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Rhodopsin-Phospholipid Complexes in Apolar Solvents: Formation and Properties

A. Darszon,* M. Philipp,** J. Zarco, and M. Montal*

Departamento de Bioquimica, Centro de Investigacion y de Estudios Avanzados, Instituto Politecnico Nacional, Mexico, 14, D.F., Mexico, and Departments of Physics and Biology, University of California, San Diego, La Jolla, California 92093

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Summary. The extraction of rhodopsin-phospholipid complexes into lipid solvents is described. Two general procedures were developed: (i) the extraction of detergent-solubilized rhodopsin which involved: (a) solubilization of rhodopsin from bovine retinal rod disc membranes in detergent solutions; (b) partial removal of detergent from solubilized rhodopsin; (c) recombination with a defined lipid mixture by sonication of lipid and protein in salt media; and (d) sequential extraction into organic solvents. (ii) The direct transfer of rhodopsin from purified retinal rod disc membranes into apolar solvents which involved: (a) resuspension of membranes in hexane containing phospholipids; (b) sonication; (c) charge neutralization with salts; and (d) sequential extraction into organic solvents. The extraction yield was dependent on several parameters such as: phospholipid to rhodopsin ratio, sonication, type of solvent, and others. The composition of the extracts regarding the molar ratio of phospholipid, detergent, and rhodopsin varied according to the extraction protocol. The spectral characteristics of rhodopsin in the organic phase were similar to those of native rhodopsin in disc membranes. The capacity to regenerate the characteristic dark absorbance maximum at ~485 nm after incubation of dark chromophore (9-cis retinal) with bleached rhodopsin was preserved to about 70%. The general applicability of these techniques in functional reconstitution studies of membrane systems is considered.

Visual excitation is initiated with the absorption of light by the membrane-bound visual pigment rhodopsin (Wald, 1968). Rhodopsin can absorb single quanta of light and initiate the electrical response in photoreceptor cells (Hecht, Schlaer & Pirenne 1942; for review, *see* Ebrey & Honig, 1975). Rhodopsin, beyond acting as a phototransducer, is also the major structural protein component of photoreceptor cell membranes (Papermaster & Dreyer, 1974). Although much is known about the physiology of retinal photoreceptors, the coupling mechanism of

* *Present Address:* Departments of Physics and Biology, University of California, San Diego, La Jolla, California 92093.

** *Present Address:* Department of Immunology, National Institute for Medical Research, The Ridgeway, Mill Hill, London, NW7 1AA, England.

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the rhodopsin photochemical changes to the cell electrical response remains mysterious (Hagins & Yoshikami, 1975).

Our approach to the problem has been to reassemble purified rhodopsin in bilayer membrane systems which permit the use of many techniques that are inaccessible in retinal photoreceptor cells (Montal, 1974). This requires a method whereby both the lipid and the protein are assembled into a bilayer membrane in a concerted way. A possible means to achieve this was established by demonstrating that the partition of lipoprotein complexes between aqueous and nonaqueous phases is strongly favored towards the latter, provided the overall charge of the complex is neutralized with Ca^{++} (Gitler & Montal, 1972; Montal & Korenbrot, 1973; Darszon, Montal & Philipp, 1977).

Here we report the extraction of rhodopsin-phospholipid complexes in organic solvents. We describe two distinct, independent but complementary procedures to transfer rhodopsin from aqueous into nonaqueous phases. First, rhodopsin is solubilized from retinal rod disc membranes and, after recombination with phospholipids in an aqueous phase, is extracted into an organic solvent. This complex derived from a purified protein in detergent contains residual surfactant. In contrast, the second method involves the direct transfer of rhodopsin from purified retinal rod disc membranes into a nonaqueous phase containing phospholipids. This preparation has never been in contact with detergents but is derived from the native membrane where rhodopsin is less purified.

We describe a systematic evaluation of the variables which determine optimum and reproducible performance; we present evidence concerning the composition of the extracts and the preservation of the rhodopsin spectral characteristics. The advantages and shortcomings of the procedures and the general application of the principles developed to membrane reconstitution studies are considered.

Methods and Materials

Materials. The following materials were obtained from the indicated sources:

Dark adapted bovine retinas (Hormel Co., Austin, Minn.); L-α-lecithin from soybeans and 9-cis retinal (Sigma Chemical Co., St. Louis, Mo.); egg lecithin (Applied Science Labs, State College, Pa.); digitonin (Merck, Dohny, West Point, Pa. 19486); cetyltrimethyl-ammonium bromide, CTAB[1] (British Drug Houses, Ltd., England); ^{14}C-CTAB (Amersham/Mass-

1 *Abbreviations used:* ROS – rod outer segments; ΔA_{500} – difference in absorbance at 500 nm between dark and bleached rhodopsin samples; CTAB – cetyltrimethylammoniumbromide.

achusetts); Bio-Beads SM-2 (Bio Rad Laboratories, Richmond, Calif.); spectro quality *n*-hexane and diethyl ether (Merck). All other reagents were of the highest purity commercially available. Glass redistilled water was used throughout.

All of the procedures were performed under dim red light at 4 °C unless otherwise stated. Rod outer segments (ROS) from dark adapted bovine retinas were isolated by sucrose flotation and purified in a discontinuous gradient (McConnell, 1965; Papermaster & Dreyer, 1974). The mitochondrial contamination was estimated to be less than 5% (Papermaster & Dreyer, 1974; Zimmerman, Daemen & Bonting, 1976). Purified ROS were solubilized into 50 mM CTAB (Bridges, 1957) buffered with 66 mM phosphate, pH 7.0, or with 10 mM imidazole-HCl when phosphate analysis was carried out. The ROS solution was routinely centrifuged at $27{,}000 \times g$ for 20 min and the supernatant used thereafter. The rhodopsin concentration in the supernatant was determined by measuring the difference in absorbance ΔA at 500 nm (ΔA_{500}) between dark and bleached samples, assuming an extinction coefficient of 42,000 M^{-1} cm^{-1} (Wald & Brown, 1953) and a molecular weight of 40,000 daltons (*cf.* Ebrey & Honig, 1975). Typically the ratio of absorbance at 280 and 500 nm, A_{280}:A_{500}, was ~2.6 and the rhodopsin concentration was 2.5 mg/ml.

