Preparation and Characterization of Cytochrome c Oxidase Vesicles with High Respiratory Control*

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1. Isolation of cytochrome c oxidase which yields high respiratory control ratios in reconstituted vesicles is reported. Fusion of reconstituted vesicles with low and high respiratory control ratio suggests that lack of respiratory control is caused by faulty incorporation of protein rather than by the presence of a proton leak.

2. Digestion of cytochrome c oxidase with chymotrypsin removed some contaminating polypeptides without damaging reconstitution of cytochrome c oxidase vesicles. Urea-dodecyl sulfate gel electrophoresis revealed the presence of six subunits in the digested protein.

3. A method is described by which the orientation of cytochrome c oxidase in the membrane was evaluated. With cytochrome c oxidase as the only protein, the enzyme was assembled in the mitochondrial orientation (right-side-out). In the presence of cytochrome c there was considerable scrambling with about 50% of the enzyme in the right-side-out orientation.

4. Phospholipid requirements for the preparation of vesicles with high respiratory control differed considerably with the method of reconstitution. Marked variation in the sensitivity to detergents was observed dependent on the reconstitution procedure. Oxidation of reduced cytochrome c by vesicles reconstituted by the incorporation procedure was markedly stimulated by about 0.05% Tween 80. This respiration was blocked by 2 mM dicyclohexylcarbodiimide but not by rutamycin and was completely restored by uncouplers of oxidative phosphorylation. This phenomenon was also seen with vesicles reconstituted by cholate dialysis.

5. Fractionation of cytochrome c oxidase vesicles on Ficoll gradients revealed that cytochrome c oxidase reconstituted by incorporation was associated with only a small fraction of the phospholipid population. The isolated protein-free liposomes were incapable of incorporating cytochrome c oxidase when exposed to a second incubation with the enzyme.

Cytochrome c oxidase is the terminal electron carrier of the mitochondrial respiratory chain. The enzyme from bovine heart mitochondria is a multisubunit complex consisting of six subunits that span the inner mitochondrial membrane asymmetrically (1) as required by the chemiosmotic hypothesis (2). The enzyme was reconstituted into liposomes (3-5) which catalyzed little electron transport unless both the membrane potential and the pH gradient were collapsed by the combined action of valinomycin and nigericin in the presence of K+ or by other appropriate ionophores. Respiration in these vesicles was measured by addition of cytochrome c and ascorbate. Since neither ascorbate nor cytochrome c penetrated through the phospholipid bilayer, only the mitochondrial orientation (right-side-out) of cytochrome c oxidase was measured under these conditions. On the other hand reconstitution of the third site of oxidative phosphorylation (6) demanded the opposite operation of cytochrome c oxidase (inside-out), which was achieved at the functional level by elimination of all external cytochrome c and by the use of a hydrophobic reductant for the internal cytochrome c.

Although it was clear from these experiments that both right-side-out and inside-out orientation can be achieved by the cholate dialysis procedure of reconstitution, the factors that determine orientation were not defined nor was there an unambiguous method available for the quantitative estimation of the modes of orientation.

It is the purpose of this paper to report on the reconstitution of cytochrome c oxidase into vesicles with high respiratory control ratios by a variety of procedures. The orientation of the enzyme in the membrane and the separation of proteoliposomes from protein-free liposomes will be described. It will also be shown that a cytochrome c oxidase preparation treated with chymotrypsin to remove contaminating polypeptides is reconstitutively fully active.

EXPERIMENTAL PROCEDURES

Materials

Cholic and deoxycholic acids were obtained from Matheson, Coleman and Bell and purified as previously described (7). Enasol 1139 (polyoxyethylene sorbitan monooleate) was provided by Kao Soap Co. (Tokyo). Tween 80 (polyoxyethylene sorbitan monooleate), cytochrome c (type VI), dicetylphosphate, and valinomycin were purchased from Sigma. Chymotrypsin, crystallized and salt-free, was a product of Worthington Biochemical Corporation. Dicyclohexylcarbodiimide was purchased from Schwarz/Mann; 1799 was provided.

1 The abbreviations used are: 1799, bis-(hexafluoroacetonyl)acetone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DCCD, dicyclohexylcarbodiimide.
by Dr. Peter Heytler, E. I. DuPont. Stearylamine was a product of the Shell Oil Co. Cardiolipin, phosphatidylserine, phosphatidylino-
sitol, and phosphatidic acid were purchased from Avanti Biochemi-
cals, Inc. and were at least 98% pure. Phosphatidylethanolamine and
phosphatidycholine were purified from bovine heart mitochondria according to published procedures (8). Asolectin (crude soybean phospholipids) was purchased from Associated Concentrates, Woodside, Long Island, N. Y.; and partially purified as previously described (9). [3H]Triolein, purchased from Applied Science Labora-
tories and purified as described (10), was a gift from Dr. D. Zilversmit, Cornell University. Ficoll was purchased from Pharma-
ciaz, dialyzed against distilled water, and lyophilized before use. Electrophoresis reagents were obtained from Bio-Rad.

