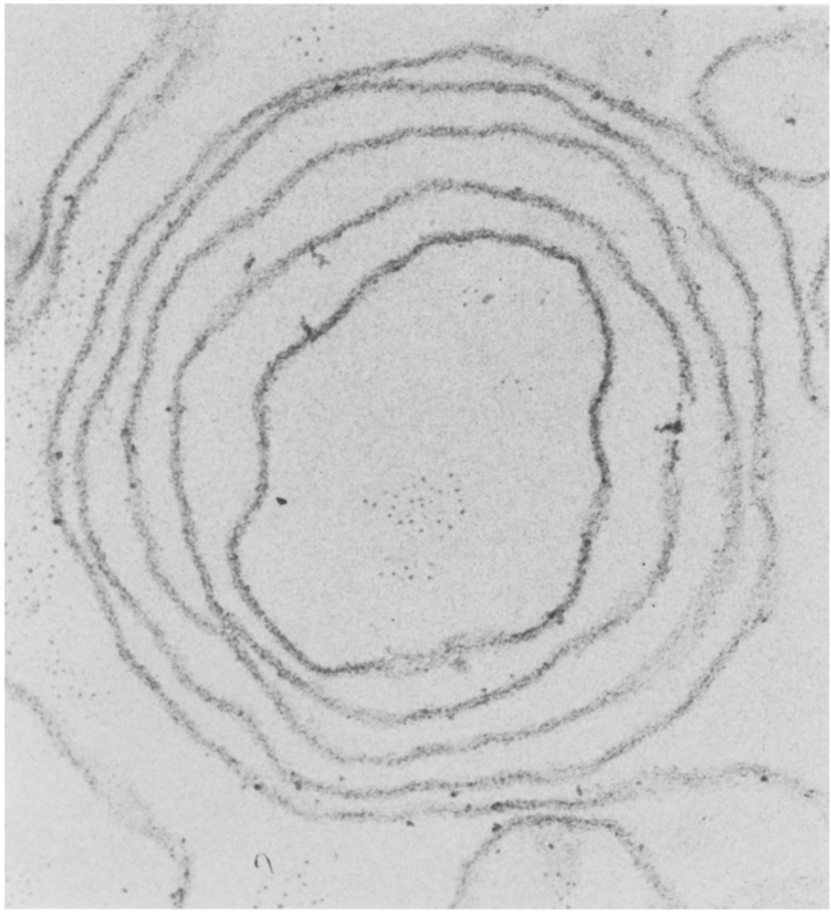


Figure 12. Thin section of the reconstituted membranous cytochrome oxidase. x 160,000. Photo by Dr. J. D. Hall.

complex still retained its "unit" membrane structure as seen in thin sectioning shown in Fig. 15. The membranes, however, were no longer in the vesicular form. Figure 16 shows that membranes can be formed by combination of the same enzyme protein and diolein, which has the hydrophobic part of phosphatidyl choline but no ionic group, by the same method as described for phospholipids. No membrane vesicles were seen but the complex was shown to be "unit" membrane. A control section showed that diolein alone is not in membrane form (Fig. 17).

Hydrogenated mixed phospholipids or phosphatidyl choline, prepared by exposure of lipid to hydrogen gas in the presence of platinum oxide

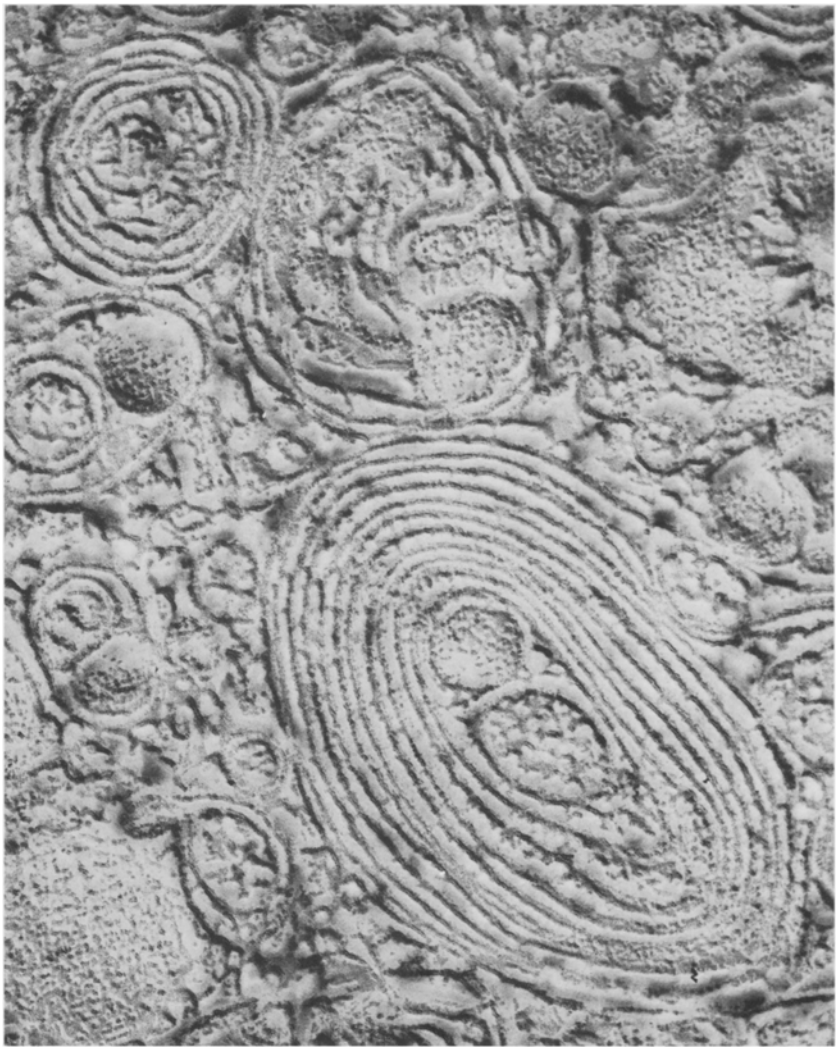


Figure 13. Freeze-etch micrograph of the reconstituted membranous cytochrome oxidase. Sample was frozen on a copper disk and fractured in a freezing microtome as described by Moor and Mühlethaler [57]. x 120,000. Photo by Dr. J. D. Hall.

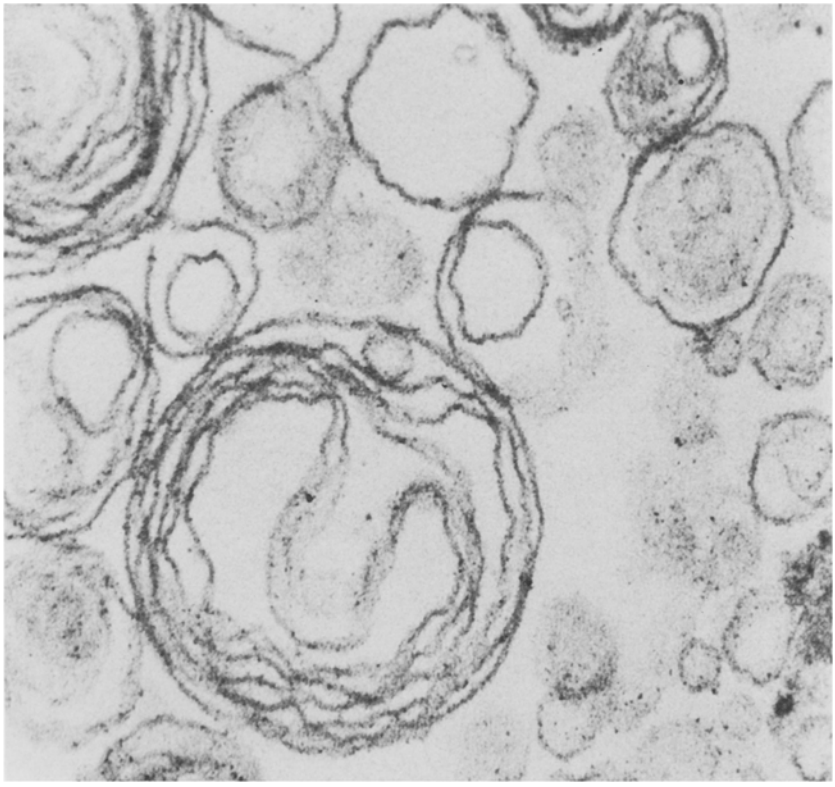


Figure 14. Thin section of phosphatidyl choline-cytochrome oxidase membrane. x 108,000. Photo by Mrs. L. Maculay.

does not yield membrane structure with cytochrome oxidase at all as seen by negative staining and sectioning techniques (Fig. 18).

Though some detergents (e.g. Emasol, Tween) can activate the lipid-depleted cytochrome oxidase to the same level as mixed phospholipids can, only phospholipids can form membrane vesicles with cytochrome oxidase. No membrane or aggregation could be seen in the detergent treated lipid-depleted cytochrome oxidase.

