Rhodopsin–Lipid Associations in Bovine Rod Outer Segment Membranes. Identification of Immobilized Lipid by Spin-Labels†

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ABSTRACT: Rhodopsin–lipid interactions have been studied in bovine rod outer segment (ROS) membranes by using spin-labels. Spin-labeled fatty acid, sterol, phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, and phosphatidic acid molecules all display a two-component spectrum when probing ROS membranes. One of the spectral components represents 33–43% of the total spectral intensity and is characteristic of a strongly immobilized nitroxide spin-label. This immobilized component is resolved from −4 to 37 °C. The remaining 67–57% of the integrated spectral intensity has a very similar form to the spectra of the same spin-labels in bilayers of extracted ROS membrane lipid. A small selectivity for the immobilized regions of ROS membranes is shown by phosphatidylserine, while the fatty acid, phosphatidylcholine, phosphatidylethanolamine, and sterol spin-labels partition almost equally into these regions. The selectivity is not solely due to the head-group charge on phosphatidylserine since the negatively charged phosphatidylglycerol and phosphatidic acid spin-labels do not display an enhanced selectivity. These results are interpreted in terms of two populations of lipid existing in ROS membranes, the major one being fluid bilayer in exchange with and surrounding the immobilized lipid which is in direct contact with rhodopsin. On the basis of available information on the size of rhodopsin, it is calculated that the immobilized lipid is sufficient to form a single complete shell around the protein.

Many important biological functions are performed by proteins organized in lipid bilayer membranes (Sandemann, 1978), and an understanding of the interactions between these proteins and their lipids is therefore of particular importance. Rod outer segment (ROS[1]) membranes are well suited to the study of the interactions between integral membrane proteins and the lipid matrix for a variety of reasons. Firstly, rhodopsin constitutes 85–90% of the total membrane protein of bovine ROS membranes (Montal & Korenbrot, 1976; O'Brien, 1978; Daemen, 1973; Papermaster et al., 1976), and therefore any protein–lipid interactions will, in all probability, be directly due to rhodopsin–lipid associations. Such a level of single protein enrichment is normally achieved by reconstitution, recombination, or specific enrichment procedures which suffer from the hazards of either protein aggregation or denaturation or both and of detergent removal, which is often a long and incomplete process. ROS membranes are isolated without the use of detergents or of enrichment processes involving delipidation or protein loss.

Secondly, a limited amount of structural information for rhodopsin is available. This has come from X-ray diffraction studies (Charbre, 1975), X-ray scattering (Sardet et al., 1976) and neutron-scattering (Osborne et al., 1978) data, and ultracentrifugation experiments (Lewis et al., 1974), and some dimensional information has been determined (Sardet et al., 1976; Osborne et al., 1978). Such parameters are useful when interpreting observations of the interactions between lipids and integral membrane proteins in structural terms.

Thirdly, ROS membranes are particularly interesting since rhodopsin, within its membrane environment, is responsible for the primary step in visual perception, leading from light absorption to nerve excitation. This process involves both the full photolytic cycle of rhodopsin, including regeneration, and the consequent modulation of the cytoplasmic activity of an internal transmitter through conformational changes taking place in rhodopsin (Saibil et al., 1976; Liebman et al., 1974; Downer & Englander, 1975; McDowell & Williams, 1976; Ostroy, 1977; Hubbell et al., 1977). The membrane lipid could be involved in both of these stages: in stabilizing the structure of rhodopsin during the various states of the photolytic and regenerative cycles and in regulating the conformational changes leading to the transmitter response.

In the present work, we demonstrate the existence of an immobilized lipid component in ROS membranes as monitored by a number of lipid spin-labels. This immobilized lipid is revealed as one component of a distinct two-component ESR spectrum of spin-labeled ROS membranes, the observation of which in itself demonstrates that the two components exist for longer than $\sim 10^{-8}$ s. Similar experiments with bilayers of extracted ROS lipid show only a one-component spectrum, typical of the spin-labels undergoing anisotropic motion in a lipid bilayer. Such fluid bilayer spectra closely resemble the other components observed from spin-labeled ROS membranes. It is suggested that the "immobilized" component arises from motionally perturbed bilayer lipid. From spectral subtraction and integration it is shown that the amount of immobilized lipid is ~24 lipid molecules/protein, which is probably sufficient to form a single shell around rhodopsin, the major protein of ROS membranes. Experiments with fatty acid, sterol, and a number of phospholipid spin-labels demonstrate a limited preference for the immobilized region by phosphatidylserine molecules. The organization in the ROS membrane, the composition of the immobilized lipids around rhodopsin, and the approximate size of the intramembranous portion of rhodopsin are discussed.

Experimental Section

Rod Outer Segment Membranes. Bovine retinas were collected from dark-adapted eyes and the rod outer segments

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[1] Abbreviations used: 14-SASL, 14-(4′,4′-dimethyloxazolidinyl-*N*-oxy)stearic acid; 14-PCSL, 14-PESL, 14-PSSL, 14-PGSL, and 14-PASL, β-14-(4′,4′-dimethyloxazolidinyl-*N*-oxy)stearoyl-γ-palmitoyl-α-phosphatidylcholine, -phosphatidylethanolamine, -phosphatidylserine, -phosphatidylglycerol, and -phosphatidic acid, respectively; ESR, electron spin resonance; ROS, rod outer segment; DPH, 1,6-diphenyl-1,3,5-hexatriene; NMR, nuclear magnetic resonance; DTT, dithiothreitol.