Preparations

Cytochrome c oxidase from bovine heart mitochondria was iso-
lated by several procedures (5, 11–17). Three of these preparations (11, 14, 15) were further purified as follows (all steps at 0 to 4º). Enzyme (30 to 50 mg/ml) stored at -70º in 0.25 M sucrose was thawed and diluted to 5 mg/ml with buffer to give a final concentra-
tion of 0.1 M NaP, (pH 7.4) in 1.5% cholate. Saturated ammonium sulfate (pH 7.4) at 0º was added with stirring to give 25% saturation. The slightly turbid mixture was allowed to stand 30 min and the precipitate was removed by centrifugation at 39,000 x g for 20 min. The enzyme remaining in the supernatant was collected by precipi-
tation at 35% saturation of ammonium sulfate and centrifugation as described above. The precipitate was dissolved in 0.1 M NaP, (pH 7.4), 1.0% Emesol 1130 to give a final volume one-half that of the starting material. This material (5 to 8 mg/ml) was then fractionated by stepwise precipitation of enzyme with 2% saturation increments of ammonium sulfate from 25 to 28% saturation. The precipitates collected by centrifugation were dissolved in 0.25 M sucrose and analyzed for heme a content and respiratory control. Fractions with respiratory control ratios above 0 were stored at -70º.

Protein

Protein was determined according to Lowry et al. (18) in the presence of 0.4% sodium deoxycholate with bovine serum albumin as a standard.

Heme a Determinations

Heme a content was determined as described by Kuboyama et al. (15) using a millimolar extinction coefficient of 12 for the reduced minus oxidized difference spectra at 605 nm.

Reconstitution Methods

Cholate dialysis was performed as described (4). Cholate Dilution (4) - Phospholipids were sonicated (10 to 15 min) at 25 mg/ml in 50 mM KP, (pH 7.5). To 0.2 ml of this suspension 10 µl of a 20% solution of sodium cholate and 5 µl of cytochrome c oxidase in 0.1 M Hepes, 0.1 mM EDTA (pH 7.4). The assay for the isolated enzyme contained 25 mM potassium ascorbate, 1 mg of cytochrome c, and 1 mg of sonicated phospholipids/ml. The assay of reconstituted cyto-

Phospholipids were prepared by sonication at 25 mg/ml with 50 mM KP, phospholipid:protein ratios and the mixtures were incubated 30 min

Total Phospholipid Determinations

Phospholipids were extracted from enzyme and liposomal frac-
tions with methanolchloroform (1:1). Total phosphorus was deter-

Thin Layer Chromatography

Silica Gel 60 F-254 precoated thin layer chromatography plates (Brinkmann Instruments) were developed with either chloromethanolwater (65:25:4) or chloroform:methanol:water:acetic acid (65:43:3:1). Chromatograms were visualized both by iodine vapor and sulfuric acid charring.

Proteolytic Digestion

Chymotrypsin (10 µg/mg of cytochrome c oxidase at 5 to 10 mg/

Gel Electrophoresis

Gel electrophoresis with dodecyl sulfate (23) and with urea/dode-
cyl sulfate (24) was performed as described. For the latter procedure we used 12.5% acrylamide, 0.83% bisacrylamide gels (9 x 0.5 cm) at 50 V and about 2 mA/gel tube for 17 h. Gels were fixed, stained, and destained as described (24) except that fixing and staining was for 4 h at 23º. Gel scans were obtained at 560 nm with a Gilford spectrophotometer equipped with linear scanner. Apparent molecular weights were calculated from a standard curve of ovalbumin, chymotrypsinogen, myoglobin, cyto-

Ficoll Discontinuous Gradients

From a stock solution of 30% Ficoll (w/v) plus 50 mM KP, (pH 7.5)
dilutions were made with 50 mM KP, to give 15, 10, 8, 6, and 5% solutions. Gradient layers were carefully pipetted at room tempera-
ture with 1.8 ml for each layer in SW 41L cellulose nitrate tubes (12 ml capacity). After temperature equilibration at 4º for 4 h, 0.5- to 1.0 ml aliquots of liposomes (25 mg of phospholipid/ml) in 50 mM KP, (pH 7.5) were carefully applied to the top of the gradient. The tubes were then centrifuged in a SW 41L swinging bucket rotor at 150,000 x g for 17 h at 4º. The Ficoll layers were collected from above with a 3 ml syringe with a bent tip needle. Fractions (0.05- to 0.4 ml aliquots) were analyzed for activity and respiratory control. Phospholipid recoveries were determined with [14C]triolein (12 x 10⁶ cpm/mg of phospholipid) as a marker by scintillation counting of 0.02 ml of the original sample, 0.1 ml of the 5% Ficoll layer, and 0.5-

