Spin-Label Studies of Protein–Protein Interactions in Retinal Rod Outer Segment Membranes. Saturation Transfer Electron Paramagnetic Resonance Spectroscopy†

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ABSTRACT: Rhodopsin in disc membrane fragments from bovine retina was labeled with a maleimide spin label on hydrophilic sites of the protein, and saturation transfer electron paramagnetic resonance spectra were recorded. This technique allows the measurement of the rotational diffusion of membrane-bound proteins and consequently the detection of any restriction in the mobility. Protein–protein interactions in the disc membranes induced by various treatments of the membranes were systematically analyzed by this technique. In large-size membrane fragments, the fast rotation of rhodopsin, first observed by R. A. Cone ((1972) *Nature (London), New Biol. 236*, 39–43) ($\tau \simeq 20$ μs), is found also by saturation transfer spectroscopy. A brief sonication of the membranes decreases the size of the vesicles but leaves the saturation transfer spectra unchanged, thus confirming that the motion observed is due to the rotation of the proteins and not to tumbling of the vesicles. The addition of sucrose reduces the apparent motion of the protein, while glutaraldehyde (5%) stops the rotation completely ($\tau > 10^{-3}$ s). Illumination for a few seconds has no effect on the saturation transfer spectra. However, prolonged illumination at 37 °C results in a progressive immobilization of the proteins. This immobilization is completed within 30 min at 37 °C but requires several hours at 20 °C. Very likely the observed phenomena reflect artificial protein aggregation. The protein–protein interactions seen in such experiments do not appear to be physiologically relevant. Progressive removal of the lipids by treatment with phospholipase A_2, followed by several washes with bovine serum albumin, slows down the motion of the proteins. When approximately 70% of the lipids has been removed, no more motion can be detected. Relipidation by fusion with egg lecithin vesicles does not restore the initial motion. The relationship between the mobility of the proteins and the amount of lipid might reflect an average viscosity depending upon the lipid to protein ratio. However, the nonreversibility of this phenomenon suggests an increasing protein aggregation induced by delipidation. The general conclusion is that a minimum amount of phospholipid is required (a) to provide a low viscosity allowing for protein mobility and (b) to protect against protein–protein aggregation. There is no need for tight binding of phospholipids to the proteins to achieve such goals.

Rhodopsin is the first membrane-bound protein whose rotational diffusion constant has been measured. Brown (1972) and Cone (1972) showed by studying photodichroism that the protein rotates rapidly in the plane of the membrane. Other techniques were later employed to investigate the rotational mobility of intrinsic membrane proteins containing no natural chromophore. In particular, Cherry has developed the use of the triplet state of phosphorescent labels and found that the rotational diffusion of proteins such as "band 3" in red blood cells can be relatively small (Cherry et al., 1976). Spin labeling could not be used for such measurements until Hyde & Dalton (1972) introduced the saturation transfer technique. Indeed classical EPR[1] of nitroxides is only sensitive to motion corresponding to correlation times smaller than 10^{-6} s. The saturation transfer technique, if advantage is taken of the second harmonic display, 90° out of phase, can be used to study motions in the correlation-time range of $10^{-6} \leq \tau \leq 10^{-3}$ s, even with biological samples, i.e., with a relatively low concentration of spin. This technique was employed by Thomas et al. (1975) to study myosin and recently the ATPase from sarcoplasmic reticulum (Thomas & Hidalgo, 1979). In preliminary reports we have shown the potentiality of saturation transfer to discriminate between membrane-bound proteins immobilized in the plane of the membrane and those free to diffuse (Baroin et al., 1977; Rousselet & Devaux, 1977). We have found that there is a drastic difference between the saturation transfer spectra obtained with spin-labeled rhodopsin in the discs and with spin-labeled cholinergic receptors in *Torpedo* membranes. Yet classical EPR gave rise to identical spectra with both samples. The comparison with reference spectra corresponding to isotropic motions allowed an approximate estimation of the correlation times involved. A value of about 20 μs was found for rhodopsin in bovine retina. This value is in good agreement with Cone's results obtained with a different technique on frog retinas. Therefore the type of analysis of saturation transfer spectra proposed by Thomas et al. (1976) for isotropic motions seems to be applicable to membranes also. However, at this point in the development of the theoretical treatment of saturation transfer spectroscopy, it seems better to interpret modifications of the spectra rather than to give absolute values of correlation times.