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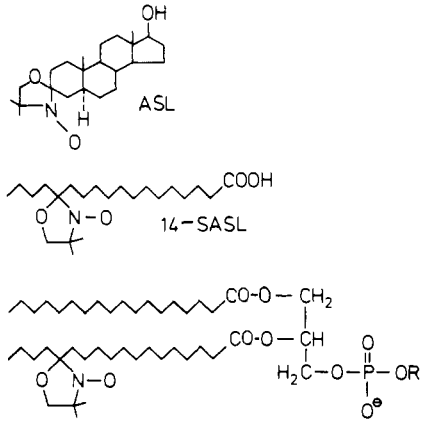
isolated as described by Papermaster & Dreyer (1974), with some slight modifications. All operations were performed in darkness or under dim red light at 4 °C as rapidly as possible. The retinas were homogenized by vigorous shaking in a sucrose solution (45% w/v) buffered with 70 mM sodium phosphate (pH 7.0) containing 1 mM $MgCl_2$ and 1 mM DTT. After centrifugation (7700*g* for 5 min), the supernatants were diluted threefold with 70 mM sodium phosphate buffer (pH 7.0) and recentrifuged (7700*g* for 5 min). The pellets were rehomogenized in sucrose solution (d = 1.10 g/mL), layered on top of a discontinuous gradient prepared in 1 mM $MgCl_2$, 0.1 mM EDTA, and 70 mM sodium phosphate (pH 7.0) buffer with sucrose density steps of 1.14 (5 mL), 1.13 (15 mL), and 1.11 (15 mL) g/mL, and then centrifuged (28000*g* for 90 min) at 4 °C. The ROS band at the 1.11–1.13-g/mL interface was recovered and either frozen under nitrogen in foil-wrapped vials or used immediately.

Fresh or thawed membranes were washed twice by centrifugation and resuspension in 10 mM maleate buffer (pH 7.0) containing 60 mM NaCl and 10 mM KCl. The A_{280}/A_{500} absorption ratio of the membranes in 1% cetyltrimethylammonium bromide was routinely found to be between 2.3 and 2.5 (De Grip et al., 1972; Papermaster & Dreyer, 1974).

Lipid Extraction. Total ROS membrane lipid extraction was performed on unbleached membranes in the presence of 1% sodium cyanoborohydride to give a retinal-free lipid extract (Fager et al., 1972; Stubbs et al., 1976). The lipids were stored dry under nitrogen at −20 °C.

Spin-Labels. The stearic acid spin-label, 14-SASL, was



R = H	14-PASL
R = $(CH_2)_2-\overset{+}{N}H_3$	14-PESL
R = $(CH_2)_2-\overset{+}{N}(CH_3)_3$	14-PCSL
R = $CH_2-\underset{\underset{COO^-}{|}}{CH}-\overset{+}{N}H_3$	14-PSSL
R = $CH_2-CHOH-CH_2OH$	14-PGSL

synthesized from the corresponding ketomethyl stearate essentially by the method of Hubbell & McConnell (1971). The phosphatidylcholine spin-label, 14-PCSL, was made by using the method of Boss et al. (1975). Other phospholipid spin-labels, 14-PESL, 14-PASL, 14-PSSL, and 14-PGSL, were made by enzymatic head-group exchange of the choline head group of 14-PCSL, catalyzed by phospholipase D (Ito & Ohnishi, 1974; Comfurius & Zwaal, 1977). Chromatographic purification was carried out with basic silicic acid columns (Watts et al., 1978) except for 14-PSSL, which was purified on Whatman CM-52 gel (Comfurius & Zwaal, 1977). The androstanol spin-label, ASL, was purchased from Syva, CA. Spin-labels were kept in stock solutions of $CHCl_3$ at 1 mg/mL for 14-SASL and 14-PSSL and ethanol at 4 mg/mL for the other spin-labels.

Spin-Labeling. Extracted lipid (0.5 mg) in organic solvent was mixed with lipid spin-label and the solvent removed by a nitrogen gas flow. After removal of all the solvent under vacuum for at least 3 h, a lipid dispersion was made by vortex mixing 50 μL of malate–NaCl–KCl buffer with the dried lipid. The dispersion was transferred to a sealed off 100-μL capillary pipet for ESR measurements. In all experiments the spin-label concentration in the lipid membranes was 1 to 2 mol % of the total membrane lipid.

Spin-labels were incorporated into ROS membranes by one of two methods. In the first method, washed ROS membranes (0.2 mg of rhodopsin) were suspended in malate–NaCl–KCl buffer (2 mL) and shaken with a dried film of the spin-label (2 μg for 14-SASL or 6–8 μg for 14-PSSL) on the inside of a glass tube. For the 14-SASL experiment, the membrane was centrifuged (30000*g* for 10 min) and the pellet used for measurement. After incubation (30 min at room temperature), the membrane labeled with 14-PSSL was centrifuged (30000*g* for 10 min) and washed extensively to remove spin-label vesicles formed spontaneously during labeling. Membrane pellets were transferred to sealed off 100-μL capillary pipets for ESR measurements. Labeling using this procedure for the phospholipid spin-labels gave a low level of label incorporation, most of the label being removed as vesicles during washing. After an initial fast uptake of label, further incorporation occurred by prolonging the incubation period (2 to 3 days at 10 °C) followed by extensive washing. Therefore, a second labeling method using ethanol was used for all the other labels to improve label incorporation into the ROS membranes, to minimize spin-label vesicle formation and thus the number of washings necessary, and to make incubation of the membranes unnecessary. This procedure is not suitable for the 14-PSSL because of its very low solubility in ethanol.

Thus, the spin-label (1 μL of 4 mg/mL in ethanol) was added to the washed ROS membranes (0.2 mg of rhodopsin) in 12 mL of buffer, centrifuged (30000*g* for 10 min), and washed 3 times, the final pellet being used for ESR measurements. Control experiments using both methods of spin-labeling for the 14-SASL and 14-PCSL gave identical spectral features, except for the difficulty with the 14-PCSL of removing the underlying broad one-line spectrum from spin-label vesicles and its low level of incorporation. In comparison with the labeling procedure used for the 14-PSSL, removal of the PCSL vesicles was much more difficult, possibly because charged lipids tend to form rather small vesicles which will not be centrifuged with the ROS membrane pellet but rather remain in the supernatant which is removed during washing.