RESULTS AND DISCUSSION

Characterization of Cytochrome c Oxidase Preparations

The reconstitutive properties of several enzyme preparations are summarized in Table I. Enzymes exposed to deoxycholate
zymes (17, 26) gave rise to much cleaner preparations (Fig. or to 3% cholate at 30° (13) gave low respiratory control ratios (1 to 2) upon reconstitution into liposomes. Enzymes prepared with cholate gave ratios of 3 to 6. This includes preparations delipidated by relatively mild procedures (5, 16). All preparations showed similar major subunit patterns on dodecyl sulfate-polyacrylamide gels (23) with varying amounts of high molecular weight contaminants (Fig. 1). Refractionation (see "Experimental Procedures") of the enzyme yielded vesicles devoid of some of the polypeptide bands that appear on urea sodium dodecyl sulfate gels (24, 28, 29). It can be seen that Band IIb was proportionately reduced by >50% as determined by relative staining intensity and Band Va was completely eliminated. Time courses of chymotrypsin digestion of the enzyme revealed (data not shown) that Band Va was rapidly digested with greater than 90% reduction in staining intensity in about 30 min. Initially Band IIb was also rapidly digested with reduction in staining intensity leveling off after 15 min. Since the specific activity and heme α content increased proportionately with digestion and since the digested and reconstituted enzyme showed no loss of respiratory control, we conclude that Band Va is not an essential subunit of the enzyme. Since Band IIb appears to be more than 50% digested it seems also unlikely that this polypeptide is a poorly staining subunits or function at substoichiometric levels. Moreover, it is conceivable that other methods of resolution might reveal additional components. For the time being we conclude that a protein with six subunits is reconstitutively active.

**Phospholipid Requirements for Reconstitution**—Five methods of reconstitution of membrane proteins into liposomes have been developed in this laboratory. All are effective for cytochrome c oxidase and yield respiratory control ratios greater than 4. The method of sonication (20) was not used frequently since losses of cytochrome c oxidase activity up to 50% were observed.

Table IV gives a summary of the observed phospholipid requirements for the four methods of reconstitution. Reconstitution of cytochrome c oxidase with respiratory control was obtained with either phosphatidylcholine or phosphatidylethanolamine in combination with an acidic phospholipid or diacetylphosphate. The greatest dependency on acidic phospholipids was observed with the incorporation procedure (5) or when phosphatidylycholine was used alone by any method of reconstitution. Exceptions to dependency on acidic phospholipids are liposomes with phosphatidylethanolamine:phosphatidylcholine (4:1) which gave high control ratios by either cholate dialysis or dilution. Pure phosphatidylethanolamine also appears to yield vesicles with high control ratios when prepared by cholate dilution. The phosphatidylethanolamine used was better than 98% pure as analyzed by thin layer chromatography (see "Experimental Procedures") in two different solvent systems. That phosphatidylethanolamine:phosphatidylycholine (4:1) mixtures eliminated the need for acidic phospholipid has previously been reported for the reconstitution of site III oxidative phosphorylation (6).

There is evidence that phosphatidylethanolamine prepara-

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**Table I**

**Reconstitutive properties of cytochrome c oxidase preparations**

| Preparations | Number of Preparations | Detergents and isolation conditions | Heme α | Specific activity | Respiratory control ratio |
|--------------|------------------------|-----------------------------------|--------|------------------|----------------------------|
|              |                        |                                    |        |                  | n mole/mg protein          | pmol oxygen/min/mg         |
| Wharton and Tsagoloff (1961) | 2 | Deoxycholate | 6-9 | 12-19 | 1-2 |
| Capaldi and Hyashi (1972) | 2 | Deoxycholate and cholate | 8-10 | 15-24 | 1-1.5 |
| Tsagoloff and MacLennan (1965)* | 3 | 3% cholate, 2 h at 30° | 8-10 | 16-21 | 1-2 |
| Yonetani (1966)* | >10 | Cholate and Tween 80 | 6-8 | 18-25 | 3-6 |
| Kuboyama, Yong, and King (1972)* | >10 | Cholate and Emasol 1130 | 9-12 | 25-35 | 3-6 |
| Yu, Yu, and King (1975) | 3 | Cholate | 9 | 12 | 25-35 | 3-6 |
| Eytan, Matheson, Carroll, Schatz, and Racker (1975)** | 5 | Cholate and digestion | 11-14 | 25-36 | 3-6 |
| Yamamoto and Okunuki (1972)** | >10 | Cholate and Emasol 1130 | 9-12 | 25-36 | 6-12 |

* Determined polarographically after cholate dialysis reconstitution with asolectin as described under "Experimental Procedures.”

