Schachter, D. (1984) *Hepatology (Baltimore) 4*, 140–151.
Schachter, D., & Shinitzky, M. (1977) *J. Clin. Invest. 59*, 536–548.
Schachter, D., Cogan, U., & Abbot, R. E. (1982) *Biochemistry 21*, 2146–2150.
Seeman, P. (1972) *Pharmacol. Rev. 24*, 583–655.
Sha'afi, R. I., Gary-Bobo, C., & Solomon, A. K. (1969) *Biochim. Biophys. Acta 173*, 141–152.
Shinitzky, M., & Barenholz, Y. (1974) *J. Biol. Chem. 249*, 2652–2657.
Shinitzky, M., & Inbar, M. (1976) *Biochim. Biophys. Acta 511*, 125–140.
Shinitzky, M., & Barenholz, Y. (1978) *Biochim. Biophys. Acta 515*, 367–394.
Storch, J., Schachter, D., Inoue, M., & Waikoff, A. W. (1983) *Biochim. Biophys. Acta 727*, 209–212.
Thulborn, K. R., & Sawyer, H. (1978) *Biochim. Biophys. Acta 511*, 125–140.
Thulborn, K. R., Treloar, E., & Sawyer, W. H. (1978) *Biochem. Biophys. Res. Commun. 81*, 42–49.
Thulborn, K. R., Tilley, L. M., Sawyer, W. H., & Treloar, E. (1979) *Biochim. Biophys. Acta 558*, 166–178.
Van Blitterswijk, W., Van Hoeven, R. P., & Van der Meer, B. W. (1981) *Biochim. Biophys. Acta 644*, 323–332.
Van Os, C. H., Wiedner, G., & Wright, E. M. (1979) *J. Membr. Biol. 49*, 1–20.
Vincent, M., DeForesta, B., Gallay, J., & Alfsen, A. (1982) *Biochem. Biophys. Res. Commun. 107*, 914–921.
Wang, J. H., Robinson, C. V., & Edelman, I. S. (1953) *J. Am. Chem. Soc. 75*, 466–470.
Wanner, A. (1977) *Am. Rev. Respir. Dis. 116*, 73–125.
Welsh, M. J., Widdicombe, J. H., & Nadel, J. A. (1980) *J. Appl. Physiol.: Respir., Environ. Exercise Physiol. 49*, 905–909.
Worman, H. J., & Field, M. (1984) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 43*, 315.
Worman, H. J., & Field, M. (1986) *J. Membr. Biol.* (in press).
Yoneda, K. (1976) *Am. Rev. Respir. Dis. 114*, 837–842.
Zlatkis, A., Jak, B., & Boyle, A. J. (1953) *J. Lab. Clin. Med. 41*, 486–492.

Reconstitution of Membrane Proteins: Catalysis by Cholesterol of Insertion of Integral Membrane Proteins into Preformed Lipid Bilayers†

Anthony W. Scotto and David Zakim*

Division of Digestive Diseases, Department of Medicine, Cornell University Medical College, New York, New York 10021

Received August 13, 1985

ABSTRACT: The presence of cholesterol in small unilamellar vesicles (ULV) of dimyristoylphosphatidylcholine (DMPC) catalyzes fusion of the vesicles at temperatures below the upper limit for the gel to liquid-crystalline phase transition of the DMPC. The extent to which ULV grow depends on the concentration of cholesterol in the vesicles and on temperature. Maximum growth occurs at 21 °C. It decreases as the temperature is lowered below 21 °C. Growth does not occur at temperatures above the phase transition. In addition, the presence of cholesterol in ULV of DMPC catalyzes the insertion of integral membrane proteins into the vesicles. Thus, bacteriorhodopsin from *Halobacterium halobrium*, UDPglucuronosyltransferase (EC 2.4.1.17) from pig liver microsomes, and cytochrome oxidase from beef heart mitochondria formed stable lipid–protein complexes spontaneously when added to ULV containing cholesterol at temperatures under which these vesicles would fuse. Incorporation of these proteins into the ULV of DMPC did not occur in the absence of cholesterol or in the presence of cholesterol when the temperature of the system was above that for the phase transition. It appears that cholesterol lowers the energy barrier for fusion of ULV of DMPC and for insertion of integral membrane proteins into these bilayers. Studies with bacteriorhodopsin suggest that the energy barrier for insertion of proteins into ULV containing cholesterol is smaller than the energy barrier for fusion of the ULV with each other.

We have described recently a simple method for reconstituting pure integral membrane proteins into preformed unilamellar bilayers (ULV)[1] of phosphatidylcholine (Scotto & Zakim, 1985). Microsomal UDPglucuronosyltransferase, mitochondrial cytochrome oxidase, and bacteriorhodopsin were incorporated into preformed ULV of dimyristoylphosphatidylcholine (DMPC), for example, when relatively small amounts of myristate (as little as 0.1 mol %) were present in the ULV and the temperature of the system was below that for the gel to liquid-crystalline phase transition. The importance of myristate in the bilayers of DMPC was its promotion of fusion of the ULV (Kantor & Prestegard, 1975), although incorporation of proteins into the ULV appeared to be an event separate from fusion of the ULV (Scotto & Zakim, 1985). We concluded that conditions for fusion of ULV also allowed for the facile insertion into bilayers of large, integral membrane proteins independently of fusion of the ULV. These obser-

† This work was supported by a grant from the National Science Foundation (PCM 8311967) and in part by a grant from the National Institutes of Health (BRSG S07 RR05396).

* Address correspondence to this author.

[1] Abbreviations: ULV, unilamellar vesicles or bilayers; DMPC, dimyristoylphosphatidylcholine; UDP, uridine 5′-diphosphate; Tris, tris(hydroxymethyl)aminomethane; DEAE, diethylaminoethyl; TCA, trichloroacetic acid.

0006-2960/86/0425-1555$01.50/0

vations not only appeared to indicate a useful method for reconstituting membrane proteins but also may be important eventually for enhancing understanding of how membrane proteins insert posttranslationally into membranes in vivo and how membrane proteins recycle in such processes as the endocytic pathway.