Protein Specificity in Membrane Formation

Denatured proteins might be expected to form membranes with phospholipids because of exposure of more hydrophobic regions on the

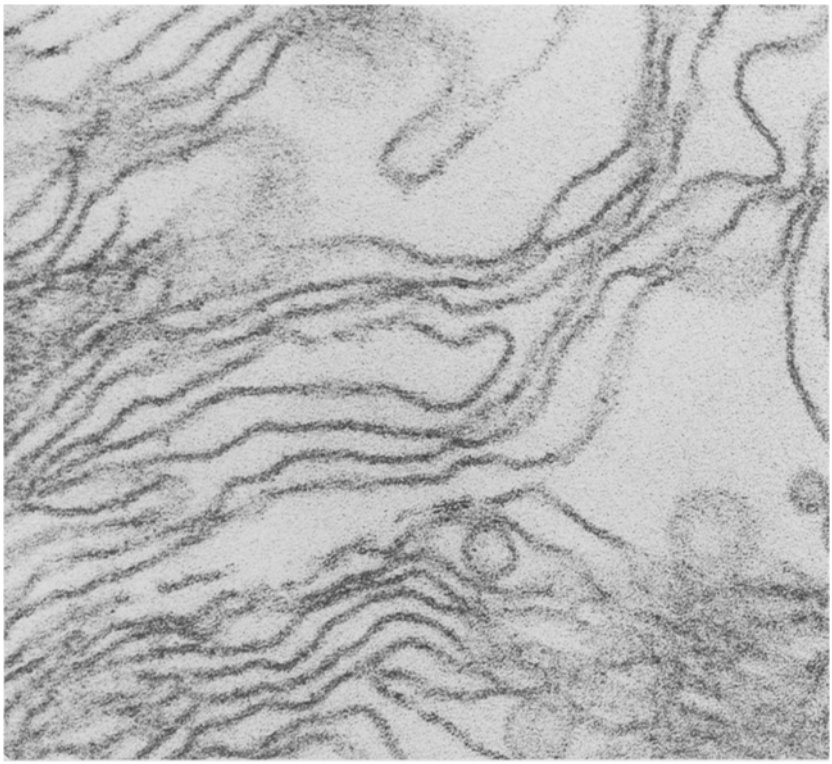


Figure 15. Phospholipase C digested phosphatidyl choline-cytochrome oxidase membrane. Reconstituted membrane phosphatidyl choline was digested at 30° by 200 μg of *Clostridium welchii* phospholipase C per mg of cytochrome oxidase membrane protein for 80 min in the presence of 2 mM $CaCl_2$. Phospholipase C specifically removes the ionic group of the phosphoryl choline. x 140,000.

denatured protein. Bovine serum albumin and cytochrome oxidase were denatured by heat treatment in boiling water for 10 min and then mixed with phospholipids. Membrane formation was not observed with either denatured protein. Phosphorus analysis disclosed that there was no phospholipid bound to the heat denatured cytochrome oxidase. Chloroform-methanol-ammonia extracted cytochrome oxidase can bind phospholipids, but the resultant complex does not have membranous structure.

Cytochrome oxidase can be separated into two protein fractions by detergent treatment in the presence of reducing agents. One fraction contains a large protein (55,000 MW) which has no cytochrome or copper. The other fraction contains three low molecular weight proteins

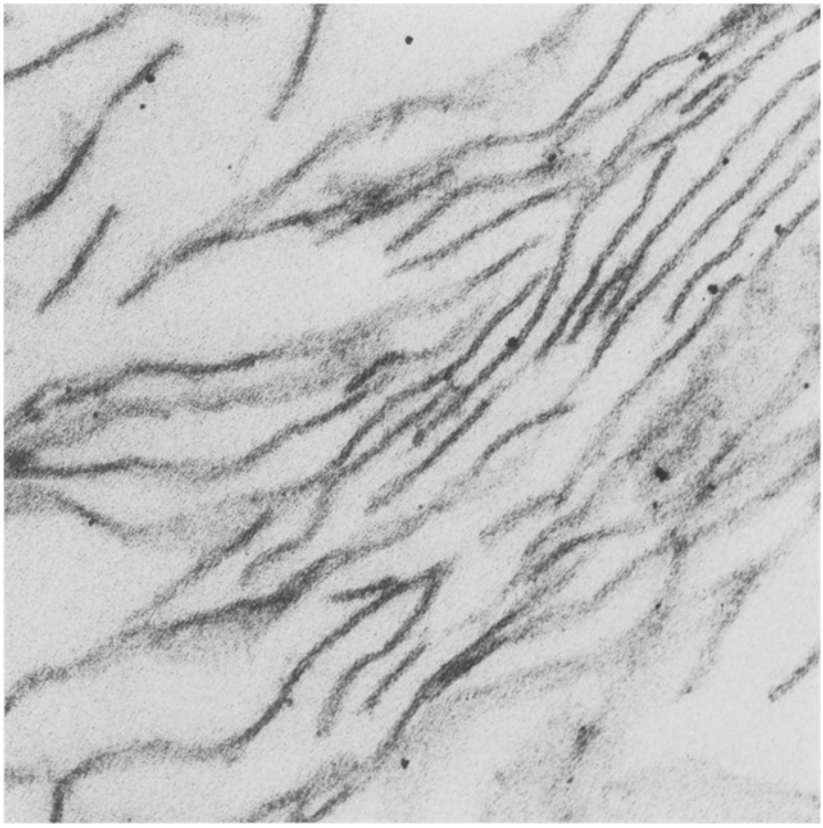


Figure 16. Thin section of diolein-cytochrome oxidase membrane Diolein-cytochrome oxidase was prepared by the same method as the reconstituted membranous cytochrome oxidase. x 160,000.

(20,000; 15,000; 12,000 MW) and contains cytochrome *a* copper and cardiolipin. Only the cytochrome containing fraction forms membrane with phospholipid which looks like the cytochrome oxidase membrane shown in Fig. 8 except that the white spots are smaller (30-50 Å). The 55,000 MW protein forms large globular lumps with phospholipid which do not have membrane structure.

Effect of pH, Ionic Strength, Detergents and Some Other Agents on Membrane Formation

Breakdown or formation of membranes can be revealed by following the turbidity change. The lipid-depleted cytochrome oxidase at the

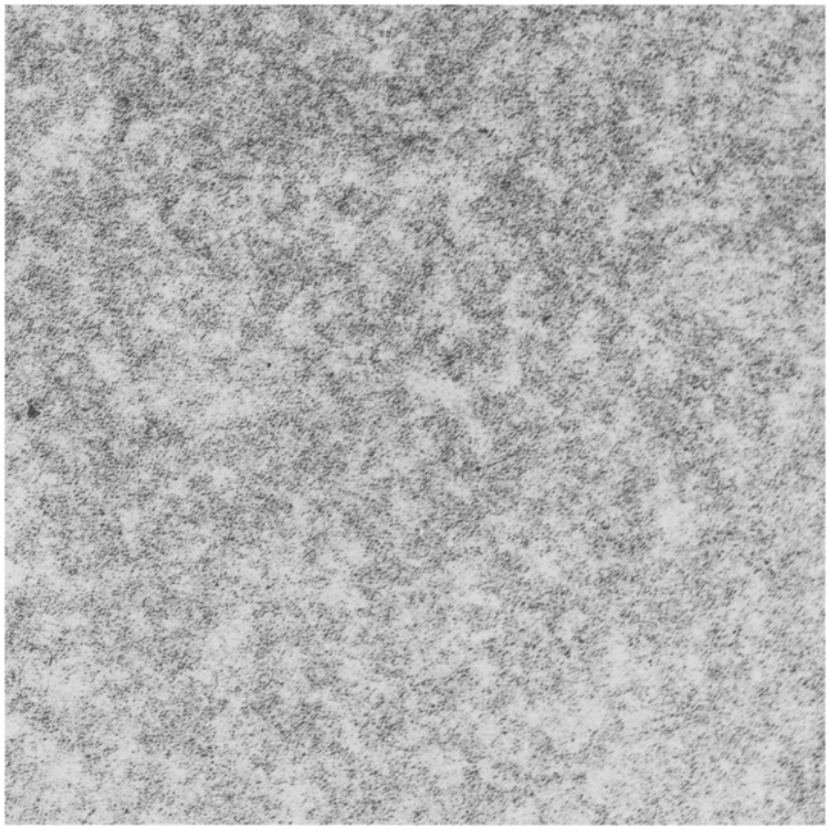


Figure 17. Thin section of diolein. Note lack of membrane structure. x 160,000.

concentration of 1 mg/ml gave an optical density of 0.15-0.20 at 520 mμ. Turbidity of the protein solution increased rapidly upon addition of phospholipids. Figure 19 shows the effect of pH of the enzyme solution on membrane formation of cytochrome oxidase with phospholipids. Final concentration of cytochrome oxidase was adjusted to 1 mg/ml and pH was adjusted with slow addition of diluted HCl or KOH. After incubating for 5 min at 23°, 14 μg phosphorus of mixed mitochondrial phospholipids was added. Readings were taken after 30 min incubation at 23°. Highest turbidity was obtained at pH 4.2. Membrane formation was inhibited at pH's lower than 3 or higher than 10. Cytochrome oxidase itself or phospholipids alone, however, at the same pH gave OD of 0.08 and 0.28 respectively. Upon addition of the component, phospholipids or the enzyme, the OD increased to the

amount indicated in Fig. 19. The pH 4.2 treated cytochrome oxidase still had 80% of the untreated enzyme activity after readjusted back to neutral pH.

The effect of ionic strength was evaluated in the similar manner. No matter whether Tris-HCl or potassium chloride was used, the optimal ionic strength for membrane formation was found at $\mu = 0.1$ (Fig. 20).

TABLE II. Effect of KCN, CO and dithionite on membrane formation

	Optical density 520 mμ
Oxidase (1 mg/ml)	0.22
PLP (30 μg P/ml)	0.05
Oxidase + PLP	1.12
Oxidase + KCN (1 m*M*)	0.18
Oxidase + KCN + PLP	0.99
Oxidase + carbon monoxide	0.80
Oxidase + CO + PLP	1.18
Oxidase + $Na_2S_2O_4$	0.82
Oxidase + $Na_2S_2O_4$ + PLP	1.16

Readings were taken after 30 min incubation with PLP. Approximately 0.7 mg Triton/mg protein in the system. Carbon monoxide was bubbled through enzyme for 2 min.

Table 2 shows the effect of cyanide, carbon monoxide and dithionite on membrane formation. None of these compounds inhibited the interaction of phospholipids and cytochrome oxidase, although carbon monoxide and dithionite increased turbidity of the enzyme to some extent before addition of phospholipids.