* Either Yonetani or Kuboyama and King preparations were used as starting material.
fig. 1. Dodecyl sulfate gel scans of cytochrome c oxidase preparations. Dodecyl sulfate gel (23) scans of (A) Wharton and Tzagoloff (11), (B) Yonetani (14), (C) Kuboyama et al. (15), and (D) Yamamoto and Okunuki (17), cytochrome c oxidase preparations (10 to 15 μg protein) were used as described under "Experimental Procedures."

tions exhibit acidic properties by pH titrations of both monolayer surface potentials and electrophoretic mobility (30). It was postulated that phosphatidylethanolamine was labile giving as hydrolytic products either free fatty acids or phosphatidic acid, but it was not reported that these breakdown components were actually observed. We have, in fact, often detected phosphatidic acid in both synthetic and highly purified natural phosphatidylethanolamine preparations. It is therefore possible that some cleavage of this phospholipid takes place during sonication and subsequent incubation for reconstitution.

Additional evidence of a requirement for a net negative charge in the reconstitution of cytochrome c oxidase vesicles comes from experiments with stearylamine. As shown in Fig. 3, at a fixed level of added acidic phospholipid, the presence of stearylamine during reconstitution inhibited respiratory control at low concentrations and oxidase activity at high concentrations. It can be seen in Fig. 4 that the inhibition by stearylamine of both respiratory control and oxidase activity was reversed by dietylphosphate. The loss of oxidase activity with excess stearylamine seems to be a function of reconstitution rather than a direct inhibitory effect since enzyme added to preformed liposomes with the same amounts of stearylamine gave full activity.

Orientation of Cytochrome c Oxidase in Reconstituted Vesicles—In order to determine the orientation of cytochrome c oxidase reconstituted into liposomes, it was necessary to compare the uncoupled rate of respiration driven by external ferrocytochrome c to the activity obtained with ferrocytochrome c after disruption of the liposomes by a detergent which did not inhibit enzyme activity. Of several detergents tested, Tween 80 was the only one that opened the vesicles without inhibiting oxidase activity. Table V shows the activity of cytochrome c oxidase vesicles in the presence of uncouplers
TABLE II
Purification and cholate dialysis reconstitution or refractionated cytochrome c oxidase

Refractionation was carried out as described under "Experimental Procedures." Specific activity and respiratory control were determined polarographically after cholate dialysis reconstitution with asolectin. Refractionation of Kuboyama and King preparations (about 10 nmoles of heme a/mg of protein) yielded fractions with high respiratory control (>6) containing 11 to 12 nmoles of heme a/mg of protein and catalyzing a rate of respiration up to 36 μatoms of oxygen/min/mg.

| Fraction | Total protein | Total heme a | % Yield heme a | Heme a | Specific activity | Respiratory control ratio |
|----------|---------------|--------------|----------------|--------|------------------|--------------------------|
| Starting material (Yonetani preparation) | 670 mg | 4450 nmoles | 100 | 6.64 | 22 | 5.1 |
| Ammonium sulfate precipitate (0-25% saturation) from 1.5% cholate | 200 mg | 1120 nmoles | 25 | 5.64 | 18 | 1.5 |
| Ammonium sulfate precipitate 0-28% saturation | 12 mg | 20 nmoles | 0.4 | 1.8 | 7 | 1.0 |
| 28-30% saturation | 22 mg | 110 nmoles | 2.5 | 5.1 | 20 | 3.2 |
| 30-32% saturation | 199 mg | 1880 nmoles | 42 | 9.5 | 28 | 9.5 |
| 32-34% saturation | 90 mg | 790 nmoles | 18 | 8.9 | 27 | 10.1 |
| 34-36% saturation | 29 mg | 180 nmoles | 4 | 6.36 | 21 | 7.9 |

TABLE III
Fusion of cytochrome oxidase vesicles with high and low respiratory control ratios

Reconstitution was performed by the cholate dialysis procedure as described under "Experimental Procedures" with a phospholipid mixture containing 70% asolectin plus 30% phosphatidylserine. Hydrophobic protein vesicles were reconstituted as previously described (21) with 3 mg of protein/ml.

| Vesicles used for fusion | I | II | Respiration |
|-------------------------|---|----|-------------|
| High respiratory control vesicles (5 μl) | None | Hydrophobic protein vesicles (5 μl) | 21 | 180 | 8.7 |
| High respiratory control vesicles (5 μl) | Hydrophobic protein vesicles (5 μl) | +Low respiratory control vesicles (5 μl) | 62 | 180 | 2.9 |
| High respiratory control vesicles (5 μl) | Hydrophobic protein vesicles (10 μl) | None | 90 | 180 | 2.0 |
| High respiratory control vesicles (5 μl) | None | Low respiratory control vesicles (5 μl) | 61 | 230 | 3.8 |
| None | Low respiratory control vesicles (5 μl) | None | 60 | 60 | 1.0 |

Fig. 2. Urea-dodecyl sulfate gel scans of cytochrome c oxidase before and after treatment with chymotrypsin. Urea-dodecyl sulfate gels of the Kuboyama et al. (15) preparation (A) before and (B) after a 2-h digestion with chymotrypsin were analyzed as described under "Experimental Procedures" with phenylmethylsulfonl fluoride added before denaturation. Aliquots of 20 μg of protein were electrophoresed as described (24).

and in the presence of 3% Tween 80. Also shown are the activities obtained with enzyme added to preformed liposomes. Since the observed activities were in good agreement with each other we conclude that reconstitution of the enzyme is unidirectional with full accessibility to external ferrocytochrome c. This result has been obtained with all the methods of cytochrome c oxidase reconstitution.

