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Dynamics of proteoliposome formation

Intermediate states during detergent dialysis

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1. The intermediate structures formed during dialysis of mixtures of cholate, phospholipid and cytochrome *c* oxidase were analysed by gel chromatography and electron microscopy. Measurements of trapped phosphate and the degree of respiratory control were used to assess the integrity of the vesicular structures formed. Protein orientation in the bilayer was monitored by the accessibility of cytochrome *c* to cytochrome *c* oxidase. 2. The results indicate that proteoliposome formation by the detergent-dialysis procedure takes place in three distinct stages. In the first stage, cholate/phospholipid and cholate/phospholipid/protein micelles coexist in solution and grow in size as the detergent is slowly removed. At a detergent/phospholipid molar ratio of about 0.2, micelle fusion results in the formation of large bilayer aggregates permeable to both phosphate and cytochrome *c*. It is at this stage that cytochrome *c* oxidase is incorporated into the bilayer. In the final stage of dialysis the bilayer sheets fragment into small unilamellar vesicles. 3. The orientation of membrane protein in the final vesicles appears to be determined by the effect of protein conformation on the initial curvature of the bilayer sheets during the fragmentation process.

INTRODUCTION

Phospholipid vesicles incorporating purified membrane protein (proteoliposomes) have become of considerable importance in the study of vectorial reactions catalysed by membrane-associated enzymes (Racker, 1976; Kagawa, 1978; Eytan, 1982; Wrigglesworth, 1984; Casey, 1984). The restoration of an original vectorial process by purification of the individual components followed by reconstitution into proteoliposomes can make it possible to identify various interactions between the different components of a system and evaluate the importance of asymmetry and orientation of the components in the membrane. Such information is also important to take full advantage of the possibility of introducing ligands for surface receptors into liposomes to target entrapped materials to specific sites in the body for therapeutic use (Gregoriadis, 1984).

Several methods have been used to prepare proteoliposomes with membrane protein incorporated into the lipid bilayer. The most successful have been those involving an initial dispersal of the lipid and protein in detergent followed by removal of the detergent by dialysis (Racker, 1972), by gel filtration (Brunner *et al.*, 1976; Bangham, 1982) or by adsorption (Kasahara & Hinkle, 1977; Rogner *et al.*, 1979). However, the mechanism of vesicle formation by any of these methods is not known. The classical method of producing small unilamellar liposomes is by sonication, and Fromherz & Ruppel (1985) have suggested a two-stage process where fragmentation of multibilayer aggregates of phospholipid into open sheets of bilayer is followed by spontaneous closure of the planar discs into closed shells. The presence of small amounts of detergent can slow down the second stage by shielding the non-polar acyl chains of the lipid from water. According to Lasic (1982), a similar effect is responsible for vesicle formation from lipid/detergent mixtures. As detergent is removed, mixed lipid/detergent micelles grow in size, with detergent concentrating at the disc edges until there is not enough detergent to shield the exposed circumference. At this stage the discs begin to curve and eventually collapse into vesicles.

Reconstitution from detergent mixtures can yield proteoliposomes of various sizes and compositions. These variations appear to depend more on the detergent and the particular procedure used than on the lipid composition (Zumbuehl & Weder, 1981; Eytan, 1982). Thus cholate mixtures yield a proteoliposome population with vesicles of smaller average diameter than do mixtures dispersed in octyl glucosides (Zumbuehl & Weder, 1981). Removal of detergent by dialysis or gel filtration generally results in smaller vesicles than removal by adsorption (Rogner *et al.*, 1979), although the rate of detergent removal during dialysis can affect the size and multilamellar nature of the final preparation (Brunner *et al.*, 1976). Schurtenberger *et al.*, (1984) have shown that vesicle radius can be selectively varied during the dialysis procedure by predilution of the mixed micellar solution of the bile salt and phospholipid.

The presence of protein introduces more uncertainties. The orientation of incorporated protein in the bilayer appears to depend both on the nature of the protein and on the final size of the vesicle (Casey, 1984). Some proteins, for example the mitochondrial ATP synthase (Sone *et al.*, 1977), incorporate into vesicles with their catalytic sites in a unidirectional orientation in the membrane, whereas other proteins, for example the lactose/H^+ carrier (Seckler & Wright, 1984), incorporate in a random (50:50) orientation. Close packing and membrane curvature in minimal-size vesicles may force a normally randomly incorporated protein, such as cytochrome oxidase, to assume a more unidirectional orientation (Wrigglesworth, 1985).

Uncertainties about the mechanism of vesiculation

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and the various factors affecting liposome size and the orientation of protein within the bilayer are important facts missing from an understanding of proteoliposome formation. The present paper reports the results of an analysis of the intermediate stages during proteoliposome formation by the detergent-dialysis method. Particular attention has been paid to the micelle–vesicle transition stage, which appears to take place via intermediate sheet-like structures that subsequently break up into small unilamellar vesicles.

MATERIALS AND METHODS

Cytochrome *c* oxidase was prepared from ox heart by the method of Kuboyama *et al.* (1972), with Tween 80 substituting for Emasol, and was stored at −70 °C until use. The final preparations of the enzyme had haem *a*/protein ratios of about 10 μmol/g.