In the present article, we further investigate the rotation of rhodopsin in disc membranes. Evidence of protein–protein interactions, corresponding to dramatic changes in the saturation transfer spectra, can be shown in various situations. Prolonged illumination and lipid depletion are two ways of achieving such results. We will attempt to discuss the biological relevance of these artificially provoked protein–protein interactions.

Materials and Methods

Membrane Preparation and Control. Rod outer segment membranes were isolated from cattle retinas as described by Osborne et al. (1974). Membranes in 20 mM sodium phosphate buffer (pH 7.4) were used fresh or stored in liquid

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[1] Abbreviations used: MSL, 3-maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl; NEM, *N*-ethylmaleimide; EPR, electron paramagnetic resonance; BSA, bovine serum albumin; DTT, dithiothreitol; $NaDodSO_4$, sodium dodecyl sulfate.

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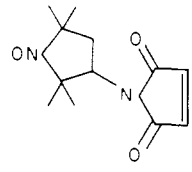
nitrogen before use. Unless otherwise specified, experiments were carried out under dim red light. The rhodopsin concentration was determined by the absorption at 500 nm, after solubilization of an aliquot in 3% Ammonyx L-0. Ten percent acrylamide–$NaDodSO_4$ gel electrophoresis experiments were performed according to Weber & Osborn (1969). DTT was purchased from Sigma, and NEM was from Merck. Lipid phosphorus was determined by the method of Rouser et al. (1969). All experiments were carried out under argon and argon was bubbled through all buffers. Freeze-fracture electron microscopy was performed on fresh and thawed membranes to control the morphological aspect of membranes after storage at liquid nitrogen temperature. The distribution of particles appeared very similar in both samples. The size of vesicles was determined by metal shadowing. An average diameter of 5000 Å was found, i.e., slightly below the diameter of intact discs. Occasionally membranes were dissolved in 30 mM aqueous octyl glucoside solution (Sigma).

Delipidation. Delipidation was performed using lyophylized *Crotalus adamanteus* snake venom (Sigma) as a source of phospholipase A_2. Rod outer segment membranes were diluted to a final concentration of 0.8 mM phospholipid in 5 mM Tris-HCl, pH 7.2, 1 mM $CaCl_2$. Snake venom (10 μg/mL) was added and the mixture left to incubate at 15 °C with gentle stirring. To stop the reaction, 5 mM EDTA was added at 0 °C and the suspension was washed in the presence of EDTA in 2% fatty acid free BSA buffered solution.

Sonication. Sonication was performed with an apparatus from Ultra-Son, Annemasse, 150 TS; a small tip was used, together with low power (~40 W).

Glutaraldehyde Treatment. Membranes were incubated with fresh glutaraldehyde (TAAB), 5%, at 4 °C overnight at pH 7.5. Membranes treated with glutaraldehyde did not penetrate the $NaDodSO_4$ gels.

Spin-Labeling Experiments. 3-Maleimido-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl (MSL) was purchased from Syva (Palo-Alto). Infrared spectroscopy showed no trace of the isomaleimide.



MSL

The detailed experimental conditions for the labeling of rod outer segment membranes with spin-label MSL have been described elsewhere (Baroin et al., 1977). EPR experiments were performed with a Varian E109 connected to a Tektronix computer 4051 (16K memory) and provided with a temperature control system. Fifty-microliter quartz cells were used together with a quartz Dewar for temperature control. For the saturation transfer experiments, the exact microwave field strength received by the sample was calibrated with Fremy's salt (see Thomas et al., 1976). Conditions for the second harmonic out-of-phase spectra are described in Baroin et al. (1977). The Tektronix computer was used to perform accumulation, double integration of the first harmonic spectra, and subtractions of spectra.

EPR experiments were routinely performed with a dim red light only. In order to study the modifications induced by bleaching, the cell was directly illuminated with a tungsten lamp through a special aperture of the cavity (i.e., without having to move the cell).

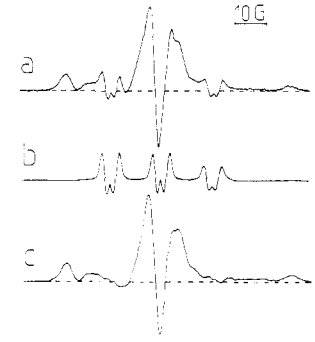


FIGURE 1: Saturation transfer spectra of spin-labeled rhodopsin (second harmonic, 90° out of phase, microwave power 32 mW, modulation 5 G, modulation frequency 50 KHz). (a) MSL bound to rhodopsin in disc membrane fragments at 20 °C; (b) MSL in a mixture of glycerol–water (50:50) at 20 °C; (c) combination of a and b, so as to minimize the contribution of the weakly immobilized component on spectrum a. The electronic manipulation enables one to obtain the pure saturation transfer spectrum of MSL tightly bound to rhodopsin. This procedure is probably safer for the membranes than any combination of chemical preincubation followed by repeated extensive centrifugations of the membranes.