On the other hand, reconstitution of oxidative phosphorylation (6) suggested that some of the cytochrome c oxidase must have been oriented inside-out. Tests with the individual components used in these reconstitution experiments revealed that cytochrome c was responsible for the scrambling of oxidase. When 80 μM or more ferricytochrome c was present during reconstitution the rates of oxidation obtained in the presence of 3% Tween 80 were from 30 to 60 ng atoms of oxygen/min higher than the uncoupled rates (Fig. 5A). There was also a loss of total activity approaching 70% at 140 μM ferricytochrome c and at concentrations above 100 μM of ferricytochrome c uncouplers no longer stimulated. Reconstitution with ferrocytochrome c did not induce random orientation or loss of activity as shown in Fig. 5B. The vesicles that were reconstituted with ferricytochrome c were capable of setting up a membrane potential as measured by anion uptake by the procedure of Jasaitis et al. (31). The vesicles that were reconstituted with ferricytochrome c showed a transmembranous inside-out orientation of cytochrome c oxidase was achieved under these conditions.

Sensitivity of Reconstituted Vesicles to Detergents and
TABLE IV
Phospholipid requirements for cytochrome c oxidase reconstitution

Reconstitutions were performed as described under "Experimental Procedures." The enzyme was reconstituted with the designated phospholipids at a phospholipid:protein ratio of 20. Acidic phospholipids used were: phosphatidylserine, cardiolipin, phosphatidylinositol, and phosphatic acid.

| Phospholipid composition                                      | Respiratory control ratios of vesicles obtained by various reconstitution procedures |
|---------------------------------------------------------------|-----------------------------------------------------------------------------------|
|                                                              | Cholate dialysis | Cholate dilution | Low detergent incorporation | Incorporation |
| Asolectin                                                     | >4               | >4               | Not tested                 | 2-4          |
| Phosphatidylethanolamine or phosphatidylcholine plus          | >4               | >4               | >4                         | 1-2          |
| 5 mole % acidic phospholipid                                  |                    |                  |                            |              |
| Phosphatidylethanolamine or phosphatidylcholine plus          | >4               | >4               | 1-2                        | >4           |
| 10 to 30 mole % acidic phospholipid                           | <4               | >4               | 2-4                        | 1-2          |
| Phosphatidylethanolamine:phosphatidylcholine (4:1)            | >4               | >4               | 2-4                        | 1-2          |
| Phosphatidylethanolamine                                     | 2-4              | >4               | 2-4                        | 1-2          |
| Phosphatidylcholine                                           | 1-2              | 1-2              | 1-2                        | 1-2          |
| Acidic phospholipid                                           | 1-2              | 1-2              | 1-2                        | 1-2          |
| Phosphatidylethanolamine or phosphatidylcholine plus          | >4               | >4               | Not tested                 | >4           |
| 10 to 30 mole % dicetylphosphate                              |                    |                  |                            |              |

FIG. 3. The effect of stearylamine on the reconstitution of cytochrome c oxidase into liposomes. Phosphatidylethanolamine:phosphatidylcholine (4:1) with 5% cardiolipin (7.5 μmoles of total phospholipids), plus the indicated micro moles of stearylamine were used to prepare cytochrome c oxidase vesicles by cholate dialysis (in a final volume of 0.3 ml with a phospholipid:protein ratio of 20). After overnight dialysis against 50 mM KP (pH 7.5), 10-μl aliquots were assayed for activity (open circles) and for respiratory control ratios (RCR, closed circles) as described under "Experimental Procedures."

DCCD — We have observed marked differences in the effectiveness of Tween 80 depending on the method of reconstitution. As shown in Fig. 6, liposomes prepared with the same phospholipids and enzyme but reconstituted by the incorporation procedure were much more sensitive to Tween 80 as compared to vesicles prepared by the cholate dialysis procedure. Also shown is that 1 mM CaCl₂ gave substantial protection against the detergent. This protective effect was eliminated by subsequent addition of 1 mM EDTA. Residual cholate present in the dialyzed vesicles (32) did not seem to be responsible for the resistance of the vesicles obtained by the dialysis procedure since no change in sensitivity was noted with 40 μg of cholate added/mg of phospholipid for 20 h at 4°C after incorporation of cytochrome c oxidase.

