Biochimica et Biophysica Acta 812 (1985) 369–376
Elsevier

BBA 72410

Rhodopsin-egg phosphatidylcholine reconstitution by an octyl glucoside dilution procedure

Marilyn L. Jackson * and Burton J. Litman **

Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, VA 22908 (U S A)

(Received May 8th, 1984)
(Revised manuscript received October 1st, 1984)

Key words: Rhodopsin, Phosphatidylcholine, Reconstitution; Octyl glucoside

The transmembrane protein bovine rhodopsin was reconstituted with egg phosphatidylcholine (PC) by using a modified detergent dilution technique employing the nonionic detergent octyl-β-D-glucoside (octyl glucoside). Using this technique, reconstituted membranes having molar phospholipid/protein ratios between 60:1 and 255:1 were prepared. This is in contrast to the results obtained when an octyl glucoside dialysis technique was employed (Jackson, M.L. and Litman, B.J. (1982) Biochemistry 21, 5601–5608). In the latter case, the highest molar phospholipid/protein ratio that could be obtained when reconstituting rhodopsin with egg PC was approximately 50:1. Reconstituted vesicles prepared by the octyl glucoside dilution technique were examined by negative stain and freeze-fracture electron microscopy, and it was found that the vesicles were unilamellar providing the molar PC/protein ratio was below about 200:1, whereas in preparations having ratios higher than this, a significant number of the vesicles were multilamellar. The mean vesicle diameter showed no trend based on the molar PC/protein ratio within the range of 82:1 to 186:1. The mean diameters of the preparations were between 520 and 850 Å. Approximately equal numbers of protein particles were observed on the concave and convex fracture faces of the freeze-fracture micrographs of the reconstituted membranes which is indicative of a symmetric distribution of the protein across the bilayer.

Introduction

The nonionic detergent octyl glucoside has been widely used in dialysis procedures to reconstitute proteins into phospholipid vesicles [1–6]. This procedure yields a heterogeneous vesicle population under certain conditions [1,2,4]. Alternatively, lack of complete protein incorporation into vesicles is also seen [3,7]. The octyl glucoside dialysis reconstitution of bovine rhodopsin with egg phosphatidylcholine (PC) is an example of conditions where two vesicle populations are obtained [4]. Multilamellar vesicles with a molar PC : protein ratio in the range of 30 : 1 to 50 : 1 comprise the major population even when the initial ratio is as high as 300 : 1, while the second population is predominately PC vesicles containing small amounts of protein. Since variable phospholipid : protein ratios are required to study phospholipid-protein and protein-protein interactions in model membranes, we have investigated the ability of an octyl glucoside dilution procedure to achieve the desired variable ratios. This procedure was found to yield small unilamellar rhodopsin/egg PC vesicles. A result which is in agreement with the previous

* Present address Laboratory of Cellular Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.
** To whom correspondence should be addressed.

Abbreviations: PC, phosphatidylcholine; octyl glucoside, octyl-β-D-glucoside; DTAB, dodecyltrimethylammonium bromide; $R_{eff(p)}$, free micellar detergent/phospholipid ratio

report of De Grip et al. [8]. Our current studies demonstrated that PC/protein ratios can be systematically varied. The dilution procedure may be valuable in the reconstitution of other phospholipid/protein systems where previous octyl glucoside dialysis procedures have yielded heterogeneous end products.

Material and Methods

Octyl glucoside synthesis. Octyl glucoside was synthesized as described by Noller and Rockwell [9] with several modifications. The octyl glucoside exhibited only one spot by thin-layer chromatography when developed twice in a solvent system of ethyl acetate/methanol (4:1, v/v) and visualized by spraying with 5% $K_2Cr_2O_7$/40% H_2SO_4 and heating in an oven.

Phospholipid preparation. Egg PC was purchased from Avanti Biochemicals and its purity was established by thin-layer chromatography. An aliquot of the egg PC in chloroform was transferred to a lyophilization tube and dried under a stream of N_2. The phospholipid film was either placed under vacuum overnight to remove residual chloroform, or redissolved in cyclohexane and lyophilized overnight.

Buffer preparation. The buffer used for reconstitution and subsequent studies was 50 mM Tris and 50 mM sodium acetate, adjusted to pH 7.0 at room temperature with HCl (50 mM Tris-acetate, pH 7.0). To inhibit phospholipid and sulfhydryl oxidation, the buffer was bubbled with N_2 prior to use.