The reason addition of myristate to ULV of DMPC is fusogenic below the phase transition is not known with certainty, but there is lateral phase separation in such mixtures (Hauser & Guyer, 1979; Hauser et al., 1979). Interestingly, in this regard are reports of spontaneous incorporation of membrane-bound proteins into preformed bilayers under conditions that also are expected to introduce packing defects or boundary defects, as, for example, freeze-thawing of vesicles, addition of fatty acids, or the use of mixed bilayers that show lateral separation of phases (Eytan & Broza, 1978; Eytan et al., 1976; Jain & De Haas, 1983; Jain et al., 1982). Apoproteins from serum lipoproteins have also been shown to associate with lipid bilayers under conditions that lattice defects are present in the bilayers either because the bilayers were at the gel to liquid-crystalline transition temperature or because cholesterol was present (Massey et al., 1984; Pownall et al., 1978, 1981). The use of such conditions for effecting reconstitution of pure integral membrane proteins has been viewed as limited to the reconstitution of the specific membrane-bound protein studied. Our previous work suggests, however, that the phenomena reported for a variety of proteins (Eytan & Broza, 1978; Eytan et al., 1976; Jain & De Haas, 1983; Jain et al., 1982; Massey et al., 1984; Pownall et al., 1978, 1981) may be generally important. In order to extend this idea we have been studying whether integral membrane proteins insert spontaneously into ULV comprising mixtures of lipids with demonstrated lateral phase separation at some temperature. We report in this paper results from studies of mixtures of cholesterol and DMPC.

Materials and Methods

Materials. DMPC was purchased from Sigma. All other chemicals used were the best available commercial grades.

Preparation of Vesicles of DMPC. DMPC was purified according to the method of Colacicco (1972). DMPC (70 mg) in chloroform was added to a glass tube, solvent was removed under a stream of dry nitrogen, and the residue was dispersed in 10 mM Tris, pH 8.0 (30 °C), 100 mM KCl, and 1 mM ascorbate. The mixture was sonicated at 30 °C in a glass tube suspended in a water bath. Sonication at this temperature, which was above the phase-transition temperature for DMPC, ensured that the vesicles were annealed (Lawaczeck et al., 1976). Sonication was carried out for 30 min with a Heat Systems W225 sonicator under a stream of dry argon. The standard tip was used at 30% of maximum power. The output was pulsed so that power was delivered for 60% of total time of sonication. Samples appeared clear at the end of sonication. When vesicles containing cholesterol were prepared, cholesterol dissolved in chloroform (10 mg/mL) was added to a stainless steel tube. Trace amounts of [^{14}C]cholesterol were added in order to simplify quantitation of the ratio of cholesterol to phospholipid in the final liposomal products. DMPC in chloroform then was added. Solvent was removed under a stream of dry nitrogen, leaving a film of cholesterol and DMPC on the vessel wall. The dried residue was hydrated for 1 h in 10 mM Tris, pH 7.5 (30 °C), 100 mM KCl, and 1 mM ascorbate. Vesicles were prepared by sonication, as described above. Sonicated vesicles were centrifuged for 30 min at 39 000 rpm in a type 40 rotor to remove titanium particles and any multilamellar vesicles that may have remained at the end of sonication. Temperature during centrifugation was 37 °C. The concentration of DMPC was determined after centrifugation by assay of inorganic phosphorus; concentrations of cholesterol were determined in separate aliquots by counting in a liquid scintillation counter.

Density Gradient Centrifugation. Lipid vesicles, lipid–protein complexes (proteoliposomes), and proteins were separated from each other by centrifugation on glycerol gradients containing 10 mM Tris, pH 8.0. The compositions of gradients are given in the text and legends to figures and tables. The gradients contained 0.5 mL of fluorinert FC-40 at the bottom of the tubes. The gradients were centrifuged at 34 000 rpm for 17 h at 30 °C in a Spinco SW41 rotor unless noted otherwise. Gradients were fractionated from the top with an ISCO Model 185 density gradient fractionator.

Purification of Bacteriorhodopsin. Halobacterium halobrium strain E1 was a generous gift of Drs. W. Stoeckenius and R. Bogomolni. The bacteria were grown and purified essentially according to the method of Oesterhelt and Stoeckenius (1982).

Proton Pumping. Proton pumping of bacteriorhodopsin was measured in a water-jacketed glass chamber at 30 °C. The preparation was illuminated by a 250-W quartz–halogen bulb with a Corning 3-69 filter. The light-dependent change in pH was measured as described by Racker and Stoeckenius (1974) in the presence of 150 mM KCl, pH 6.2.

Purification of UDPglucuronosyltransferase. Enzyme was purified from pig liver and assayed as described by Hochman and Zakim (1983). All experiments were carried out with the type of pure enzyme designated as GT_{2P} (Hochman & Zakim, 1983).

Purification of Cytochrome Oxidase. A stabilized, freeze-dried preparation of beef heart cytochrome oxidase was obtained from Biozyme Laboratories Ltd., Great Britain. The preparation was resuspended in 20 mM sodium phosphate, pH 7.0, and 1% Triton X-100 and purified by DEAE chromatography according to the method of Mason et al. (1973). The pooled fractions containing the cytochrome oxidase were concentrated to 1.0 mL by negative pressure in a dialysis concentrator from Bio-Molecular Dynamics, Beaverton, OR. Excess detergent was removed from the protein by extended (250000*g* for 60 h) centrifugation at 4 °C on a 10–60% glycerol gradient containing 10 mM Tris-HCl, pH 7.4. The isolated band of soluble cytochrome oxidase was recovered from the gradient and dialyzed overnight at 4 °C against buffer before reconstitution experiments were performed.

Analytical Methods. Proteins were measured by TCA precipitation followed by analysis by the Lowry method (Peterson, 1977) or by coprecipitation with sRNA (Polacheck & Cabib, 1981) followed by protein determination with bicinchoninic acid (Pierce Chemical Co., Technical Bulletin No. 23225). Phospholipids were measured by a modification of the malachite green method of Chalvardjian and Rudniski (1970). Samples for analysis from glycerol gradients were digested with concentrated HNO_3 at an initial temperature of 95 °C. The temperature was raised gradually to 140 °C over the course of 3 h. The mixture then was taken to dryness by heating to 195 °C. Phosphorus was measured according to the method of Chalvardjian and Rudniski (1970).