DCCD is a well known energy transfer inhibitor of oxidative phosphorylation. There is evidence that this type of inhibitor acts by blocking proton translocation through the hydrophobic protein component of the oligomycin sensitive ATPase (4, 33,
TABLE V
Activities of reconstituted cytochrome c oxidase

Cytochrome c oxidase vesicles were reconstituted by cholate dialysis as described under "Experimental Procedures" with asolectin at a phospholipid:protein ratio of 20. Phospholipid activated enzyme was prepared by adding enzyme to asolectin liposomes (at 4°C) prepared by cholate dialysis to give a phospholipid:protein ratio of 20. The samples were kept at 4°C and assayed within 1 h of terminating dialysis or mixing the liposomes and enzyme. The values given represent the average of three separately reconstituted samples.

| Additions | Addition | Oxygen uptake | Respiratory control ratio |
|-----------|----------|---------------|--------------------------|
| None      |          | 43            | 43                       |
| Cytochrome c oxidase vesicles (10 µg enzyme) | Valinomycin + 1799 | 209 | 4.9 |
| Cytochrome c oxidase vesicles (10 µg enzyme) | 3% Tween 80 | 209 | 1.1 |
| Enzyme (10 µg) plus liposomes | None | 200 | 1.1 |
| Enzyme (10 µg) plus liposomes | 3% Tween 80 | 200 | 1.1 |

Fig. 6. Sensitivity of incorporation and cholate dialysis liposomes to detergent uncoupling. Cytochrome c oxidase vesicles were prepared by (A) direct incorporation or (B) cholate dialysis, with phosphatidylethanolamine:phosphatidylcholine:phosphatidylinositol (1:1:1) and 40 mM KCl, 10 mM Hepes, 0.3 mM EDTA buffer (pH 7.4) as described under "Experimental Procedures." Phospholipid:protein ratios for both preparations was 25. Assays of 10-µl aliquots were performed as described by titrating Tween 80 from a 25% stock solution with either 40 mM KCl, 10 mM Hepes (pH 7.4) (closed circles) or 40 mM KCl, 10 mM Hepes plus 1 mM CaCl₂ (pH 7.4) (open circles) as assay buffer.

FIG. 5. Reconstitution of cytochrome c oxidase into liposomes in the presence of either reduced or oxidized cytochrome c. Cytochrome c oxidase vesicles were reconstituted in a final volume of 0.6 ml with 15 µmoles of partially purified asolectin by the cholate dialysis procedure (50 mM KCl, pH 7.5). The phospholipid:protein ratio was 20 with the indicated concentrations of (A) ferricytochrome c or (B) ferrocytochrome c, maintained in the reduced form by dialysis under nitrogen with buffer containing 10 mM potassium ascorbate. Assays on 10-µl aliquots were done as described under "Experimental Procedures" in the presence of either 17 µM 1799 plus 0.5 µg of valinomycin (closed circles) or 3.0% Tween 80 (open circles). Initial rates (open squares) were determined in the absence of uncoupler or detergent.

TABLE VI
Effect of Tween 80 and dicyclohexylcarbodiimide on oxygen uptake of cytochrome c oxidase vesicles reconstituted by incorporation procedure

Cytochrome oxidase vesicles were reconstituted by the incorporation procedure as described under "Experimental Procedures" with phosphatidylethanolamine:phosphatidylcholine:phosphatidylinositol (2:2:1) and a phospholipid:protein ratio of 20.

| Additions to vesicles (10 µg enzyme) | Oxygen uptake (natoms oxygen/min) | Respiratory control ratio |
|-------------------------------------|----------------------------------|--------------------------|
| None                                | 53                               | 4.9                      |
| Valinomycin + 1799                  | 268                              | 4.9                      |
| 0.2 mM DCCD                         | 240                              |                          |
| None                                | 53                               | 4.4                      |
| 0.02% Tween 80                      | 181                              | 3.4                      |
| 0.2 mM DCCD                         | 55                               |                          |
| Valinomycin + 1799                  | 232                              | 4.2                      |
| None                                | 53                               | 4.4                      |
| 0.02% Tween 80                      | 235                              | 4.4                      |
| 0.2 mM DCCD                         | 57                               |                          |
| Valinomycin + 1799                  | 240                              | 4.2                      |
| None                                | 51                               | 3.2                      |
| 0.02% deoxycholate                  | 165                              |                          |
| 0.6 mM DCCD                         | 64                               |                          |
| Valinomycin + 1799                  | 90                               | 1.4                      |
| None                                | 55                               | 1.3                      |
| 1 µg valinomycin                    | 67                               |                          |
| 0.3 mM oleic acid                   | 200                              | 3.8                      |
| 0.4 mM DCCD                         | 200                              | 3.8                      |
34). In the course of experiments on the stimulation of cytochrome c oxidase activity by detergents in reconstituted vesicles prepared by the incorporation procedure, we observed that 0.2 mM DCCD gave almost complete inhibition of respiration in the presence of 0.02 to 0.03% Tween 80 (Table VI). Addition of valinomycin plus 1799 completely restored the original oxidation rate. Thus the system behaved as if a receptor for the energy transfer inhibitor was present. Similar effects were noted with deoxycholate instead of Tween 80 but stimulation by valinomycin plus 1799 were not as complete. Rutamycin did not imitate the effect of DCCD.