Results

Saturation Transfer Spectra of Spin-Labeled Rhodopsin in the Disc Membranes. MSL binds tightly to rhodopsin molecules, apart from approximately 10% of the probes which give rise to a loosely bound signal (Delmelle & Virmaux, 1977; Baroin et al., 1977). Double integration of the first harmonic spectra shows that between one and two spin labels per rhodopsin molecule are bound. The value of two seems to be a maximum and is consistent with the number of sulfhydryl groups found to be readily accessible to NEM (De Grip et al., 1973). Figure 1a shows the saturation transfer spectrum at 20 °C of rhodopsin in the disc membrane fragments, labeled with MSL. The spectrum is stable for hours, providing argon has been bubbled through the suspending medium. Figure 1c is the result of subtracting the saturation transfer spectrum 1b, which corresponds to MSL in a mixture of glycerol–water (50:50), from spectrum 1a. The fact that glycerol must be added confirms that the "loosely bound signal" corresponds to probes attached in some way to the membranes and not to spin labels tumbling freely in water. The spectrum 1c corresponds to the highly immobilized component and is likely to reflect the motion of the protein itself.

The following experiments will tend to demonstrate that the observed mobility of the probes does not reflect the rotation of the vesicles but indeed that of the proteins in the membrane. Our approach consisted of reducing the average size of the vesicles by a brief sonication. The average sizes of the vesicles before and after sonication are 5000 and 500 Å, respectively, as determined by electron microscopy (metal shadowing). No modifications of the saturation transfer spectra could be observed under such conditions (spectrum in Figure 2a). However, if sonication is prolonged for more than 1 min, an *increase* in the apparent rotational correlation time can be observed.

Another possible approach might have been to add sucrose to reduce the vesicle rotation. However, sucrose can also perturb directly the rotation of rhodopsin molecules due to the hydrophilic moiety protruding into the aqueous phase. Actually, sucrose does modify the shape of the spectrum in a way corresponding to an immobilization of the probe (spectrum in Figure 2b).

If membranes are incubated with 5% glutaraldehyde, the saturation transfer spectrum is dramatically changed, while the first harmonic spectrum remains unchanged (see Figure

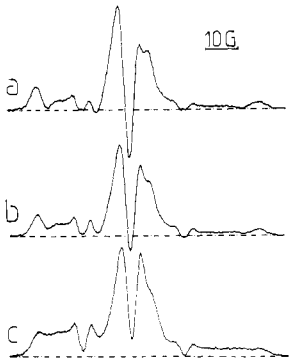


FIGURE 2: Effects of various physical and chemical modifications of the membranes on the saturation transfer spectrum. Same setting as in Figure 1. Spectra recorded at 4 °C. (a) Membranes sonicated 30 s. The spectrum is identical with the spectrum corresponding to the reference sample (unsonicated). If the sonication is prolonged, the shape of the spectrum is modified, and this indicates more immobilization. (b) Thirty percent sucrose has been added to the medium. (c) Membranes have been treated with 5% glutaraldehyde.

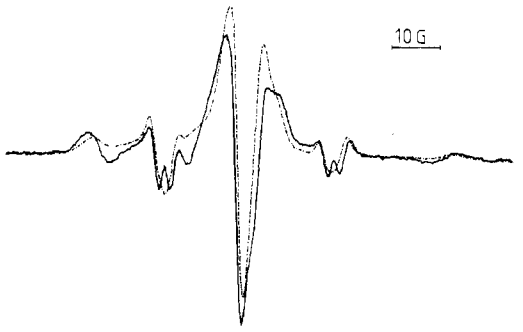


FIGURE 3: Effect of light on the saturation transfer spectra of MSL in disc membranes, at 37 °C. (Full line) Sample in the dark; (dotted line) sample illuminated continuously, temperature maintained at 37 °C. The passage from the first spectrum to the second spectrum requires about 30 min after the beginning of the illumination. The concentration of rhodopsin is approximately 300 μM. One spectrum is recorded in 8 min.