Results

Cholesterol Is a Fusogen for ULV of DMPC. Small ULV composed of phosphatidylcholine aggregate below the transition temperature (Wong & Thompson, 1982). They disaggregate when warmed to temperatures above the phase transition. We expected that cholesterol, if it were fusogenic, would facilitate fusion below the phase transition. In order

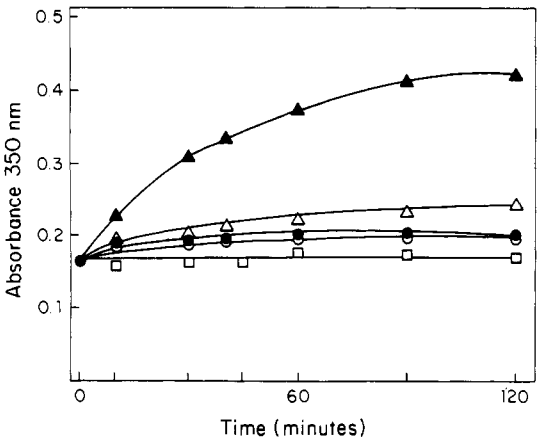

FIGURE 1: Effect of temperature on the fusion of ULV of DMPC containing 12 mol % cholesterol. ULV of DMPC containing 12 mol % cholesterol (3 mg/mol) were maintained at 37 °C prior to treatment at the indicated temperatures. The increase in turbidity which is due to vesicle growth at the indicated temperature was measured after the samples were rewarmed to 37 °C for 5 min. Temperatures were 30 °C (□), 21 °C (▲), 18 °C (△), 10 °C (●), and 5 °C (○).

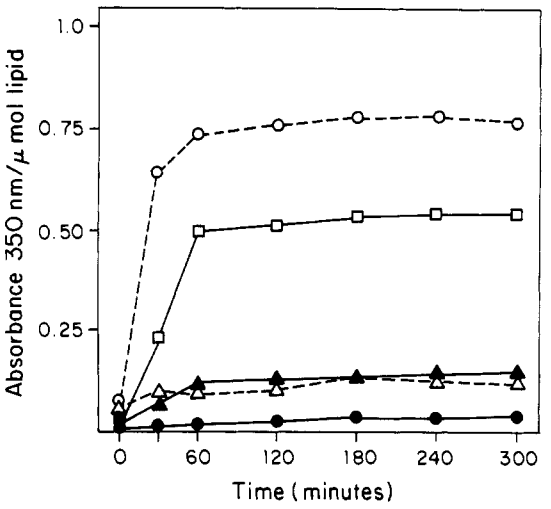

FIGURE 2: Light scattering as a function of time at 21 °C for ULV containing cholesterol. ULV containing cholesterol were prepared as under Materials and Methods. Samples were held at 21 °C for the times indicated. Samples were warmed to 37 °C for 10 min prior to determining optical density at 350 nm. Samples then were recooled to 21 °C, andd treatment was continued. Optical densities are plotted per mole of phospholipid per milliliter. The concentrations of cholesterol in mol % were 3.9 (●), 7.5 (▲), 13.8 (□), 19.9 (○), and 23.8 (△).

to use light scattering to evaluate this possibility, it was necessary to separate contributions to scattering due to true growth in size (fusion) from those due to aggregation. This was accomplished by allowing vesicles to fuse at different temperatures below the phase transition and then warming the vesicles to 37 °C. Changes in light scattering then were measured at 37 °C. Results of these experiments are shown in Figure 1. There was a time-dependent increase in light scattering (measured at 37 °C) for ULV of DMPC containing cholesterol at the temperatures indicated. The maximum change in light scattering, secondary to addition of cholesterol to ULV, occurred at 21 °C. Even at 5 °C, however, there was a small but rapid initial change in light scattering. This was followed by a stable level of light scattering. Presumably, the initial rapid increase in light scattering was due to a limited fusion of vesicles at 5 °C vs. 21 °C.

The data in Figure 2 indicate the influence of the concentration of cholesterol on fusion of ULV of DMPC at 21 °C, as reflected by light scattering. The extent of the change in light scattering was related directly to the concentration of cholesterol in the ULV, up to about 20 mol % cholesterol. The extent of light scattering declined at higher concentrations of cholesterol. Since the data suggest that fusion was complete within 60 min for all concentrations of cholesterol, it appears that the concentration of cholesterol in ULV determined the final size of fused vesicles. An effect of cholesterol concentration on the size of ULV made by cosonication of cholesterol and egg phosphatidylcholine has been demonstrated previously (Gent & Prestegard, 1974; Forge et al., 1978). The effect of cholesterol on the stable size of ULV demonstrated in the above studies was quite small, however, in the range of 0–20 mol % cholesterol, which was the range of concentration used by us. Nevertheless, it was not possible to determine from our data (Figure 2) if the effects of different amounts of cholesterol on light scattering were determined by kinetic (rates of fusion of different sized vesicles) or thermodynamic factors (effect of cholesterol concentration on the stability of ULV of different sizes). This question was not pursued further in these studies.

Incorporation of Bacteriorhodopsin into Preformed Vesicles of DMPC Containing Cholesterol. The finding that ULV of DMPC containing cholesterol could be induced to fuse below the gel to liquid-crystal phase transition, in a manner similar to that demonstrated for ULV of DMPC containing myristate, suggested that the former conditions, like the latter, would facilitate insertion of integral membrane proteins into the ULV. This idea was investigated first with bacteriorhodopsin as a prototypic integral membrane protein.

