

IDENTIFICATION AND TRANSMEMBRANOUS LOCALIZATION OF ACTIVE CYTOCHROME OXIDASE IN RECONSTITUTED MEMBRANES OF PURIFIED PHOSPHOLIPIDS BY ELECTRON MICROSCOPY

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ABSTRACT

Cytochrome oxidase vesicles with high oxidase activity and respiratory control ratio (greater than 3.5) were characterized by the freeze-etch technique for electron microscopy. By the use of this technique, cytochrome oxidase is shown to be an inner membrane particle. By locating cross-fractured vesicles in the same preparation, cytochrome oxidase particles are shown to extend across the phospholipid bilayer membranes. When cytochrome oxidase is added to preformed liposomes respiratory control is not observed, but high oxidase activity is maintained. In this preparation the cytochrome oxidase particles are located on the outer vesicle membrane surface. These observations provide direct evidence that cytochrome oxidase is found in a transmembranous position in closed, active cytochrome oxidase vesicles having respiratory control.

Reconstituted cytochrome oxidase vesicles have been used to study cytochrome oxidase activity, respiratory control, and the proton translocating system of mitochondria. From observations with valinomycin and nigericin, it has been suggested that respiratory control is a consequence of the formation of a membrane potential by the transmembranous organization of cytochrome oxidase in closed vesicular compartments (4, 10). The relationship of the cytochrome oxidase to the closed vesicle membrane is a critical test of this hypothesis. If the cytochrome oxidase is on the outside of the vesicle membrane this would exclude such a formulation, whereas a location within the membrane would be consistent with the hypothesis.

That cytochrome oxidase occupies a transmembranous position in the lipid bilayer has been sug-

gested by several workers (2, 14, 18). Indirect evidence from biochemical data and from particle and membrane measurements strongly suggests a transmembranous location. However, it is still necessary to show that the cytochrome oxidase is located in a transmembranous position in reconstituted phospholipid vesicles which exhibit cytochrome oxidase activity and respiratory control.

It will be shown in the present study that vesicles reconstituted with the enzyme exhibit particles on fracture faces of freeze-fractured membranes, whereas control preparations made with phospholipids alone contain no particles. Examination of cross-fractured vesicle membranes from preparations of cytochrome oxidase vesicles show particles in a transmembranous position.

Vesicle populations were examined by electron microscopy, using negative staining to determine

vesicle size populations. Freeze-fracturing was done to identify cytochrome oxidase in association with the bilayer membranes.

MATERIALS AND METHODS

Preparation of Cytochrome Oxidase Vesicles

The standard procedure for preparing cytochrome oxidase vesicles was the cholate dialysis method against 50 mM potassium phosphate, pH 7.55, as previously described (10). The phospholipids used were either partially purified soybean asolectin (6) or, where noted, purified soybean phospholipids (5) (phosphatidyl choline-phosphatidyl ethanolamine-cardiolipin, in a ratio of 5:5:1.5 μ mol). Two preparations of cytochrome oxidase were used in the present study (7, 19). Unless otherwise specified, the Yonetani preparation (19) was used to make the cytochrome oxidase vesicles. Liposomes were used as a control vesicle system and were prepared as described above except for omitting the cytochrome oxidase.

The salt content of the vesicle preparation used for freeze-fracturing was reduced by two methods. In the first method, the 50 mM potassium phosphate buffer was gradually reduced by dialysis to 10 mM potassium phosphate + 1 mM $MgCl_2$ over a period of 2 days, then further dialyzed for 5 days against 10 mM Tris-HCl (pH 7.4) + 1 mM $MgCl_2$ with daily changes of buffer. In the second method, the cytochrome oxidase vesicles were reconstituted as above, except that the buffer used was 0.05 mM potassium phosphate + 0.05 mM $MgCl_2$. The low salt medium prevented the formation of a salt eutectic during the initial freezing of the sample.

After dialysis, the solutions were examined in suspension or were centrifuged for 1.5 h in a no. 50 Ti rotor at 45,000 rpm in an L2-65B Beckman ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The resulting pellet or suspension was examined by negative-staining, thin-sectioning, and freeze-fracturing techniques.

Electron Microscopy Procedures

Samples of the vesicle preparations were examined by negative staining with 2% sodium phosphotungstate (pH 6.8). For this sectioning, pellets were fixed in 4% glutaraldehyde (0.1 M sodium cacodylate, pH 7.2, 0.25 M sucrose), followed by postfixation in 1% osmium tetroxide (same buffer). The fixed pellets were prestained in aqueous 1% uranyl acetate followed by dehydration through an ascending series of ethanol concentrations and two changes of propylene oxide, and were finally embedded in an Araldite-Epon plastic mixture (15). Thin sections of the plastic-embedded pellets were obtained using a diamond knife mounted on a Sorvall Porter-Blum MT-2 ultramicrotome (Dupont Instruments, Sorvall Operations, Newtown, Conn.). Sections were picked up on no. 400 copper grids and stained with methanolic

uranyl acetate alone or in combination with Reynolds' concentrated lead citrate (11).

Freeze-fracturing of the fresh pellets was done on a Balzer's model BA 360M freezing microtome (Balzer's High Vacuum Corp., Santa Ana, Calif.) at a stage temperature range of $-98^\circ C$ to $-130^\circ C$. The fractured surfaces were replicated with evaporated platinum-carbon followed by evaporated carbon. After the routine cleaning procedure, the replica was picked up on thin carbon films adhering to no. 300 copper grids.

All electron microscopy examinations of the freeze-fractured preparations were done on a JEM 100 B electron microscope at 80 kV. Negatively stained preparations and thin sections were examined on an AEI EM6B electron microscope at 60 kV or 80 kV.

Measurement of Shadowed Replicas

The freeze-fractured replicas were photographed at magnifications of 50,000 and 100,000. The micrographs were projected onto a flat screen at an additional increased magnification of 20. Shadow widths were measured and plotted in a histogram. Corrections for instrument calibration were made and applied to the final measurements.

Measurement of Negatively Stained

Vesicle Preparations

The cytochrome oxidase vesicles and the liposomes from both the purified and soybean asolectin preparations were negatively stained with 2% sodium phosphotungstate. The vesicle diameters were measured by projecting the micrographs (plate magnification of 40,000 or 60,000) onto a flat surface at an increased magnification of 20. Two orthogonal diameters were measured. Histograms of the average of the two orthogonal diameters for the four vesicle preparations were plotted and vesicle size distributions were determined. Correction for instrument calibration was applied here as previously stated.

RESULTS AND DISCUSSION

Size Distribution of the Liposome

and the Cytochrome Oxidase

Vesicle Preparations

The cytochrome oxidase vesicles prepared with either soybean asolectin or purified phospholipids had high cytochrome oxidase activity and respiratory control (Table I A).

The size distributions of the vesicles with and without cytochrome oxidase for both soybean asolectin and purified phospholipids are shown in Figs. 1 and 2, respectively. Different size distributions were observed for both phospholipid prepara-

TABLE I
Activity and Respiratory Control of the Different Cytochrome Oxidase Vesicle Preparations

Cytochrome oxidase vesicle preparation conditions	Oxygen uptake (nAO/min)		RCR	Activity (nAO/min/ml of vesicles)
	-1799	+1799		
A. Standard cytochrome oxidase vesicles				
(Soybean asolectin, 50 mM potassium phosphate)	21	98	4.7	19,500
(Purified phospholipids, 50 mM potassium phosphate)	18	86	4.8	17,100
B. Standard vesicle				
(Soybean asolectin, dialyzed against 10 mM Tris-HCl + 1 mM MgCl ₂)	9	32	3.6	6,400
C. Vesicles reconstituted				
(with soybean asolectin in 0.05 mM potassium phosphate + 0.05 mM MgCl ₂)	16	67	4.2	13,500
Liposomes dialyzed				
(against low salt medium + cytochrome oxidase added after dialysis)	94	94	1.0	18,700

The assay consists of measuring O₂ uptake by an oxygraph, in the presence of 45 mM potassium phosphate, pH 7.0, 23 mM ascorbate, and 1 mg/ml cytochrome *c*. 5 μ l of the vesicle sample was used in each assay.

1799 = 1,3-(bis-Trifluoromethyl hydroxymethyl)-acetone.

RCR = respiratory control ratio = +1799/-1799. (Represents the stimulation of respiration by an uncoupler).

tions. Vesicles reconstituted in the presence of cytochrome oxidase exhibited a larger mean size for the whole population than vesicles formed in the absence of cytochrome oxidase.