It was estimated that almost complete (>90%) incorporation of 14-SASL into ROS membranes was achieved whereas for the 14-PSSL the amount of label incorporated was much lower (10–20% of the total label added). For all the other labels, the level of labeling was ~50–60% of the total label added. The amount of label used in each experiment was chosen to give a labeling level such that 1 to 2 mol % of the membrane lipids were spin-label molecules, although no spectral differences were observed when the membrane label concentration was increased 2 or 3 times.

Electron Spin Resonance Measurements. ESR measurements were made with a Varian E-12, 9-GHz spectrometer equipped with a nitrogen gas-flow temperature regulation system. Sample capillaries prepared as stated above were accommodated within standard 4-mm quartz ESR tubes

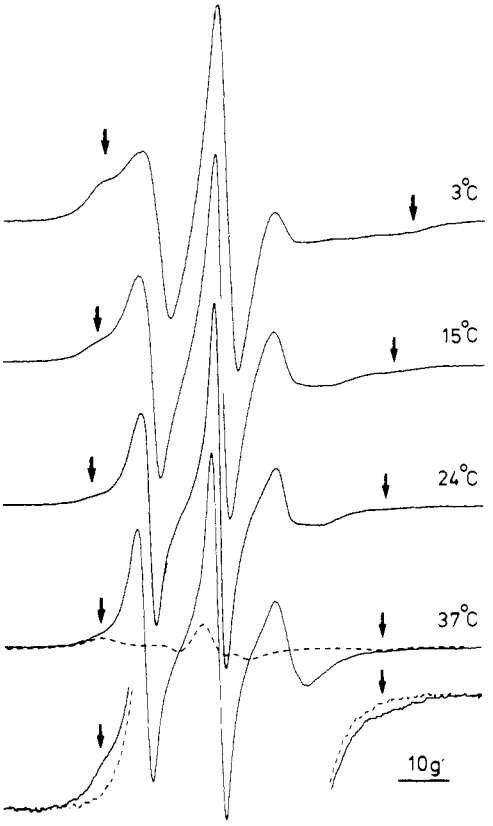


FIGURE 1: ESR spectra of 14-SASL in ROS membranes at 3, 15, 24, and 37 °C. The outer wings of the 37 °C spectrum are expanded 5 times to display the immobilized component, and the dotted lines in the expanded spectrum are from an extracted lipid spectrum which has the same spectral parameters and shape as the fluid membrane spectrum. The immobilized spectrum (dotted line) superimposed upon the 37 °C membrane spectrum has an integrated intensity of 37% of the membrane spectrum. Arrows indicate the outermost wings of the immobilized spectra, which are absent in the extracted lipid spectra.

containing silicone oil for thermal stability. Temperatures were measured with a thermocouple placed just above the cavity within the quartz tube to within ±0.15 °C. Labeling and ESR measurements were performed under dim red light.

ESR spectra were recorded and stored digitally either on paper tape by using a Hewlett-Packard data collection system (3450B/2547A/27531) or directly on disk via a PDP 11/10 dedicated computer. Spectral subtractions and integrations were performed by using programs written by W. Maschke of this institute.

Results

ESR Spectra. The ESR spectra of the 14-SASL stearic acid spin-label in ROS membranes are given in Figure 1. At all temperatures studied the membrane spectra are composed of two components, one of which corresponds rather closely with the single-component spectra of 14-SASL in aqueous bilayers of extracted ROS lipids. The second component in the membrane spectra, shown by arrows, has an outer splitting, A_{max}, of between 54 and 64 G and corresponds to a lipid population which is much more strongly immobilized than the fluid bilayer lipid (Freed, 1976).

The 14-PCSL, 14-PESL, 14-PASL, 14-PSSL, 14-PGSL, and ASL spin-labels all show a similar two-component spectrum in ROS membranes. The spectra in bilayers of the extracted lipid again give a single-component spectrum, which is similar to the fluid component in the membrane spectra. As the temperature is increased, the immobilized component in the membrane spectra becomes less obvious because the fluid component narrows much more rapidly, giving rise to much greater relative line heights for this latter component (Jost & Griffith, 1978a). Representative spectra at 15 °C for the phospholipid labels in both ROS membranes and bilayers of the extracted lipids are given in Figure 2. The immobilized component is seen in all of these membrane spectra, most clearly as a shoulder in the wings of the low-field line.

Quantitation of the Spectral Components. The proportion of the immobilized lipid, as reported by the various labels, has been obtained by spectral subtraction and double integration of the two individual components. Subtraction was performed both by using a suitable fluid spectrum (S_{lip}^{flu}) obtained from bilayers of the extracted lipid and by using a suitable immobilized spectrum (S_{sim}^{imm}) obtained by simulation.