Rhodopsin preparation. Unless otherwise noted, all procedures involving the visual pigment were performed in dim red light (Kodak red filter No. 1) at 4°C. Disks were prepared from frozen bovine retinas (American Stores or Lawson, Co.) by the Ficoll flotation procedure of Smith et al. [10]. Rhodospin was purified from octyl glucoside-solubilized disks by affinity chromatography on concanavalin A-Sepharose 4B (Pharmacia Fine Chemicals) as described by Litman [11] except that the buffer employed was 50 mM Tris-acetate (pH 7.0). The chromatography was performed at room temperature. The rhodopsin is virtually phospholipid-free as there is less than one phospholipid molecule per ten rhodopsin molecules. The column-purified rhodopsin in octyl glucoside was concentrated to 2.5–3.5 mg/ml in an Amicon ultrafiltration cell with a PM-10 membrane. The mannoside concentration was reduced by dialyzing the rhodopsin sample against an 18–20-fold excess of 30–50 mM octyl glucoside in buffer for 12–24 h. The octyl glucoside concentration of the rhodopsin solution was determined by the Anthrone assay [12] and the rhodopsin concentration was measured by determining the difference in absorbance at 500 nm (A_{500}) produced by bleaching the solubilized sample with a high intensity microscope illuminator for 60 s. An extinction coefficient of 40000 was used for rhodopsin. The spectral ratio (A_{280}/A_{500}), i.e., the ratio of the absorbances of the solubilized rhodopsin solution at 280 to that at 500 nm, was routinely in the range of 1.7–1.8.

Reconstitution preparation. Solid octyl glucoside, buffer, and column-purified rhodopsin were added to lyophilized egg PC such that the final rhodopsin concentration, as determined by the ΔA_{500}, was 60 μM. The calculated starting phospholipid/protein ratio was varied from 80:1 to 300:1. The initial octyl glucoside concentration employed was dependent upon the calculated phospholipid/protein ratio and was calculated so that the free micellar detergent/phospholipid ratio, $R_{eff(p)}$ [4] of the solubilized starting mixture was 6.0. This corresponds to octyl glucoside concentrations of 63 mM for a starting phospholipid:protein ratio of 80:1 to an octyl glucoside concentration of 142 mM for a starting phospholipid/protein ratio of 300:1. The sample was vortexed, allowed to completely solubilize, and then equilibrated at 4°C for a minimum of 4 h. Subsequently, this rhodopsin/egg PC solution was added dropwise to deoxygenated buffer which was being rapidly stirred. Unless indicated otherwise, the rate at which the rhodopsin/egg PC solution was added to the buffer was 20 ml/h. The volume of buffer used for dilution was the amount required to bring the final octyl glucoside concentration to 10 mM. Upon dilution, the $R_{eff(p)}$ is -9 or less, which corresponds to a subsolubilizing octyl glucoside concentration [4,13], i.e., dilution to 10 mM octyl glucoside insures that all the PC in mixed micelles will have been converted to vesicles. To remove the detergent, the dilute reconstituted vesicles were dialyzed at 4°C

against at least an 18-fold excess of buffer for 18–36 h with two or three changes of buffer. Finally, the reconstituted membranes were concentrated to approx. 2.0 mg rhodopsin/ml in an Amicon ultrafiltration cell by using a PM-10 membrane.

Sucrose density centrifugation. An aliquot of the reconstituted membrane sample was reserved for analysis, and the remainder was loaded onto 17 ml 0–40% or 0–50% continuous sucrose density gradients prepared in 50 mM Tris-acetate (pH 7.0). Normally, a vesicle suspension containing 4–7 mg of rhodopsin was layered on top of each gradient. The gradients were centrifuged in an SW-27 rotor at 25000 rpm for 8–13 h. The bands, which could be observed in dim red light, were harvested from the gradients using a Pasteur pipette. Sucrose removal was accomplished by dialysis against an excess of buffer.