Suspensions of bile salt and phospholipid were prepared by dispersing 0.1 g (approx. 0.13 mmol) of dry asolectin (L-α-phosphatidylcholine, Sigma type IV-S) in 2 ml of 0.1 M-potassium phosphate buffer, pH 7.0, containing 0.116 M-sodium cholate (Sigma Chemical Co., Poole, Dorset, U.K.) by sonication for 2 min. The samples were dialysed, at 4 °C with gentle stirring, against 100 vol. of 0.1 M-potassium phosphate buffer, pH 7.0, containing sodium cholate at various constant concentrations. The dialysis medium was changed after approx. 2, 4, 16 and 22 h. After dialysis, the samples were analysed by gel filtration on a 30 cm × 3.8 cm^2 column of Sepharose 2B equilibrated in 0.1 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EGTA and sodium cholate to the same concentration as the dialysis medium. The absorption difference between 350 nm and 500 nm was measured on the eluted fractions. A linear relationship between this absorption difference and phospholipid concentration, measured by organic phosphate, was found over the fractionation range of Sepharose 2B, but this did not apply to absorbance measurements on material excluded from the gel, which was eluted in the void volume.

For samples containing cytochrome *c* oxidase, the enzyme was added to the initial phospholipid/bile-salt mixture to a final concentration of approx. 8 μM (a phospholipid/protein weight ratio of approx. 40:1). Cytochrome *c* oxidase content was analysed by spectral measurements at 420 nm on the eluted samples.

Sidedness of cytochrome *c* oxidase incorporated into the membrane preparations was assessed by the reducibility of the enzyme, under anaerobic conditions, by cytochrome *c* plus ascorbate as described by Wrigglesworth (1978). Trapped volumes were calculated from the measured phosphate concentration remaining in the preparations after further prolonged dialysis against 70 mM-potassium sulphate as described previously (Wrigglesworth, 1985). Respiratory control was measured by using an oxygen electrode and calculated from the rates of O_2 uptake in the absence and presence of valinomycin and carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone with vesicles respiring in the presence of ascorbate, *NNN′N′*-tetramethyl-*p*-phenylenediamine and cytochrome *c*.

Samples for freeze-fracture electron microscopy were frozen in liquid N_2 'slush' (−210 °C) before fracture and replication. Samples for shadowing were sprayed on to carbon-coated grids and rotary-shadowed with platinum at low angle before examination in a Phillips 301 transmission microscope.

RESULTS

Detergent/lipid mixtures

Gel-permeation analysis of mixtures of sodium cholate and asolectin, at different stages of dialysis to remove detergent, shows the presence of several distinct states during liposome formation (Fig. 1). At detergent/phospholipid molar ratios greater than 0.2, the phospholipid is retarded on the column and is eluted close to the bed volume. According to Mazer *et al.* (1980), bile-salt/phospholipid mixtures form small disc-like micelles at high detergent/lipid ratios (> 1.0), and these structures progressively grow in size as detergent is removed. A progressive size increase in the phospholipid structures can be seen in Fig. 1 as the detergent concentration is lowered from 116 mM to 12 mM. The structures seen in these samples by electron microscopy (Fig. 2*a*) are approximately disc-shaped with a range of radii from 6 to 25 nm. The size of these cholate micelles can be contrasted with that seen from micelles of Triton and asolectin at detergent/phospholipid ratios of approximately the same value, where the radii range up to 150 nm (Figs. 1*a* and 2*b*).

At a critical cholate/phospholipid ratio, in this case about 0.1, large structures form and are eluted in the void volume on the gel column (Fig. 1*b*). Electron microscopy shows these to comprise large bilayer sheets (Fig. 2*c*). As the detergent concentration is further lowered, multilamellar closed vesicles form (Figs. 2*d* and 2*e*), which finally break up into small unilamellar vesicles (Fig. 2*f*) eluted in the mid-size range on the Sepharose column (Fig. 1*b*). Freeze-fracture electron microscopy tends to underestimate the size of these vesicles because of the lack of fracture planes falling exactly across the vesicle diameter (Wrigglesworth, 1985).

Detergent/lipid/protein mixtures

In the absence of phospholipid, cytochrome *c* oxidase suspended in 116 mM-sodium cholate is eluted from a gel column, equilibrated at the same detergent concentration, in an earlier fraction than in the absence of detergent (Fig. 3*a*), indicating the formation of detergent/protein micelles. The presence of phospholipid in the initial mixture, before gel filtration slightly increases the size of these micelles, but it is noticeable that separate detergent/lipid micelles of smaller size coexist in the same mixture (Fig. 3*a*). As the cholate concentration is lowered, the two micelle populations, detergent/protein and detergent/lipid, remain separate until a critical detergent concentration for bilayer formation is reached. At this stage, the cytochrome *c* oxidase incorporates into the large sheets of bilayer and elutes in the void volume (Fig. 3*b*). Full dialysis of the initial detergent/lipid/protein mixture, to remove the detergent, finally results in the formation of small unilamellar vesicles with the enzyme incorporated into the bilayer, as described previously (Wrigglesworth, 1985).