The vesicles reconstituted with purified phospholipids (Fig. 2) showed two distinct size distributions. This was more apparent in the liposome preparation than in the vesicle population reconstituted in the presence of cytochrome oxidase. The size distribution of vesicles prepared from the soybean asolectin did not exhibit a bimodal size distribution.

Ratios of the two orthogonal dimensions measured for each vesicle size in the four vesicle populations indicated that the population was composed of near spherical vesicles (Table II), the ratio for a perfect spherical vesicle being 1. Cytochrome oxidase vesicles prepared from the purified phospholipids had a tendency to aggregate. This was observed only in negatively stained preparations and may have resulted from the interaction of the negative stain with the vesicle population. The aggregation phenomenon was not observed in thin-sectioned vesicle populations or in freeze-fractured preparations. Vesicles showing no apparent distortion due to aggregation were used for the size measurements.

Figure 3 illustrates representative negatively stained samples from each of the four vesicle preparations. There were no multivesicular structures found in vesicle preparations formed in the presence of cytochrome oxidase, and only a few were found in the controls (liposomes) by either freeze-etching or thin sectioning. Large but infrequent sheetlike structures appeared to be present in all preparations in the presence or absence of cytochrome oxidase. Centrifugation at 45,000 rpm in a no. 50 Ti rotor, for 30 min eliminated sheetlike structures and gave a supernatant population of smaller vesicles (16). These vesicles had an improved respiratory control ratio, confirming that only the vesicles are associated with this phenomenon.

Particulate Cytochrome Oxidase and its Location in Cytochrome Oxidase Vesicle Preparations by Freeze-Fracture

Two purified cytochrome oxidase preparations and their suspending media without cytochrome oxidase were examined by freeze fracture. Fig. 4 *a* and *b* illustrates the freeze-fracture surfaces in the

SOYBEAN ASOLECTIN PHOSPHOLIPIDS

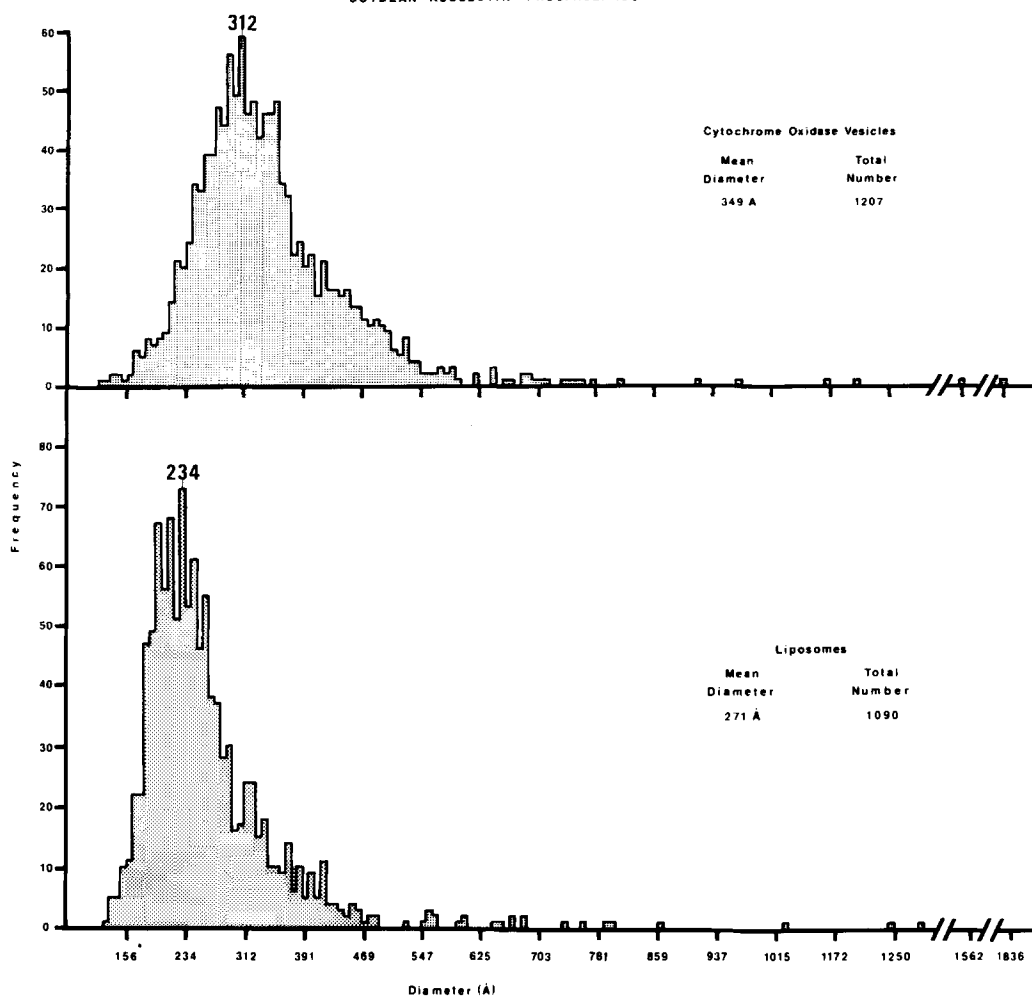


FIGURE 1 Distribution of diameters of vesicles prepared from soybean asolectin phospholipids reconstituted in the presence (cytochrome oxidase vesicles) and absence (liposomes) of cytochrome oxidase.

suspending media of 50 mM sodium phosphate (pH 7.4) + 0.25% Emasol 1130, and of 250 mM sucrose, respectively, which do not contain cytochrome oxidase. Fig. 4 *c* and *d* shows the presence of particles in the purified cytochrome oxidase preparations. Cytochrome oxidase can be identified as a particle by the freeze-fracture technique.

It has been shown that freeze-fracturing cleaves membranes internally through their hydrophobic interior (1, 9, 17). Freeze-fractured cytochrome oxidase vesicles from a centrifuged pellet in 50 mM potassium phosphate show particles on the internal membrane fracture faces (Fig. 5 *a, c*).

TABLE II
Vesicles Reconstituted from Soybean Asolectin and Purified Phospholipids in the Presence and Absence of Cytochrome Oxidase

Vesicle preparation	Lipid source	No. of counts	Average diam-eter ratio
Liposome	Soybean asolectin	400	1.2
Cytochrome oxidase Liposome	Soybean asolectin	400	1.2
	Purified phospholipid	604	1.1
Cytochrome oxidase vesicle	Purified phospholipid	465	1.2

These particles are not present on the control vesicle membrane fracture faces (Fig. 5 *b, d*). These results were obtained with both the soybean asolectin and the purified phospholipids. It can be concluded that cytochrome oxidase can be an inner membrane particle (9, 17).

By simultaneously freeze-fracturing two cytochrome oxidase preparations and a centrifuged

cytochrome oxidase vesicle preparation, followed by platinum-carbon shadowing, it was possible to compare the particle shadow width populations in the three samples. The particle shadow width means (Fig. 6) of the three preparations are the same within the experimental error of the measurements. In addition, the distribution curves are similar for all three sample populations. The right-