Determination of phospholipid: Protein ratios. The phospholipid/protein ratios (mol/mol) of the reconstituted membrane samples were determined by phosphate [14] and total protein [15] analysis. Bovine serum albumin was used as a standard for the Lowry protein analysis. The Lowry procedure was modified by the addition of sodium dodecyl sulfate such that its final concentration in the assay mixture was approx. 1.2%. In our experiments, the concentration of column purified rhodopsin, as determined by ΔA_{500}, modified Lowry, and total nitrogen measurements agreed within 5%. A molecular weight of 39000 was used for rhodopsin [16]. The rhodopsin concentration in reconstituted membrane samples was determined by the modified Lowry procedure rather than the ΔA_{500}, as some protein denaturation may occur during the reconstitution process. The phospholipid : protein ratio of the reconstituted vesicle band obtained from the sucrose density gradient is referred to as the final phospholipid/protein ratio. The phospholipid : protein ratio of the aliquot reserved prior to sucrose density gradient centrifugation was also determined and is referred to as the initial phospholipid/protein ratio.

Electron microscopy. For negative staining, reconstituted membrane samples with rhodopsin concentrations between 0.3 and 0.6 mg/ml (0.5 mM in phospholipid) were dropped onto carbon coated Formvar grids using a loop drop technique. The grids were stained with 2% ammonium molybdate, air dried, and viewed in a JEOL-100CX electron microscope.

A standard freeze-fracture procedure was employed [17]. The reconstituted membrane sample was pelleted by centrifugation at $60000 \times g$ for 15 min and resuspended in buffer to yield a phospholipid concentration of approx. 10 mM. The sample was bleached prior to freezing.

Results

Rhodopsin-egg PC reconstituted membranes were prepared by the octyl glucoside dilution technique described under Materials and Methods. Briefly, an octyl glucoside-solubilized solution of column-purified rhodopsin and egg PC was added to a volume of buffer, at a rate of 20 ml/h, such that the octyl glucoside concentration was reduced to 10 mM. The detergent was then removed by dialysis. After dialysis, the recovery (mean % recovery ± S.D. for 14 preparations) was determined to be 86 ± 10% for the protein and 86 ± 15% for the phospholipid. The reconstituted membranes were subjected to sucrose density gradient centrifugation. Only one major band was observed on the gradient, and its position depended on the phospholipid : protein ratio of the reconstituted vesicle sample. Although the band was tight, protein and phospholipid were frequently detected at sucrose densities lower than that of the main band, indicating that the dilution reconstitution procedure produces an inhomogeneous population of reconstituted vesicles. Only reconstituted membranes collected from the banded portion of the gradient were subsequently characterized. The overall recovery (mean % recovery ± S.D. for 14 preparations) was calculated from the amount of material loaded on the gradient and that obtained from the band on the gradient and was determined to be 58 ± 10% and 54 ± 11% for the protein and phospholipid, respectively.

The spectral ratio (A_{280}/A_{500}) of the reconstituted membranes was determined before and after the sucrose density gradient centrifugation step. Prior to the gradient step, the spectral ratios of the reconstituted vesicles were usually 2.5 or less, but subsequent to sucrose density gradient centrifugation and dialysis to remove the sucrose,

the spectral ratios were routinely in the range of 2.0–3.0. A comparison of the rhodopsin concentration as determined by the method of Lowry et al. [15] and by ΔA_{500} measurement indicates that both protein denaturation and phospholipid peroxidation contribute to the increase in the spectral ratio. A comparison of the rhodopsin concentration, as determined by the Δ_{A500} and the modified Lowry procedure, in several reconstituted vesicle preparations, indicates that between 2 and 10% of the total rhodopsin present may be denatured. Such measurements made before and after sucrose density gradient centrifugation indicate that when denaturation occurred, it was during this procedure. The remaining change in the spectral ratio may be attributable to lipid peroxidation.

The phospholipid : protein ratio of the major band obtained from the sucrose gradient (final phospholipid/protein ratio) is plotted in Fig. 1 vs. the phospholipid/protein ratio of the reconstituted membranes prior to the centrifugation step (initial phospholipid/protein ratio). When the initial phospholipid/protein ratio ranged from 56 : 1 to 311 : 1, the corresponding final phospholipid/protein ratio ranged from 60 : 1 to 255 : 1. This data can be fitted to a least squares line having a slope = 0.718, intercept = 27.7, and a correlation coefficient = 0.9665. There is a better correlation between the initial and final ratios at lower phospholipid/protein ratios. It should be noted that the final ratio of 255 : 1 does not necessarily represent the highest ratio that can be attained since initial phospholipid/protein ratios higher than 311 : 1 have not been examined.