Solubilization of membranous cytochrome oxidase and turbid detergent-free, lipid-depleted cytochrome oxidase (small fragments of "unit" membrane structure, see Fig. 5) by some agents is summarized in Table 3. One percent deoxycholate or 8 M urea solubilized 43% and 59% of the membranous cytochrome oxidase, respectively, and 19% and 47% of the detergent-free, lipid-depleted preparation, respectively, while sodium dodecyl sulfate solubilized both to the same extent. On the other hand, Triton X100 either with or without KCl was a better solubilizing agent for the lipid-depleted membrane than phospholipids containing membranes.

Figure 21 shows solubilization of two type of membranes by pH. High optical density was obtained at pH 4.5 for phospholipids-containing reconstituted membranous cytochrome oxidase and at pH 8.1 for the lipid-depleted membrane enzyme. Effect of pH on the phospholipids-containing reconstituted membranous enzyme shows a pattern very

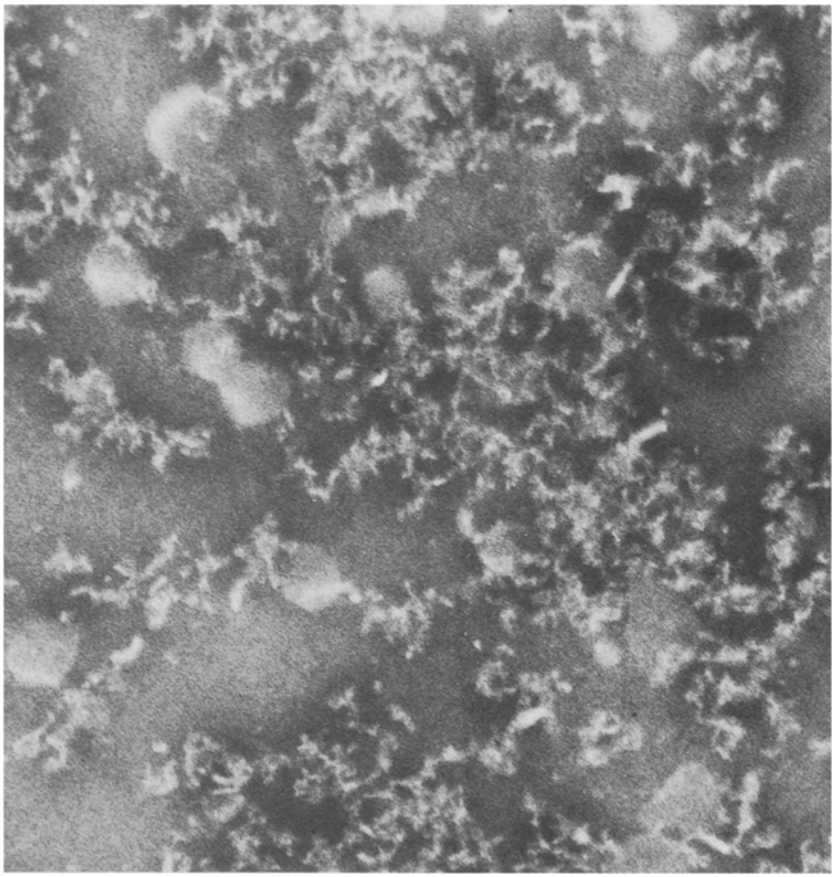


Figure 18. Hydrogenated phospholipid-cytochrome oxidase complex.
a. negatively stained, x 160,000.
b. thin section, x 160,000.

similar to that observed in membrane formation study (Fig. 19). Both acidic (lower than pH 3.0) and alkaline pH (higher than 12.0) could dissolve both membranes.

Fluorescence Hydrophobic Probe

The dye ANS (8-anilo-1-naphthalene-sulfonic acid) has been employed as an indicator of conformational changes in proteins where exposure of hydrophobic groups to the dye results in an enhancement of

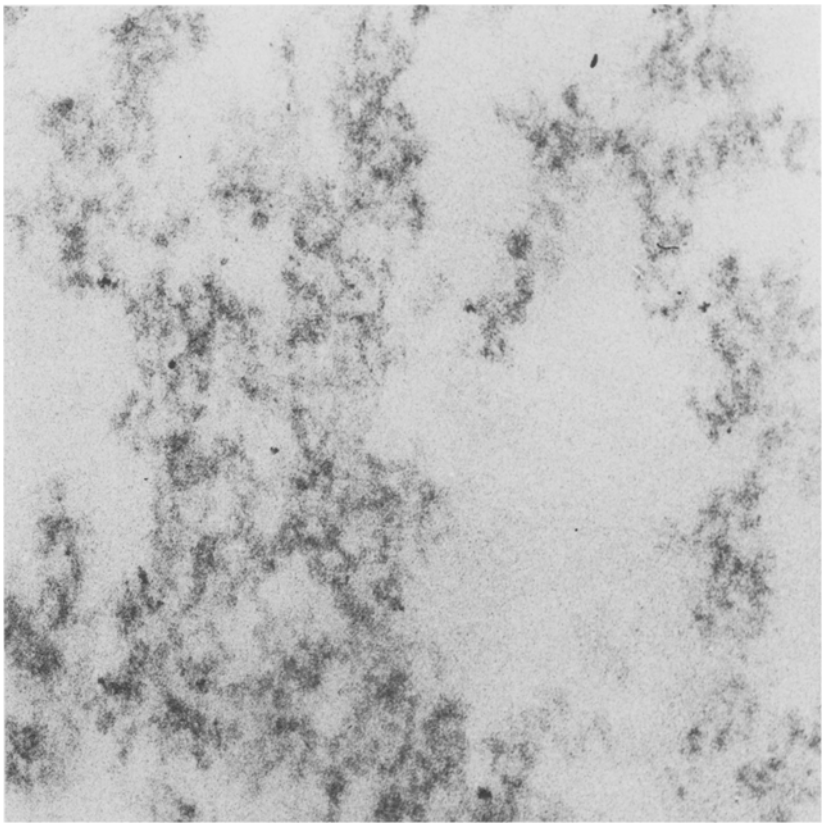


Fig. 18b.

fluorescence [22, 23]. It is not fluorescent in water but becomes fluorescent in such hydrophobic environments as organic solvents and the nonpolar regions of proteins [22, 24, 25]. ANS was used in this study to reveal hydrophobic environment changes when cytochrome oxidase interacted with phospholipids to form membranes.

Table 4 summarizes the fluorescence of ANS in solution containing cytochrome oxidase protein, phospholipids or ethanol at 485 mμ with excitation at 380 mμ. Cytochrome oxidase protein or phospholipids alone gave fluorescence values of 81 and 30, respectively. Upon addition of phospholipids to enzyme one would expect an increase of fluorescence if hydrophobic contribution from phospholipids and the enzyme to the environment is the sum of each component. ANS, in the presence of both phospholipids and the enzyme, however, only gave

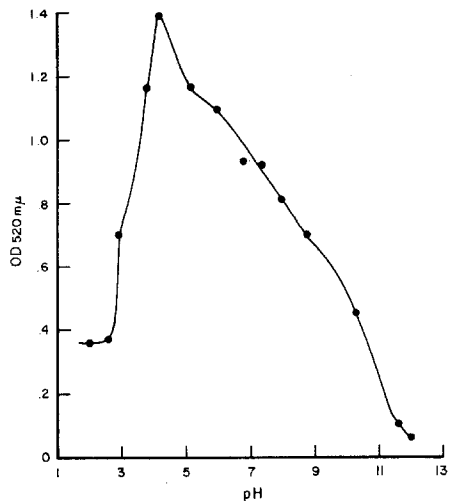


Figure 19. Effect of pH on phospholipids-cytochrome oxidase interaction in membrane formation. Concentration of lipid-depleted cytochrome oxidase was adjusted to 1 mg/ml and pH was adjusted with slow addition of 0.5 N HCl or KOH. After incubating the enzyme for 5 min at 23°, 14 μg phospholipid phosphorus was added to 1 ml of the enzyme solution. Readings were taken after 30 min at 23°.

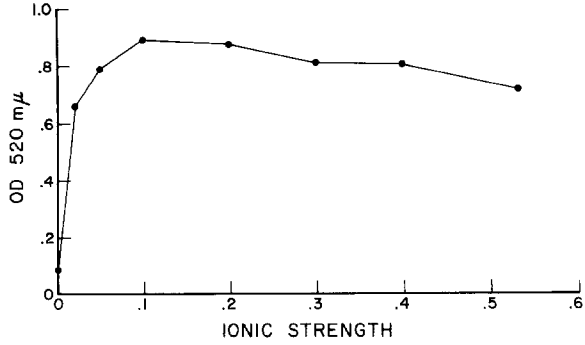


Figure 20. Effect of ionic strength on phospholipids-cytochrome oxidase interaction in membrane formation. Lipid-depleted cytochrome oxidase was adjusted to protein concentration of 1 mg/ml at different ionic strengths (adjusted with Tris-HCl, pH 7.4). OD readings were taken at 30 min at room temperature after addition of 14 μg phospholipid phosphorus to 1 ml enzyme.