Bacteriorhodopsin (0.25 mg) was mixed at 37 °C with ULV of DMPC (3.5 mg) containing 12 mol % cholesterol. The mixture then was cooled to 21 °C by immersion in a water bath at this temperature. Aliquots were removed at 21 °C at different intervals, rewarmed to 37 °C, and layered on top of 10–60% glycerol gradients, which were centrifuged for 1 h at 37 °C. The gradients were fractionated and analyzed for protein and phospholipid. These analyses revealed that all the bacteriorhodopsin became associated with DMPC. Control experiments carried out as above showed that bacteriorhodopsin did not become associated with DMPC when the ULV lacked cholesterol. Analysis of data for the ratios of lipid to protein in fractions containing bacteriorhodopsin–DMPC complexes, after mixing protein with ULV of DMPC plus 12 mol % cholesterol, indicated that the formation of functionally significant lipid–protein complexes occurred as a two-stage process. Thus, all the bacteriorhodopsin became associated with ULV within about 3 min or less. For example, the ratio of moles of DMPC to moles of bacteriorhodopsin was 13 for DMPC mixed with protein at 37 °C. This ratio was 44 after density gradient fractionation of mixtures of phospholipid and protein treated at 21 °C for 3 min. Subsequent to this initially rapid incorporation of bacteriorhodopsin into ULV, the lipid–protein complexes became progressively enriched in phospholipid. The ratio of moles of DMPC to moles of bacteriorhodopsin in complexes isolated by density gradient centrifugation was 76 after treatment of mixtures of lipid and protein for 10 min at 21 °C and 159 and 279, respectively, after treatment of mixtures at 21 °C for 30 and 120 min. The concentration of cholesterol in the DMPC–bacteriorhodopsin complexes isolated by density gradient centrifugation was identical with that of the original ULV. The purified bacteriorhodopsin used in these experments (mixed with DMPC at 37 °C) contained 3–5 mol of residual phospholipid per mole of protein. The zero-time sample of bacteriorhodopsin in Table I contained more phospholipid than this, but the amount of phospholipid in this sample was not significant for function

Table I: Incorporation of Bacteriorhodopsin into ULV of DMPC Containing Variable Amounts of Cholesterol[a]

concn of cholesterol (mol %)	ratio of lipid to protein in complexes (mol/mol)	
	fusion for 1 min at 21 °C	fusion for 10 min at 21 °C
0	11	13
0.1	<20	32
1	<20	54
3	<20	104
6	56	97
12	99	134
18	102	222

[a] Bacteriorhodopsin (0.25 mg) was added to ULV of DMPC (3 mg) containing the indicated amounts of cholesterol. The samples initially were at 37 °C. The temperature then was lowered to 21 °C for 1 min or 10 min, and the mixtures were rewarmed to 37 °C. Subsequently the mixtures were layered on glycerol gradients, as under Materials and Methods, and the lipid–protein complexes were separated by centrifugation for 1 h at 39 000 spm in an SW41 rotor. Temperature during centrifugation was 37 °C. Lipid–protein complexes were collected and analyzed as under Materials and Methods.

of bacteriorhodopsin in that it did not reconstitute proton pumping (see below). Bacteriorhodopsin added to ULV at 37 °C, but not cooled to 21 °C, sedimented to the bottom of the density gradient.

Incorporation of Bacteriorhodopsin into ULV of DMPC as a Function of the Concentration of Cholesterol and Temperature. We examined the effect of a broad range of concentrations of cholesterol on the formation of bacteriorhodopsin–DMPC complexes. Presented in Table I are the phospholipid to protein ratios of complexes of bacteriorhodopsin and DMPC isolated from 10–60% glycerol gradients. These data show that insertion of bacteriorhodopsin into ULV occurs at low concentrations of cholesterol. In fact, protein inserted into ULV at concentrations of cholesterol that did not appear to catalyze fusion of ULV. No change in light scattering as a function of time was observed, for example, when ULV containing 1 mol % cholesterol were treated at 21 °C for several hours. Liposomes made by cosonication of phospholipids and cholesterol are quite heterodisperse in size (Forge et al., 1978), however; hence it was possible that bacteriorhodopsin interacted initially with only a small fraction of the ULV containing 0.1 or 1 mol % cholesterol (Table I). Fusion of a small subset of relatively small ULV containing these amounts of cholesterol conceivably could go undetected in the light-scattering experiments. The relationship between the potential for fusion of a subset of ULV present in small amounts and insertion of bacteriorhodopsin into ULV was investigated by allowing ULV containing 0.1 or 1 mol % cholesterol to fuse by treating them for 2 h at 21 °C. Subsequently, bacteriorhodopsin was added and treatment continued for an additional 2 h. Bacteriorhodopsin, under these conditions, did not become incorporated into ULV (data not shown). This result confirmed the idea that bacteriorhodopsin interacted initially with a subset of the ULV containing 0.1 and 1 mol % cholesterol and that the interacting subset of ULV were removed from the system under conditions that promoted fusion of ULV.

The bacteriorhodopsin formed a complex with ULV containing 13.8 mol % cholesterol when a mixture of protein and ULV was treated at 5 °C. There was, however, an important difference between results at 5 °C and 21 °C. There was a reasonably rapid incorporation of bacteriorhodopsin into ULV of DMPC at 5 °C; but after initial incorporation, there was no subsequent growth of the vesicles as was observed at 21 °C. Thus, the ratio of moles of DMPC to moles of bacteriorhodopsin in complexes isolated by density gradient centrifugation was 23.5 after treatment at 5 °C for 10 min or 240 min prior to centrifugation. This amount of phospholipid was sufficient to reconstitute proton pumping. It seems, therefore, that bacteriorhodopsin can insert into ULV at 5 °C but that the ULV cannot fuse with each other (Figure 1) or with complexes of bacteriorhodopsin–ULV. In fact, we found, as in the experiments at 21 °C in which the concentration of cholesterol was relatively low, that bacteriorhodopsin interacted at 5 °C only with a subset of ULV. It is noteworthy, nevertheless, that the behavior of the system at 5 °C vs. 21 °C could be used to turn on or turn off the growth in the ratio of protein to lipid in bacteriorhodopsin–ULV complexes. For example, when vesicles containing bacteriorhodopsin prepared at 5 °C were warmed to 21 °C, there was a rapid and progressive increase in the ratio of lipid to protein in isolated protein–lipid complexes (data not shown).