Since the carboxyl ester linkage of oleic acid to polyoxyethylene sorbitan of Tween 80 and the free carboxyl group of deoxycholate are the most likely groups that could react with DCCD, free oleic acid at concentrations up to 1.6 mM was tested. It has been previously observed (35) that 0.3 mM oleic acid serves as an uncoupler in the presence of valinomycin. However, as shown in Table VI, DCCD had no effect on the rate of respiration induced by oleic acid.

These experiments show that extreme caution must be used in the interpretation of data obtained with DCCD as an energy transfer inhibitor.

Isolation and Characterization of Liposomes Containing Cytochrome c Oxidase – One of the questions that has been raised with respect to reconstitution experiments is why high ratios of phospholipid to protein (about 20 w/w) are required for optimal reconstitution (4, 5). We considered the possibility that the enzyme was preferentially incorporated into a subpopulation of liposomes. Attempts to separate such a subpopulation on a sucrose gradient failed probably because of osmotic effects. When Ficoll was substituted for sucrose a subpopulation of cytochrome c oxidase vesicles was isolated. As shown in Fig. 7, most of the cytochrome c oxidase activity (>80%) was associated with only 10 to 20% of the total phospholipid when either cholate dialysis or incorporation was used for reconstitution. The phospholipid:protein ratios

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\text{Phospholipid:Protein Ratio (w/w)} = \frac{\text{Phospholipid (PL) in mg}}{\text{Protein (Protein) in mg}}
\]

Fig. 7. Fractionation of cytochrome c oxidase liposomes by Ficoll discontinuous gradient centrifugation. Incorporation cytochrome c oxidase vesicles were performed as described under "Experimental Procedures." with phosphatidylethanolamine-phosphatidycholine-phosphatidylinositol (1:1:1) plus 5% phosphatidylcholine in 50 mM KCl (pH 7.5). After reconstitution, phospholipid:protein ratio (w/w) was determined for cytochrome c oxidase vesicles. The phospholipid:protein ratio (w/w) was 20 giving a respiratory control ratio (RCR) of 5.3 for the reconstituted enzyme.

Thin layer chromatography of the original phospholipid mixture of the liposomes with or without cytochrome c oxidase, revealed identical phospholipid compositions. This showed that the selection process was not a function of a differential distribution of a major phospholipid component.

![Fig. 8. Time course of cytochrome c oxidase reconstitution by cholate dilution. Cholate dilution cytochrome c oxidase vesicles prepared with phosphatidylethanolamine-phosphatidylcholine (1:1) plus 5% phosphatidylinositol (50 mM KCl, pH 7.5) were prepared and assayed after the indicated incubation time, as described under "Experimental Procedures." Phospholipid (PL):protein ratios obtained by varying the amount of phospholipid were 25 (closed circles), 5 (open circles), and 2.5 (open squares). Results were plotted on a % incorporation scale (taking the initial rate as unincorporated enzyme activity) with the corresponding respiratory control ratios (RCRs) given on the right. The "zero time" is actually a sample taken about 30 s after mixing.](http://www.jbc.org/)

![Fig. 9. Efficiency of cytochrome c oxidase reconstitution into liposomes by cholate dilution, cholate dialysis, and incorporation without detergents. Reconstitution efficiencies at various phospholipid:protein ratios were determined for cytochrome c oxidase vesicles prepared by cholate dilution (open squares), cholate dialysis (closed circles), and incorporation (open circles). Cholate dilution vesicles were prepared and assayed after a 10-min incubation at 0° as described in the legend of Fig. 8. Cholate dialysis vesicles were prepared with phosphatidylethanolamine-phosphatidylcholine (1:1) plus 5% phosphatidylinositol in a final volume of 0.2 ml (50 mM KCl, pH 7.5) as described under "Experimental Procedures." Cytochrome c oxidase vesicles were prepared by incorporation with phosphatidylethanolamine-phosphatidylcholine (1:1) plus 20% phosphatidylinositol by mixing 0.2 ml of sonicated liposomes (in 50 mM KCl, pH 7.5) with small aliquots of concentrated enzyme (10 mg/ml) to give the indicated phospholipid:protein ratios. After a 30-min incubation at 20°, 10-μl aliquots were assayed as described under "Experimental Procedures." Similar results were obtained by varying the amount of phospholipid with a constant amount of enzyme. Results are plotted as in Fig. 8. RCR, respiratory control ratio.](http://www.jbc.org/)
Cytochrome c oxidase vesicles were reconstituted with phosphatidylethanolamine:phosphatidy[13](2:1) plus a trace amount of [13]triolein by the incorporation procedure as described under "Experimental Procedures." The amount of enzyme added was varied to give phospholipid:protein ratios ranging from 5 to 40. Following reconstitution for 30 min at 20°C the vesicles were cooled to 4°C and applied to separate Ficoll gradient tubes and centrifuged as described under "Experimental Procedures." Fractions corresponding to the Ficoll discontinuous gradient layers were collected and aliquots assayed for activity and respiratory control. Phospholipid recoveries were monitored by [13]triolein counts. Fractions marked * were stimulated 4- to 5-fold by incubation with phospholipid and cholate as described in the text.