The following general protocol was used to prepare rhodopsin-lipid complexes derived from purified rhodopsin: detergent-solubilized rhodopsin (equivalent to 0.75 mg) was incubated with Bio-Beads SM-2 (90 mg) for a given period of time (*see* Fig. 2) at the indicated temperature. Bio-Beads were previously washed as indicated by Holloway (1973) and Feldberg (1974). Thereafter, rhodopsin was mixed with a dispersion of partially purified soybean phospholipids in 1.0 ml of 0.1 M KCl, 0.01 M imidazole-HCl buffer, pH 7.0 at a defined concentration. This phospholipid mixture contains about 37% phosphatidylcholine, 34% phosphatidylethanolamine, 8% cardiolipin, 14% monophosphoinositides, 4% phosphatidylserine, and others (Kagawa & Racker, 1971; O'Brien, Costa & Ott, 1977). The mixture was sonicated by immersing the test tube in a water bath sonicator (Bransonic, Heat Systems Ultrasonic, Plainview, N.Y., power output of 100 W) for a defined time at 4 °C. Then, 0.1 ml of 0.1 M $CaCl_2$ and 1 ml of hexane were added to the vesicle suspension. The tube was vigorously mixed in a vortex mixer for 4 min, and the two phases were separated in a clinical centrifuge for 1 min. The hexane phase was removed and a second extraction performed with 1 ml of ether (diethylether); the suspension was mixed for 3 min and the two phases were separated by centrifugation.

The following protocol was followed to transfer rhodopsin directly from ROS membranes into organic solvents: ROS membrane aliquots in 10 mM tris-acetate buffer, pH 7.4, containing about 0.75 mg of rhodopsin were centrifuged at $27{,}000 \times g$ for 20 min, the supernatant removed, and the pellet frozen in liquid nitrogen for storage at −70 °C. A ROS pellet, with the indicated amount of rhodopsin, was resuspended in 1 ml of hexane containing partially purified soybean phospholipids. The resulting ROS dispersion in hexane was immediately sonicated for a given time in a water bath sonicator. Thereafter, 0.1 ml of the indicated salt was added and the mixture stirred in a vortex mixer for 30 sec, unless otherwise stated. The preparation was centrifuged in a clinical centrifuge (2 min at maximum speed) and the hexane phase removed for later analysis. The remaining ROS-containing aqueous emulsion was subsequently extracted with 1 ml of pure ether (diethylether) in a vortex mixer for 3 min. The suspension was centrifuged (1 min) and the ether phase removed.

Absorption spectra of 0.5-ml aliquots of the first hexane extraction and the second ether extraction were recorded with a Cary 14 spectrophotometer (Applied Physics Corp., Monrova, Calif.) in 1-cm path length cells by using the corresponding solvent in the reference cell. White light was used to bleach the samples. Samples were also analyzed for phospholipid phosphorous according to Dawson (1960) and for protein according to Lowry *et al.* (1951). Radioactivity was measured in a Packard Scintillation Counter,

utilizing detergent-based toluene liquid scintillation solution (Klip, Darszon & Montal, 1976).

Rhodopsin regeneration in ROS discs was assayed as follows: an aliquot of the preparation was dissolved in 50 mM CTAB, 66 mM phosphate buffer, pH 7, to measure the rhodopsin concentration. The preparation was then bleached completely and incubated at 4 °C in the dark for 15 hr with 9-cis retinal (a twofold excell on a molar basis) (Hubbard & Wald, 1952). Samples of the incubated preparation were dissolved in the same CTAB solution, and the iso-rhodopsin concentration was measured (ΔA_{485}) (Hubbard & Wald, 1952).

Regeneration of rhodopsin from ether extracts of CTAB-solubilized rhodopsin prepared as indicated in Table 1 legend was assayed as follows: the solvent was layered over a salt aqueous medium containing 1% digitonin and evaporated under a stream of nitrogen. Thereafter, an absorption spectrum of the dispersion was taken, the sample bleached and incubated in the dark with a twofold molar excess of 9-cis retinal for 15 hr at 4 °C. Rhodopsin regeneration in extracts derived from membrane-bound rhodopsin prepared as indicated in Table 2 legend was assayed as follows: 0.5-ml aliquots of the ether extract were freed of solvent under a stream of nitrogen. The residue was thereafter dissolved in 1 ml of 0.066 M phosphate buffer, pH 7.0, containing 2% digitonin (purified according to Bridges, 1977) and the dark spectrum recorded. Next, the sample was totally bleached with white light and incubated in the dark with a fourfold excess (molar basis) of 9-cis retinal, for 36 hr at 22 ± 2 °C. In both preparations, the spectrum was measured and the extent of absorbance recovery at 485 nm (isorhodopsin – Hubbard & Wald, 1952) was compared to that recorded prior to bleaching and expressed as the percentage of regeneration.

Results

The assay for rhodopsin-phospholipid complex formation is the yield of rhodopsin extraction into the solvent as measured by the difference in absorbance between dark and bleached samples. We proceed to describe the influence of the experimental conditions on the extraction yield because this is dependent on several parameters at different stages of the procedure.

Extracts Derived from Detergent-Solubilized Rhodopsin

Partial Detergent Removal. The rate of CTAB removal from rhodopsin by Bio-Beads at 24 °C is illustrated in Figure 1. Radioactively-labeled ^{14}C-CTAB was used: detergent removal was as effective in the presence as in the absence of rhodopsin, and no differences were detected if opsin (the bleached protein moiety of rhodopsin) was used instead.

The amount of residual detergent critically modifies the extraction yield as indicated by its dependence on the incubation time in Bio-Beads

Table 1. Dependence of the extract characteristics on the combinations between hexane (H) and ether (E) in two subsequent extractions[a]

First extraction	H	H	E	E
ΔA_{500}	0.023 ± 0.022 $n=23$	0.023 ± 0.022 $n=23$	0.08 ± 0.05 $n=7$	0.08 ± 0.05 $n=7$
Phospholipid/rhodopsin mole ratio	9188	9188	4900	4900
Detergent/rhodopsin mole ratio	6522	6522	2925	2925
Second extraction	**H**	**E**	**E**	**H**
ΔA_{500}	0.006 ± 0 $n=2$	0.181 ± 0.041 $n=23$	0.002 ± 0.002 $n=2$	0.004 ± 0.005 $n=2$
Phospholipid/rhodopsin mole ratio	9666	889	60000	17666
Detergent/rhodopsin mole ratio	7050	548	28222	16833

[a] The incubation time in Bio-Beads was 7 min at 24 °C, phospholipid concentration 10 mg/ml in a 0.1 M KCl solution, the final $CaCl_2$ concentration was 10 mM and the sonication time 4 min. The ΔA_{500} is expressed as the mean ± 1 SD, n=number of experiments.

(Fig. 2*A*); both the first hexane and the second ether extracts are shown. The profile of the extracts is drastically different: the hexane extract exhibits saturation attaining a maximum at about 30 min, whereas the ether extract displays a bell-shaped pattern with a maximum at about 7 min. Likewise, the extent of extraction in the optimal condition is different: hexane, $\Delta A = 0.022 \pm 0.021$ ($n=20$),[2] whereas ether, $\Delta A = 0.183 \pm 0.043$ ($n=20$). Therefore, the ether extraction is more effective.