Reconstituted membrane preparations were studied by negative stain electron microscopy. Representative negative stain electron micrographs of reconstituted vesicle preparation having final phospholipid/protein ratios of 86 : 1, 147 : 1, and 186 : 1 are shown in Figs. 2A, B, and C, respectively. It can be seen that the membranes are predominately vesicular; however, there is a wide distribution in the vesicle diameters. The mean vesicle diametrs and the corresponding standard deviations, as determined by electron microscopy, of seven preparatioans are listed in Table I. The wide distribution in vesicle diameters are reflected in the large standard deviations. In the range of

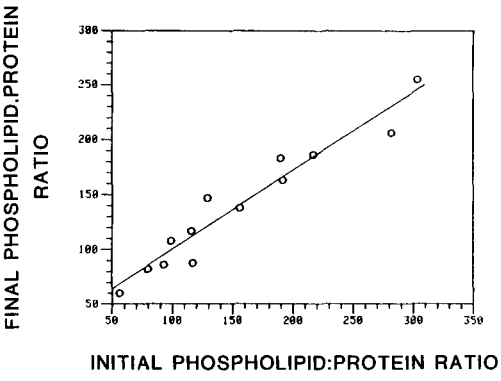

Fig. 1. The molar phospholipid/protein ratio of the reconstituted membranes after sucrose density gradient centrifugation (final phospholipid/protein ratio) vs the ratio of the reconstituted membranes prior to the centrifugation step (initial phospholipid/protein ratio)

phospholipid/protein ratios examined, i.e., 82 : 1 to 186 : 1, the recombinant vesicle diameters range from 520 to 850 Å; however, there does not appear to be a trend based upon the final phospholipid/protein ratio.

Reconstituted membrane preparations were also examined by freeze-fracture electron microscopy. It was found that at phospholipid/protein ratios below 200 : 1, the membranes are predominately unilamellar. At higher phospholipid/protein ratios, many of the vesicles are multilamellar; however, it is interesting to note that they usually contain only two or three lamellae. Fig. 2D is a representative freeze-fracture micrograph of a pre-

TABLE I

MEAN VESICLE DIAMETER AS A FUNCTION OF PHOSPHOLIPID/PROTEIN RATIO

Values are the means ± S D, number of vesicles measured in parentheses

Ratio [a]	Mean diameter (Å)
82:1	610 ± 200 (251)
86·1	720 ± 230 (539)
117 1	570 ± 150 (251)
131 1	560 ± 210 (508)
138 1	850 ± 250 (560)
147·1	520 ± 210 (307)
186 1	630 ± 170 (524)

[a] Molar phospholipid/protein ratio of the vesicle preparation determined after sucrose density gradient centrifugation.