Formation of closed vesicles

The micelle–bilayer transition appears to take place, under the present conditions, at a critical cholate concentration of around 8 mM (a cholate/phospholipid

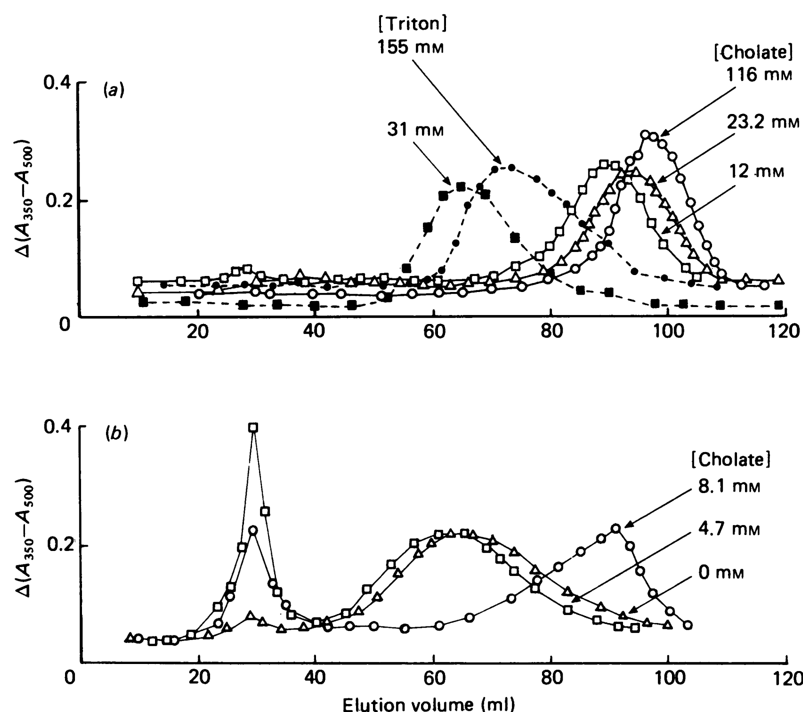

Fig. 1. Analysis of lipid/cholate mixtures on gel-filtration columns equilibrated at different cholate concentrations

Suspensions of sodium cholate (116 mM) and asolectin (65 mM) were dialysed to various final cholate concentration. Portions (2 ml) of the final dialysed preparations were analysed on Sepharose 2B columns (30 cm × 3.8 cm²) equilibrated with 0.1 mM-potassium phosphate buffer, pH 7.0, containing 1 mM-EGTA and sodium cholate at the same concentration as in the final dialysed preparation. The absorbance difference between 350 nm and 500 nm was measured on the eluted fractions and is plotted against elution volume. (*a*) Samples dialysed to and columns equilibrated in sodium cholate concentrations of 116 mM (○), 23.2 mM (△) and 12 mM (□). (*b*) Samples dialysed to and columns equilibrated in sodium cholate concentrations of 8.1 mM (○), 4.7 mM (□) and 0.0 mM (△). Also shown are the analyses of mixtures of asolectin (65 mM) in 155 mM- (●) and 31 mM- (■) Triton X-100 on Sepharose 2B columns equilibrated in 0.1 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EGTA and Triton X-100 at the same concentrations as the mixtures.

ratio of approx. 0.1) and was not noticeably affected by the presence of protein at the protein concentrations used. A closer investigation was made of the properties of the large bilayer structures initially formed from micelle fusion that are eluted in the void fraction of the Sepharose column. From Table 1, it can be seen that these structures, present at cholate concentrations of 8.1 mM, do not have any significant ability to trap inorganic phosphate, in contrast with the few large bilayer sheets present in samples dialysed to zero cholate concentration whose trapped volume assessed from trapped phosphate is 1.1 litre·mol^{-1}. The trapped volume of the small unilamellar vesicles formed after exhaustive dialysis at zero detergent concentration is found to be 0.69 litre·mol^{-1}, which gives an average external diameter of 33 nm. The few small vesicles formed in the 8.1 mM-cholate mixture also retain some ability to trap inorganic phosphate, as can be seen from the apparent trapped volume of 0.49 litre·mol^{-1}. Some leakage of phosphate from these vesicles might be expected at the relatively high cholate concentration present in the mixture.