2c for the modifications of the saturation transfer spectrum). These modifications were already pointed out in our preliminary report (Baroin et al., 1977). In our hands, the modifications induced by 5% glutaraldehyde were greater than those in a subsequent paper by Kusumi et al. (1978).

In this original report, we discussed the effect of changing the temperature. No discontinuity appeared in the progressive decrease of τ, deduced from the saturation transfer spectra.

Effects of Illumination on the Saturation Transfer Spectra of Spin-Labeled Rhodopsin. The effect of light was examined at different temperatures by direct illumination of the samples in the cavity. Fixed values of the field were selected first and any modification taking place within a minute or two after illumination could be observed. However, no such modifications occurred, even at 37 °C. Subsequently entire spectra were recorded with different scans of 8 min. Figure 3 shows the saturation transfer spectra before and after 45 min of illumination at 37 °C. No further modifications take place if illumination is prolonged. To achieve a comparable modification at 20 °C requires about 1.5 h.[2] No effect was detected at 4 °C, as reported by Baroin et al. (1977). In all these cases, the first harmonic spectrum remains unchanged. However, if membranes are dissolved with detergent, the extreme splitting for the unbleached membranes is 63.5 G, at

[2] This delay is reduced considerably if argon is not used to protect the membranes.

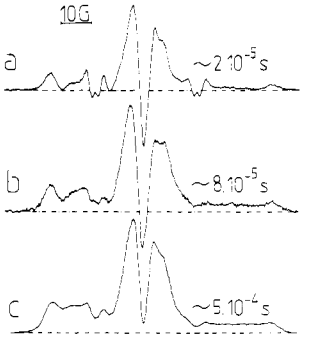


FIGURE 4: Effect of lipid depletion on the saturation transfer spectra of MSL in disc membranes. Temperature is 20 °C. Concentration of rhodopsin, 200 μM. (a) Native membranes; (b) membranes from which 30 to 40% phospholipids have been removed; (c) 60–70% lipids removed. Approximate values of the rotational correlation times indicated on the spectra correspond to the average values obtained from a line-shape analysis of the type proposed by Thomas et al. (1976) for isotropic samples.

20 °C, whereas membranes bleached (under drastic conditions) give rise after solubilization to a splitting of 65.5 G, a value to be compared with the splitting obtained with glutaraldehyde-treated membranes, solubilized with detergent: 67 G.

Effect of Lipid Depletion on the Saturation Transfer Spectra of Spin-Labeled Rhodopsin. Partial removal of the phospholipids from the disc membranes was performed with phospholipase A_2 treatment followed by washes with BSA. Saturation transfer EPR spectroscopy was performed on the resulting samples. Freeze-fractured electron microscopy was also performed on a few samples. When 60–70% of the lipids is removed, electron microscopy shows that membranes are severely perturbed but that vesicles still exist. The size of the particles may have significantly increased, although it is hard to conclude definitely on this point. Polyacrylamide gel electrophoresis with and without DTT indicated no differences with the control membranes; i.e., only one major band appears, corresponding to a molecular weight of about 40 000. This is very important since it shows (a) that the phospholipase A_2 had no protease activity (no small subunits appearing on the gels) and (b) that no disulfide bridges are involved during the protein aggregation since no larger oligomers are formed. The saturation transfer spectra are shown in Figure 4. Numbers superimposed on the spectra are approximate rotational correlation times, deduced from the analysis of the spectra following the method of Thomas et al. (1976). The conventional EPR spectrum is practically unaffected by the lipid depletion. Addition of octyl glucoside gave the same first harmonic and second harmonic out of phase spectra for delipidated membranes and the control membranes (extreme splitting: 63.5 G). Delipidated membranes were incubated with sonicated egg lecithin at 30 °C for about an hour and centrifuged. It is found that the lipid to protein ratio can be increased again to approximately the initial value in the disc membrane but that the saturation transfer spectrum of the initial sample is not restored.