TABLE III. Solubilization of particulate cytochrome oxidase

Agents	Percentage solubilized	
	Membranous cytochrome oxidase (1 mg/ml)	Detergent-free lipid-depleted cytochrome oxidase (0.84 mg/ml)
Emasol-1130		
0.05%	0	1.0
0.1%	2.1	4.6
0.5%	6	12
1.0%	8	14
Deoxycholate		
0.15%	–	1
0.1%	15	9
0.5%	27	13
1.0%	43	19
Sodium dodecyl sulfate		
0.005%	45	1
0.01%	51	18
0.05%	89	93
0.10%	93	94
Triton X100		
0.05%	14	–
0.13%	16	75
1.0%	55	84
Triton X100 (0.13%) plus KCl		
0.1 *M*	25	87
0.5 *M*	76	89
1.0 *M*	76	89
KCl		
0.1 *M*	0	0
0.5 *M*	14	3
1.0 *M*	14	6
EDTA		
0.05 *M*	0	0
0.1 *M*	0	1
UREA		
1 *M*	12	3
4 *M*	47	24
8 *M*	59	47
Guanidine HCl		
0.5 *M*	0	6

Percentage solubilized was calculated as $\frac{\text{OD of control} - \text{OD of experiment}}{\text{OD of control}}$ where the same amount of protein in 0.02 *M* Tris HCl was the control.

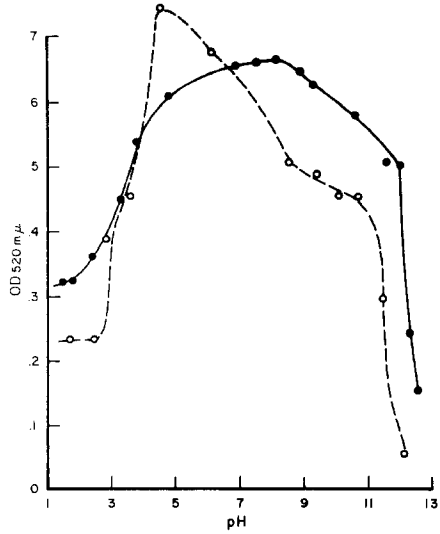


Figure 21. Solubilization of cytochrome oxidase membranes by pH treatment. Detergent-free, lipid-depleted cytochrome oxidase (heavy circles) was adjusted to protein concentration of 0.42 mg/ml with various pH treatment (with 0.5 N KOH or HCl). Reconstituted membranous cytochrome oxidase (open circles) was tested in the same manner at protein concentration of 0.8 mg/ml.

TABLE IV. The effect of protein, phospholipid or ethanol on ANS fluorescence

Sample	Arbitrary fluorescence units
Lipid-depleted cytochrome oxidase (0.46 mg/ml)	81.0
PLP (15 μg P/ml)	30.0
Lipid-depleted cytochrome oxidase + PLP	61.5
Ethanol (50%)	24.2
Ethanol (94%)	245.0
ANS in buffer	0

The experiment was carried out in 0.02 *M* Tris-HCl, pH 7.4 at 25°. Concentration of ANS (8-anilino-1-naphthalene-sulfonic acid) was 0.05 m*M*. Fluorescence of mixture of cytochrome oxidase and PLP was taken at 10 min after addition of PLP to the enzyme. The excitation was 380 mμ, and the emission was 485 mμ. Fluorescence units were taken arbitrarily.

fluorescence value of 61.5 which was even less than that induced by the protein alone.

Figure 22 shows fluorescence of ANS in a fixed amount of the lipid-depleted cytochrome oxidase with addition of increasing amount of phospholipids. Fluorescence decreased upon addition of phospholipids and then slowly increased with higher amounts of phospholipids. The control experiments, which are also shown in Fig. 22, eliminated the possibility of the decrease of ANS fluorescence was due to interaction of Triton and phospholipids instead of the protein-phospholipids interaction in question. Phospholipids increased ANS fluorescence linearly in the absence of protein. These were completely different patterns from that obtained by interaction of cytochrome oxidase and phospholipids. When a fixed amount of phospholipid was added to increasing amounts of cytochrome oxidase, fluorescence increase was also less than in the absence of phospholipids (Fig. 23). These data indicate that either protein conformation is changed or hydrophobic groups of proteins are involved in the interaction with phospholipids.

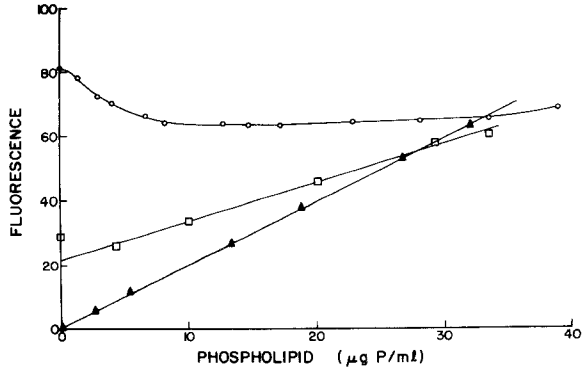


Figure 22. Effect of phospholipids on the fluorescence of ANS. Circles: phospholipids added to 0.46 mg of the lipid-depleted cytochrome oxidase in a total volume of 1 ml. Square: phospholipids added to 0.30 mg Triton X100 in a total volume of 1 ml which is equivalent to the Triton concentration of the lipid-depleted cytochrome oxidase. Triangles: phospholipids added to 0.02 M Tris HCl, pH 7.4. Experimental condition was same as Table 4.

Infrared Spectroscopy

Infrared spectroscopy has been employed to provide information about protein conformation in certain membranes, including myelin [26, 27], erythrocyte [26, 28, 29], plasma membranes and endoplasmic reticulum of Ehrlich ascites carcinoma [30, 31] and mitochondrial membranes [32, 33].

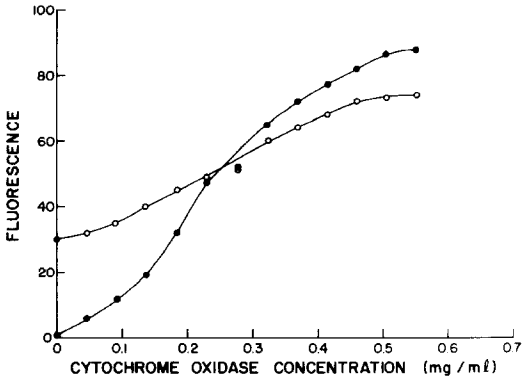


Figure 23. Effect of cytochrome oxidase on the fluorescence of ANS. Heavy circles: cytochrome oxidase added to buffer containing ANS. Open circles: cytochrome oxidase added to 15 μg phospholipids phosphorus in the total volume of 1 ml containing ANS. Experimental condition was same as Table 4.

The infrared spectrum of the reconstituted membranous cytochrome oxidase is shown in Fig. 24. The peak at 1652 cm^{-1} corresponds to the C=O stretching frequency of the amide I band which is associated with an α-helical or random coil conformation, or both, while that at 1630 cm^{-1} is correlated with the β-configuration [34, 35]. The amide II band at about 1535 cm^{-1} does not allow distinction between the α- and β-conformations. The spectrum of the reconstituted membranous cytochrome oxidase does not show appreciable difference from that of ETP (Fig. 26). Lipid extraction of both membrane films abolishes the band at 1740^{-1} due to C=O stretching frequency in fatty acid esters (Figs 25 and 27).

The absorption band at 720 cm^{-1} has been assigned to the main methylene (CH_2) rocking mode [36]. Presence or absence of the 720 cm^{-1} band in the spectra of biological membranes thus provides an indication of the organization of the lipid chains. Comparing spectra of membranous cytochrome oxidase (Fig. 24), ETP (Fig. 26) and mixed total mitochondrial phospholipids (Fig. 28), which was used in the reconstitution of the cytochrome oxidase membranes, one can see a peak at 720 cm^{-1} in the phospholipid spectrum but not in cytochrome oxidase or in ETP membranes. The frequencies associated with the phosphate group at about 1225 cm^{-1} and about 1060 cm^{-1} are present in both phospholipids and membranes. Extraction of membranes with chloroform-methanol (2:1, v/v) causes the disappearance of these two phosphate group frequencies as well as 1740 cm^{-1} band.

The infrared spectrum of the detergent-free, lipid-depleted cytochrome oxidase is indistinguishable from that of solvent extracted membranes. It also lacks all bands of lipids.

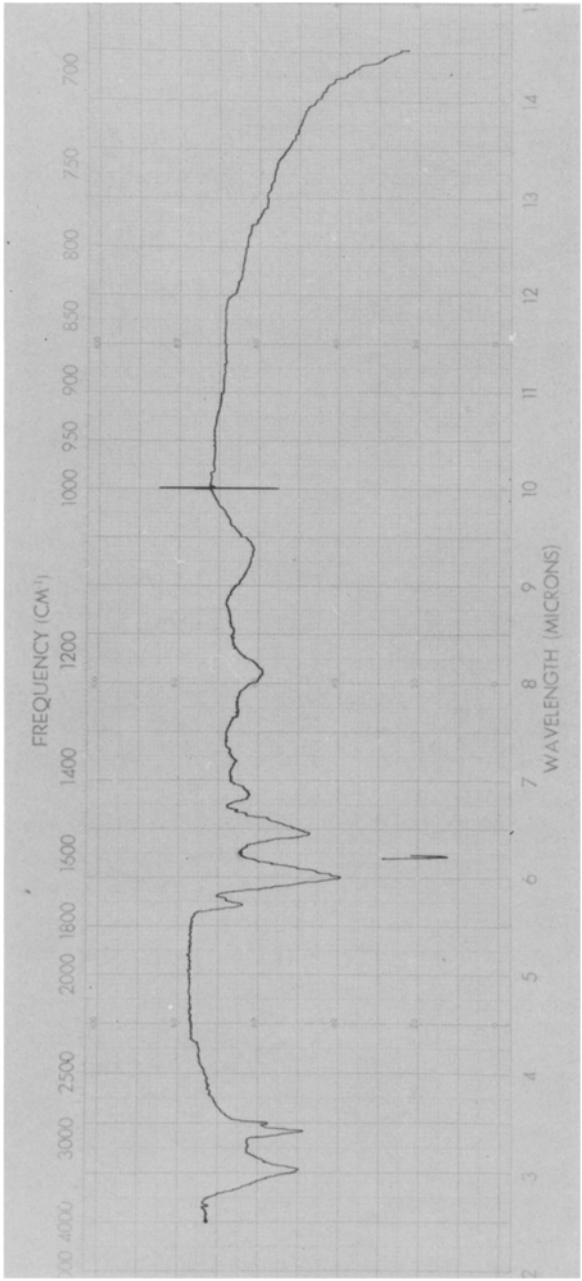


Figure 24. Infrared spectrum of reconstituted membranous cytochrome oxidase.