The large bilayer sheets initially formed at the higher cholate concentration also appear to be topologically open to large molecules such as cytochrome *c*. As can be seen in Fig. 4, ferrocytochrome *c*, in the absence of O_2, can reduce all the cytochrome *c* oxidase molecules in the fraction. At this stage, the results of gel filtration and electron microscopy indicate that the protein has incorporated into the bilayer. Cytochrome *c* oxidase is a transmembrane protein with its cytochrome *c*-reaction site on one side only of the native mitochondrial membrane. When the purified enzyme is incorporated into large liposomes, such as are formed by brief sonication of phospholipid and protein, the molecules have been shown to orientate in a random manner into the bilayer such that half have their cytochrome *c*-reaction site exposed to the external medium and the other half have their cytochrome *c*-reaction site inaccessible to externally added ferrocytochrome *c* (Wrigglesworth, 1978; Nicholls *et al.*, 1980). In the present case it might be possible that a non-random incorporation of the protein occurs into topologically closed bilayer sheets, with all the cytochrome *c*-binding sites of the enzyme exposed to external reductant. However, this would require a subsequent randomization of the protein molecules by a 'flip-flop' mechanism since, as the detergent concentration is further lowered, only 50% of the cytochrome *c* oxidase molecules incorporated into the bilayer sheets at zero detergent concentration can be reduced by ferrocytochrome *c*. The addition of a membrane-permeable reductant, *NNN′N′*-tetramethyl-*p*-phenylenediamine, is required before complete reduction of the oxidase population can be achieved. These

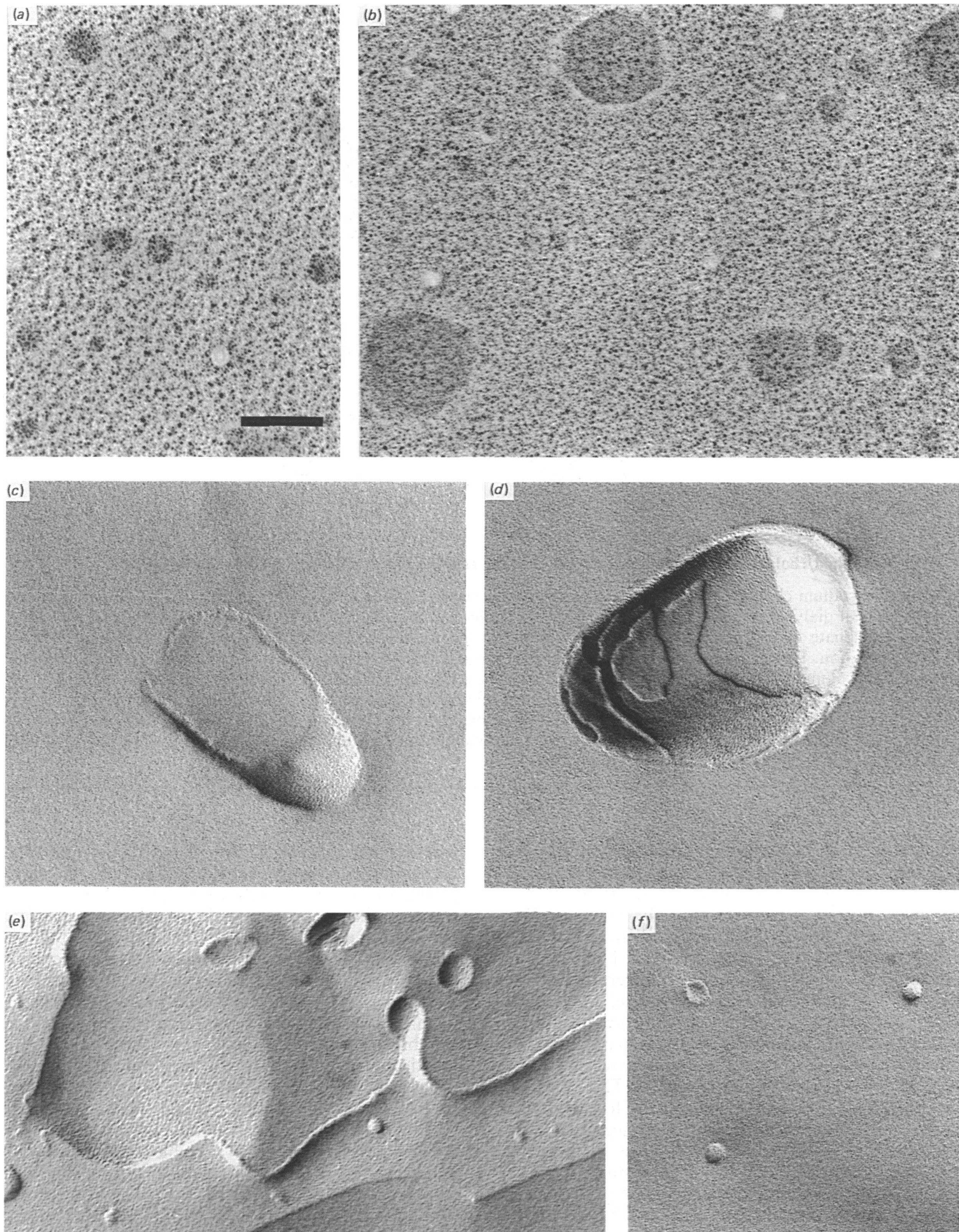

Fig. 2. Intermediate structures formed during dialysis of lipid/cholate mixtures

(*a*) Rotary-shadowed preparation of a mixture of asolectin (65 mM) and sodium cholate (116 mM). (*b*) Rotary-shadowed preparation of a mixture of asolectin (65 mM) and Triton X-100 (30 mM). (*c*) Freeze-fracture preparation of the void-volume fraction from Sepharose 2B gel chromatography of a mixture of asolectin (65 mM) and sodium cholate (116 mM), dialysed to a final concentration of 8.1 mM-cholate and analysed on a gel-filtration column equilibrated to 8.1 mM-cholate on 0.1 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EGTA. (*d*) and (*e*). Freeze-fracture preparations of the void-volume fraction from Sepharose 2B gel chromatography of a mixture of asolectin (65 mM) and sodium cholate (116 mM), dialysed to