Is the Energy Barrier for Fusion between ULV Containing Cholesterol the Same as the Energy Barrier for Incorporation of Bacteriorhodopsin into These ULV? The experimental results described in the preceding section reveal a complex relationship between the size of vesicles, the concentration of cholesterol, and fusion between vesicles or between vesicles and bacteriorhodopsin. As mentioned already, one of the unknowns in this system is whether the dependence of the change in light scattering in Figure 2 on the concentration of cholesterol reflects kinetic or thermodynamic constraints. Given the properties of ULV containing cholesterol, i.e., that stable size depends on the concentration of cholesterol and that the ULV are polydisperse (Gent & Prestegard, 1974; Forge et al., 1978), it would be technically extremely difficult to sort out the relationship between insertion of bacteriorhodopsin into ULV and the exact size and concentration of cholesterol in the vesicles. We therefore considered an alternative approach to examining this question in order to elaborate further on the mechanism of spontaneous insertion of proteins into preformed bilayers. We questioned whether the bacteriorhodopsin could be modified to cause insertion into ULV under conditions that did not allow insertion of sheets of purple membranes. The technique chosen for modifying bacteriorhodopsin was sonication in order to comminute the size of the aggregated bacteriorhodopsin. The experimental question that was proposed was whether sonicated bacteriorhodopsin would incorporate into ULV under conditions where the unsonicated preparation would not. The ULV for this experiment were prepared as follows. ULV (9000 nmol) containing 2.9 mol % cholesterol were mixed with bacteriorhodopsin (96 nmol), and the mixture was treated for 105 min at 21 °C. At this time an additional 96 nmol of bacteriorhodopsin was added, and treatment at 21 °C continued for 15 min. The second addition of bacteriorhodopsin was made to ensure that any subset of ULV capable of fusing with it would be completely used. The mixture then was layered on a glycerol gradient, which was centrifuged for 1 h at 37 °C at 39 000 rpm in an SW41 rotor. After centrifugation, there was a band of bacteriorhodopsin at the bottom of the gradient corresponding to essentially phospholipid-free bacteriorhodopsin. Another band of bacteriorhodopsin was floating at the middle of the gradient. ULV containing no protein, the "remnant" vesicles, floated at the top of the gradient (10% glycerol). These "remnants" were collected by side puncture of the centrifuge tube and mixed with either untreated bacteriorhodopsin, bacteriorhodopsin sonicated for 6 min, or bacteriorhodopsin sonicated for 20 min. These mixtures were treated at 21 °C for 60 min and layered over 10–75% glycerol gradients. The separations

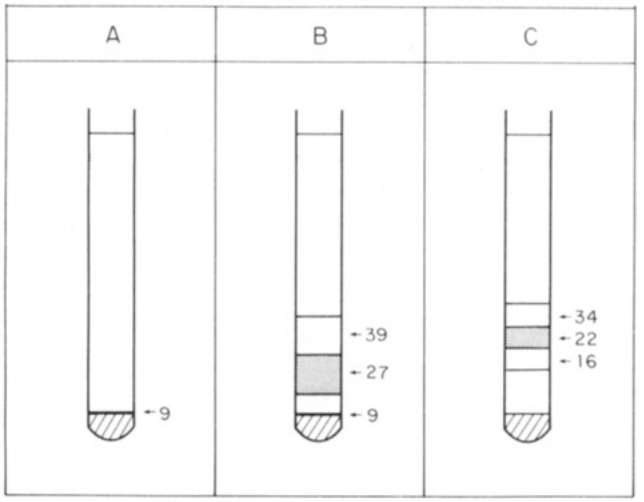

FIGURE 3: Incorporation of bacteriorhodopsin into remnant vesicles of DMPC containing 3.9 mol % cholesterol. Remnant vesicles were prepared as described in the text. Each gradient depicted below contained 9.6 nmol of protein and 1128 nmol of DMPC. Prior to mixing protein and lipid, bacteriorhodopsin prepared as under Materials and Methods was not treated (A) or was sonicated for 6 min (B) or 20 min (C) by using a 0.5-in. tip at 30% full power and 50% pulse. Mixtures of ULV and protein were treated at 21 °C for 60 min, warmed to 30 °C, and layered on 10–75% glycerol gradients. Gradients were centrifuged for 60 h at 39000 rpm in a SW41 rotor at 30 °C. The gradients were fractionated from the top. Phospholipid was determined as phospholipid phosphorus, and bacteriorhodopsin was quantitated as under Materials and Methods. The darkened area at the bottom of the gradients represents bacteriorhodopsin that did not become reconstituted with lipids. The stippled areas represent reconstituted bacteriorhodopsin–lipid complexes. Numbers to the right of each gradient are the ratios of lipid to protein (mol/mol) in the indicated fraction of the gradient. The bacteriorhodopsin used in this experiment had a residual lipid phosphorus content of 3.6 mol per mole of protein. The stripped areas at the bottom of the tube represent the cushion of fluorinert.

of lipids and proteins on these gradients are depicted in Figure 3. The important result is that sonicated bacteriorhodopsin formed lipid–protein complexes with the "remnant" vesicles whereas unsonicated bacteriorhodopsin did not. The durations of sonication also influenced results. After 6 min of sonication, about 30% of bacteriorhodopsin was found as lipid–protein complexes; but all the protein was found associated with remnants for bacteriorhodopsin that had been sonicated for 20 min. The complexes of lipid and protein formed in Figure 3 (panels B and C), with lipid to protein ratios greater than 16, were active in proton pumping.