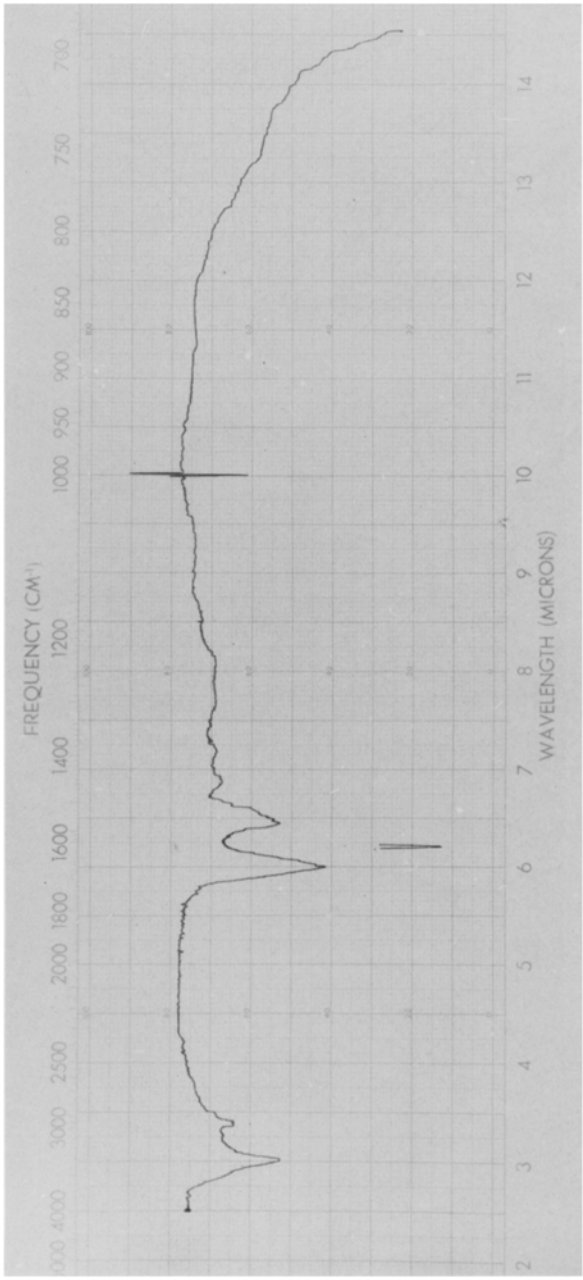


Figure 25. Infrared spectrum of solvent extracted reconstituted membranous cytochrome oxidase.

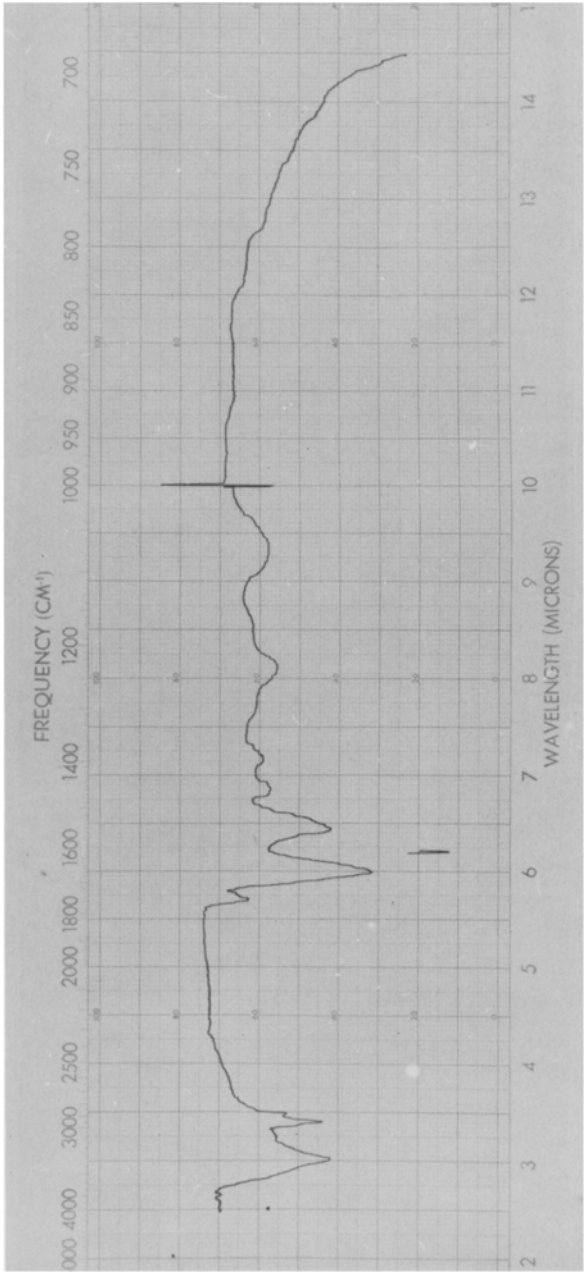


Figure 26. Infrared spectrum of electron transport particles (ETP).

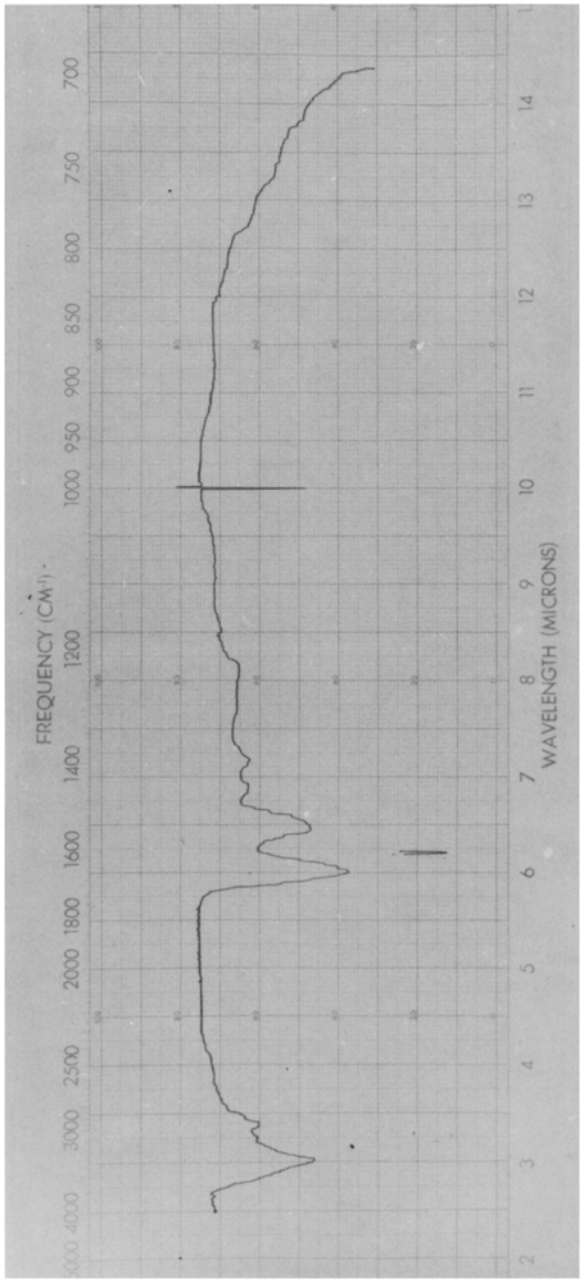


Figure 27. Infrared spectrum of solvent extracted electron transport particles.

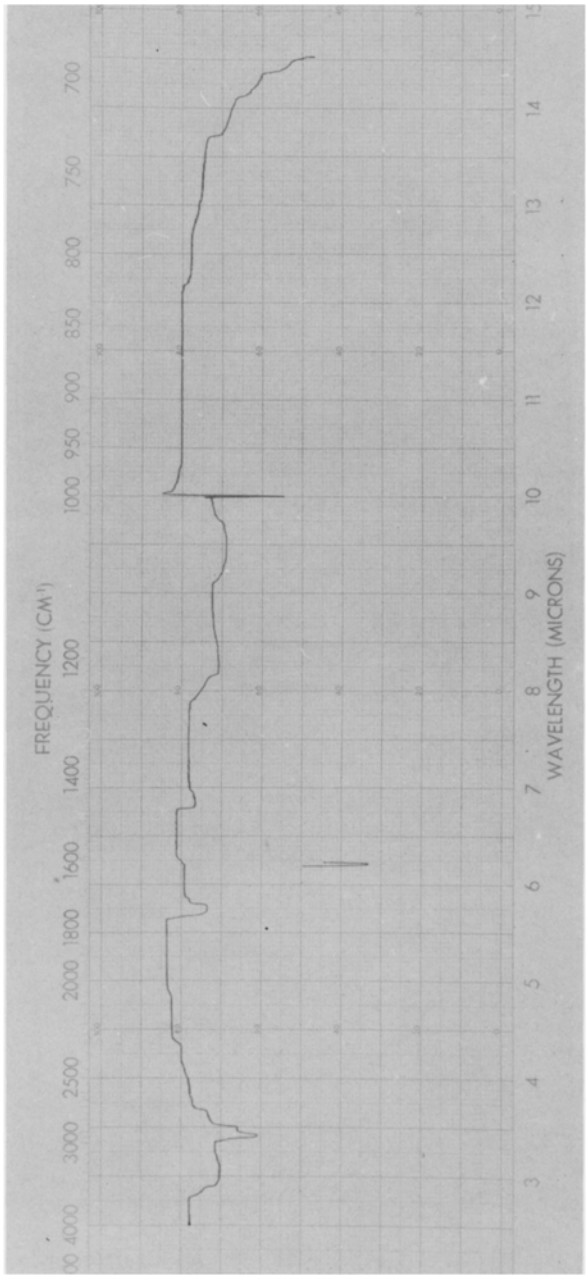


Figure 28. Infrared spectrum of mixed mitochondria phospholipids.