Other combinations of hexane and/or ether in the two subsequent extractions were explored and the results are shown in Table 1. It is clear that the best combination is a first extraction with hexane and a second one with ether. Several organic solvents such as chloroform, methylene-chloride, butanol and their mixtures, denature rhodopsin and only alkanes, such as pentane, hexane, heptane, and octane, and ether were suitable to extract unbleached rhodopsin.

The organic phase contains, in addition to rhodopsin (and opsin), phospholipid and residual detergent. The opsin content of the second ether extract was estimated to be between 20–30%, based on the Lowry protein assay and the ΔA_{500}. The phospholipid to rhodopsin molar ratios

2 Mean ± SD; n=number of experiments.

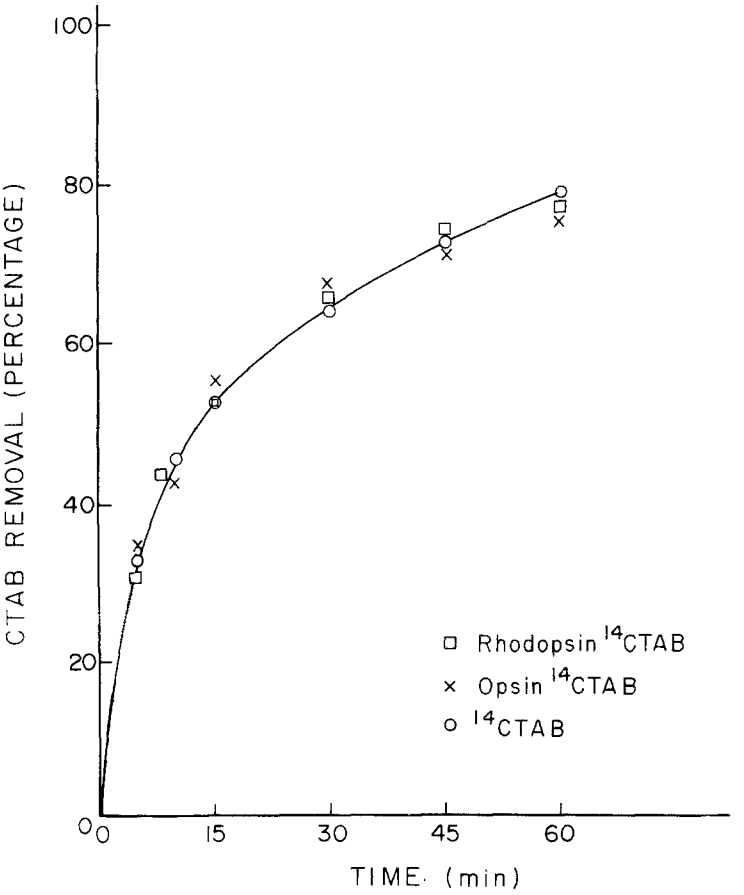

Fig. 1. Time course of CTAB removal by Bio-Beads SM-2 at 24 °C. The time course of CTAB removal is not modified by the presence of rhodopsin or opsin

(calculated from direct phospholipid phosphate analysis) for the hexane and ether extracts are about 9000 and 1000, respectively (*see also* Table 1). The two extracts also differ in the detergent content (measured with ^{14}C-CTAB) as shown in Figure 2*B*: at the condition of maximum extraction the hexane phase contains between 20–25% of the original detergent concentration, while the ether has about 15%. No CTAB extraction into either hexane or ether was detected in the absence of lipid and rhodopsin.

Recombination with Phospholipids by Sonication. Two important variables must be considered in the recombination step, i.e., the lipid to protein ratio and the sonication period.

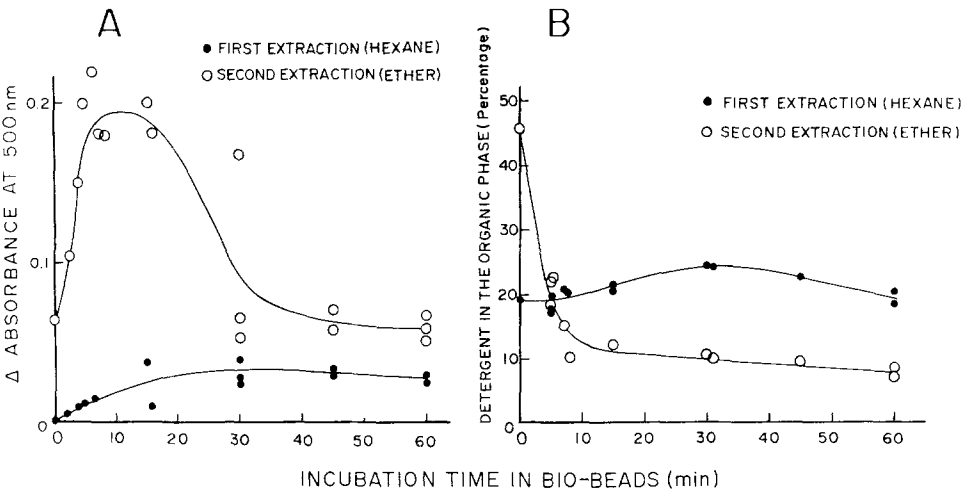

Fig. 2. (*A*): Extent of rhodopsin extraction into hexane and ether as a function of the incubation time of rhodopsin in Bio-Beads SM-2 at 24 °C. The phospholipid concentration was 10 mg/ml, the final $CaCl_2$ concentration was 10 mM, and the sonication time 4 min. (*B*): Residual CTAB extracted with rhodopsin into hexane and ether as a function of the incubation time of rhodopsin in Bio-Beads SM-2. Other conditions as in *A*

The dependence of rhodopsin extraction on the concentration of added soybean phospholipid is illustrated in Figure 3, where the phospholipid was varied, maintaining the rhodopsin and CTAB concentrations constant, as well as the sonication time; the hexane extract shows saturation at a phospholipid/protein ratio of 17 (w/w), while in ether a maximum is obtained at a ratio of 13. The extraction yield in the optimal condition is at least a factor of 4 greater in the second extraction. Thus, the second ether extraction provides a preparation with higher rhodopsin content and lower phospholipid and detergent concentration.

The effect of sonication time on the extent of extraction is shown in Figure 4. A very low extraction into hexane ($\Delta A_{500} \sim 0.005$) occurs if no sonication is performed and it increases with sonication time attaining saturation at about 10 min ($\Delta A_{500} \sim 0.04$). In contrast, the second extraction into ether is considerable even without sonication ($\Delta A_{500} \sim 0.1$) and it increases reaching saturation at about 4 min sonication time ($\Delta A_{500} \sim 0.2$). Therefore, in the experiments reported where other variables were studied the sonication time was kept constant at 4 min.