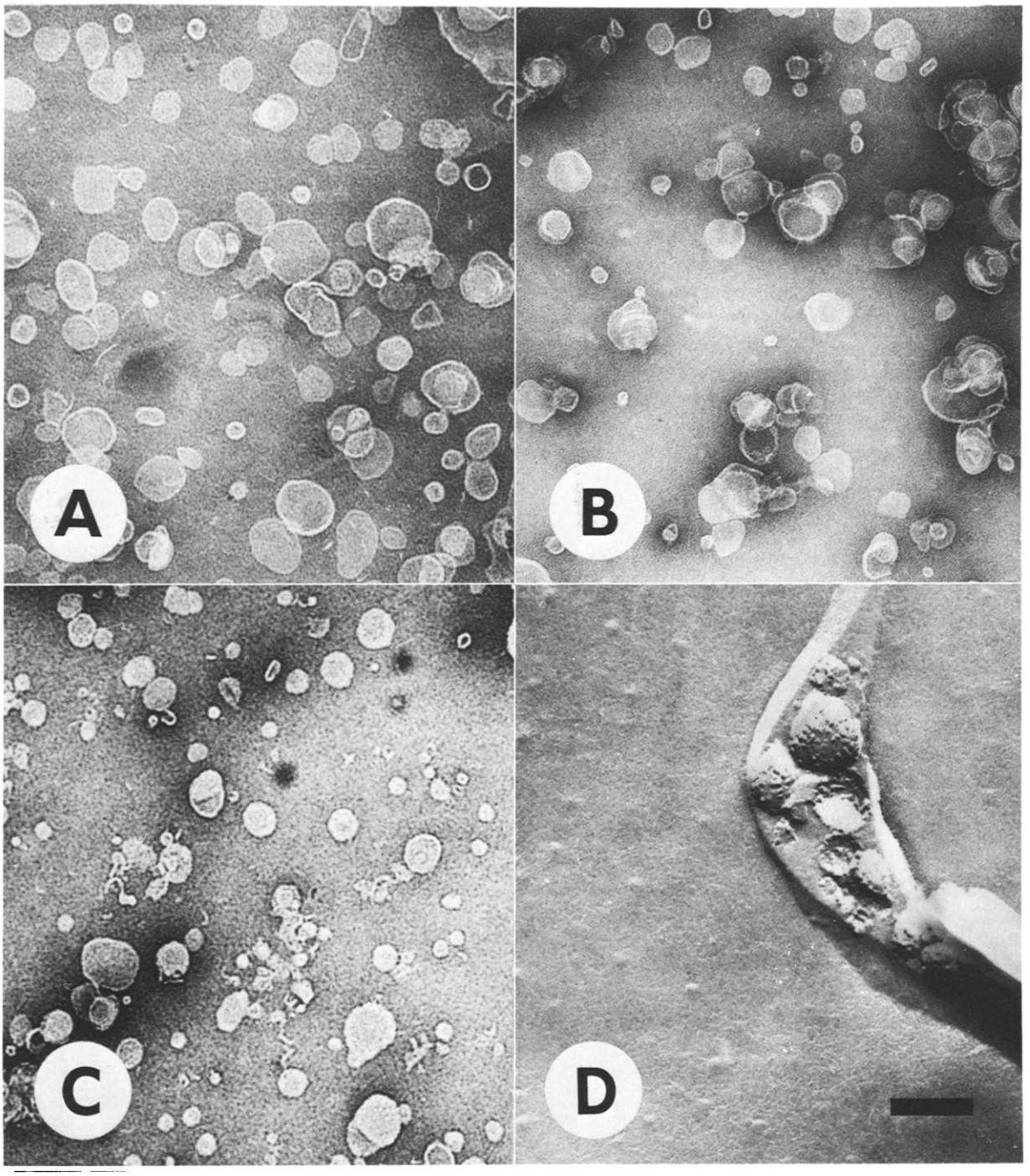

Fig 2. Electron micrographs of reconstituted membranes (A)–(C) examined by negative staining Vesicle preparations have final phospholipid/protein ratios and mean diameters of (A) 86·1, 717 Å, (B) 147 1, 522 Å, (C) 186 1, 633 Å. (D) Freeze-fracture electron micrograph of a vesicle preparation having a final phospholipid/protein ratio of 88 1. Bar represents 1600 Å.

TABLE II

EFFECT OF DILUTION RATE ON VESICLE PROPERTIES

Values are the means ± S D., number of vesicles measured in parentheses.

Dilution rate (ml/h)	Ratio [a]	Mean diameter (Å)
5	111:1	640 ± 190 (556)
10	116 1	740 ± 210 (517)
20	117:1	570 ± 150 (251)

[a] Molar phospholipid/protein ratio of the vesicle preparation determined after sucrose density gradient centrifugation

paration having a phospholipid : protein ratio of 88 : 1. Protein particles can be observed in approximately equal numbers on both convex and concave fracture faces of the unilamellar vesicles. These particles appear to be about 100 Å in diameter.

Most reconstituted membranes were prepared using a dilution rate of 20 ml/h. In one set of experiments, the dilution rate was varied to determine what affect, if any, this would have on the recombinant vesicle properties. Aliquots of an octyl glucoside-solubilized rhodopsin/egg PC solution, having a calculated phospholipid/protein ratio of 100 : 1, were added dropwise to buffer at rates of 5, 10, and 20 ml/h. Subsequent steps were performed as described under Materials and Methods. The three recombinant membranes samples were examined by negative stain electron microscopy. There were no discernable differences in the micrographs of the recombinant vesicles prepared at 5, 10, and 20 ml/h. The mean diameters, as determined from the negative stain micrographs, ranged from 570 to 740 Å (Table II); however, the variation was random, and there was not a trend based on the dilution rate. The differences in the mean diameters are not significant given the large standard deviations. Furthermore, the final phospholipid/protein ratio is, within experimental error, the same for all three samples (see Table II).