Circular Dichroism

The cotton effects due to the peptide bond in the ultraviolet region, as studied by optical rotatory dispersion (ORD) and circular dichroism (CD) have recently been proved extremely valuable in characterizing the molecular conformation of polypeptides and soluble proteins [30, 37, 38]. The characteristic circular dichroism values for an α-helix are negative extrema at 222 mμ and 208 mμ, a crossover at 201 mμ, and a positive peak at 191 mμ. These values are shifted to higher wavelengths in turbid suspensions [60]. Figure 29 contains circular dichroism curves

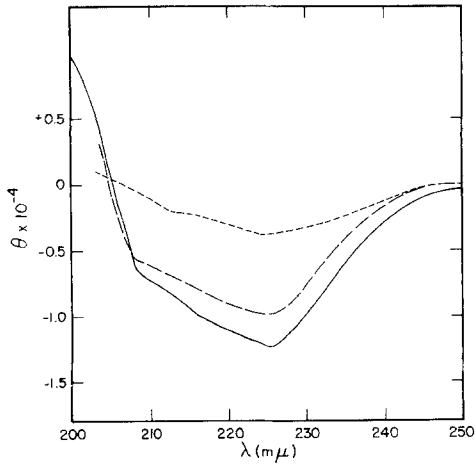


Figure 29. Circular dichroism of membranous and lipid-free oxidase. – – – – – –, detergent-free, lipid-depleted cytochrome oxidase; —— —— reconstituted membranous cytochrome oxidase; ———————— ETP.

for the reconstituted membranous cytochrome oxidase, the detergent-free, lipid-depleted cytochrome oxidase and ETP. The negative extrema of long wavelength for both the reconstituted cytochrome oxidase membranes and ETP membranes are at 225 mμ while that for the lipid-depleted enzyme is at 224 mμ. The circular dichroism curves of the lipid-depleted and the membranous cytochrome oxidase, and ETP membranes exhibit features qualitatively similar to those reported for red blood cell membranes [37], Ehrlich ascites carcinoma membranes [30], and mitochondrial and submitochondrial particles [39], namely, a characteristic α-helix curve shifted to longer wavelengths. The detergent-free, lipid-depleted cytochrome oxidase exhibits a decreased dichroism from that of membranous enzyme and ETP, and the negative extremes occur at 224 mμ and 215 mμ.

Discussion

The capability of cytochrome oxidase to form membrane was demonstrated earlier by McConnell *et al.* [40] and Jacobs *et al.* [56]. Sun *et al.* [7] discussed the relationship between ionic strength and membrane formation of the enzyme. Green and his associates [1] proposed that protein forms a bulk phase in the absence of phospholipids and the lipid-free protein organizes to form membranes after reintroduction of phospholipids. The lipid-depleted cytochrome oxidase which has only one to two molecules of cardiolipin per molecule of cytochrome oxidase apears as 90 Å particles. Extensive washing and diethyl ether extraction remove essentially all Triton from the lipid-depleted cytochrome oxidase (0-0.03 mg Triton/mg protein was detected). Negatively stained samples of these preparations show aggregation of 90 Å particles while thin section electron micrographs show 500-1000 Å long "unit" membranes with 75-100 Å in thickness. This demonstrates a reaggregation of a solubilized enzyme at extremely low lipid content (1.3-1.8 μg P/mg Protein) to form a "unit" membrane structure. Grula *et al.* [41] solubilized acetone-ammonia-water or methanol extracted plasma membranes of *Micrococcus lysodeikticus* with treatment of sodium dodecyl sulfate. These "stripped subunits" reaggregated into membranous sheets that also possess a three-layered "unit" membrane structure.

Upon addition of phospholipid micelles to the regular lipid-depleted cytochrome oxidase a structural transformation parallel with increase of enzyme activity is observed. The 90 Å enzyme particles or rod-like assemblies swell to approximately 200 Å in width and 50-60 Å protein particles are loosely packed into a sheet of membrane upon addition of phospholipids. Further addition of phospholipid micelles allows a more even dispersion of the enzyme in the form of mosaic membrane vesicles with 50-60 Å globules. Once the phospholipids required for membrane formation and maximal activity is reached, further addition of phospholipids does not increase the activity. With the addition of excess phospholipids to the membrane protein more than required for the membrane formation, phospholipid micelles can be seen around the edge of membrane vesicles and concomitantly the excess phospholipids also spread over the membrane surface. Consequently, the globular particles in the membrane take on a smaller size (30-50 Å) compared to the regular 50-60 Å particles.

Sonication facilitates the interaction of cytochrome oxidase protein and phospholipids to form membranes. After removal of unbound phospholipids and free cytochrome oxidase, a constant value of 60-65 g Atom phosphorus/mole cytochrome oxidase is obtained. This membrane represents a tightly packed structure as is found in the membranous form prepared by the Triton X114 procedure (Triton X114 membranous

cytochrome oxidase). Negatively stained electron micrographs of both Triton X114 membranous and reconstituted membranous cytochrome oxidase preparations show 50-60 Å spots on the surface. It is likely the Triton X114 membranous enzyme still bears some relation to the structure of native cytochrome oxidase in mitochondria because it is obtained before the solubilization of the enzyme. The reconstituted membranous cytochrome oxidase represents a reconstructed membrane structure similar in appearance to the original cytochrome oxidase membranes. Both thin sectioned and freeze etched micrographs of these two membranous preparations show 50-60 Å cross section.

The 90Å particles characteristic of the lipid-depleted enzyme are equivalent to 290,000 daltons if the protein density is assumed as 1.25. Based on the molecular weight of 72,000 for a monomer of cytochrome oxidase [12] the lipid-depleted cytochrome oxidase may exist as a tetramer. Seki and Oda [42] suggested the ellipsoid particles, measuring about 80-90 Å in diameter, which they observed in Okunuki's preparation [43] were the main and smallest structural component of the highly active preparation of cytochrome oxidase (the "unit particle of cytochrome oxidase" or "UPCO") and they were the organizing unit of polymers. They concluded that the 80-90 Å particles are the smallest unit of active cytochrome oxidase. Our lipid-depleted cytochrome oxidase prepared by the Triton method also shows 90 Å particles, but they are not active. Treatment of the lipid-depleted cytochrome oxidase with Emasol does not change the 90 Å particles to any measurable extent but they are active (this is equivalent to Okunuki's preparation which is solubilized in Emasol). Seki and Oda [42] also suggested that the particles observed in the green membrane was a subunit of the "UPCO" and that two such particles fused to form a "UPCO". However, the lipid titration study on the cytochrome oxidase structure seems to indicate that the 90 Å globules swell up to a size of 200 Å and more than two 50 Åparticles appear on dissociation of one of the original 90 Å particles. Ball *et al.* [44] suggested that cytochrome oxidase might be a tetrapolymer consisting of four identical hemoprotein molecules. Takemori *et al.* [45] also suggested that their Emasol solubilized cytochrome *a* was a pentamer based on sedimentation experiments. After cytochrome oxidase is reorganized into membrane, the globule size appears to be 50-60 Å on the negative staining surface and 50-55 Å in thickness in thin section which is equivalent to the molecular weight of 50,000-85,000. This value agrees with the molecular weight of 72,000 suggested by Criddle and Bock [12]. Takemori *et al.* [45] suggested that cytochrome oxidase existed as a monomer with only one heme group per molecule in mitochondrial particles. It tended to polymerize after extraction from mitochondria with bile salt and purification with ammonium sulfate. Similar polymerization probably also occurs in the lipid-depleted preparation and "depolymerization" occurs again during

reconstitution. The monomer or the 50-55 Å subunits may be a complex of the 3 heme *a* proteins (MW 23,000, 17,000, 12,000) and core protein IV (MW 55,000) [10, 58, 59].

Solubilization of membranous cytochrome oxidase and reconstitution of the membrane from the dispersed soluble lipid-depleted enzyme are diagrammatically summarized in Fig. 30. The protein particles (50 Å, monomer units) of the membranous cytochrome oxidase (the membranous cytochrome oxidase) form tetramers when the enzyme becomes soluble and lipids are removed (the lipid-depleted cytochrome oxidase).

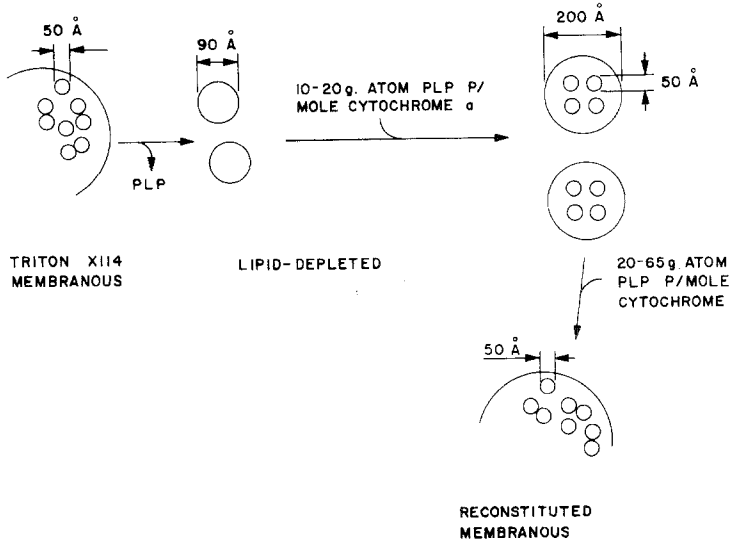


Figure 30. Diagram of solubilization and reconstitution of cytochrome oxidase membranes.