Characterization of Subpopulations of Cytochrome c Oxidase Liposomes Prepared by Incorporation Procedure—Reconstitution by the incorporation procedure revealed that only a 10% subpopulation of liposomes allowed incorporation of the enzyme. It can be seen from Table VII that fractionation of vesicles reconstituted at phospholipid:protein ratios between 40 to 5 yielded liposome bands (5% Ficoll layers) containing 87 to 90% of the phospholipids and less than 12% of the activity. Most of the activity was associated with about 10% of the original phospholipids used for reconstitution.

| Reconstitution phospholipid: protein ratio | Recovery % Phospholipid | Recovery % Activity | Recovery % Respiratory Control |
|-------------------------------------------|-------------------------|---------------------|-------------------------------|
| % Phospholipid | Activity Ratio | % Phospholipid | Activity Ratio | % Phospholipid | Activity Ratio |
|----------------|---------------|-----------------|-----------------|----------------|---------------|
| 5 %Ficoll Layer | 88 | 12 | 9.4 | 90 | 12 | 9.3 | 89 | 10 | 8.7 |
| 6 | 8 | 36 | 9.0 | 6 | 27 | 6.6 | 2 | 32 | 6.4 |
| 8 | 3 | 36 | 6.6 | 1 | 15 | 3.8 | 1 | 20 | 5.2 |
| 10 | 1 | 13 | 3.8 | 0.5 | 5 | 4.0 | 1 | 10 | 2.8 |
| 15 | 0.5 | 2 | 1.7 | 0.5 | 1 | 1.0 | 0.5 | 1 | 1.2 |
| 30 | 0 | 1 | 1.5 | 0.5 | 1 | 1.0 | 0.5 | 1 | 1.2 |

This possibility was also unlikely since a high phospholipid:protein ratio was required with a large variety of phospholipid mixtures (see Table IV).

Other possibilities that were considered are selection of asymmetrically assembled liposomes, differences in membrane curvature, or phospholipid packing density.

Reconstitution of Vesicles at Low Phospholipid:Protein Ratios—In the course of experiments exploring these possibilities it was noted that low phospholipid:protein ratios can be used in the cholate dilution procedure provided exposure to 1% cholate was limited to 10 min as shown in Fig. 8. These observations led us to modify the cholate dialysis technique in order to remove cholate as quickly as possible. This was done by working rapidly, dialyzing immediately after the addition of enzyme, and by reducing the sample volume from 0.6 to 0.2 ml, thereby increasing the surface during dialysis. As shown in Fig. 9, good reconstitution efficiency was obtained at phospholipid:protein ratios of 2.5 with both cholate dialysis and cholate dilution procedures. Similar results were obtained with several other phospholipid mixtures. However, the incorporation procedure still required phospholipid:protein ratios of 20 (Fig. 9). We therefore pursued the problem of the properties of vesicles prepared by the incorporation procedure.
During the first incorporation, cytochrome c oxidase vesicles were reconstituted (same preparation as described in Table VII) at phospholipid:protein ratios of 40, 20, and 5. The liposomes obtained from these preparations after Ficoll gradient centrifugation were assayed by incorporation with fresh cytochrome c oxidase in discerning between the different populations of liposomes.

Liposomes that had no exposure to cytochrome c oxidase were used as controls. This was particularly striking with liposomes isolated from experiments with a phospholipid:protein ratio of 5. These results show that incorporation was limited to 10% of the original phospholipid vesicles and that about 90% of the liposomes were not suitable for reconstitution. This is not due to the presence or absence of some component in the liposome band since after extraction and resonication these phospholipids were again suitable for incorporation of cytochrome c oxidase. We were therefore left with two alternatives, either a difference in the size of the competent vesicles or in the packing of the phospholipids. Thus far we have been unable to detect reproducible differences in the properties of the competent and incompetent liposome. The answer to this puzzling question must await the development of physicochemical methods that are as sensitive as cytochrome c oxidase in discerning between the different populations of liposomes.

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