Discussion

In this paper, we describe the preparation of rhodopsin/egg PC vesicles by using an octyl glucoside dilution procedure. The advantages of this procedure are that unlike the octyl glucoside dialysis procedure [4], vesicles of variable phospholipid/protein ratios can be achieved (Fig. 1); exhaustive dialysis is not required as is necessary with the cholate dialysis procedure [18]; and the vesicles are small (mean diameters vary from 520–850 Å), which is advantageous for optical measurements, without having to sonicate as is necessary for vesicles obtained from either a tridecyltrimethylammonium bromide [19] or dodecyltrimethylammonium bromide (DTAB) [20] dialysis procedure. In addition, the negative stain electron micrographs show that the mean vesicle diameter of the reconstituted preparations is not dependent upon the molar phospholipid/protein ratio of the preparation within the range of ratios studied here. Finally, since octyl glucoside is a mild detergent, little protein denaturation occurs.

When the native rod outer segment mebrane is subjected to freeze-fracture electron microscopy, particles are observed on only the concave fracture face [21]. In contrast, freeze-fracture particles are found on both the concave and convex fracture faces of the reconstituted membranes prepared by the octyl glucoside dilution procedure described in this paper. The latter is also true of reconstituted vesicles formed by nonyl glucoside dialysis [8], sodium cholate dialysis [18], DTAB dialysis [21], and a solvent extraction-evaporation-hydration procedure [22]. Furthermore, the diameter that we determined for the freeze-fracture particles is in excellent agreement with values of 80–110 Å previously reported [8,21,22]. The presence of freeze-fracture particles on both fracture faces is indicative of a symmetric distribution of the protein across the bilayer, i.e., in the reconstituted membranes, the rhodopsin molecules no longer have a vectorial orientation.

The rate of dilution was varied to determine if this would affect the size and/or other properties of the reconstituted vesicles. Nordlund et al. [23] used an ethanol injection procedure to prepare unilamellar egg PC/egg PE vesicles. Large vesicles were obtained by slowly injecting the phospholipid dissolved in ethanol into KCl at a rate of less than 3 ml/h; whereas, a rapid injection produced small vesicles. As indicated in the results, variation in the dilution rate (from 5 to 20 ml/h) didn't signifi-

cantly change the mean diameter or final phospholipid/protein ratio of the reconstituted vesicles containing rhodopsin.

Egg PC-rhodopsin reconstituted vesicles of varying phospholipid/protein ratios between 60 : 1 and 255 : 1 were prepared using the octyl glucoside dilution technique described above. The results obtained using this technique are in contrast to those obtained using an octyl glucoside dialysis procedure. In the latter case, the ratio of the recombinants obtained from sucrose density gradient centrifugation (final phospholipid/protein ratio) was in the range of 30 : 1 to 50 : 1, even when the starting phospholipid/protein ratio was as high as 300 : 1 [4].

The major difference between the dialysis and dilution technique is the rate at which the detergent concentration is lowered. Previous studies have indicated that rhodopsin/egg PC/octyl glucoside micelles are more stable than egg PC/octyl glucoside micelles [4]. Apparently when the octyl glucoside is removed by dialysis, the process is slow enough that the difference in stability of the two types of micelles leads to disruption of egg PC/octyl glucoside micelles prior to rhodopsin/egg PC/octyl glucoside micelle disruption. Initially, phospholipid-rich vesicles are formed; subsequently, protein-rich vesicles are formed as the octyl glucoside concentration is lowered. On the other hand, removal of the octyl glucoside by dilution is so rapid that disruption of both micelle types is simultaneous, resulting in a mixing of their components. Thus, the resulting reconstituted vesicles are more nearly a reflection of the overall composition of the starting solution.