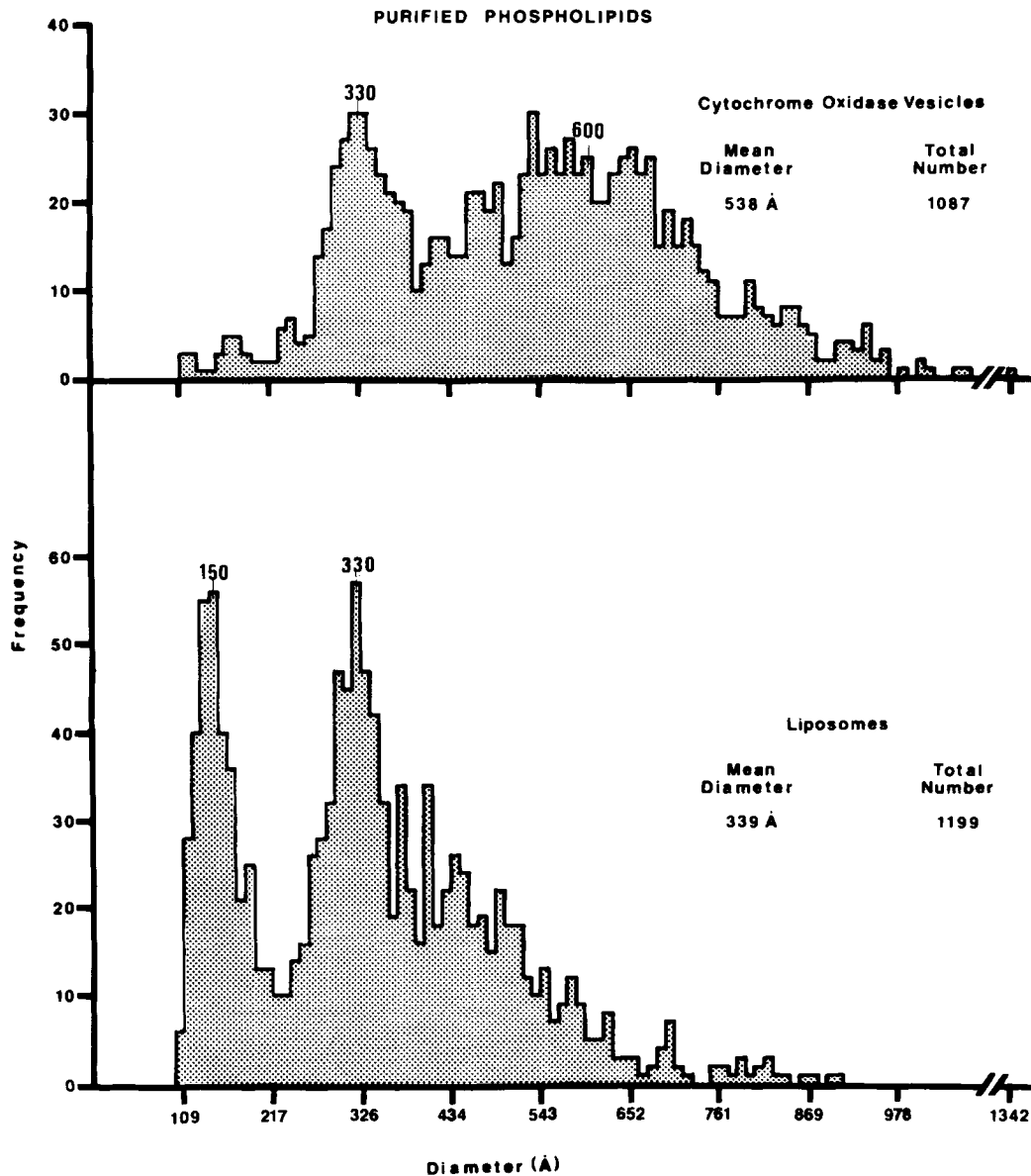


FIGURE 2 Distribution of diameters of vesicles prepared from purified phospholipids reconstituted in the presence (cytochrome oxidase vesicles) and absence (liposomes) of cytochrome oxidase.

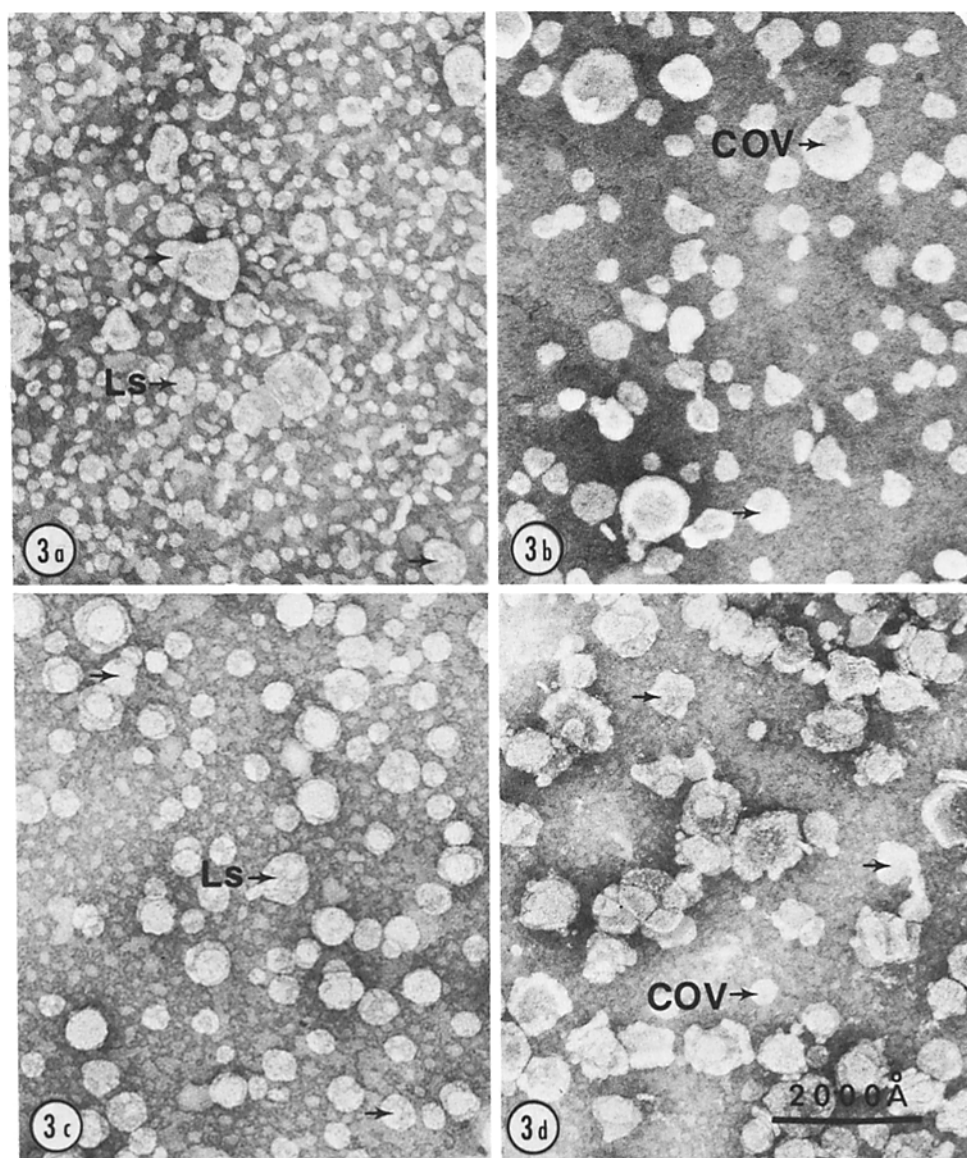


FIGURE 3 Vesicle population negatively stained with 2% sodium phosphotungstate, pH 6.8. $\times 100,000$. (a) Liposomes (*Ls*) prepared from soybean asolectin. (b) Cytochrome oxidase vesicles (*COV*) prepared from soybean asolectin. (c) Liposomes (*Ls*) prepared from purified phospholipids. (d) Cytochrome oxidase vesicles (*COV*) prepared from purified phospholipids.

skewed shaped of the three curves has been observed before (3, 8, 12) and is characteristic of platinum-carbon shadow width distributions of homogeneous particles. The results confirm that only the particles added from the purified cytochrome oxidase preparation are found on the membrane fracture faces and that all the inner

membrane particles in cytochrome oxidase vesicles are cytochrome oxidase. Since no differences were observed to be due to phospholipid content, using either purified phospholipids or soybean asolectin, and since the soybean asolectin was readily available, it was used exclusively for subsequent freeze-fracture experiments.