Typical subtractions ($S_{mem} - S_{lip}^{flu}$) are given in Figures 3 and 4. It was found that the best match to the membrane fluid component was obtained with a fluid bilayer spectrum

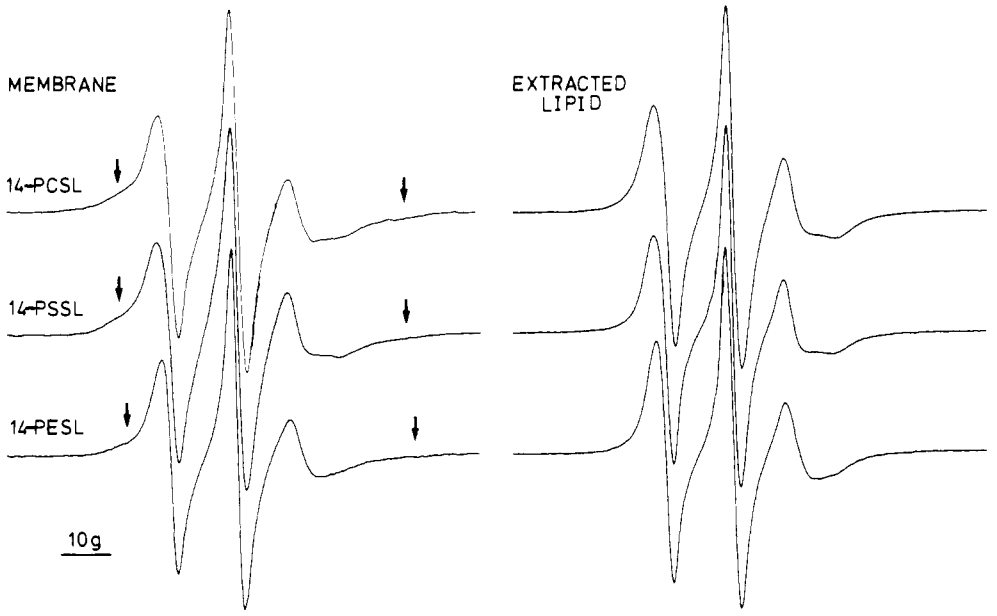


FIGURE 2: ESR spectra of 14-PCSL, 14-PSSL, and 14-PESL at 15 °C in ROS membranes and extracted lipid. Arrows indicate the outer wings of the immobilized components in the membrane spectra.

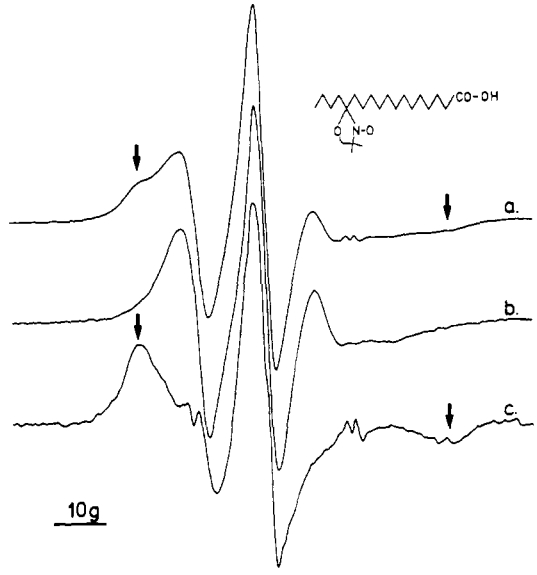


FIGURE 3: ESR spectra of 14-SASL in (a) ROS membranes at 3 °C and (b) lipid bilayers at 0 °C to give the spectrum used for subtraction from spectrum a to yield (c) the immobilized lipid spectrum. A free spin-label spectrum has been subtracted from spectrum a, and the spectra are normalized with respect to their height.

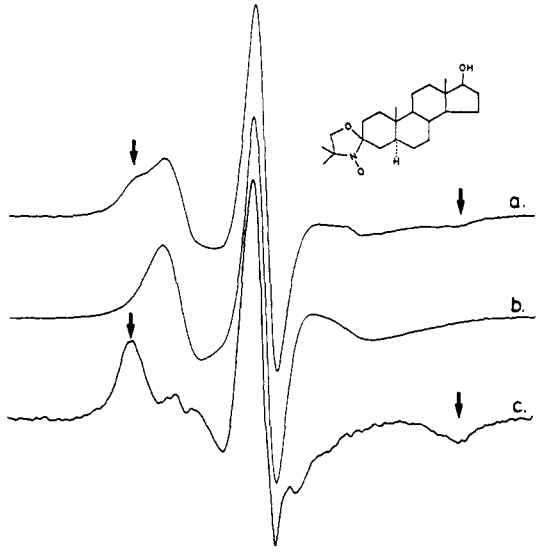


FIGURE 4: ESR spectra of ASL in (a) ROS membranes at 3 °C and (b) lipid bilayers at 5 °C to give the spectrum used for subtraction from spectrum a to yield (c) the immobilized lipid spectrum. Spectra are normalized with respect to their height.

recorded at a somewhat different temperature. For example, a S_{lip}^{flu} spectrum recorded at 0 °C and a S_{mem} spectrum recorded at 3 °C were used for the subtraction in Figure 3, and for this same type of subtraction for S_{mem} recorded at 21 °C, the S_{lip}^{flu} spectrum at 15 °C was found to be most suitable. Reasonable subtraction end points were only achieved at lower temperatures by this method, since at higher temperatures it was impossible to obtain a good fit to the line width and line shape of the fluid components. The percentage of spin-label intensity in the immobilized component ($f \times 100$), deduced from these subtractions, is given in Table I.

Typical subtractions ($S_{mem} - S_{sim}^{imm}$) using an immobilized spectrum are given in Figure 5. Two similar, but differently derived, S_{sim}^{imm} spectra were used: one a simulation in the slow-motion regime from Mason & Freed (1974) and the other a rigid-limit spectrum with suitably adjusted line widths simulated by using the program of Schindler & Seelig (1973). In both cases the outer lines were scaled to match the splitting

Table I: Percentage ($f \times 100$) of Immobilized Lipid in ROS Membranes at Different Temperatures Measured by Spectral Subtraction

	3 °C	15 °C	24 °C	37 °C
14-SASL	37 ± 5[a]	37 ± 4	37 ± 4	37 ± 4
	37 ± 3[b]	37 ± 4		
ASL	44 ± 4[a]	41 ± 4	41 ± 4	38 ± 3
	38 ± 3[b]	37 ± 4	37 ± 6	
14-PCSL	42 ± 4[a]	38 ± 3	37 ± 3	37 ± 3
	39 ± 4[b]			
14-PESL	38 ± 5[a]	37 ± 4	37 ± 4	38 ± 3
14-PSSL	45 ± 4[a]	43 ± 4	44 ± 3	44 ± 3
	45 ± 5[b]			
14-PASL	36 ± 5[a]	35 ± 5	33 ± 4	32 ± 4
	30 ± 6[b]			
14-PGSL	37 ± 3[a]	37 ± 3	37 ± 3	36 ± 3
	38 ± 3[b]			

[a] From subtraction of an immobilized spectrum from the membrane spectrum. [b] From subtraction of a lipid spectrum from the membrane spectrum.