It is likely that differences in micelle stability similar to those described above for the rhodopsin/egg PC system are responsible for the observation of a limiting ratio of 100 : 1 in the octyl glucoside dialysis reconstitution of bacteriorhodopsin with egg PC [5]. In contrast, micelle stability doesn't appear to be responsible for the heterogeneous populations obtained when reconstituting the Semliki forest virus since multiple populations are obtained with both octyl glucoside dialysis and dilution procedures. In this case, the two populations appear to arise from two different mechanisms, the predominant one being insertion of the virus subsequent to vesicle formation [24]. When glycophorin is reconstituted by octyl glucoside dialysis into egg PC vesicles only 25–50% of the protein is incorporated into vesicles [3], which may attributable to glycophorin's aqueous solubility. While the products of reconstition are dependent upon many factors including the protein and phospholipid, the octyl glucoside dilution procedure ought to be a valuable method for the reconstitution of phospholipid/protein systems where an octyl glucoside dialysis procedure has failed to yield the desired end products.

Acknowledgements

We thank Charles Frazier for excellent technical assistance, Sidney Breese and Bonnie Sheppard of the Central Electron Microscopy Facility of the University of Virginia for the negative stain electron microscopy studies, and Margaretta Allietta and Dr. Thomas Tillack for the freeze-fracture electron micrographs. This work was supported by National Institutes of Health Grant EY00548 and National Science Foundation Grant PCM80-12028.

References

1 Helenius, A., Fries, E. and Kartenbeck, J (1977) J Cell Biol. 75, 866–880
2 Petri, W.A., Jr and Wagner, R.R. (1979) J Biol. Chem 254, 4313–4316
3 Mimms, L.T., Zampighi, G., Nozaki, Y., Tanford, C. and Reynolds, J.A. (1981) Biochemistry 20, 833–840
4 Jackson, M.L. and Litman, B.J. (1982) Biochemistry 21, 5601–5608
5 Klausner, R.D., Berman, M., Blumenthal, R., Weinstein, J.N and Caplan, S.R. (1982) Biochemistry 21, 3643–3650
6 Shelton, R L., Jr. and Langdon, R G. (1983) Biochim Biophys. Acta 733, 25–33
7 Van der Steen, A.T M., Taraschi, T.F., Voorhout, W.F. and De Kruijff, B. (1983) Biochim. Biophys. Acta 733, 51–64
8 De Grip, W.J., Olive, J. and Bovee-Geurts, P.H M. (1983) Biochim. Biophys. Acta 734, 169–179
9 Noller, C.R. and Rockwell, W.C (1938) J Am. Chem. Soc 60, 2076–2077
10 Smith, H.G , Jr., Stubbs, G.W. and Litman, B.J. (1975) Exp Eye Res. 20, 211–217
11 Litman, B.J. (1982) Methods Enzymol. 81, 150–166
12 Spiro, R.G (1966) Methods Enzymol. 8, 3–26
13 Jackson, M.L , Schmidt, C.F., Lichtenberg, D , Litman, B J. and Albert, A D (1982) Biochemistry 21, 4576–4582
14 Bartlett, G.R. (1959) J. Biol. Chem. 243, 466–468
15 Lowry, O.H., Rosebrough, N.J., Farr, A.L and Randall. R J. (1951) J. Biol. Chem. 193, 265–275

16 Ovchinnikov, Y A (1982) FEBS Lett 148, 179–191
17 Schullery, S E , Schmidt, C F , Felgner, P , Tillack, T.W and Thompson, T.E (1980) Biochemistry 19, 3919–3923
18 Fung, B , K -K. and Hubbell, W L. (1978) Biochemistry 17, 4403–4410
19 O'Brien, D F , Costa, L F and Ott, R A (1977) Biochemistry 16, 1295–1303
20 Hong, K. and Hubbell, W.L (1972) Proc Natl Acad Sci USA 69, 2617–2621
21 Chen, Y.S and Hubbell, W L. (1973) Exp Eye Res 17, 517–532
22 Darzon, A., Vandenberg, C.A., Schonfeld, M , Ellisman, M H , Spitzer, N C. and Montal, M (1980) Proc. Natl Acad Sci USA 77, 239–243
23 Nordlund, J.R , Schmidt, C F , Dicken, S N and Thompson, T E (1981) Biochemistry 20, 3237–3241
24 Helenius, A , Sarvas, M and Simons, K (1981) Eur. J Biochem 116, 27–35