Discussion

In a previous report (Baroin et al., 1977), we discussed the applicability of the saturation transfer technique to the study of the rotational diffusion of membrane-bound proteins such as rhodopsin. It was shown that the same empirical parameters used by Thomas et al. (1976) to analyze the spectra corresponding to isotropic motions can also be used to obtain approximate values of the correlation times in the case of anisotropic motions of the type experienced by membrane-bound proteins. In the present discussion, we will interpret

the modifications appearing in the shape of the saturation transfer spectra as qualitative indications only of the changes in the mobility of the proteins. It will never be essential to our discussion to assess an accurate value of τ to each spectrum. Therefore we feel safe in using this type of analysis. Finally it should be recalled that the classical EPR spectra of spin-labeled rhodopsin were unchanged during the various treatments discussed. This, by itself, justifies the use of the saturation transfer technique.

Effect of Illumination. If illumination takes place at 37 °C, a significant modification of the saturation transfer spectrum can be obtained (Figure 3). Crude interpretation of the spectra suggests that the correlation time τ changes from 10–20 μs to approximately 70–80 μs.[3] The most likely explanation of this modification, taking place on a slow time scale, is that proteins tend to aggregate. The fact that a limit is reached, within 30–60 min, suggests an association of, at the most, three or four proteins cross-linked in a limited number of sites rather than the formation of continuous domains of closely packed proteins. It is known that the reactivity of SH groups on rhodopsin is increased by bleaching (De Grip et al., 1975) which suggests an increase in their accessibility. Furthermore, bleached rhodopsin molecules are very unstable in detergents where they readily aggregate irreversibly. Bleaching probably exposes cystein groups that are responsible for the cross-linking of proteins.

The aggregation of rhodopsin from bovine retina does not seem to occur below 20 °C. Although there is no clear phase transition of the lipids in such a range of temperature, Chabre (1975) has indicated that rigid domains tend to appear below 20 °C. If these domains correspond to a crystallization of phospholipids around the proteins, rather than a lipid protein segregation, then this artificial halo of rigid lipids formed at low temperature could protect the proteins from the formation of disulfide bridges.

The observations reported here are therefore attributed to nonphysiological interactions between proteins, rendered fragile by prolonged illumination. These results should not be compared with the observations of Hubbell et al. (1977) using very different spin labels attached to a different site of rhodopsin. The probes in this latter case are not completely immobilized and the spectra do not reflect the motion of the proteins but rather are characteristic of the environment of the specific type of probe. The modifications seen by these authors are therefore attributed to a change of protein conformation and not to an increased immobilization of rhodopsin.

Effect of the Delipidation of Disc Membranes on the Rotational Diffusion of Rhodopsin. We have shown (see Figure 4) how the saturation transfer spectra of spin-labeled rhodopsin membranes are modified when lipids are progressively removed. Again, the type of modifications shown in Figure 4 suggests an increase in the rotational correlation time on going from a to c. A simple comparison with the spectra obtained with 5% glutaraldehyde (Figure 2c) shows that the removal of 60–70% of lipids produces an immobilization comparable to what is obtained by a cross-linking of the proteins. Very similar results were obtained by Thomas & Hidalgo (1978) with spin-labeled Ca^{2+}-ATPase reconstituted with a small amount of lipid. The intermediate case (spectrum b, Figure 4) suggests an intermediate immobilization. However, the spectrum is somewhat ambiguous. Two interpretations of this spectrum are possible and both may be correct. It is possible that the average viscosity of the remaining lipid phase has increased, as suggested by Cherry et al. (1977) for bacteriorhodopsin reconstituted with various amounts of lipid. Then spectrum b should be attributed to a homogeneous sample of high viscosity. But even electron-microscopy does not allow us to rule out the possibility of inhomogeneous samples. In this case, spectrum b would have to be attributed to a mixture of spectrum a and spectrum c. Both possibilities are compatible with the shape of spectrum b.

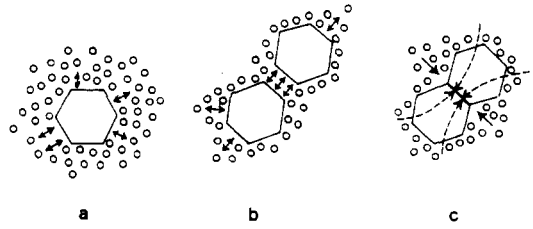


FIGURE 5: A simple model to explain protein aggregation. Intrinsic proteins are represented with an arbitrary hexagonal cross-section and phospholipids are represented by circles. The "holes" or short lifetime defects corresponding to temporary unoccupied phospholipid sites are schematized by the following sign (↔). If all the phospholipids diffuse rapidly in the plane of the membrane, a protein is never perfectly covered by a closed shell of lipids. However, the interaction between two proteins, unless disulfide bridges are formed, is only stabilized if large areas of the two proteins interact. This requires the simultaneous absence of a large number of phospholipids from the surrounding of two neighboring proteins. This event has a low probability in native membranes (a). It becomes a nonnegligible event if the ratio of lipids to proteins is reduced (b). Once the association between two proteins has been formed, phospholipids are not efficient for disrupting the binding because the access between the proteins is limited in the bidimensional space experienced by phospholipids (→) (c). On the contrary, detergents can be inserted readily because they move in three dimensions (- - →).