Proton Pumping by Reconstituted Bacteriorhodopsin. Data for proton pumping by bacteriorhodopsin reconstituted into ULV of DMPC plus cholesterol are shown in Table II. These data show the bacteriorhodopsin reconstituted into ULV by the method used in Figure 2 functions as a proton pump. The relationship between efficiency of pumping and reconstitution into vesicles is complex, however. For example, after 2 min of treatment at 21 °C, mixtures of bacteriorhodopsin and DMPC plus 3 mol % cholesterol pump protons far less efficiently vs. mixtures of bacteriorhodopsin and DMPC plus 12 mol % cholesterol. It was demonstrated in separate experiments that all the bacteriorhodopsin was associated with lipid in less than 2 min when bacteriorhodopsin was added to DMPC plus 3 mol % cholesterol. Hence, the differences in efficiency of pumping for ULV containing 3 mol % vs. 12 mol % cholesterol could not be related to failure of incorporation of a portion of the bacteriorhodopsin into lipid–protein complexes. The data indicate, instead, that the time-dependent increase in the efficiency of pumping is due to some change

Table II: Proton Pumping of Bacteriorhodopsin Reconstituted in ULV of DMPC Containing Cholesterol[a]

time of treatment at 21 °C	proton pumping	
	3 mol % cholesterol	12 mol % cholesterol
20 s		3.7
30 s	0.5	
1 min		21.6
2 min	0.8	
5 min	8.9	
10 min	22.1	31.6
30 min	21.6	21.3
60 min	10.8	16.1

[a] Bacteriorhodopsin (0.5 mg) was incorporated into ULV of DMPC (6 mg) containing either 3 or 12 mol % cholesterol. The mixtures were treated at 21 °C for the indicated times and then rewarmed to 30 °C. Proton pumping was measured as described under Materials and Methods. Units of proton pumping are ng of H^+/mg of protein.

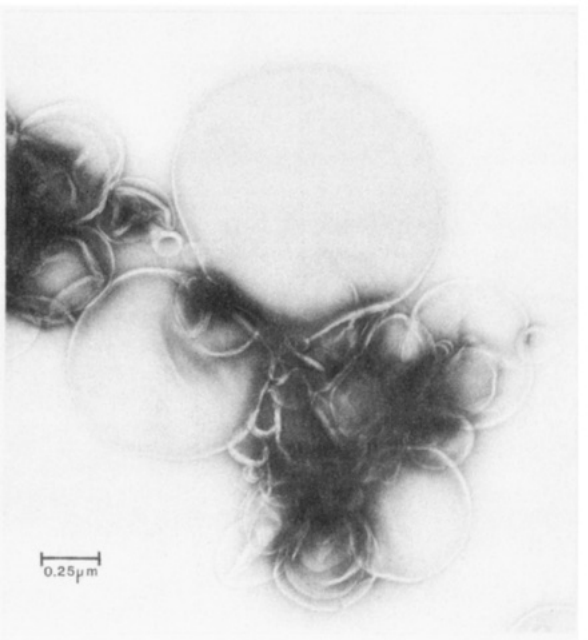

FIGURE 4: Electron microscopy of bacteriorhodopsin–phospholipid complexes. Bacteriorhodopsin was mixed with ULV of DMPC plus 12 mol % cholesterol. After 60 min at 21 °C, the protein–lipid complexes were separated from protein-free ULV by gradient centrifugation as in Figure 5. The protein–lipid complexes were obtained by side puncture of the tube and prepared for electron microscopy by negative staining with 5% uranyl acetate of samples adhered to glow-discharged carbon film grids.

in the character of the bacteriorhodopsin–ULV complexes.

Electron Microscopy of Bacteriorhodopsin–Lipid Complexes. The appearance of proteoliposomes prepared from bacteriorhodopsin and ULV of DMPC plus 12 mol % cholesterol is shown in Figure 4. This preparation was made by mixing lipid and protein for 60 min at 21 °C. Lipid–protein complexes were isolated by density gradient centrifugation. The vesicles in this figure range in size from 0.1 to >1 μm. As compared with proteoliposomes made with DMPC containing myristate as fusogen, the vesicles in Figure 4 appear more rigid and smooth (Scotto & Zakim, 1985). Also, the vesicles in Figure 4 are not as large as proteoliposomes made from bacteriorhodopsin and ULV of DMPC plus myristate. This last finding could be an artifact, however, due to breakage of rigid vesicles during isolation from the gradient.

Incorporation of Microsomal UDPglucuronosyltransferase and Mitochondrial Cytochrome Oxidase into ULV of DMPC Containing 12 mol % Cholesterol. The data in Figure 5 indicate that these proteins incorporate into ULV of DMPC

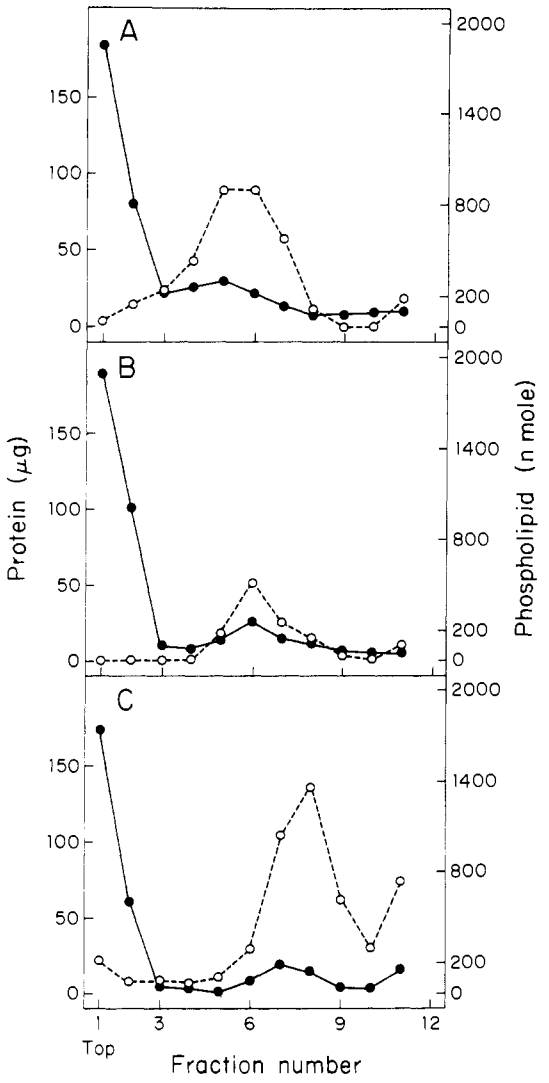

FIGURE 5: Incorporation of integral membrane proteins into ULV of DMPC containing 12 mol % cholesterol. ULV of DMPC containing 12 mol % cholesterol (3 mg) were mixed at 30 °C with 250 μg of bacteriorhodopsin (A), 213 μg of UDPglucuronosyltransferase (B), or 155 μg of cytochrome oxidase (C). The mixtures were treated at 21 °C for 30 min and returned to 30 °C, after which they were separated by centrifugation for 17 h at 34000 rpm in a SW41 rotor. The gradients consisted of a 10–60% glycerol containing 10 mM Tris-HCl, pH 7.5 at 30 °C. The gradients were fractionated from the top. Phospholipids (●) and proteins (○) were determined as under Materials and Methods.