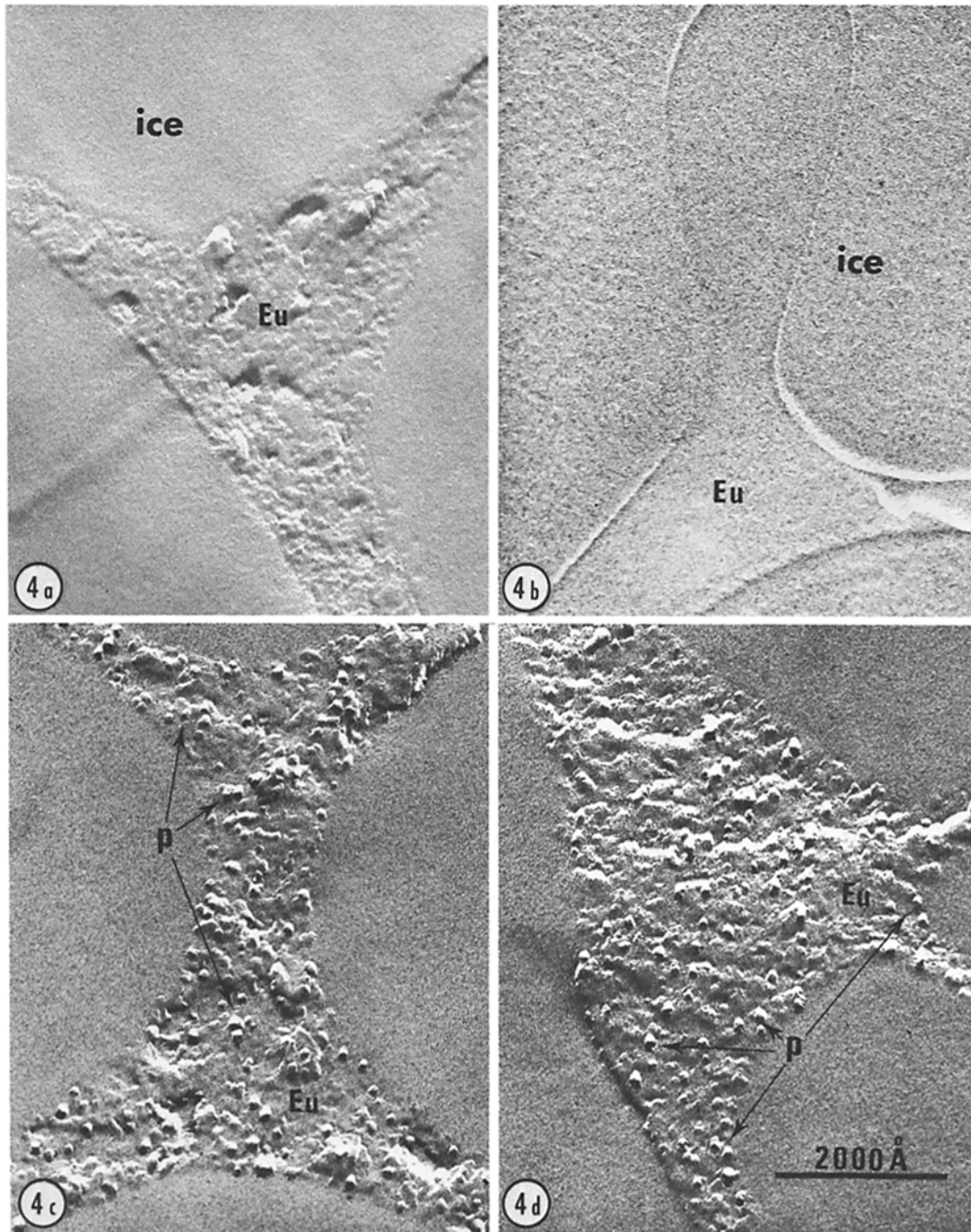


FIGURE 4 Freeze-fracture of 50 mM sodium phosphate, pH 7.4, + 0.25% Emasol 1130, and 250 mM sucrose at -115°C in the absence and in the presence of cytochrome oxidase particles (*p*), respectively. Eutectic, (*Eu*). $\times 120,000$. (a) 50 mM sodium phosphate + 0.25% Emasol 1130. (b) 250 mM sucrose. (c) 50 mM sodium phosphate + 0.25% Emasol 1130 plus cytochrome oxidase. (d) 250 mM sucrose plus cytochrome oxidase. Shadow direction for all the freeze-fracture figures originates from the bottom of the figure.

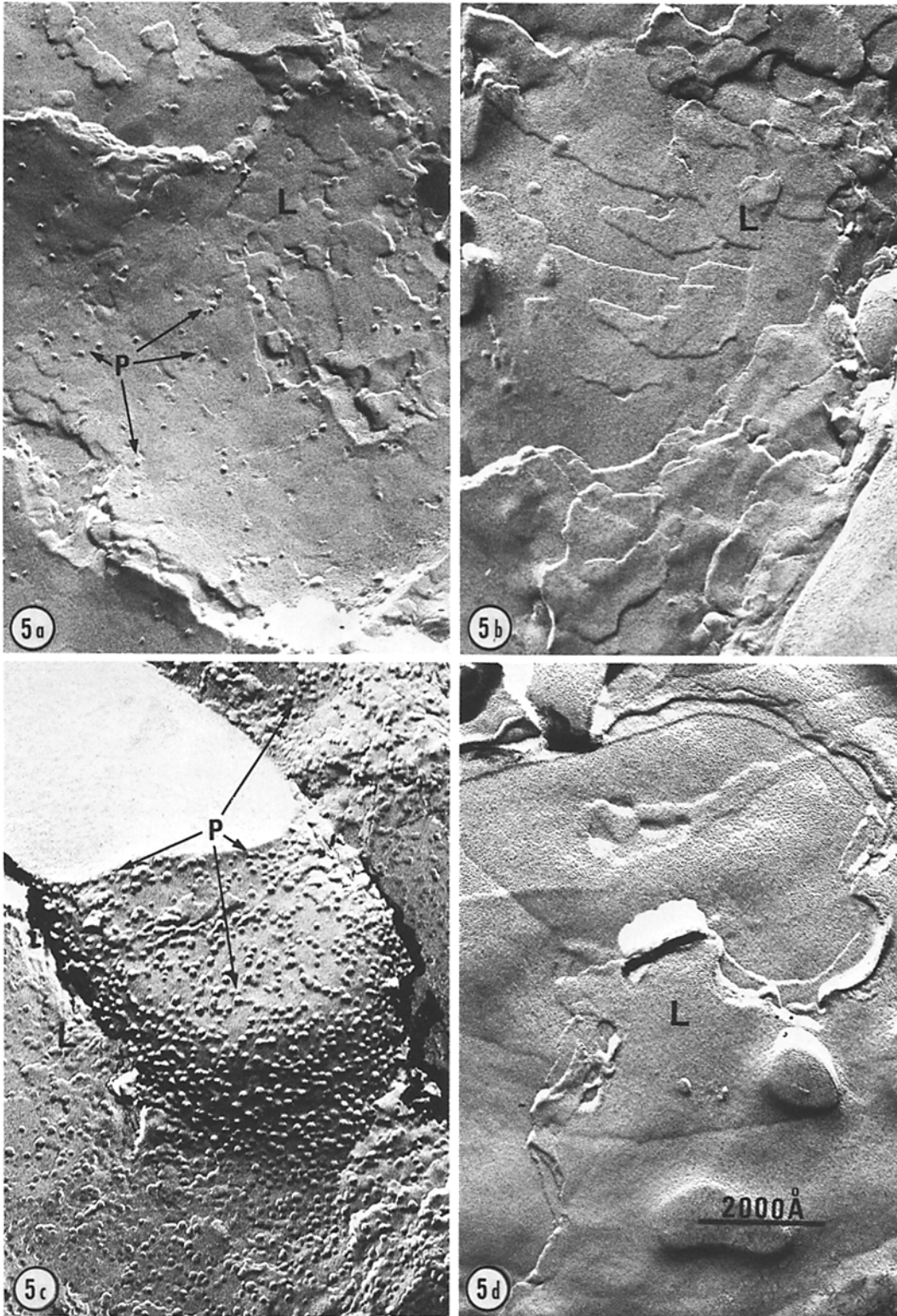


FIGURE 5 (a) Freeze-fracture of centrifuged and pelleted cytochrome oxidase vesicles prepared from purified phospholipids (-115°C); cytochrome oxidase particles (*p*), lipids (*L*). $\times 100,000$. (b) Freeze-fracture of centrifuged and pelleted liposomes prepared from purified phospholipids (-120°C); lipids (*L*). $\times 100,000$. (c) Freeze-fracture of centrifuged and pelleted cytochrome oxidase vesicles prepared from soybean asolectin phospholipids (-130°C); cytochrome oxidase particles (*p*), lipids (*L*). $\times 100,000$. (d) Freeze-fracture of centrifuged and pelleted liposomes prepared from soybean asolectin phospholipids (-130°C); lipids (*L*). $\times 100,000$.