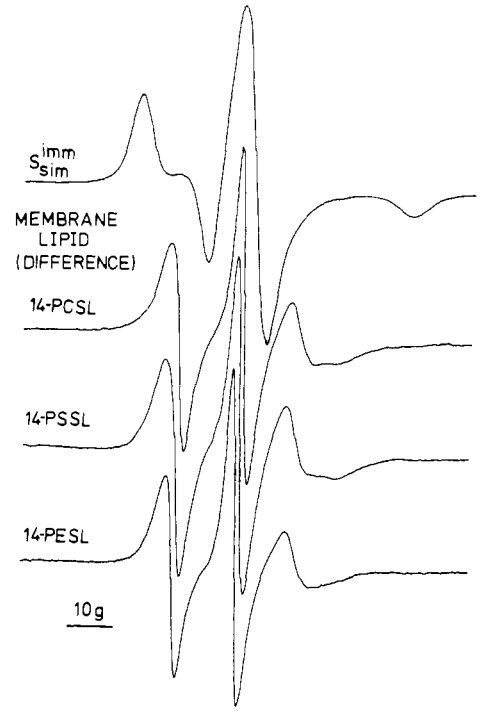


FIGURE 5: Difference spectra of the 14-PCSL, 14-PESL, and 14-PSSL spin-labels in ROS membranes at 15 °C. The membrane lipid spectra are obtained by subtraction of the immobilized component, S_{sim}^{imm} (from Mason & Freed, 1974), after suitable scaling of the outer peak positions, from the membrane spectra shown in Figure 2. The amount of integrated immobilized spectrum subtracted, f, is given in Table I. Spectra are normalized with respect to their height.

of the immobilized component in the membrane spectra, the latter being found to vary somewhat with temperature. The end points in these spectral subtractions were determined by comparison with a very similar lipid spectrum, both at fivefold vertical expansion (Jost & Griffith, 1978b). Values of the percentage of the immobilized component obtained from these subtractions using the two immobilized spectra were much the same and are given in Table I.

An exhaustive series of subtractions was performed at different temperatures for the 14-SASL and ASL labels. The results are summarized in Figure 6, which shows the agreement between the two subtraction methods over the temperature range for which they overlap and that the proportion of immobilized lipid remains approximately constant over the temperature range 0–37 °C, in spite of the relatively large

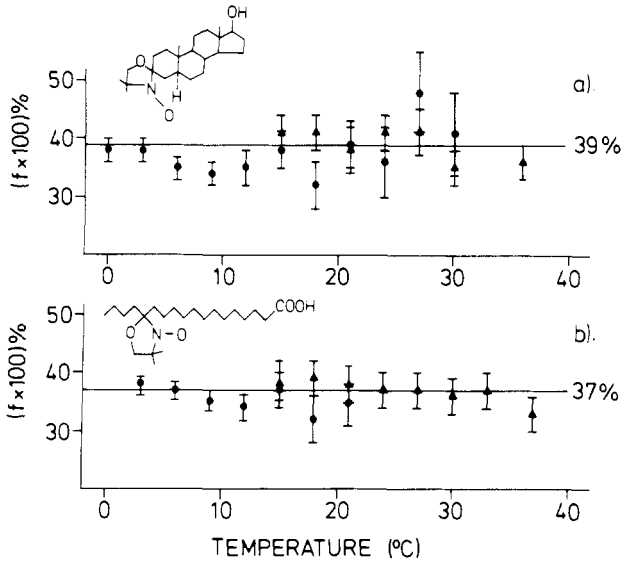


FIGURE 6: Percentage of integrated spectral intensity in the immobilized component of ROS membranes being probed by (a) ASL and (b) 14-SASL from 0 to 37 °C. The two methods of subtraction are described in the text: (●) by fluid spectrum subtraction to reveal an immobilized spectrum and (▲) by immobilized spectrum subtraction to reveal a fluid spectrum.

changes taking place in the spectral line shape.

Discussion

The present work indicates the presence of an immobilized lipid component in addition to the fluid bilayer lipid component in the bovine rod outer segment membranes studied. The spectral characteristics of this immobilized component indicate that it has a motional correlation time of the order of or greater than 3×10^{-8} s and a lifetime of greater than $\sim 10^{-8}$ s. It does, however, exchange with the fluid lipid on a longer time scale because the spin-labeled lipids are found to exchange into this region between the time of the labeling procedure and the spectral measurement. The immobilization is specifically induced by the membrane protein (80–90% rhodopsin) since it is not detected in bilayers of the extracted lipids. Possible reasons for the origin of the immobilized lipid are the occurrence of protein aggregation which traps lipid molecules within the aggregates, a protein-induced lipid lateral phase separation, or a more direct interaction between the lipid molecules and the hydrophobic surface of protein monomers or small oligomers, such as has been suggested for the boundary layer lipid in cytochrome oxidase (Jost et al., 1973; Knowles et al., 1979).