These tetramers swell up to a larger sheet upon addition of phospholipids and "dissociate to 50 Å subunits (the reconstituted membranous cytochrome oxidase).

Earlier investigators [46] claimed that phospholipids were not specific activators of cytochrome oxidase because they could simply be replaced by Emasol or Tween. Though Emasol can activate the lipid-depleted cytochrome oxidase to the same level as phospholipids can, it does not form membrane structure with cytochrome oxidase. Both Emasol and phospholipids decrease the Km for the cytochrome oxidase to a similar degree and kinetically they exhibit the same pattern of effects on cytochrome oxidase [47], but Emasol does not play a structural role as phospholipids do. None of the detergents (Emasol, Tween, deoxy-

cholate, cholate, sodium dodecyl sulfate or Triton), lysophosphatides, or fatty acids form membrane with cytochrome oxidase.

Purified phospholipids, i.e. phosphatidyl choline, phosphatidyl ethanolamine and cardiolipin are able to reconstitute membrane structure when combined with lipid-depleted cytochrome oxidase as well as the mixed total mitochondrial phospholipids. Well characterized purified membrane protein plus purified phospholipid make the phosphatidyl choline-cytochrome oxidase membrane suitable for study as a model membrane. Effects of various phospholipases on phosphatidyl choline have been well established. Removal of some groups from this lipid in the membrane can be used to indicate which group is involved in interaction. The structure of the membranous vesicles of the phosphatidyl choline-cytochrome oxidase complex appears to be the same as the structure of the reconstituted mixed phospholipids-cytochrome oxidase complex. Digestion of this membrane phosphatidyl choline by phospholipase C results in release of phosphoryl choline, the ionic group of the lipid, into solution. In spite of the removal of 80% of the ionic groups of the lipid by the action of phospholipase C, electron micrographs of the negatively stained complex do not show any difference from undigested control phosphatidyl choline-cytochrome oxidase membrane. Lenard and Singer [48] also reported that treatment by phospholipase C to remove 69 to 74% of the ionic groups from phospholipids of red blood cell, did not affect the membrane structure as evidenced by phase microscopy and circular dichroism measurement. The digested phosphatidyl choline-cytochrome oxidase complex of this study still retains its triple layers of so-called "unit-membrane" appearance in thin section. However, the membrane vesicles are broken and membranes are elongated and cross fused as seen in thin section. The fact that ionic groups of phospholipids are not necessary for membrane formation can further be seen by combination of diglyceride with cytochrome oxidase. Combination of cytochrome oxidase with diolein, a lipid which has fatty acid chains similar to phosphatidyl choline but no ionic group, results in a membrane structure. These membranes are not in vesicular form but have the appearance of "unit membrane". Green and Tzagoloff [1] suggested that lipid may determine the curvature of the plane of association of membranes, which could account for the formation of spherical (vesicular) structures. The formation of spherical structure or vesicular apearance might be facilitated by the ionic groups of phospholipids.

Without ionic groups lipid is still able to form membrane-like structure with protein (in the instance of diolein). In other words, the hydrophobic regions of fatty acid chains are required for membrane formation. Investigation was further done to determine whether unsaturation of fatty acid chains is essential for membrane formation. Incapability of membrane formation of hydrogenated phospholipids

with cytochrome oxidase supports the concept [49] that unsaturation of fatty acid chains of phospholipids is essential for membrane formation.

It has been proposed that lipid is bound to protein in membranes predominantly through hydrophobic interactions [50]. Effects of organic solvents and detergents on the extraction of lipids and solubilization of membrane proteins constitute strong evidence for hydrophobic bonding between these membrane components. Theoretical work of Scheraga *et al.* [51] indicates that ionic and hydrogen bonds cannot provide sufficient energy to stabilize the specific conformations known to exist for proteins. However, apolar or hydrophobic bonds can provide this energy, the driving force coming from the ordering of water around exposed apolar groups causing a thermodynamically unfavorable loss of entropy. As a consequence, the apolar groups tend to rearrange in such a way that they cluster together, excluding water and decreasing the free energy of the system as a whole. Data presented in this study generally agree with the concept that interaction between protein and phospholipids is hydrophobic:

1. Infrared spectroscopy. Infrared spectra give some direct evidence for nonpolar interactions between membrane proteins and lipids. The frequencies associated with phosphate group at about 1225 cm^{-1} and about 1060 cm^{-1} and the main methylene (CH_2) rocking mode at 720 cm^{-1} are present in phospholipids. ETP membranes as well as the reconstituted membranous cytochrome oxidase still retain the characteristics of the phospholipid phosphate group, namely 1225 cm^{-1} and 1060 cm^{-1} bands, but the 720 cm^{-1} peak, characteristic of the associated fatty acid chains, is missing. This is consistent with Chapman's data [27] on lipid conformation in erythrocyte membranes.

2. Organic solvent and detergent effects. Organic solvents extract most of the lipid from the membrane. Detergents are used for purification of cytochrome oxidase from mitochondria and for removal of phospholipids from the enzyme. Solubilization studies on membranous cytochrome oxidase indicated that detergents, such as deoxycholate, sodium dodecyl sulfate and Triton can solubilize most of the membranes and remove most of the lipid. Since addition of detergents to the aqueous medium is considered to weaken hydrophobic bonds [52], these results are consistent with the idea that the interaction between the protein and the lipid is hydrophobic.

3. ANS fluorescence hydrophobic probe. Either phospholipids or cytochrome oxidase induces ANS fluorescence. One would expect higher fluorescence of ANS to be induced by the presence of both phospholipids and protein than by only either one of them. As a matter of fact, however, lower fluorescence of ANS is obtained when phospholipids are added to the lipid-depleted cytochrome oxidase This suggests that fewer hydrophobic groups are available to interact with ANS in the phospholipid-cytochrome oxidase complex (membrane) than

in cytochrome oxidase alone. This indicates that either hydrophobic groups of proteins and phospholipids interact with each other to form membranes and/or phospholipids change conformation of proteins so that ANS is exposed to a less hydrophobic environment.

4. Circular dichroism. The trough at 225 mμ indicates a considerable α-helix in the membranous cytochrome oxidase. This type of structure can be correlated with globular proteins in the oxidase particle.

5. Enzyme cleavage of membrane-bound phosphatidyl choline by phospholipase C. Removal of phosphoryl choline from the phosphatidyl choline-cytochrome oxidase membrane by action of *Clostridium welchii* phospholipase C supports the interpretation that polar heads of phospholipids are on the membrane surface. Examination of the treated phosphatidyl choline-cytochrome oxidase membranes by electron microscope shows that they remained as membranes.

6. Formation of "unit membrane" structure between cytochrome oxidase and diolein. Formation of complex with "unit membrane" appearance between cytochrome oxidase and diolein which does not have an ionic group indicates that lack of an ionic group does not affect membrane structure.

7. Alteration of fatty acid chains of phospholipids by hydrogenation makes the saturated phospholipids unable to react with protein to form membranes.

However, the interaction between phospholipids and protein in membrane is not simply by nonpolar groups. Ionic detergents, e.g. deoxycholate or sodium dodecyl sulfate are most effective for solubilization of membranous cytochrome oxidase and detergent-free lipid-depleted enzyme aggregate. A mixture of KCl and Triton is a better solubilizing agent than Triton alone. Ionic strength and pH also affect the interaction of phospholipids and proteins when phospholipids and the lipid-depleted cytochrome oxidase are mixed. Sun *et al.* [7] also found that in the presence of detergent, the physical state of cytochrome oxidase is affected by the ionic strength of the solution. All of these data are consistent with the concept that electrostatic binding may be involved in association of protein in addition to a primary hydrophobic association.

Two types of membranes are observed in this study. The lipoprotein complex of the lipid-depleted cytochrome oxidase which has 1-2 molecules of cardiolipin associated with each protein molecule, after removal of detergent, reaggregate to a lipid-deficient membrane. Weier and Benson [53] proposed that chloroplast membranes are aggregates of globular subunits. These lipoprotein subunits consist of a protein matrix which binds the chlorophylls and lipids by hydrophobic association with their hydrocarbon moieties. The lipid-depleted membranes of cytochrome oxidase consist of lipoprotein subunits of cardiolipin and cytochrome oxidase protein analogous to the model proposed by Weier

and Benson for the chloroplast membranes. Another type of membrane is diagrammatically presented in Fig. 31. In this model the interaction of membrane protein, cytochrome oxidase, and additional phospholipids forms a mosaic membrane. Upon addition of phospholipids to the lipid-depleted cytochrome oxidase a complex forms between these two components, and structural transformation occurs parallel to and in as pronounced a fashion as the enhancement of enzyme activity. Protein molecules of cytochrome oxidase, as membrane subunits, are assembled to form a mosaic structure with phospholipids. Cytochrome oxidase membranes of the Triton X114 membranous enzyme or the reconstituted membranous enzyme cannot be explained by the Danielli-Davson-Robertson lipid bileaflet theory. The cytochrome oxidase membrane model is rather like the mosaic membrane proposed by Wallach and Gordon [54]. They suggested that in membrane formation the various subunits are assembled to form a protein lattice penetrated by cylinders of lipids.