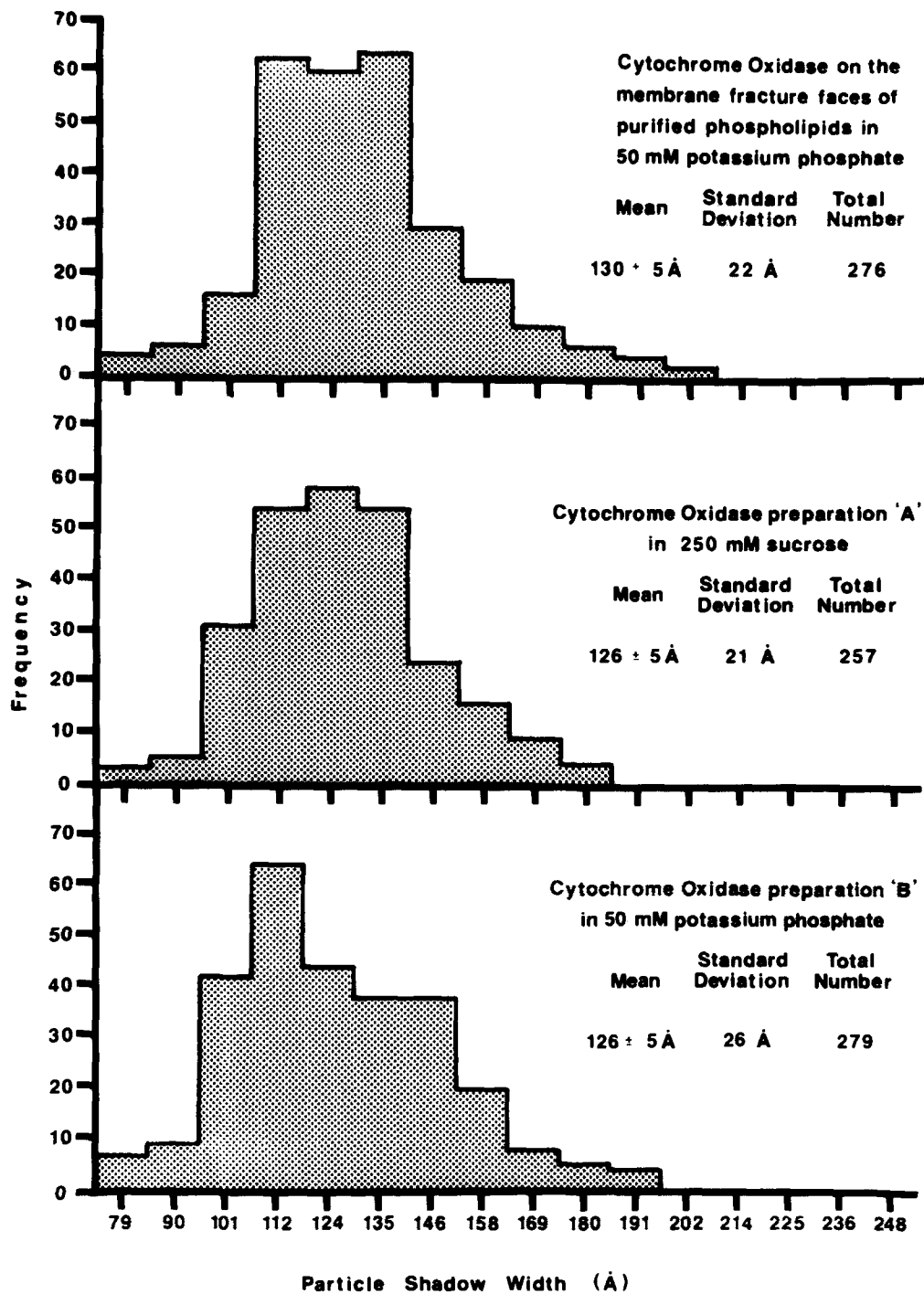


FIGURE 6 Comparison of particle shadow width distribution for cytochrome oxidase vesicles prepared from purified phospholipids, centrifuged, pelleted, and freeze-fractured (Fig. 5 a) and for freeze-fractured cytochrome oxidase preparation "A" (Fig. 4 d, reference 18) and preparation "B" (Fig. 4 c, reference 7). All three preparations were simultaneously freeze-fractured and shadowed with Pt-C.

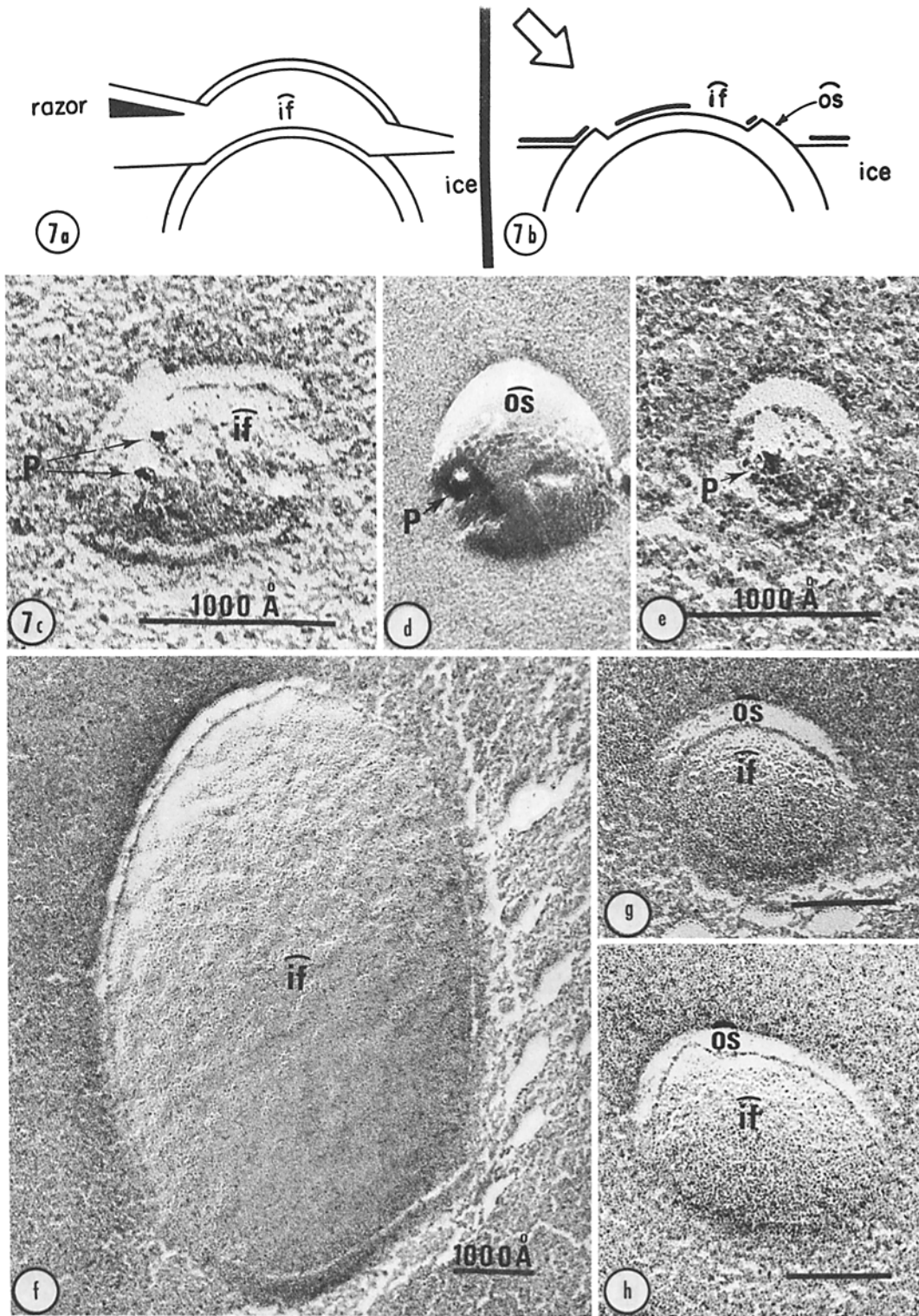


FIGURE 7 (a) Schematic presentation of the normal mode of liposome freeze-fracture. The fracture follows an interior membrane hydrophobic plane, revealing an inner vesicle membrane fracture face (*if*). (b) After the freeze-fracture step, ice sublimation reveals an outer vesicle membrane surface (*os*) surrounding the inner vesicle membrane fracture face (*if*). Pt-C shadowing at 45° accumulates on the rising slopes (heavy line) and is absent from the falling slopes. (c-e) Freeze-etched (-99°C) cytochrome oxidase vesicles prepared from soybean asolectin phospholipids. Cytochrome oxidase particles (*p*), inner vesicle membrane fracture face, (*if*), outer vesicle membrane surface, (*os*). × 300,000. (f) Freeze-etched (-99°C) liposomes made from soybean asolectin phospholipids. × 120,000. (g, h) Smaller vesicles as illustrated in (f) × 160,000.