when the latter contain cholesterol and the system is held at 21 °C. The experiment in Figure 5A is for DMPC plus bacteriorhodopsin. The experiment in Figure 5B is identical with that in Figure 5A except that UDPglucuronosyltransferase was substituted for bacteriorhodopsin. The protein in Figure 5C is cytochrome oxidase. The data in Figure 5C are different from those of Figure 5A,B in that after centrifugation of the gradients, cytochrome oxidase was present as a lipid–protein complex and as free protein. We attribute this last finding to irreversible aggregation of cytochrome oxidase during extensive dialysis to remove residual detergent. It is important to emphasize in this context that no cytochrome oxidase became associated with DMPC when it was mixed with ULV not containing cholesterol or when it was mixed with ULV of DMPC plus cholesterol at temperatures above 23 °C.

Discussion

Reconstitution of Integral Membrane Proteins Using Preformed ULV Containing Lipid Contaminants. The use of fusogens to catalyze the insertion of integral membrane proteins into preformed ULV provides a simple and mild set of conditions for reconstituting the pure, delipidated proteins. In addition, studies of this phenomenon may provide important ideas about the assembly of membranes in vivo. With regard to the simple technical aspects of reconstituting membrane-bound proteins, the present results show that the nature of the fusogen can have effects on the type of lipid–protein complex that is produced and the exact conditions needed to effect reconstitution. The temperature at which efficiency of incorporation is best is 18 °C for myristate (Scotto & Zakim, 1985) and 21 °C for cholesterol, for example. In addition, it is possible to inhibit the growth of lipid–protein complexes with cholesterol as the fusogen by lowering the temperature to about 5 °C; this does not occur when myristate is the fusogen (Scotto & Zakim, 1985). This difference could be important in reconstituting temperature-labile proteins, since with myristate as fusogen, inhibition of the growth of the lipid–protein complexes can be achieved only by increasing the temperature to above the phase transitions for ULV of DMPC. It remains to be determined why cholesterol and myristate have differing effects. Nevertheless, it seems that the technique of using fusogens to catalyze reconstitution of integral membrane proteins not only is more simple technically vs. such techniques as sonication and cholate dialysis but also provides a simple means for reconstituting membranes with a considerable degree of complexity.

Relationship between Fusion of ULV and Spontaneous Insertion of Intregral Membrane Proteins. The basis for the original studies of the effect of myristate on the reconstitution of integral membrane proteins into lipid bilayers was the observation that small amounts of residual cholate catalyzed the insertion of pure, delipidated UDPglucuronosyltransferase into preformed ULV of DMPC. This catalysis occurred under the conditions that cholate caused fusion of the ULV. We therefore were led to investigate the incorporation of integral membrane proteins into preformed ULV containing fusogens other than cholate. Myristate was chosen as the fusogen (Kantor & Prestegard, 1975). The decision to investigate the effects of cholesterol on fusion of ULV and reconstitution of lipid–protein complexes was taken because neither myristate (and probably other fatty acids) nor cholesterol appears to mix ideally with phosphatidylcholine (Hauser et al., 1979; Shimshick & McConnell, 1973; Copeland & McConnell, 1980). Bilayers containing immiscible lipids will tend to have localized packing defects, which can be the basis for such properties as fusion, unusually high permeability to electrolytes, and incorporation of proteins (Liao & Prestegard, 1980; Massari et al., 1980; Op den Kamp et al., 1975; Papahadjopoulos et al., 1973; Singer & Jain, 1980). The data in the present work extend the idea that defects in packing are important for the insertion of integral membrane proteins into preformed bilayers. The idea that fusogens catalyze fusion of ULV and spontaneous incorporation of integral membrane proteins into preformed ULV because they induce defects in packing predicts that techniques for induction of defects in membranes, other than those caused by myristate and cholesterol, also will catalyze spontaneous reconstitution of lipid–protein complexes from mixtures of ULV and pure, integral membrane proteins. This has not been examined in a systematic manner, but data in the literature validate the prediction. Cytochrome oxidase and bacteriorhodopsin can be reconstituted by freeze-thawing of mixtures of ULV and the respective proteins (Eytan et al., 1976; Van Dijck & Van Dam, 1982). Direct testing verifies too that bacteriorhodopsin inserts

spontaneously into unannealed ULV of pure DMPC prepared by sonication below the temperature for the gel to liquid-crystal phase transition (Scotto and Zakim, unpublished results). In addition, cytochrome oxidase reconstitutes spontaneously into ULV comprising mixtures of phospholipid that can be expected to display lateral separation of phases (Eytan & Broza, 1978; Eytan et al., 1976). Jain and co-workers have shown that phospholipase A_2 inserts spontaneously into ULV when the ULV contain lysophosphatides and fatty acids, which induce defects in packing (Jain & De Haas, 1983; Jain et al., 1982) or when the vesicles are unannealed (Upreti & Jain, 1980).

Mechanism of Insertion of Integral Membrane Proteins into ULV and the Properties of the Proteins. The detailed molecular events by which two separate phospholipid bilayers fuse with each other are uncertain. There is no model for fusion that can accommodate all the experimental data relating to fusion. Given the uncertainty about the mechanism of fusion between ULV, it is difficult to speculate about the energy barrier to insertion of integral membrane proteins into ULV. We believe all that can be stated is that fusogens lower the energy barrier for fusion between ULV and for insertion of integral membrane proteins into ULV. This may mean that the nature of the barrier is the same for both types of events.