It should be recalled, however, that in no case could we recover the original spectrum by addition of lipid. This is in favor of the latter interpretation and suggests an increased probability of aggregation accompanying the reduction of the lipid to protein ratio.

Stable aggregation of lipid-free proteins was also found by other authors. Van Breugel et al. (1978) have shown, using phospholipase C lipid depleted membranes, that it is not possible to reconstitute homogeneous lipid–rhodopsin vesicles in the absence of detergent. The very fact that it is possible, if detergents are added, demonstrates that there is no covalent binding involved in this latter experiment and that protein aggregation can be stable even without disulfide bridges. This remark can be extended to other intrinsic proteins. For example, Jost et al. (1977) had to use a lipid-rich cytochrome oxidase preparation in order to incorporate by fusion spin-labeled phosphatidylcholine in the absence of detergents. In the last paragraph, we will discuss the implications of these findings on the role of lipids in biological membranes.

How Protein Aggregation Can Be Prevented. In a recent article, Chapman et al. (1977) have discussed the solubility of gramicidin A in lipids. Models illustrating how the polypeptides aggregate when lipids are crystallizing at low temperatures are shown. The importance of the ratio of lipid to polypeptide is pointed out. We want to show now that, if the ratio of lipids to proteins is high enough, phospholipids will assure the bidimensional solubility of proteins and that no requirement for a stable boundary layer exists. Because of its size, an intrinsic protein is surrounded by a large number of phospholipids (20–30?). But, if we accept the hypothesis of Brown et al. (1977) that all phospholipids in the rod outer

[3] With the present knowledge on saturation transfer spectroscopy, it cannot be ruled out that a reorientation of the labels is, at least partially, responsible for the change observed on the saturation transfer spectrum under illumination of the sample.

segment membrane diffuse with nearly the same lateral diffusion coefficient, then thermodynamic fluctuations dictate that a certain number of "sites" must be temporarily vacant around each protein at a given moment. In other words, there are a certain number of phospholipid "holes" around a protein (see Figure 5a). In normal membranes, the probability that several holes will be next to each other is very low. In order to bind two proteins by van der Waals forces, a large number of phospholipids must be accidentally and simultaneously vacant on two close protein surfaces (Figure 5b). This will only happen with a high probability if the ratio of lipid to protein is low. Whenever two proteins come into contact (Figure 5c), the binding energy is much greater than between two phospholipids or one phospholipid and one protein and, therefore, the association is stable. A whole set of phospholipids is required to disrupt the protein–protein interaction. But their concomitant effect is very unfavorable in this two-dimensional system. Only detergent can break the protein–protein interaction efficiently because detergent, being amphiphilic, moves in the three-dimensional space.

To summarize, interactions between lipids and proteins should be viewed in the first approximation as a direct transposition of the interactions between solutes and solvents. It is well-known in a three-dimensional system that, if the amount of solvent is reduced, then the solute will precipitate (or crystallize); furthermore if the amount of solvent is now increased, it may take a very long time (or heat and stirring) to solubilize the solute again.

The general conclusion of this article is that a minimum amount of phospholipid is required to provide the protein's two-dimensional mobility in membranes. Two reasons can be advanced: (a) the viscosity of the lipidic phase varies with the ratio of lipid to protein (this has already been suggested by other authors); (b) a minimum ratio of lipid to protein is necessary (and sufficient) to prevent proteins from aggregating. This minimum may very well be chosen by natural membranes. It is remarkable that the ratio of phospholipid to protein is, in most cases, close to 100/1 (mole to mole). This ratio corresponds to more than one single phospholipid shell around an average intrinsic protein, such as rhodopsin; however, it does not allow for the large area of typical bilayer structure. In the next paper (Favre et al., 1979), we will show that the physical state of the hydrocarbon chains, in direct contact with rhodopsin in the rod outer segment membranes, are not very different from the physical state of the so-called bulk lipid hydrocarbon chains.

Acknowledgments

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