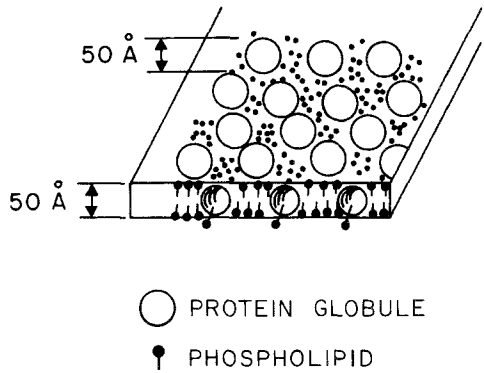


Figure 31. Model mosaic cytochrome oxidase membrane. The lipid in the protein globule indicates tightly bound cardiolipin whereas other lipid is in bilayer between the protein globules.

In the proposed mosaic membrane model the particles observed in the negatively stained cytochrome oxidase membranes are lipoprotein complexes. These complexes are made of cardiolipin and cytochrome oxidase protein. The model also interprets the black areas among the particles as phospholipids. According to the model the higher the lipid content in the membrane, the smaller should be the ratio of globular (unstained) to nonglobular (stained) regions. The surface area ratios of globular to nonglobular regions of the negatively stained electron micrographs agree well with the theoretical value on the basis of the mosaic structure assumption (Table 5).

TABLE V. Surface area ratios of protein globules to phospholipids of the model mosaic membranes

Phospholipid content of reconstituted membranes (g atom P/mole cytochrome *a*)	Ratio of globular (unstained) to nonglobular (stained) region[a]	Calculated area ratio of protein globules to phospholipids based on mosaic structure[b]
32.0	1.56	1.75
36.8	1.34	1.52
51.0	1.04	1.10

[a] Obtained by weighing enlarged photographic paper after unstained and stained areas being cut apart.

[b] Calculation based on the surface area of one phospholipid molecule to be 70 $Å^2$ [55], protein globules to be 50 Å sphere.

Evidence that the white spots in the negatively stained membrane are protein also comes from the fact that the isolated low molecular weight proteins obtained by fractionation of cytochrome oxidase (MW 2000-25,000) [10] form a membrane with smaller white spots (30 Å) than found on the original oxidase membrane (50-60 Å).

In essence the mosaic model which we propose agrees with the model proposed by Vanderkooi [61] for cytochrome oxidase. We seldom see the regular spacing of protein units in our membrane preparations which may reflect procedural differences or differences in subunit composition of our oxidase. The thickness of our membrane preparations is also more consistent with an oxidase unit 50-60 Å thick with a corresponding molecular weight approaching 100,000 rather than an 85 Å thick unit of molecular weight 220,000.

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References

1. D. E. Green and A. Tzagoloff, *J. Lipid Res.*, 7 (1966) 587.
2. J. Lenard and S. J. Singer, *Proc. Nat. Acad. Sci., U.S.*, 56 (1966) 1828.
3. D. F. H. Wallach and P. H. Zahler, *Proc. Nat. Acad. Sci., U.S.*, 56 (1966) 1552.
4. D. Chapman and D. F. H. Wallach, in: *Biological Membranes*, D. Chapman (ed.), Academic Press, New York, 1968, p. 125.
5. H. Löw and I. Vallin, *Biochim. Biophys. Acta*, 69 (1963) 361.

6. T. F. Chuang, F. F. Sun and F. L. Crane, *J. Bioenergetics*, 1 (1970) 227.
7. F. F. Sun, K. S. Prezbindowski, F. L. Crane and E. E. Jacobs, *Biochim. Biophys. Acta*, 153 (1968) 804.
8. W. H. Vanneste, *Biochim. Biophys. Acta*, 113 (1966) 175.
9. T. Yonetani, *J. Biochem. (Tokyo)*, 46 (1959) 917.
10. T. F. Chuang and F. L. Crane, *Biochem. Biophys. Res. Commun.*, 42 (1971) 1076.
11. T. Yonetani, *J. Biol. Chem.*, 236 (1961) 1680.
12. R. S. Criddle and R. M. Bock, *Biochem. Biophys. Res. Commun.*, 1 (1959) 138.
13. S. Fleischer and B. Fleischer, in: *Methods in Enzymology*, R. W. Estabrook and M. Z. Pullman (eds), Vol. X, Academic Press, New York, 1967, p. 407.
14. P. S. Chen, T. Y. Toribara and H. Warner, *Anal. Chem.*, 28 (1956) 1756.
15. W. P. Cunningham and F. L. Crane, *Plant Physiol.*, 40 (1965) 1041.
16. J. H. Luft, *J. Biophys. Biochem. Cytol.*, 9 (1961) 409.
17. E. S. Reynolds, *J. Cell Biol.*, 17 (1963) 208.
18. F. L. Crane, C. J. Arntzen, J. D. Hall, F. J. Ruzicka and R. A. Dilley, in: *Autonomy and Biogenesis of Mitochondria and Chloroplasts*, Symposium of Australian Academy of Science, Canberra, 1969, p. 53.
19. L. R. Fowler, S. H. Richardson and Y. Hatefi, *Biochim. Biophys. Acta*, 96 (1962) 103.
20. Y. C. Awasthi, T. F. Chuang, T. W. Keenan and F. L. Crane, *Biochim. Biophys. Acta*, 226 (1971) 42.
21. T. F. Chuang, Y. C. Awasthi and F. L. Crane, *Fed. Proc.*, 29 (1970) 540a.
22. G. Weber and D. J. R. Laurance, *Biochem. J.*, 51 (1954) xxi.
23. W. O. McClure and G. M. Edelman, *Biochemistry*, 5 (1965) 1908.
24. G. Weber and L. B. Young, *J. Biol. Chem.*, 239 (1964) 1415.
25. G. H. Dodd and G. K. Radda, *Biochem. Biophys. Res. Commun.*, 27 (1967) 500.
26. A. H. Maddy and B. R. Malcolm, *Science*, 150 (1965) 1616.
27. D. Chapman, *The Structure of Lipids*, Methuen, London, 1965.
28. A. H. Maddy and B. R. Malcolm, *Science*, 153 (1966) 212.
29. D. Chapman, V. B. Kamat and R. J. Levene, *Science*, 160 (1968) 314.
30. D. F. H. Wallach and P. H. Zahler, *Proc. Nat. Acad. Sci., U.S.*, 56 (1966) 1552.
31. D. F. H. Wallach and P. H. Zahler, *Biochim. Biophys. Acta*, 150 (1968) 186.
32. D. F. H. Wallach, J. M. Graham and B. R. Fernback, *Arch. Biochem. Biophys.*, 131 (1969) 322.
33. J. M. Graham and D. F. H. Wallach, *Biochim. Biophys. Acta*, 193 (1969) 225.
34. T. Miyazawa and E. R. Blout, *J. Am. Chem. Soc.*, 83 (1961) 712.
35. A. Elliott and E. J. Ambrose, *Nature*, 165 (1950) 921.
36. L. J. Bellamy, *Infrared Spectra of Complex Molecules*, Methuen, London, 1958, p. 27.
37. J. Lenard and S. J. Singer, *Proc. Nat. Acad. Sci., U.S.*, 56 (1966) 1828.
38. S. Beychok, *Ann. Rev. Biochem.*, 37 (1968) 437.
39. D. W. Urry, M. Mednicks and E. Bejnarowicz, *Proc. Nat. Acad. Sci., U.S.*, 57 (1967) 1043.
40. D. G. McConnell, A. Tzagoloff, D. H. Machennan and D. E. Green, *J. Biol. Chem.*, 241 (1966) 2473.
41. E. A. Grula, T. F. Butler, R. D. King and G. L. Smith, *Canadian J. Microbiol.*, 13 (1967) 1499.
42. S. Seki and T. Oda, *Arch. Biochem. Biophys.*, 138 (1970) 122.
43. K. Okunuki, T. Sekuzu, T. Yonetani and S. Takemore, *J. Biochem. (Tokyo)*, 45 (1958) 847.
44. E. G. Ball, C. G. Strittmatter and O. Cooper, *J. Biol. Chem.*, 193 (1951) 635.
45. S. Takemori, I. Sekuzu and K. Okunuki, *Biochim. Biophys. Acta*, 51 (1961) 464.

46. Y. Orrii and K. Okunuki, *J. Biochem. (Tokyo)*, **58** (1965) 561.
47. T. F. Chuang and F. L. Crane, in preparation.
48. J. Lenard and S. J. Singer, *Science*, **159** (1968) 738.
49. A. A. Benson, in: *Membrane Models and the Formation of Biological Membranes*, L. Bolis and B. A. Pethica (eds), North Holland, Amsterdam, 1968, p. 190.
50. D. E. Green and S. Fleischer, in: *Metabolism and Physiological Significance of Lipids*, R. M. C. Dawson and D. N. Rhodes (eds), John Wiley, New York, 1964, p. 581.
51. H. A. Scheraga, G. Nemethy and J. Z. Steinberg, *J. Biol. Chem.*, 237 (1962) 2560.
52. A. D. Brown, *J. Mol. Biol.*, 12 (1965) 491.
53. T. E. Weier and A. A. Benson, *Amer. J. Bot.*, **54** (1967) 389.
54. D. F. H. Wallach and A. Gordon, *Fed. Proc.*, 27 (1968) 1263.
55. D. M. Small, *J. Lipid Res.*, 8 (1967) 551.
56. E. E. Jacobs, E. C. Andrews, W. P. Cunningham and F. L. Crane, *Biochem. Biophys. Res. Commun.*, 25 (1966) 96.
57. H. Moor and K. Mühlethaler, *J. Cell Biol.*, 17 (1963) 609.
58. P. G. Shakespeare and H. R. Mahler, *J. Biol. Chem.*, **246** (1971) 7649.
59. G. Schatz, G. S. P. Groot, T. Mason, W. Rouslin, D. C. Wharton and J. Saltzgaher, *Fed. Proc.*, 31 (1972) 21.
60. D. W. Urry, *Biochim. Biophys. Acta*, **265** (1972) 115.
61. G. Vanderkooi, *Ann. N.Y. Acad. Sci.*, 195 (1972) 6.