Control saturation transfer ESR experiments on covalently labeled rhodopsin in the present membrane preparations yielded results similar to those of other workers (Baroin et al., 1977; Kusumi et al., 1978; Cone, 1972), indicating that rhodopsin rotates relatively fast in the membrane, with a correlation time at 20 °C of ~ 20 μs. This would be inconsistent with extensive protein aggregation. Control freeze–fracture electron microscopy experiments yielded results similar to those of Olive et al. (1978), giving no evidence for extensive aggregation. Independent chemical cross-linking experiments (Brett & Findlay, 1979; F. J. M. Daemen, personal communication) and energy-transfer experiments (Ebrey, 1971) show that rhodopsin is present as the monomer in the disk membrane, with no evidence for aggregation. A decrease in the correlation distance between rhodopsin molecules with decreasing temperature, corresponding to a condensing or freezing of the lipid chains, has been observed in X-ray diffraction studies of bovine disk membranes (Charbre, 1975). Even if this does represent a partial protein segregation, it does not correlate with the presence of the immobilized lipid since this is observed with approximately constant intensity throughout the whole temperature range from 0 to 37 °C.

A gross protein-induced phase separation seems unlikely in view of the highly unsaturated and heterogeneous lipid composition of the membranes (Anderson & Maude, 1970; Anderson & Sperling, 1971; Miljanich et al., 1979). The temperature dependence of the fluid lipid component shows no clear evidence of features associated with lateral phase separation or phase transitions in the disk membrane, in common with the fluorescent probe measurements of Stubbs et al. (1976). The lack of any systematic or marked temperature dependence in the proportion of the immobilized lipid component is again inconsistent with a simple lipid-phase separation. Some evidence for a phase separation between fluid and "less fluid" lipid has come from ^{1}H NMR measurements on bovine disk membranes (Brown et al., 1977a). However, this "less fluid" lipid does not correspond to the immobilized lipid observed here since it was also observed with the extracted lipid alone and was shown to have a strong temperature dependence in the amount detected.

Rhodopsin has been shown to be a transmembrane protein (Chen & Hubbell, 1973; De Grip et al., 1973; Raubach et al., 1974; Jan & Revel, 1974; O'Brien, 1978), and thus immobilization of the lipid by direct contact with the hydrophobic surfaces of the protein seems a possible explanation for the origin of the immobilized spin-label component. In this case, it would be expected that the fraction of immobilized lipid would be relatively insensitive to temperature, as is observed, but would be directly correlated with the size of rhodopsin. Taking the molecular weight of rhodopsin as 37 000 (Lewis et al., 1974; Montal & Korenbrot, 1976; O'Brien, 1978) and the lipid/protein ratio in bovine disk membranes as 1.35 w/w (Daemen, 1973; Van Breugel, 1977), we calculated that the value of $f = 0.37$ in Table I corresponds to $n_b \approx 24$ immobilized lipids per protein. For proteins which protrude to similar extents from the membrane, the number of lipid molecules which can be accommodated around the intramembranous portion of the protein will be approximately proportional to the square root of the protein molecular weight. A value of $n_b \approx 55$ has been obtained for the number of immobilized lipids associated with cytochrome oxidase (Jost et al., 1973; Knowles et al., 1979), giving a ratio of 0.44 between the number of immobilized lipids associated with rhodopsin and cytochrome oxidase, respectively, compared with a ratio of 0.43 between the square roots of their molecular weights.

Information on the overall shape and size of rhodopsin has come from X-ray scattering (Sardet et al., 1976) and neutron scattering (Osborne et al., 1978) measurements of the radius of gyration of the protein. Calculated values for the numbers of lipid molecules in the first three shells surrounding rhodopsin for the three possible shapes suggested by Sardet et al. (1976) are given in Table II. According to these estimates, the number of immobilized lipids, $n_b = 24$, would be sufficient to form a single, immobilized boundary layer in direct contact with the hydrophobic surface of the protein. Some caution is necessary, however, in such interpretations since the detailed shape of rhodopsin is not yet known, although a cylindrical shape has been suggested by Albert & Litman (1978) from proteolysis of rhodopsin and analysis of the fragments by circular dichroism. Even though rhodopsin is a relatively small membrane protein, it is possible that the intramembranous surface is invaginated and the immobilized lipid may be

Table II: Calculated Radii and Numbers of Molecules in the Lipid Shells Surrounding Rhodopsin[a]

shell, i	ellipsoid[b]		cylinder[c]		dumbbell[d]	
	r_i (Å)	n_i	r_i (Å)	n_i	r_i (Å)	n_i
1	18.6 ± 1.4	24 ± 2	18.1 ± 1.8	24 ± 2	21.4 ± 1.3	28 ± 2
2	23.4 ± 1.4	31 ± 2	22.9 ± 1.8	30 ± 2	26.2 ± 1.3	34 ± 2
3	28.2 ± 1.4	37 ± 2	27.7 ± 1.8	36 ± 2	31.0 ± 1.3	41 ± 2

[a] Calculated from the X-ray scattering measurements of Sardet et al. (1976). Radii are calculated to the center of a lipid chain (diameter = 4.8 Å) at a height of ±17 Å from the center of the membrane. [b] Assuming a prolate ellipsoid for rhodopsin with axes of 33.7, 33.7 (±3.5), and 125 (∓25) Å. [c] Assuming a cylinder with a diameter and height of 31.4 (±3.5) and 96.6 (∓20) Å. [d] Assuming a dumbbell shape with a maximum length and diameter of the spheres of 92 (±6) and 41.4 Å.

accommodated within the invaginations rather than covering the whole of the surface.

The number of the lipids in the fluid component can be calculated from Table I (fraction $1 - f = 0.63$) to be 42/rhodopsin. Comparison with Table II shows that, allowing for interstitial positions, this will constitute one further lipid shell around rhodopsin. Since the spectra of this component are not exactly identical with those of bilayers of the extracted lipid at the same temperature (cf. Figures 2 and 5), it is clear that the effect of the protein extends out to this second shell. Recent experiments with cytochrome oxidase–lipid complexes suggest that this perturbation of the bilayer can extend out to four to six shells from the protein (Knowles et al., 1979).