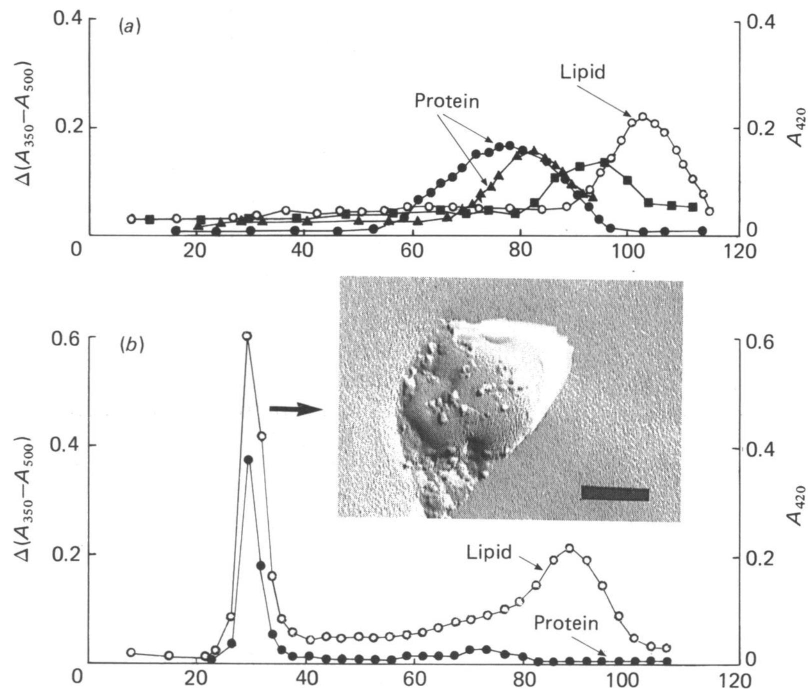

Fig. 3. Analysis of lipid/cholate/protein mixtures by gel filtration on columns of Sepharose 2B equilibrated at different cholate concentrations

(*a*) Elution profile of cytochrome *c* oxidase from a Sepharose 2B column equilibrated in 0.1 M-potassium phosphate buffer, pH 7.0 ■). The column (30 cm × 3.8 cm^2) was loaded with 2 ml of a suspension of cytochrome *c* (8.4 mM) in 0.1 M-potassium phosphate buffer, pH 7.0, containing 0.25% Tween 80. Also shown are the elution profiles of suspensions of the enzymes in 0.1 M-potassium phosphate buffer, pH 7.0, containing 116 mM-sodium cholate (▲) or 116 mM-sodium cholate plus 65 mM-asolectin (●) after passage down columns equilibrated in 0.1 M-potassium phosphate buffer, pH 7.0 containing 116 mM-sodium cholate. The asolectin content of the fractions from the lipid mixture is also indicated (○). (*b*) A mixture of asolectin (65 mM), sodium cholate (116 mM) and cytochrome *c* oxidase was dialysed against 0.1 mM-potassium phosphate buffer, pH 7.0, containing 8.1 mM-sodium cholate. The final suspension was analysed by gel filtration on a Sepharose 2B column equilibrated in 0.1 M-potassium phosphate buffer, pH 7.0, containing 8.1 mM-sodium cholate. The fractions were monitored for cytochrome *c* oxidase (●) and asolectin (○) content. The inset shows a freeze-fracture electron micrograph of the void-volume fraction; the bar represents 100 nm.

Table 1. Physical and kinetic parameters of dialysed fractions of cholate/lipid/cytochrome *c* oxidase mixtures

Two suspensions of cholate (0.116 M), asolectin (65 mM) and cytochrome *c* oxidase (8 μM) were dialysed to a final concentration of 8.1 mM- and 0 mM-cholate respectively. Each sample was then analysed on a Sepharose 2B column appropriately equilibrated to the same cholate concentration. The fractions collected for analysis were the void-volume fraction (between 25 ml and 40 ml on the eluate scale in Fig. 1) and the vesicle fraction (between 55 ml and 75 ml on the eluate scale in Fig. 1). Accessibility to ferrocytochrome *c* was assessed as indicated in Fig. 4. The details of other measurements are as given in the Materials and methods section.

	Void-volume fraction		Vesicle fraction	
	0 mM-Cholate	8.1 mM-Cholate	0 mM-Cholate	8.1 mM-Cholate
Trapped volume (litre·mol^{-1})	1.1±0.2	0.1±0.1	0.69±0.3	0.49±0.3
Ferrocytochrome *c* accessibility (%)	56±5	98±4	70±7	84±15
Respiratory control ratio	1.9±0.3	1.2±0.2	5.2±1.5	1.2±0.2

results, together with the phosphate-trapping experiments, are more simply explained by assuming that the bilayer sheets initially formed from micelle fusion at a critical cholate concentration of about 8 mM are topologically open.