Because exterior vesicle surfaces are obscured by freeze fracturing in the presence of the eutectic-forming 50 mM potassium phosphate, cytochrome oxidase vesicles and control liposomes reconstituted in the presence of 50 mM potassium phosphate were extensively dialyzed against 10 mM Tris-HCl (pH 7.4) + 1 mM MgCl₂ (13). These vesicles still maintained a good respiratory control ratio, although total activity had been reduced compared to the standard preparation (Table I B). Uncentrifuged samples from the two vesicle preparations were freeze-fractured and slightly etched. Fig. 7 *a* and *b* schematically illustrates the membrane-fracturing process and the subsequent shadow build-up on the exposed inner fracture surface of the vesicles. Particles were observed only on the fracture faces of the cytochrome oxidase vesicles (Fig. 7 *c-e*) and none were seen on the fracture faces of the control vesicles (Fig. 7 *f-h*).

Since particles are absent from liposomal membrane fracture faces and are within the size range

of the cytochrome oxidase particles in Fig. 6, the membrane fracture face particles are identified as cytochrome oxidase.

Transmembranous Location of Cytochrome Oxidase in Freeze-Fractured Membranes of Reconstitutively Active Cytochrome Oxidase Vesicles

It has been shown that cytochrome oxidase is an inner membrane particle. However, to prove that it is transmembranous, cross-fractured vesicles must be found showing particles extending across the cross-fractured vesicle membrane. This type of vesicle fracturing depicted in Fig. 8 *a* is a relatively frequent occurrence. The fact that the inner vesicle ice is removed with the upper fracture surface was surprising. Shadowing of the cross-fractured vesicles is schematically shown in Fig. 8 *b*. A number of cross-fractured vesicles showing the full cross-

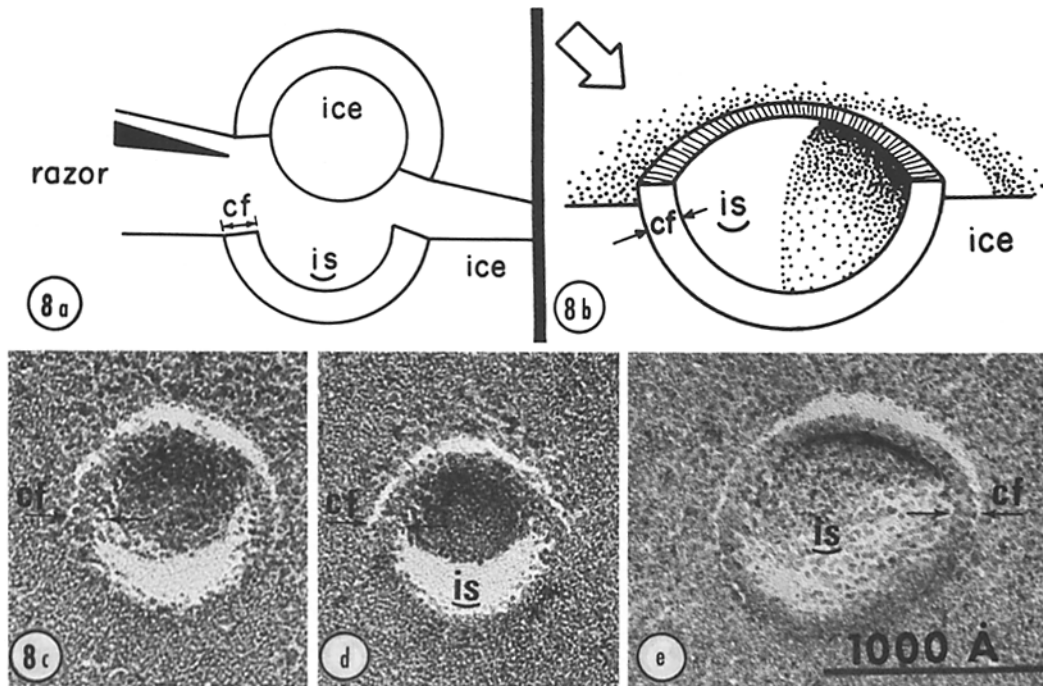


FIGURE 8 (a) Schematic representation of the vesicle cross-fracture process. As the vesicle fractures below its geometrical center, the inner ice sphere is constrained and removed with the upper fracture surface. By this process, an inner vesicle membrane surface (*is*) and a cross-fractured membrane (*cf*) can be seen. (b) Schematic representation of a freeze-etched, cross-fractured vesicle after Pt-C shadowing. Inner vesicle membrane surface (*is*), cross-fractured membrane, (*cf*). (c-e) Examples of freeze-etched (-99°C), cross-fractured liposomes of soybean asolectin phospholipids. $\times 300,000$.

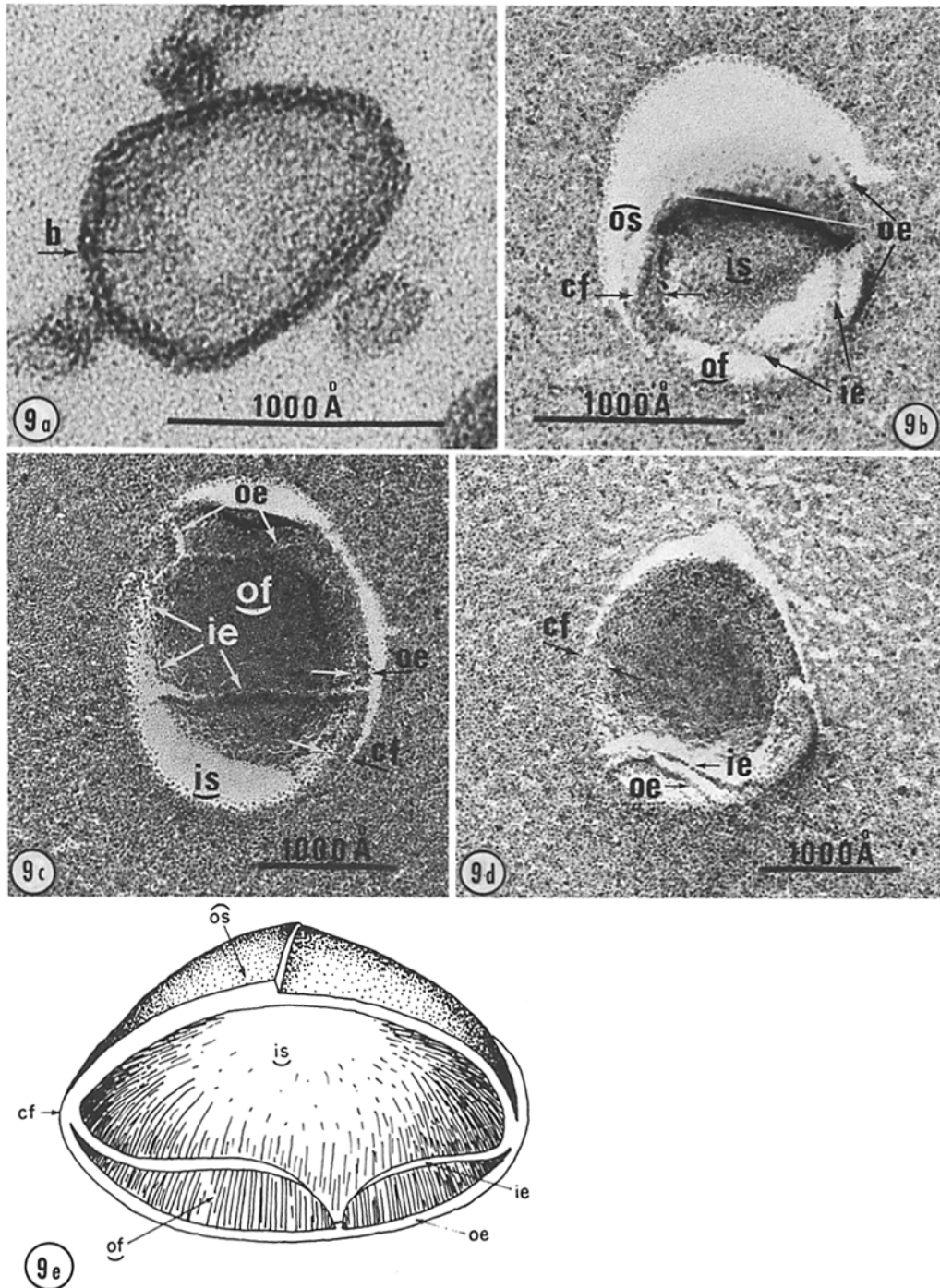


FIGURE 9 (a) Thin section of a liposome prepared from soybean asolectin phospholipids. Poststained with methanolic uranyl acetate and Reynolds' lead citrate. Membrane bilayer (b). $\times 400,000$. (b) Cross-fractured and freeze-etched (-99°C) liposome of soybean asolectin phospholipids showing a bilayer structure of the cross-fractured membrane (cf). Inner vesicle bilayer membrane edge (ie), outer vesicle bilayer membrane edge (oe), outer vesicle membrane fracture face (of). $\times 300,000$. (c, d) Freeze-fractured liposomes, similar to (b) but showing examples of the bilayer membrane features of the liposomes. Outer vesicle membrane surface (os), inner vesicle membrane surface (is). (e) Schematic interpretation of fracture edges and surfaces observed in (b).