The values in Table I display a small but definite preferential immobilization of phosphatidylserine relative to the other lipids tested. A preference of the antenna bacteriochlorophyll binding proteins in the photosynthetic bacterium *Rhodopseudomonas sphaeroides* for negatively charged lipids has recently been demonstrated by using spin-labels (Birrell et al., 1978). Such a partial specificity of phosphatidylserine for rhodopsin is also consistent with a direct interaction of the immobilized lipid with the protein, although the selectivity does not appear to be simply charge mediated, as shown by the results in Table I for the phosphatidylglycerol and phosphatidic acid spin-labels. The degree of specificity can be accounted for either by a 34% increase in the effective binding constant [cf. Griffith & Jost (1978)], corresponding to an increased free energy of binding of 175 cal/mol for PS relative to the other lipids, or, at the opposite extreme, by the existence of ~0.7 specific PS-binding site per rhodopsin which is inaccessible to the other lipids. For either of these extremes, the number of unlabeled parent lipid molecules immobilized per rhodopsin can be approximated by $f^i \times n^i_t$, where f^i is the immobilized fraction of molecules of the ith lipid class given in Table I and n^i_t is the total number of ith class molecules per rhodopsin. From Table I and the lipid composition of ROS disk membranes (Daemen, 1973), it can be estimated that 11 phosphatidylcholine, 9 phosphatidylethanolamine, 4 phosphatidylserine, and 1 cholesterol molecules per rhodopsin are immobilized. Since rhodopsin (Jan & Revel, 1974; Worthington, 1974; Olive & Benedetti, 1974; O'Brien, 1978) and the ROS lipids (Crain et al., 1978) are asymmetrically distributed across the membrane of the disk, the distribution of the different lipid classes is estimated to be that indicated diagrammatically in Figure 7. Figure 7 has been constructed assuming that the spin-labels have been incorporated into both sides of the ROS membrane fragments and that the distribution of the membrane lipids is identical with those of the spin-labels of the same type. This latter assumption has not been demonstrated in a mixed lipid membrane, but at least

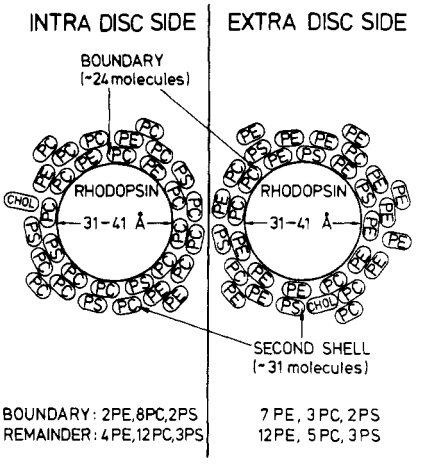


FIGURE 7: Diagrammatic indication of the instantaneous disposition of the phospholipid molecules in bovine ROS disk membranes around one rhodopsin molecule. The relative numbers of molecules in the first and subsequent shells are obtained from the present spin-labeling studies. The trans-bilayer distribution is obtained from the chemical labeling experiments of Crain et al. (1978), and the dimensions of rhodopsin are obtained from Sardet et al. (1976). The dynamic nature of the membrane is not indicated but is discussed in the text.

it has been shown that spin-labeled phosphatidylcholine molecules do not preferentially compete for boundary sites against unlabeled molecules in reconstituted cytochrome oxidase–phosphatidylcholine complexes (Griffith & Jost, 1978; Knowles et al., 1979).

The results summarized in Figure 7 can be compared to the other studies on the lipid–protein interactions in rod outer segment disk membranes. Pontus & Delmelle (1975) estimated that only two-thirds of the lipids in ROS disk membranes are in a fluid state, with the remaining 34% of nonfluid lipids correlating rather well with the immobilized component observed in the present study. Both Pontus & Delmelle (1975) and Baroin et al. (1977) have observed lipid immobilization in partially delipidated ROS disk membranes; this possibly corresponds to enrichment in the immobilized component observed here, but may also result from additional protein-packing restrictions or aggregation arising from the delipidation process. In reconstituted rhodopsin–lipid membranes, Hong & Hubbell (1972) have observed an increase in order parameter of the spin-labeled lipid chains with increasing protein content of the membranes. This immobilization of the lipid chains by rhodopsin could be the result of both the perturbation of the more distant lipid shells and also the presence of an unresolved immobilized component. Stubbs et al. (1976) have measured the fluorescence anisotropy of the probe diphenylhexatriene (DPH). They report a fourfold greater effective microviscosity in bovine disk membranes than in bilayers of the extracted lipids, corresponding to an increased immobilization due to interactions of the lipid chains with rhodopsin. On the basis of the effects of energy transfer to the retinal, it was suggested that the immobilization was experienced by all of the membrane lipid rather than being restricted to the shell in closest contact with rhodopsin. However, the fluorescence energy-transfer range of rhodopsin–DPH ($R_0 \sim 48$ Å) is large when compared with the radii of the lipid shells given in Table II, and the measurement involves the comparison of bleached and unbleached membranes. Thus, it is not entirely clear how this observation correlates with the resolution of an immobilized lipid component in the present work.

The above studies all involved measurements which are sensitive to a time scale similar to that of the present ESR

work. In contrast, high-resolution ^1H NMR experiments by Brown et al. (1977a,b) on sonicated bovine ROS disk membranes have led to the suggestion that rhodopsin affects only the high-frequency motions of the lipid chains, and the slower processes, namely, exchange between lipid molecules, may be unchanged. This would not be inconsistent with the present spin-label results, since, as mentioned above, the lower limit on the lifetime of the immobilized lipid component is $\sim 3.10^{-8}$ s, whereas the rate of exchange by lateral diffusion in pure lipid bilayers is $\sim 10^7$ s^{-1}. It should be pointed out that only 20–40% of the ROS membrane lipid contributed to the high-resolution spectrum in this ^1H NMR study, and it is not clear in what way the rhodopsin interacts with the remaining 60–80% of the lipid which gives rise to the very broad NMR spectrum.