Extraction into Organic Solvents. It was originally considered that the partition of the rhodopsin-lipid ensemble into the solvent required

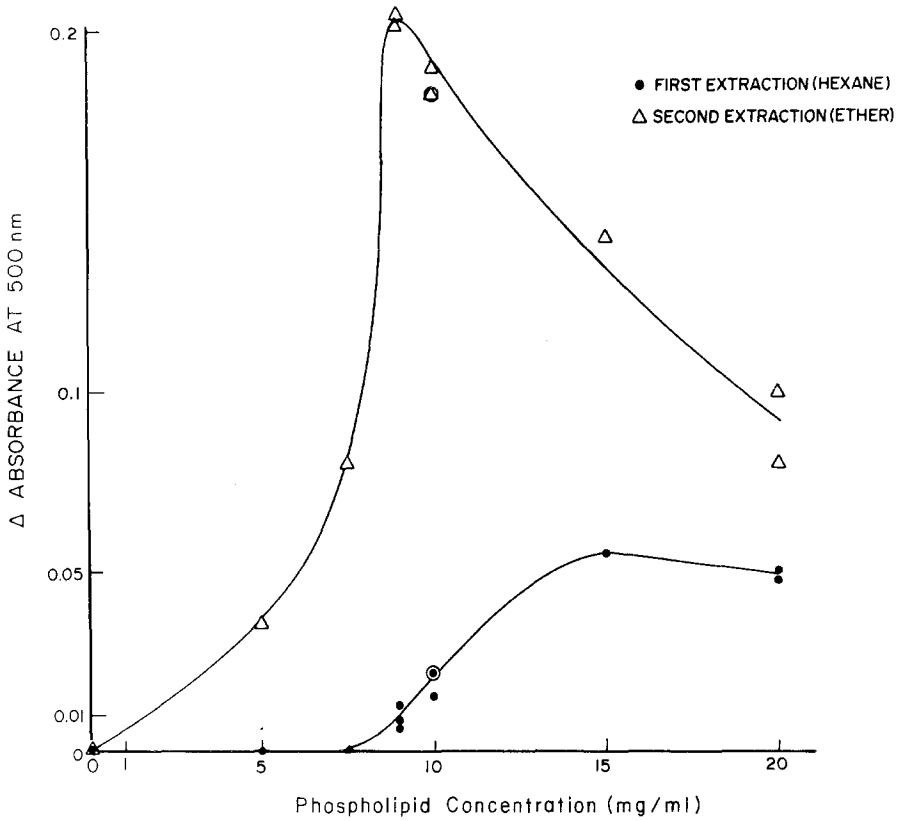

Fig. 3. Extent of rhodopsin extraction into hexane and ether as a function of the concentration of added phospholipid. The circled triangle and black point represent the average of 20 extractions performed at 10 mg/ml of added lipid. The incubation time in Bio-Beads was 7 min at 24 °C. The final $CaCl_2$ concentration was 10 mM and the sonication time 4 min

the overall charge neutralization of the complex; this was achieved with counterions, Ca^{++} being the most effective (Gitler & Montal, 1972; Montal & Korenbrot, 1973). This point was re-examined for the first and second extractions of the CTAB-solubilized rhodopsin; both extractions were independent of the Ca^{++} concentration. The lack of requirement for Ca^{++} suggests that the cationic detergent CTAB, which remains associated to the complex, fulfills the role of counter-ion, allowing the formation of ion pairs and thus the extraction into the solvent.

A significant variable in the extraction step is the mixing time (in a vortex mixer) after the addition of Ca^{++} and hexane to the dispersion. As illustrated in Figure 5, no extraction of rhodopsin to hexane occurred when the mixing period was 10 sec, but it increased linearly with time

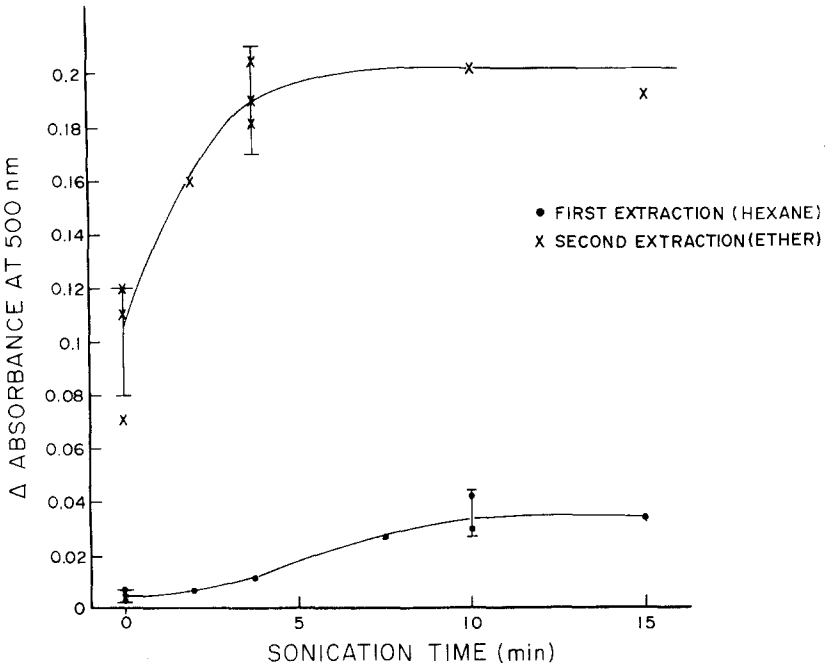

Fig. 4. Extent of rhodopsin extraction into hexane and ether as a function of the sonication time. Rhodopsin was incubated with Bio-Beads for 7 min at 24 °C, the phospholipid concentration was 10 mg/ml, and the final $CaCl_2$ concentration was 10 mM

up to a $\Delta A_{500} \sim 0.04$ at 4 min. Simultaneous measurements of phospholipid extraction into hexane showed that even with a short 10-sec mixing, the phospholipids partitioned into hexane irrespective of rhodopsin, and thereafter the partition increased with mixing time. Thus, the effect of the first hexane extraction is to reduce the amount of free phospholipid available, allowing the second extraction to be low in phospholipid and high in rhodopsin content. This is clearly indicated in Figure 5 where, for example, at a mixing time of 2 min the first extraction exhibits a $\Delta A_{500} \sim 0.02$ and phospholipid content of 116 μg/ml, whereas the second extraction presents a $\Delta A_{500} \sim 0.24$ with phospholipid content of ~112 μg/ml.