One difficulty in work with pure integral membrane proteins is that the proteins aggregate, and because of the methods used in purification schemes, these proteins can be contaminated with residual detergents and phospholipids. It is important to consider, in the context of the current work, whether any of these variables could affect the results reported. We believe the evidence on this question is clear. Bacteriorhodopsin is purified in the absence of detergent. Moreover, the size of bacteriorhodopsin appears not to influence its capacity for inserting into ULV of DMPC because sheets of this protein, as well as monomers (Scotto & Zakim, 1985), incorporate into ULV under identical conditions. The states of aggregation of purified UDPglucuronosyltransferase and cytochrome oxidase are unknown. In view of the data for bacteriorhodopsin, however, the aggregation of these proteins is unlikely to be a significant factor for their insertion into ULV. We think it is worthwhile to mention in this regard the observation that integral membrane proteins in a cell also may occur as aggregates prior to insertion into mitochondria (Zimmerman & Neupert, 1980).

The UDPglucuronosyltransferase and cytochrome oxidase used in our experirments contained residual detergent. Control experments were carried out to exclude that the residual detergent established conditions for fusion of the ULV. We cannot exclude, with certainty, that the residual detergent influenced the structure of these proteins so as to give them the properties required for insertion into ULV. Detergent was not needed, however, for insertion of bacteriorhodopsin; complete resolution of the significance of residual detergent will depend on further work to establish the structural features of integral membrane proteins that are important for spontaneous insertion into ULV. It seems clear in this regard that these proteins contain structural information that directs insertion because the orientation of the inserted protein is nonrandom.

ACKNOWLEDGMENTS

The expert technical assistance of Deborah Goodin is gratefully acknowledged. The method of reconstitution described in this paper has been assigned to the Cornell Research Foundation.

Registry No. EC 2.4.1.17, 9030-08-4; DMPC, 18194-24-6; cytochrome oxidase, 9001-16-5; cholesterol, 57-88-5.

REFERENCES

Chalvardjian, A., & Rudniski, E. (1970) *Anal. Biochem. 36*, 225–226.
Colacicco, G. (1972) *Biochim. Biophys. Acta 266*, 313–319.
Copeland, B. R., & McConnell, H. M. (1980) *Biochim. Biophys. Acta 599*, 95–109.
Eytan, G. D., & Broza, R. (1978) *J. Biol. Chem. 253*, 3196–3202.
Eytan, G. D., Matheson, M. J., & Racker, E. (1976) *J. Biol. Chem. 251*, 6831–6837.
Forge, A., Knowles, P. F., & Marsh, D. (1978) *J. Membr. Biol. 41*, 249–263.
Gent, M. P. N., & Prestegard, J. H. (1974) *Biochemistry 13*, 4027–4033.
Hauser, H., & Guyer, W. (1979) *Biochim. Biophys. Acta 553*, 359–363.
Hauser, H., Guyer, W., & Howell, K. (1979) *Biochemistry 18*, 3283–3291.
Hochman, Y., & Zakim, D. (1983) *J. Biol. Chem. 258*, 4143–4146.
Jain, M. K., & De Haas, G. H. (1983) *Biochim. Biophys. Acta 736*, 157–162.
Jain, M. K., Egmond, M. R., Verheij, H. M., Apitz-Castro, R., Dijkman, R., & De Haas, G. H. (1982) *Biochim. Biophys. Acta 688*, 341–348.
Kantor, H. L., & Prestegard, J. H. (1975) *Biochemistry 14*, 1790–1795.
Lawaczeck, R., Kainosho, M., & Chan, S. I. (1976) *Biochim. Biophys. Acta 443*, 313–330.
Liao, M., & Prestegard, J. H. (1980) *Biochim. Biophys. Acta 599*, 81–94.
Mason, T. L., Poyton, R. D., Wharton, D. C., & Schatz, G. (1973) *J. Biol. Chem. 248*, 1346–1354.
Massari, S., Arslan, P., Nicohusi, A., & Colonna, R. (1980) *Biochim. Biophys. Acta 599*, 118–126.
Massey, J. B., Gotto, A. M., Jr., & Pownall, H. J. (1984) *Biochim. Biophys. Acta 794*, 137–141.
Oesterhelt, D., & Stoeckenius, W. (1982) *Methods Enzymol. 88*, 667–678.
Op den Kamp, J. A. F., Kauertz, M. T., & Van Deenen, L. L. M. (1975) *Biochim. Biophys. Acta 406*, 169–177.
Papahadjopoulos, D., Jacobson, K., Nir, S., & Isac, T. (1973) *Biochim. Biophys. Acta 311*, 330–348.
Peterson, G. L. (1977) *Anal. Biochem. 83*, 346–356.
Polacheck, I., & Cabib, E. (1981) *Anal. Chem. 117*, 311–314.
Pownall, H. J., Massey, J. B., Kusserow, S. L., & Gotto, A. M. (1978) *Biochemistry 17*, 1183–1188.
Pownall, H. J., Par, Q., Hickson, D., Sparrow, J. T., Kusserow, S. L., & Massey, J. B. (1981) *Biochemistry 20*, 6630–6635.
Racker, E., & Stoeckenius, W. (1974) *J. Biol. Chem. 249*, 662–663.
Scotto, A. W., & Zakim, D. (1985) *Biochemistry 24*, 4066–4075.
Shimshick, E. J., & McConnell, H. M. (1973) *Biochemistry 12*, 2351–2360.
Singer, M. A., & Jain, M. K. (1980) *Can. J. Biochem. 58*, 815–821.
Upreti, G. C., & Jain, M. K. (1980) *J. Membr. Biol. 55*, 113–123.
Van Dijck, P. W. M., & Van Dam, K. (1982) *Methods Enzymol. 88*, 17–25.
Wong, M., & Thompson, T. E. (1982) *Biochemistry 21*, 4133–4139.
Zimmerman, R., & Neupert, W. (1980) *Eur. J. Biochem. 109*, 217–229.