Recently, Favre et al. (1979) have studied the lipid environment of rhodopsin by using spin-labeled fatty acids covalently attached to hydrophobic sites on the protein. Their results with labels far removed from the point of attachment suggest an environment with fluidity similar to that observed for the second lipid shell (or intermediate between the first and second shells) in the present study. Spectra of labels close to the point of attachment, on the other hand, give immobilized spectra, indicative of a lipid region motionally restricted by rhodopsin on the conventional ESR time scale, consistent with the present results. Favre et al. (1979) also point out conditions under which they induce a separate immobilized component: delipidation, prolonged bleaching, and high label concentrations; none of these conditions apply to the present study.

The possible functional significance of the immobilized lipid associated with rhodopsin is at present unclear. However, since the thermal stability of rhodopsin, the completion of the photolytic cycle, and the regeneration of rhodopsin from opsin all require the presence of a lipid membrane (Hubbell, 1975; Van Breugel, 1977), it may be that the immobilized component is that part of the lipid which stabilizes the rhodopsin in its active form. In addition, the immobilized lipid may be involved in the conformational changes which take place in rhodopsin following light illumination leading to the nervous response (Abrahamson, 1973; Abrahamson & Fager, 1973; Lamola et al., 1974a,b).

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Rearrangement of Immunoglobulin Genes†

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ABSTRACT: The extent of rearrangements of immunoglobulin genes was investigated. The deoxyribonucleic acids (DNAs) of four κ-chain-producing myelomas (MOPC-167, MOPC-41, MOPC-21, and MOPC-321), one λ-chain myeloma (S178), Krebs ascites cells, liver, spleen, and thymus were digested with six different restriction endonucleases (*Kpn*I, *Eco*RI, *Pvu*II, *Xba*I, *Hin*dIII, and *Bam*HI) used alone or in all possible pairs. Following agarose gel electrophoresis and transfer to nitrocellulose filters [Southern, E. M. (1975) *J. Mol. Biol. 98*, 503], the DNAs were hybridized to one of three phosphorus-32 nick-translated probes: one for the κ-chain constant (C) region, one for the MOPC-167 κ-chain variable (V) region, and one for both the C and V regions. The probes were derived from a cloned complementary deoxyribonucleic acid from myeloma MOPC-167. It was found that liver, spleen, thymus and Krebs DNAs always produced hybridization band patterns indistinguishable from one another when a given enzyme or enzyme pair was used. One myeloma (MOPC-321) produced these same "germline" bands, but always exhibited other bands as well. The other four myelomas exhibited banding patterns which were not superimposable over the normal tissue pattern. Hybridization banding data obtained after double-enzyme digestion allowed the construction of restriction maps for sequences surrounding the C- and V-region genes in each of the DNAs. V maps for the MOPC-167 sequence were invariant in all DNAs except MOPC-167, indicating an absence of rearrangement in cells which do not express V_{167}. MOPC-167 showed single nongermline V and C maps which overlapped in such a way that the V- and C-containing fragments were about 1 kilobase (kb) apart. The 6 kb mapped at the 5′ side of the MOPC-167 V_{167} gene were identical with the sequence flanking the germline V_{167} gene. A single map, presumably in a germline configuration, was obtained for the sequences surrounding the C-region gene for the liver, spleen, thymus, and Krebs ascites cells. MOPC-321 had a map which was identical with this germline map, but it had in addition two rearranged C maps. The four other myelomas each gave one to three rearranged C maps, none of which were identical with any other C map. It was concluded that phenotypic "allelic exclusion" in myeloma cells is not necessarily correlated with the maintenance of one normal chromosome. A C-map comparison revealed that most restriction site alterations occur within 10 kb beyond the 5′ end of the restriction fragment carrying the C-region gene. Other features emerging from this study were the following. (1) All cells tested have multiple V_{167}-like sequences in identical restriction fragments. (2) S178, a λ myeloma, displays shifted κ genes. This correlates with our previous finding that S178 cells contain a low level of κ ribonucleic acid (RNA) [Storb, U., Hager, L., Wilson, R., & Putnam, D. (1977) *Biochemistry 16*, 5432]. There must therefore exist transcriptional or posttranscriptional controls in this tumor which limit the accumulation of κ mRNA but not λ mRNA. (3) The DNAs of spleen and thymus, organs which contain 87 and 99%, respectively, of cells which produce κ RNA [Storb, U. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2905], showed a germline restriction map for $C_κ$ genes, suggesting that the majority of κ genes are not rearranged in these cells or that one of the allelic $C_κ$ genes is maintained in a germline context. It is not yet known whether the differences between the myeloma cells tested and the nonmalignant lymphoid tissues are due to the polyploidy or to the differentiated plasma cell nature of the myeloma.

Studies of the genetics and protein structure of antibodies have led to the concept of "two genes–one polypeptide chain" (Dreyer & Bennett, 1965). The recent analyses of the organization of DNA sequences which contain immunoglobulin genes have supported this hypothesis and suggested that a rearrangement of sequences coding for different portions of an immunoglobulin chain has taken place in a cell which produces antibodies (Brack & Tonegawa, 1977; Brack et al., 1978; Lenhard-Schuller et al., 1978; Seidman et al., 1978a,b; Seidman & Leder, 1978; Bernard et al., 1978).

Cellular studies have shown that single lymphoid cells of an individual heterozygous for a given immunoglobulin allotype express only one allele, i.e. show "allelic exclusion" (Pernis

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