Extracts Derived from Membrane-Bound Rhodopsin

Sonication of purified retinal ROS membranes in the presence of a phospholipid solution in hexane allows the effective extraction of rho-

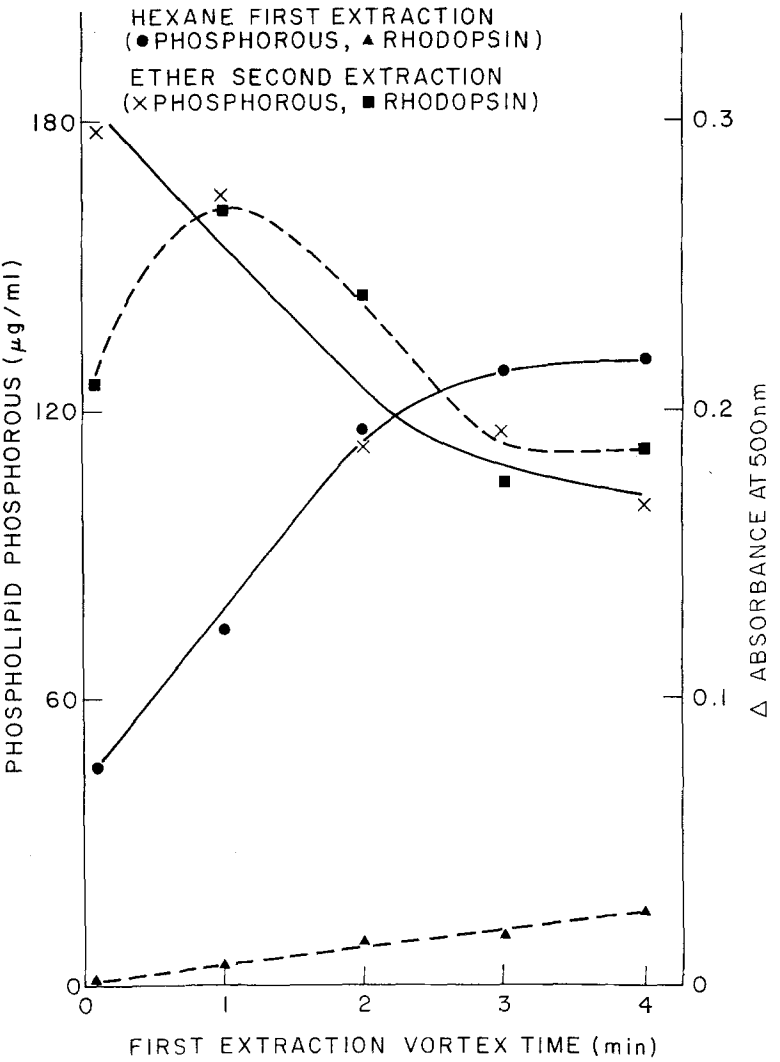

Fig. 5. Extent of rhodopsin and phospholipid extraction into hexane and ether as a function of the mixing time (in a vortex mixer) after the addition of $CaCl_2$ (10 mM final) and hexane to the dispersion. The sonication time was 4 min. All other conditions as in Fig. 4

dopsin into ether. This procedure is quick, simple, and circumvents the involvement of detergents in the preparation

Resuspension with Phospholipids in Hexane. The amount of rhodopsin transferred from ROS membranes to either hexane or ether depends on the concentration of phospholipid in the resuspending hexane solution. Other variables involved in the experimental protocol were arbitrarily

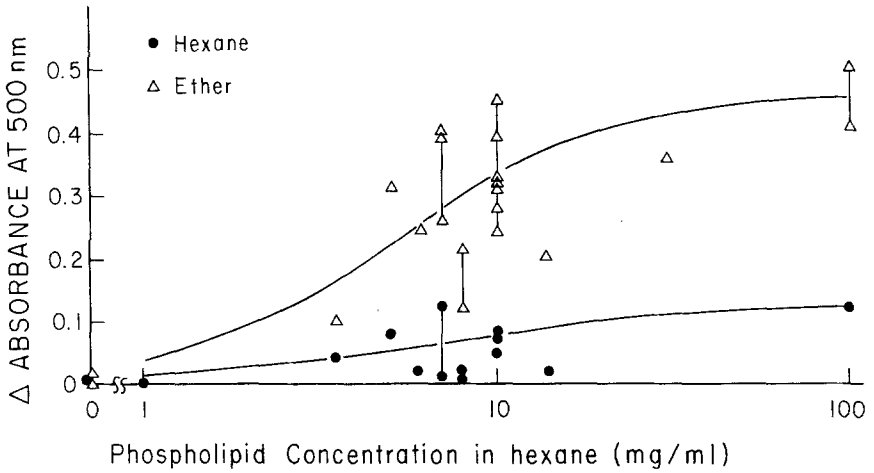

Fig. 6. Extent of rhodopsin extraction into hexane or ether as a function of the phospholipid concentration in the resuspending hexane. The sonication time was 5 min and 0.1 ml of 0.1 M $CaCl_2$ was added. Other conditions are as in *Materials and Methods*

Table 2. Rhodopsin transfer from ROS membranes into hexane (H) or ether (E) as a function of solvent combination in two subsequent extractions

First extraction	ΔA_{500}	Second extraction	ΔA_{500}
H	0.08 ± 0.03	E	0.27 ± 0.03
H	0.08 ± 0.03	H	0.00 ± 0.00
E	0.23 ± 0.1	H	0.00 ± 0.00
E	0.23 ± 0.1	E	0.21 ± 0.03

In all cases ΔA_{500} is expressed as the mean $\pm$ 1 SD of two experiments. The phospholipid concentration in the resuspending hexane was 10 mg/ml, the sonication time was 5 min, and 0.1 ml of 0.1 M $CaCl_2$ was added. Other conditions were as described in *Materials and Methods.*

fixed according to previous experience (*see* legend Fig. 6). As illustrated in Figure 6, the yield of rhodopsin extraction into either of the two solvents increases with the phospholipid concentration in hexane, reaching saturation at 15–20 mg/ml. The second ether extraction is more effective than the first one in hexane, allowing the transfer of as much as 60% of the rhodopsin originally present in the ROS pellet (0.75 mg). At the maximum of the curves, ether contains a fourfold more rhodopsin than hexane. However, if the first vortex time, that is after addition of 0.1 ml of salt solution, is increased to 2 min, hexane extracts up to 40% of the rhodopsin in the ROS pellet (*see* Table 2).