The ratio of 'externally facing' cytochrome *c* oxidase molecules in the small unilamellar vesicle fraction, formed at zero detergent concentration, is higher (Table 1), a value of 70% being found, in agreement with previous results on minimal-size vesicles (Casey *et al.*,

zero cholate concentration and analysed on a gel-filtration column equilibrated in 0.1 M-potassium phosphate buffer, pH 7.0, containing 1 mM-EGTA. (*f*) Freeze-fracture preparation of the vesicle fraction of the sample used in (*d*) and (*e*) above. All micrographs are at the same magnification; the bar represents 100 nm.

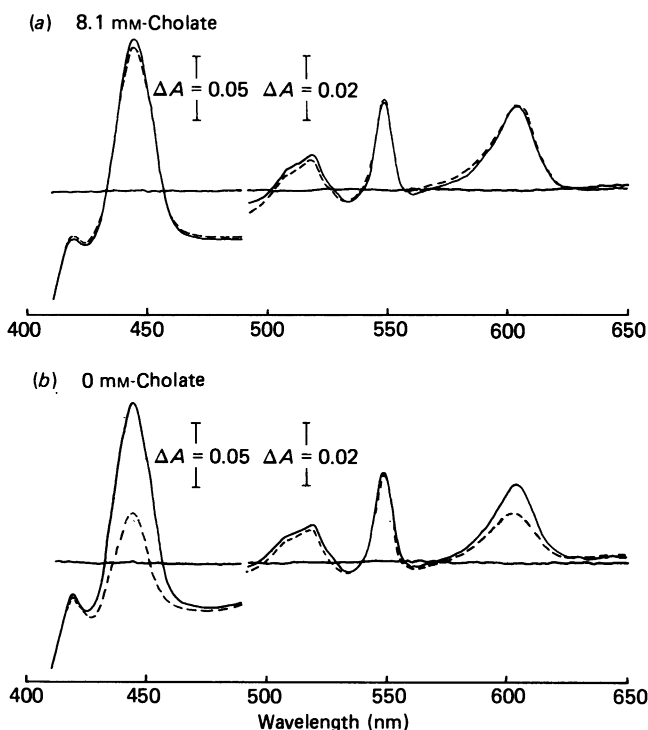

Fig. 4. Effect of *NNN'N'*-tetramethyl-*p*-phenylenediamine on the anaerobic spectrum of bilayer-sheet structures containing cytochrome *c* oxidase in the presence of ascorbate and cytochrome *c*

The void-volume fractions were taken from the gel-filtration eluate of cholate/asolectin/cytochrome *c* oxidase mixtures, as indicated in Fig. 3, dialysed to final cholate concentrations of (*a*) 8.1 mM and (*b*) 0 mM-cholate. These were suspended in 0.1 M-potassium phosphate buffer, pH 7.0, containing 10 mM-sodium ascorbate and 2 μM-cytochrome *c*. The samples were allowed to go anaerobic and the spectra scanned (----). The membrane-permeable reductant *NNN'N'*-tetramethyl-*p*-phenylenediamine (50 μM) was then added, and after 1 min the spectra were re-recorded (——).

1984; Wrigglesworth, 1985). The slightly higher value measured with the small-vesicle fraction in 8.1 % cholate could be a measure of orientation or alternatively indicate some accessibility of ferrocytochrome *c* to the vesicle interior at this detergent concentration.

As a final measure of bilayer integrity, the respiratory control of the incorporated cytochrome *c* oxidase was measured in the different fractions. Only the small-vesicle fraction formed under zero-detergent dialysis conditions exhibits any significant degree of respiratory control (Table 1).

DISCUSSION

In the present study an attempt was made to characterize the pathway of proteoliposome formation by the detergent-dialysis methods. Changes in shape and size were assessed by electron microscopy and gel chromatography on columns equilibrated at intermediate detergent concentrations. Protein incorporation into the bilayer and the formation of closed vesicles was monitored by using several of the well-characterized properties of the transmembrane protein cytochrome oxidase, as well as by direct measurements of the trapped volume. The results indicate that, with cholate as the detergent, proteoliposome formation takes place in three main stages (Fig. 5). First, disc-like micelles of cholate and phospholipid grow in size as detergent is slowly removed. Present, at the same time, is a separate population of protein-containing micelles. At a critical detergent concentration, the phospholipid/detergent micelles fuse to form large bilayer sheets. It is at this stage that protein incorporates into the bilayer. The final stage takes place as the detergent concentration is further lowered and the unstable bilayer sheets fragment into small unilamellar vesicles.