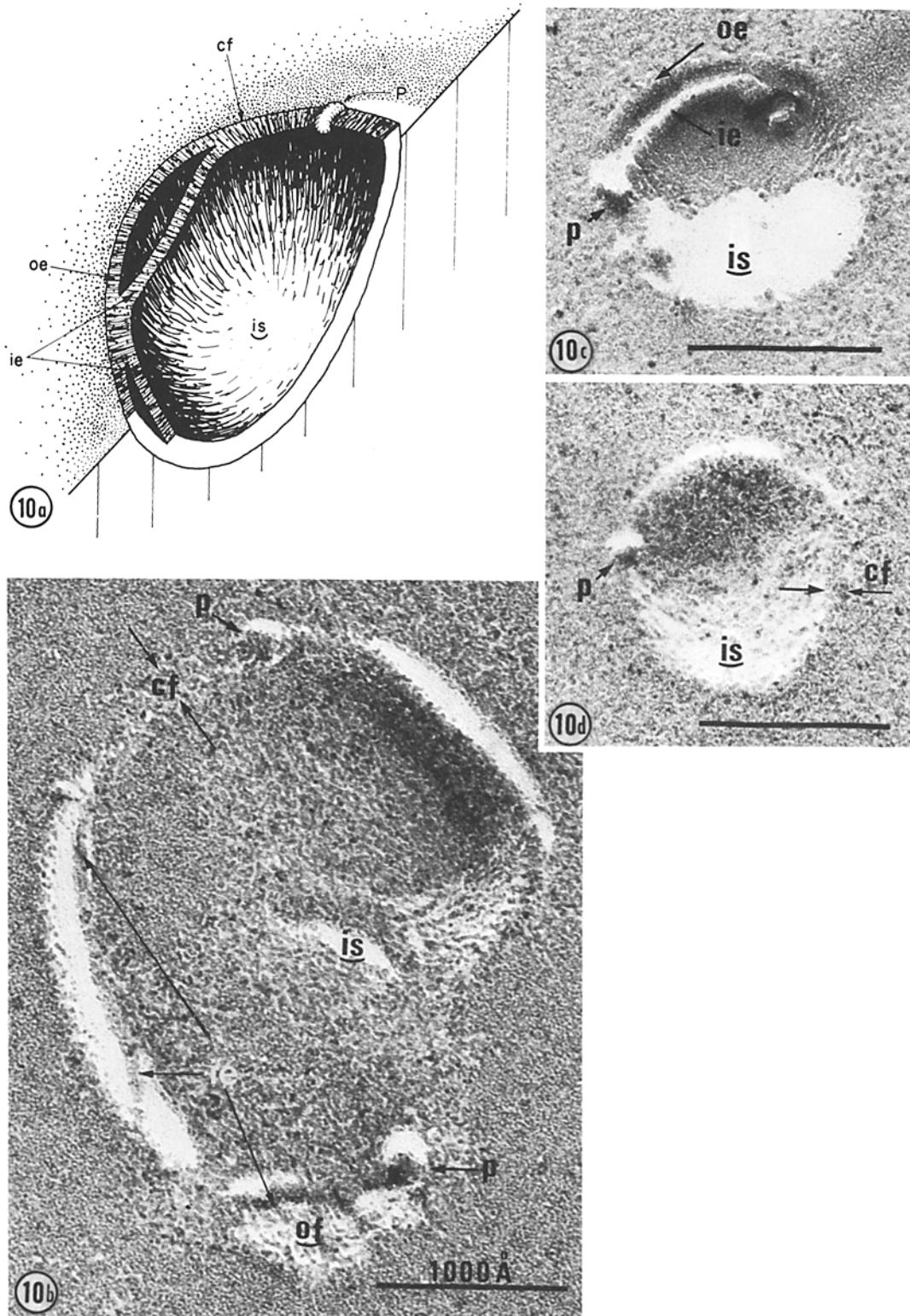


FIGURE 10 (a) Schematic drawing of the left half of the cross-fractured vesicle in Fig. 10 b. Cross-fractured membrane (*cf*), outer vesicle bilayer membrane edge (*oe*), inner vesicle bilayer membrane edge (*ie*), inner vesicle membrane surface (*is*), cytochrome oxidase particles (*p*). (b) Freeze-etched (-99°C) cross-fractured cytochrome oxidase vesicle of soybean asolectin showing a cytochrome oxidase particle (*p*) extending across the cross-fractured membrane (*cf*). Inner vesicle bilayer membrane edge (*ie*), outer vesicle membrane fracture face (*of*). $\times 300,000$. (c) Similar interpretation as for (b) except freeze-fractured at -120°C . (d) Fractured vesicle similar to that in (b) having a cytochrome oxidase particle (*p*) extending across the bilayer membrane (*cf*) of the vesicle.

fractured membrane (*cf*) and an inner vesicle membrane surface (*is*) are shown in Fig. 8 *c-e*. That the vesicles contain bilayer membranes has been confirmed by thin sectioning (Fig. 9 *a*) and freeze-etching in Fig. 9 *b-d*. By the freeze-etch technique, it was possible to distinguish internal vesicle membrane surfaces (*is*) from outer vesicle membrane fracture faces (*of*) in concave vesicle surfaces (Fig. 9 *b-e*). Half-bilayer membrane cross-fracture edges were also observed (Fig. 9 *b-d*), and Fig. 9 *b* is illustrated diagrammatically in Fig. 9 *e*.

The structural information from the smaller vesicles appears to have a content similar to that of the vesicles shown above. However, the structural features of the larger vesicles (greater than 600–700-Å diam) (shown in the figures) are less ambiguous in their interpretation.

In order to observe the full thickness of the bilayer membrane cross-fracture, the platinum-carbon shadow direction has to be tangential to the membrane edge. The cytochrome oxidase particles had to be observed in this same region so that their size could be directly compared to that of the membrane thickness (Fig. 11 *a*).

Fig. 10 *a* schematically illustrates the cross-fractured cytochrome oxidase vesicles showing a particle on a membrane cross-fracture. This drawing depicts the upper part of Fig. 10 *b*. Fig. 10 *c* and *d* also shows particles on the cross-fractured cytochrome oxidase vesicle membrane which are absent from the cross-fractured liposomal membrane (Fig. 8). In order to confirm the results of this experiment, cytochrome oxidase vesicles were reconstituted directly in a noneutectic-forming buffer of 0.05 mM potassium phosphate + 0.05 mM MgCl₂. The cytochrome oxidase activity and respiratory control of this preparation were superior to those of the extensively dialyzed cytochrome oxidase vesicle preparation in 10 mM

Tris-HCl + 1 mM MgCl₂ (Table I B, C). Cross-fractured cytochrome oxidase vesicles reconstituted in low salt show particles extending across the cross-fractured membranes (Fig. 11). The