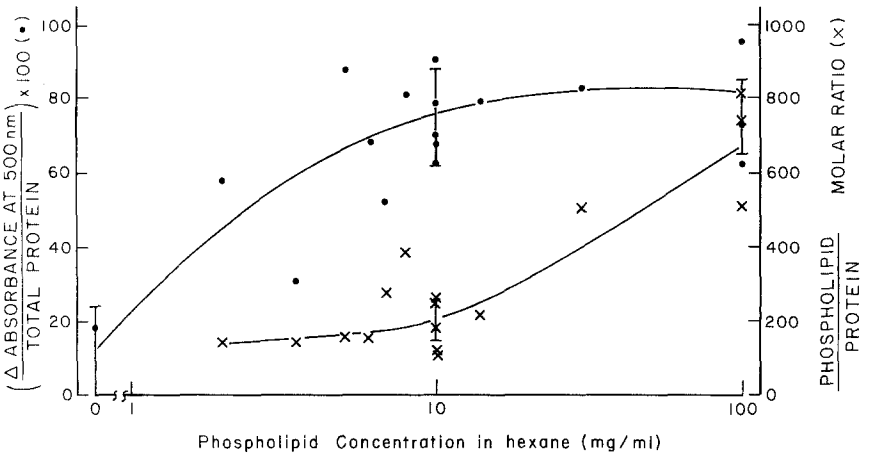

Fig. 7. Dependence of the lipid/protein molar ratio (×) and of the ratio of rhodopsin/total protein (●) extracted into ether on the phospholipid concentration in the resuspending hexane. All conditions as in Fig. 6

The phospholipid to rhodopsin ratio is an important parameter in reconstitution studies. This ratio in ROS disc membranes is in the range of 60–90 (*see* review by Daemen, 1973), and we have aimed to develop conditions that approach this limit. In doing so, we found that the extent of rhodopsin denaturation is dependent on the phospholipid concentration in the initial resuspending hexane. Figure 7 shows the measured mole ratios of phospholipid to total protein as well as rhodopsin to total protein. Notice that as the phospholipid content in hexane increases the rhodopsin to protein ratio becomes larger; this suggests that the lipid excess protects rhodopsin during the extraction, favoring the transfer of unbleached protein into the ether phase. Analysis of these curves indicates that at a concentration of 10 mg/ml the extraction efficiency is high (45%), the phospholipid to protein ratio is ~250, and the rhodopsin to protein ratio approaches the peak value, indicating that this condition yields a preparation with the lowest lipid and highest rhodopsin content.

The extraction yield increases linearly with the amount of ROS utilized at a fixed phospholipid concentration. The ROS pellet used as starting material must be hydrated since when this extraction procedure is applied to liophylized ROS, the extraction yield is virtually zero.

Sonication. Figure 8 shows the yield of rhodopsin extraction in the first hexane and second ether extracts as a function of the sonication

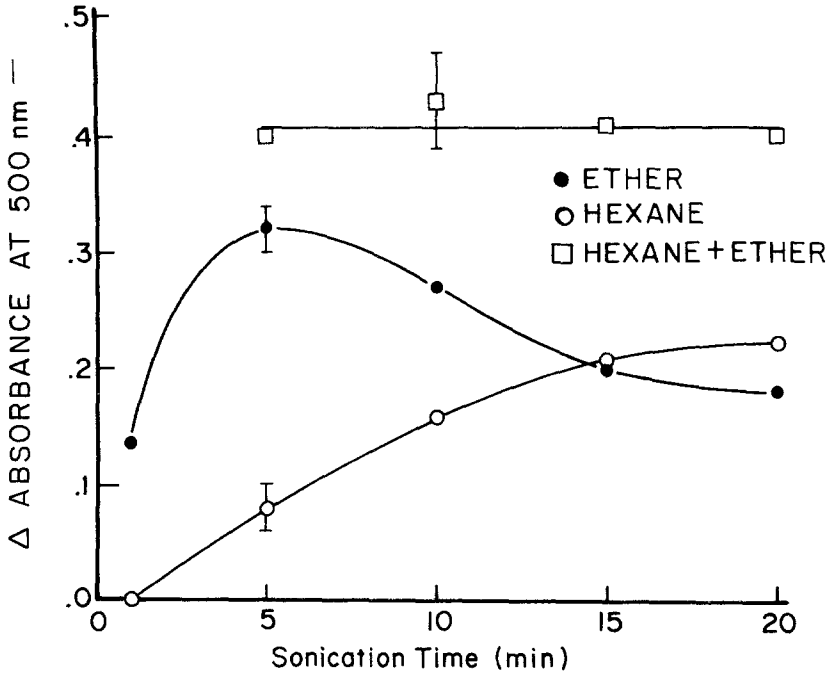

Fig. 8. Extent of rhodopsin extraction into hexane and ether as a function of the sonication time. The phospholipid concentration in the resuspending hexane was 10 mg/ml. Other conditions as in Fig. 6

time. Without sonication, the extraction yield in hexane is practically zero, whereas a significant amount of rhodopsin is extracted in ether. A sonication period of 5 min substantially increases the yield in the hexane extract and allows the maximum ether extraction. Longer sonication periods enhance the extraction of rhodopsin into hexane while reducing that into ether such that, at 15 min, the yield in hexane is larger than in ether. The total extraction yield, namely, the sum of the rhodopsin content in both solvents, remains constant after a 5-min sonication time, suggesting that beyond this critical time the total extraction is not limited by sonication.

Charge Neutralization with Salts. The next variable involved in the procedure is the salt concentration added after sonication. The extraction yield in hexane and ether is markedly dependent on the concentration of Ca^{++} as illustrated in Figure 9. The amount of rhodopsin extracted into hexane increases monotonically up to a saturating $CaCl_2$ concentration. The yield in the ether extract increases, reaching the peak at around

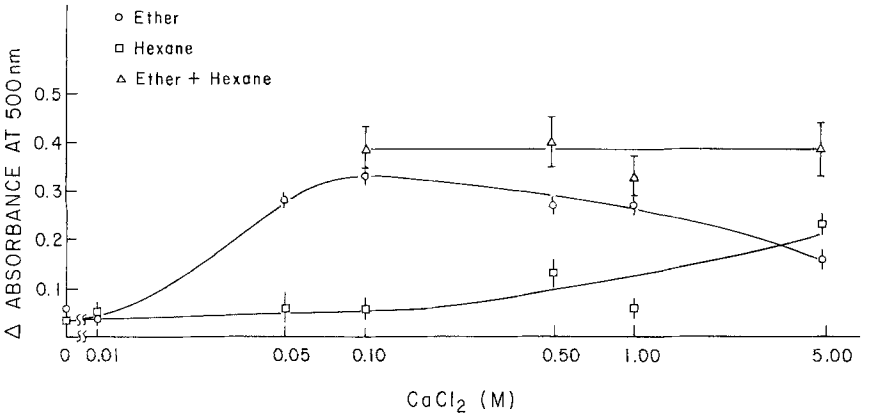

Fig. 9. Extent of rhodopsin extraction into hexane and ether as a function of the $CaCl_2$ concentration. The concentration indicated represents that of the original solution, and the volume of all the additions was 0.1 ml. The phospholipid concentration in the resuspending hexane was 10 mg/ml. Other conditions as in Fig. 6

Table 3. Characteristics of ether extracts prepared with NaCl or $CaCl_2$

	NaCl 0.3 M	*n*	$CaCl_2$ 0.1 M	*n*
ΔA_{500}	0.35± 0.04	3	0.35± 0.03	3
(ΔA_{500}/Total protein) ×100	55.1 ± 7.5	3	67.9 ± 6.0	3
Phospholipid/protein (molar ratio)	177.7 ±44.0	2	209.0 ±10.0	2

The experimental conditions were as in Table 2 using a first extraction in hexane and a second one in ether. The results of the ether extract are expressed as the mean ± 1 SD and *n* is the number of experiments.