Several critical stages can be identified in this pathway. One is the initial micelle–bilayer-sheet transition. This has been studied, in the absence of protein, by a number of workers (Mazer *et al.*, 1980; Stark & Roberts, 1984; Schurtenberger *et al.*, 1984; Fromherz & Ruppel, 1985). Below the critical concentration of bile salt for bilayer-sheet formation, phospholipid and cholate have been shown to form disc-shaped micelles in which an outer perimeter of bile-salt molecules encloses a small disc of phospholipid (Small *et al.*, 1969). As the detergent/phospholipid molar ratio is lowered, the diameter of the micellar disc increases, resulting, in the present experiments, in an earlier elution of the micelles on the gel column. The shape of the mixed micelles has previously been confirmed by light-scattering experiments (Mazer *et al.*, 1980), with evidence for some incorporation of bile salt within the disc giving rise to a divergence of micellar size. A 6-fold increase in disc size, relative to the size of a pure bile-salt micelle, is reported as the detergent/phospholipid ratio is lowered to approx. 0.5:1 (Mazer *et al.*, 1980; Stark & Roberts, 1984). Presumably, continuous association–disassociation of micelles is taking place to form an average equilibrium size at any particular detergent concentration. Both detergent and lipid retain substantial motional freedom, as assessed by n.m.r. linewidth signals, until close to the concentration of detergent where transition to large bilayer structures begins, when the bile-salt molecules become preferentially restricted (Stark & Roberts, 1984). Below this detergent concentration, the micellar system can no longer solubilize additional phospholipid, and multi-bilayer sheets are formed. In the present work, the cholate/phospholipid ratio for this transition is about 0.2:1 which can be compared with a value reported for various bile-salt/phosphatidylcholine mixtures of about 0.2–0.5:1 (Lichtenberg *et al.*, 1979; Mazer *et al.*, 1980). For pure phosphatidylcholine this ratio is a function of temperature, salt concentration and the absolute concentrations of phosphatidylcholine (Mazer *et al.*, 1980). Asolectin from soya-bean was used in the present work in order to take advantage of the reconstitution properties of cytochrome oxidase with this lipid. The preparation contains a high proportion of unsaturated fatty acyl chains (Casey, 1984), which may lower slightly the cholate/phospholipid ratio for the micelle–bilayer-sheet transition. The presence of protein does not seem materially to affect this ratio.

The present results show that the initial structures formed by micelle fusion are large bilayer sheets. This is in agreement with the predictions of Mazer *et al.* (1980) and Fromherz & Ruppel (1985) and would not support the model of a direct transition of the growing disc-like

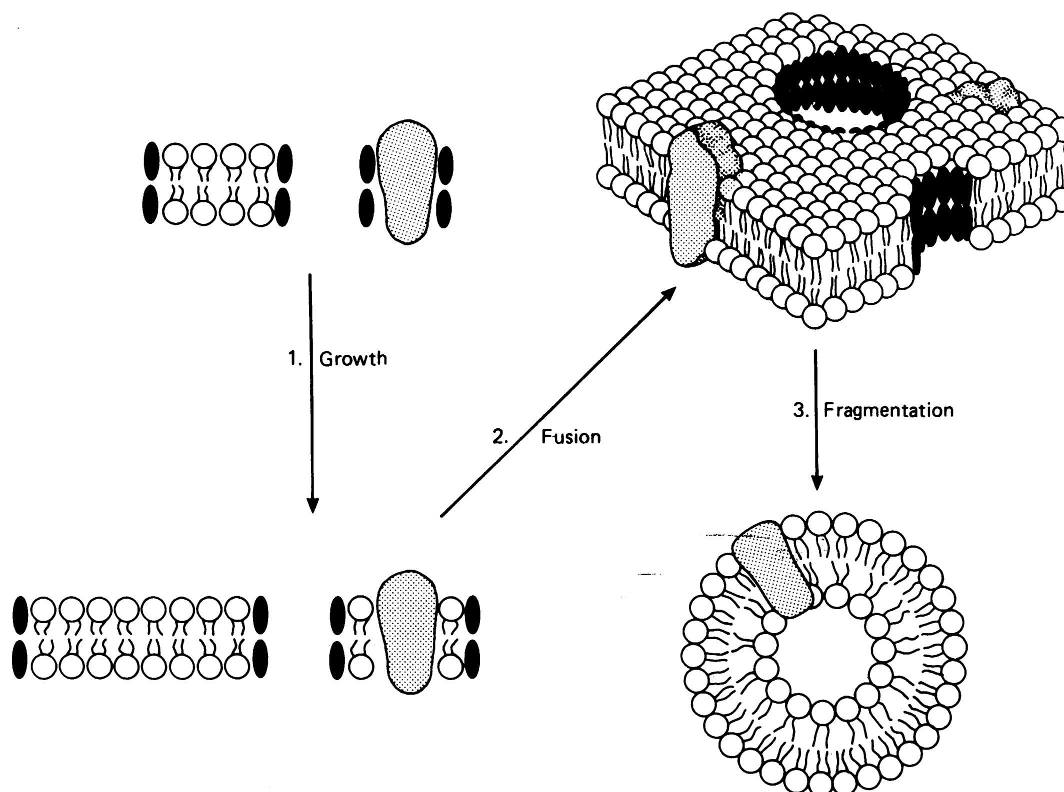

Fig. 5. Diagrammatic representation of intermediate structures during proteoliposome formation by the cholate dialysis method