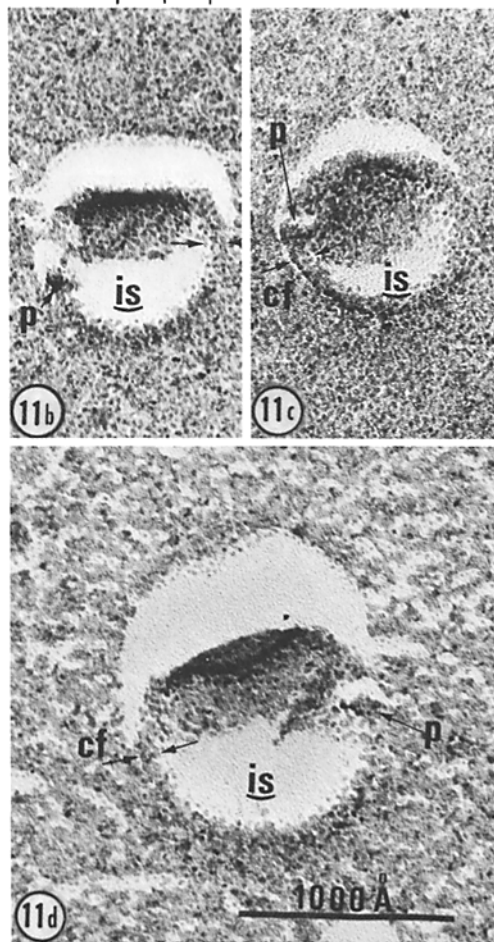
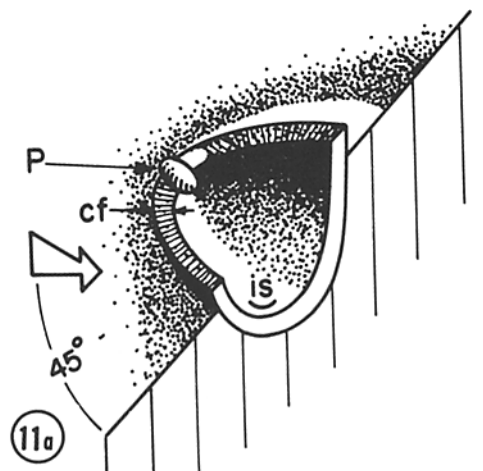


FIGURE 11 (*a*) Schematic drawing of a shadowed, freeze-etched, cross-fractured cytochrome oxidase vesicle. In order to compare the particle (*p*) size to the membrane width (*cf*), the particle must be positioned in a region where the shadow is tangential to the membrane surface so that both sides of the particle and the membrane can be viewed. (*b-d*) Freeze-etched (-98°C), cross-fractured cytochrome oxidase vesicles of soybean asolectin formed in 0.05 mM potassium phosphate + 0.05 mM MgCl₂. A cytochrome oxidase particle (*p*) is shown extending across the fracture membrane (*cf*). Inner vesicle membrane surface, (*is*). $\times 300,000$.

width of the cross-fractured membrane is about twice the width of the membrane fracture face (Fig. 8 *c-e*), and its shadowed width averages about 107 Å. The cytochrome oxidase particles average about 1.4 times the average cross-fractured membrane thickness. The results with low salt-reconstituted cytochrome oxidase vesicles confirm the observation in the extensively dialyzed cytochrome oxidase preparation that cytochrome

oxidase is found in a transmembranous location, in closed active vesicles showing respiratory control. Nonetheless, these correlative observations need to be confirmed with a closed vesicle preparation showing no respiratory control.

When cytochrome oxidase was added to a closed, liposome preparation after dialysis against the low salt medium, the biochemical data (Table I *d*) showed that the full cytochrome oxidase vesicle

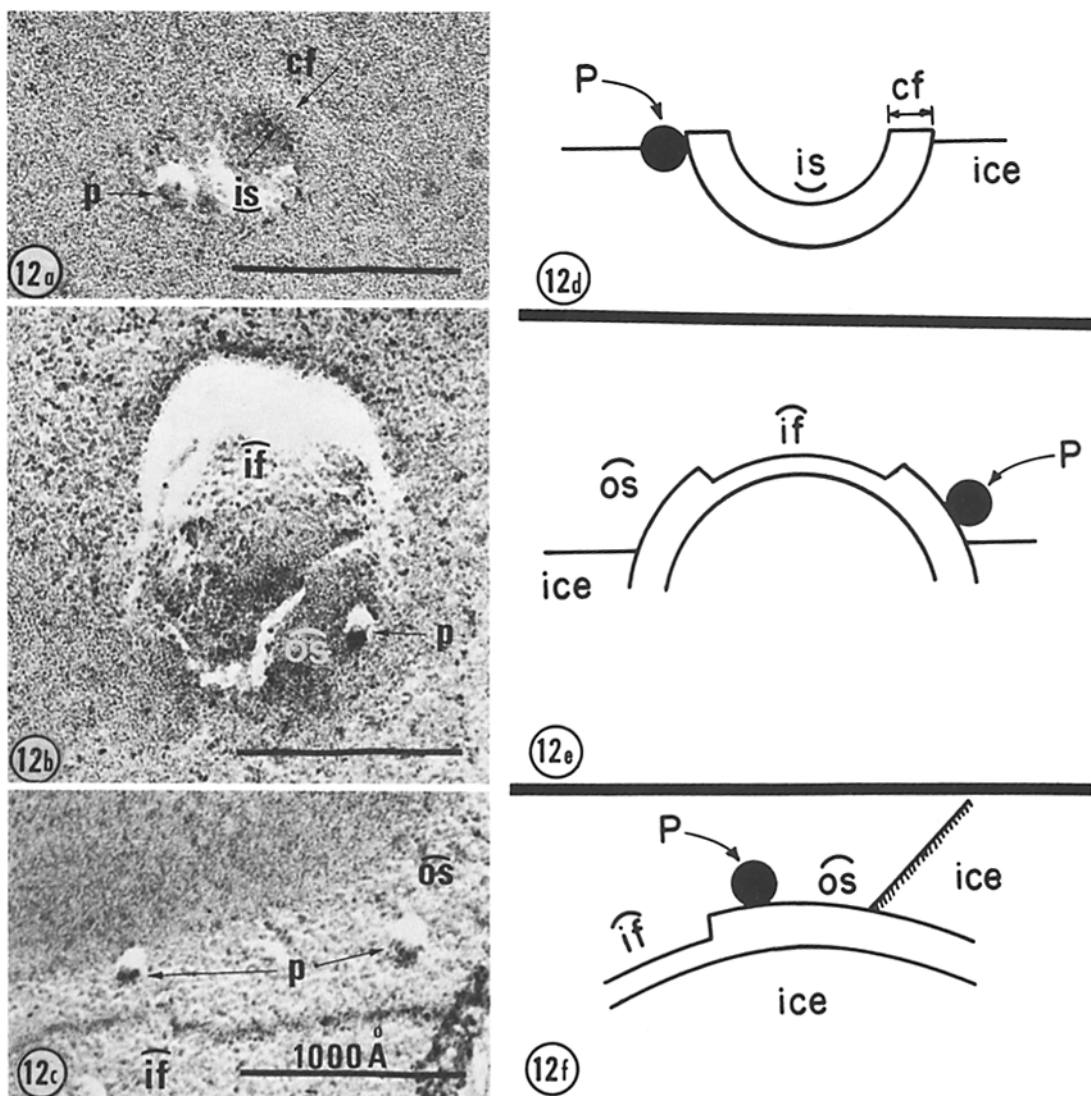


FIGURE 12 (a) Freeze-etched (-98°C), cross-fractured liposome prepared from soybean asolectin showing a cytochrome oxidase particle (*p*) on the outside of the cross-fractured membrane (*cf*). Inner vesicle membrane surface, (*is*). $\times 300,000$. (b) Freeze-etched (-98°C) liposome showing a cytochrome oxidase particle (*p*) on an outer vesicle membrane surface (*os*). Inner vesicle membrane fracture face (*if*). $\times 300,000$. (c) Situation similar to that found in (b). (d-f) Schematic interpretations of *a*, *b*, and *c*, respectively.

activity (the same as that of cytochrome oxidase vesicles plus uncouplers) could be observed, but that there was no respiratory control (RCR = 1). The lack of respiratory control in this preparation correlates with the freeze-etch observations (Fig. 12) that none of the cytochrome oxidase particles were located transmembranously with respect to the membrane, but were found on an outer vesicle membrane surface. Fig. 12 *d*-*f* illustrates diagrammatically the position of the cytochrome oxidase observed in Fig. 12 *a*-*c*, respectively. These observations provide direct evidence that cytochrome oxidase is found in a transmembranous position in closed, active cytochrome oxidase vesicles having respiratory control.

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