0.1 M $CaCl_2$, and subsequently the yield in hexane becomes the largest. As expected, this effect is not specific for Ca^{++} since comparable extractions can be obtained with Na^+, though at larger concentrations. A comparison of the extraction yield, rhodopsin/protein ratio and phospholipid/protein ratio obtained with 0.1 M $CaCl_2$ and 0.3 M NaCl is shown in Table 3.

Sequential Extraction into Organic Solvents. Table 2 illustrates all the combinations of hexane and ether in two sequential extractions. It is clear that our objective to extract a rhodopsin-lipid complex with a maximum content of unbleached rhodopsin and with a rhodopsin to phospholipid ratio approaching that present in the native membrane is achieved with the sequence of extraction described throughout, namely, first with hexane and second with ether.

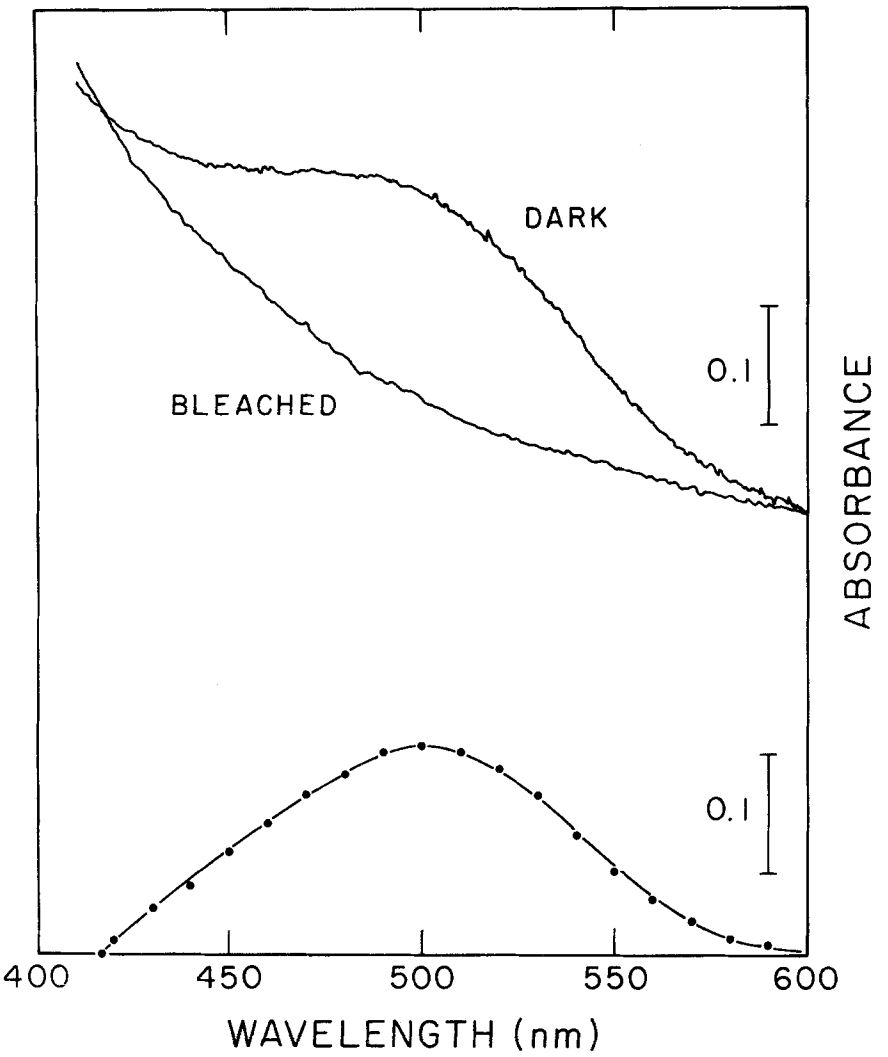

Fig. 10. Absorbance spectra of rhodopsin in ether in the dark (upper record) and bleached (lower record) states. Rhodopsin was incubated with Bio-Beads for 7 min at 24 °C, the phospholipid concentration was 10 mg/ml, and the final $CaCl_2$ concentration was 10 mM: Spectrophotometer cells of 1 cm path length were used. The corresponding absorbance difference spectrum (dark-bleached) is presented under the original spectrum. Temperature, 25 °C

Properties of Rhodopsin in Apolar Solvents. The current lack of a functional assay for rhodopsin presents a problem in evaluating the extent of denaturation induced by the extraction. We have used two operational criteria: spectral characteristics and regenerability.

The measured spectral characteristics of rhodopsin in the organic phase are similar to those of native rhodopsin in the disc membrane:

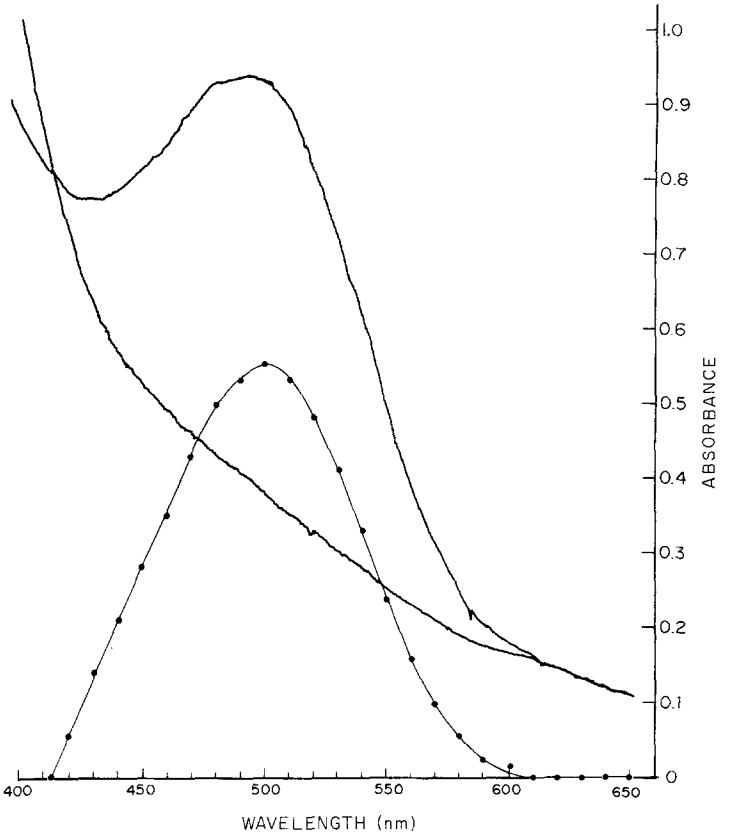

Fig. 11. Absorbance spectrum of rhodopsin in ether in the dark (upper record) and bleached (lower record) states. ROS membranes containing about 1.5 mg of rhodopsin were used. The phospholipid concentration in the resuspending hexane was 10 mg/ml. The absorbance at 650 nm is due to the turbidity of the preparation which tends to precipitate several hours after preparation. The lowest trace shows the corresponding absorbance difference spectrum (dark-bleached) of rhodopsin in ether. Temperature, 25 °C