At high detergent/phospholipid ratios, micelles of detergent plus phospholipid and of detergent plus protein coexist in solution. As the detergent concentration is lowered, these micelles grow in size until, at a critical detergent/phospholipid ratio, the micelles fuse to form large bilayer sheets. The remaining cholate molecules (⬮) concentrate at any exposed edges to form holes in the bilayer, which might act as sites for further micelle fusion. A further lowering of the detergent concentration results in fragmentation of the unstable sheets into small unilamellar vesicles.

micelles into small closed vesicles (Lasic, 1982; Casey, 1984). The large bilayer structures appear to be freely permeable to charged molecules such as phosphate and small proteins such as cytochrome *c*. The energy cost of exposing a hydrophobic edge to the aqueous phase is greater than the curvature energy for any reasonable vesicle radius (Fromherz & Ruppel, 1985; Zasadzinski, 1986). Presumably, therefore, the remaining detergent molecules concentrate at the edges of any bilayer to shield the lipid acyl chains from water and form aqueous 'holes' in the vesiculated sheets (Fig. 5). It is in these regions of the bilayer that incorporation of membrane protein could take place by fusion of the detergent/protein micelles with the bilayer boundary. The use of various detergent-like molecules to fuse purified membrane proteins with preformed bilayers may depend critically on the membrane concentration of the detergent and the ability of the detergent to provide an appropriate edge dislocation in the bilayer. For example, relatively high concentrations of short-chain phosphatidylcholines (20 mol/100 mol of total lipid) are required to facilitate the transmembrane insertion of bacteriorhodopsin into lipid bilayers (Dencher, 1986). Some proteins can themselves act as 'edge-actant'. The similarity of human high-density-lipoprotein/phosphatidylcholine discs to bile-salt/phosphatidylcholine discs has been discussed by Tall & Small (1980).

The break-up of the large bilayer sheets into small unilamellar vesicles takes place as the detergent concentration is further decreased. The large ratio of surface area to bilayer thickness makes the sheets very sensitive to shape changes and, in the absence of 'edge-actant' to stabilize any exposed edges, the sheets will soon fragment into closed vesicles even under minor mechanical stress. The efficiency of detergent removal by several different procedures has been studied by Allen *et al.* (1980). It is found that, even after extensive dialysis, the final cholate/lipid ratio of the mixture remains about 0.01:1 (Allen *et al.*, 1980; Kramer *et al.*, 1981). At this ratio, with slow dialysis, the mixture comprises mainly small unilamellar vesicles with an average diameter of 30 nm (Wrigglesworth, 1985). However, observations by Brunner *et al.* (1976) and Zumbuehl & Weder (1981) suggest that the kinetics of detergent removal in this later stage of vesicle formation can affect the resulting vesicle size.

A fast dilution of mixtures of glycocholate and phosphatidylcholine with buffer to a lower detergent concentration has been shown by Schurtenberger *et al.* (1984) to result in vesicles whose final size can be varied depending on the initial composition of the mixture. The detergent concentration can then be further lowered by dialysis without much effect on vesicle size. From the present hypothesis of vesicle formation, we would suggest that the degree of mechanical stress during the final bilayer-sheet–vesicle transition could strongly affect the vesicle size.

The presence of cytochrome oxidase in the mixture does not appear to affect significantly the final size of the liposomes reconstituted by slow dialysis of cholate/phospholipid mixtures. However, the protein does appear to affect the direction of bilayer curvature as the large bilayer sheets disrupt into smaller vesicles. The smaller the vesicle, the more unidirectional appears to be the orientation of the enzyme, with approx. 70% of the protein incorporated unidirectionally in the minimum-size vesicles. It is unlikely that transmembrane rotation of the protein would occur in pre-formed vesicles, and the most likely explanation for unidirectional orientation would be that the shape of the protein molecule, by its packing in the membrane, directs the initial curvature of the bilayer sheet as it breaks up to release the small vesicles. Formation of proteoliposomes by sonication in the absence of detergent results in a more random orientation of the cytochrome oxidase (Wrigglesworth, 1978). In this case the mechanism of fragmentation is different and probably involves sonication-induced crystalline defects in the lipid regions of the bilayer (Zasadzinski, 1986). Differently shaped membrane proteins might be expected to affect the fragmentation process during dialysis to produce proteoliposomes with a different protein orientation. Some may not impose any bilayer curvature at all. For example, Seckler & Wright (1984) report that proteoliposomes reconstituted by detergent dialysis and incorporating the lactose/H^+ carrier contain half of the incorporated protein oriented with the *C*-termini directed outward and about half oriented with the *C*-termini directed inward. Most proteins, however, appear to show preferential orientation in minimum-size vesicles when the proteoliposomes are formed by the detergent-dialysis procedure (Casey, 1984). A conclusion from the present experiments is that this preferential orientation occurs after protein incorporation, by the protein imposing a preferential curvature on the fragmenting sheets of bilayer.

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