spectra of the second-ether extractions derived from detergent solubilized rhodopsin or directly from retinal rod disc membranes are illustrated in Figures 10 and 11, respectively. The absorption spectra in the visible range of the hexane and ether extracts showed a peak with λ max at 500 nm which vanished upon illumination. Difference spectra between dark and bleached samples showed an isosbestic point around 415 nm. Accurate values cannot be claimed because of the scattering of the samples in the solvent as well as a change in scattering on bleaching.

Rhodopsin in isolated discs regenerated up to $85\% \pm 3$ ($n=5$) of its original absorbance. Rhodopsin from the second ether extraction

derived by the detergent procedure regenerated 70.51 ± 14.51 ($n=5$). Bleached rhodopsin from the second ether extract of disc membranes regenerated $75\% \pm 16$ ($n=16$). Regeneration was not observed in ether or when bleaching was performed in ether followed by the evaporation of solvent and resuspension of the residue in digitonin. Analysis of the regeneration kinetics show that the initial rate is comparable to that reported for digitonin-solubilized rhodopsin (Hubbard & Wald, 1952); however, other slow components have been observed and are currently under study.

Other experiments, reported elsewhere, have indeed established the presence of excitable rhodopsin molecules in the solvent: briefly, they show that monolayers at polar/apolar interfaces formed from rhodopsin-lipid extracts display light-induced phenomena that are accounted for by rhodopsin bleaching (Trissl, Darszon & Montal, 1977).

Discussion

The ultimate goal of our approach is to assemble a functionally active rhodopsin membrane; therefore, conditions were developed to reproducibly obtain a high rhodopsin extraction into ether ($\sim 50\%$) (Fig. 7), with a phospholipid to rhodopsin ratio (~ 400) (Fig. 7) that approaches that existent in the retinal rod disc membranes (~ 75). Rhodopsin in these extracts exhibits spectral characteristics and regeneration capability similar to rhodopsin in cell membranes and therefore constitutes an active starting point for reconstitution studies in bilayer membranes (Montal, 1974; Montal, Darszon & Trissl, 1977; Trissl *et al.*, 1977).

The procedures developed in this paper represent two independent but complementary pathways designed to extract protein lipid complexes into apolar solvents to be used as starting material for reconstitution in bilayer membranes. One modality allows the solubilization of the protein in detergents and its subsequent purification to a defined extent; after partial detergent removal and recombination with phospholipids in the aqueous phase, the lipoprotein vesicles can be partitioned into an organic phase. The major advantage of this procedure is to yield extracts derived from a purified membrane protein. In reconstitution studies of membrane systems containing several proteins this is the procedure of choice, since it is required to solubilize and purify the protein of interest and then proceed to extract it. The main limitation of this

version is the presence of residual detergent. This is overcome in the direct approach whereby the biological membrane is sonicated with lipids in hexane, thus allowing the transfer of the protein into the nonaqueous phase. This extract has never been in contact with detergents and, therefore, is instrumental in defining the role of detergents in the phenomena studied. Likewise, the detergent preparation is valuable in dissecting the involvement of one or more components in the behavior of reconstituted membranes. Thus, the two procedures complement each other.

The approach of directly transferring proteins from membranes to solvents is applicable, in principle, to any membrane system. The procedure is particularly suitable, though not exclusive, for membranes naturally enriched with a given protein such as retinal rod discs (Papermaster & Dreyer, 1974; Zimmerman *et al.*, 1976), sacroplasmic reticulum (MacLennan & Holland, 1975) or *Halobacterium halobium* (Henderson, 1977). This is, in general, rather exceptional since usually the protein of interest constitutes a small fraction of the total membrane protein. However, the availability of specific functional assays or of known end points is immensely valuable in reconstitution studies; for example, substrates and inhibitors for enzyme systems, activators and inhibitors for receptors, or even mutants for specific proteins in bacterial systems.

Concerning the extraction mechanism it is clear that supplementary phospholipids and charge neutralization are required in order to favor the partition of the protein into the solvent. This process can be schematically visualized as analogous to the formation of swollen inverted micelles where the polar entities (hydrophilic lipid and protein moieties) are segregated in an inner core and carried into the apolar solvent by the hydrocarbon coat provided through the lipid and/or surfactant acyl chains (Montal, 1976). However, the situation is more complex: the extracts, particularly in ether, are turbid and probably exist emulsified (Prince, 1977). In this respect, notice that the aqueous phase subjected to the second ether extraction is an emulsion already containing some organic solvent (hexane). This may partially account for the higher efficiency of the second ether extraction. In addition, the solvent characteristics are obviously important: the solubility of water in ether is two orders of magnitude higher than that in hexane (Riddick & Bunger, 1970). Hence, ether could allow rhodopsin to carry more water around its hydrophilic region, facilitating its partition together with lipids.

The rhodopsin-lipid complexes in apolar solvents are building blocks of bilayers and monolayers; they can be spread as a monolayer at air-water interfaces (Montal, 1974; Montal *et al.*, 1977; Trissl *et al.*, 1977)

and subsequently used to form planar bilayers by apposing two such monolayers. Alternatively, black films can be formed by spreading the extracts in the organic phase (Montal, 1974; Chien & Mueller, 1976). Bilayer vesicles can be prepared from the complex by solvent evaporation and subsequent hydration in salt media (Montal, 1974). In addition, they are interesting preparations in their own right. Studies of isolated membrane proteins in low dielectric constant media which mimic the membrane milieu, are scarce; the use of these complexes as a relevant and potentially informative model system can be foreseen.

The use of these methods to transfer biologically active proteins from membranes into apolar solvents has already proved to be successful in other membrane systems, such as cytochrome oxidase (Montal, 1974; Chien & Mueller, 1976), bacteriorhodopsin (Hwang, Korenbort & Stoeckenius, 1977), acetylcholine receptor (D. Ben-Haim and J. Lindstrom, *personal communication*), reaction centers from photosynthetic bacteria (M. Schonfeld & G. Feher, *personal communication*), and a mitochondrial anion channel (Schein, Colombini & Finkelstein, 1976). It is our hope that this paper will find primary use as a guideline to reliably extract proteins from other membranes where the approach of functional reconstitution in bilayer membranes appears promising.

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