proteins in a minimum of phospholipids. The lipid composition of rod outer segment membranes is probably optimized to achieve this function.

The Effect of Light on the Rotational Correlation Time of Rhodopsin

As pointed out earlier, the advantage of using ST-EPR is to allow the possibility of comparing rhodopsin rotational diffusion in the dark and after bleaching. If rhodopsin is exposed to a brief illumination, one finds that the ST-EPR spectra do not change. However, prolonged illumination (half an hour at 37°) is accompanied by a 2- or 3-fold increase of τc. The time scale of these experiments suggests that it is a nonphysiological event. Very likely, nonreversible cross-linking between proteins took place via disulfide bridges. It is known indeed that new SH groups become accessible after illumination of rhodopsin. If these experiments were carried out after solubilization by detergent, the protein eventually precipitated. Finally, it should be pointed out that all these experiments were carried out with rhodopsin preparations depleted from GTPase. They probably should be repeated in the presence of this intrinsic protein, which is thought to interact with rhodopsin.

Acknowledgments

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Introduction

The rhodopsin-containing disk membranes of the retinal rod outer segment (ROS) represent an attractive model for the study of lipid–protein interactions in a native biological membrane system. Rhodopsin comprises more than 95 wt.% of the ROS intrinsic membrane protein, is

1 Dedicated to the memory of Stephen Schwartz.
deeply embedded in the membrane lipid, and spans the disk membrane. The native ROS membrane phospholipid composition is well characterized, and a number of intriguing problems relating to the structural and functional role of the highly polyunsaturated ROS phospholipids are evident, which appear amenable to investigation using biophysical techniques. Finally, the photochemical behavior of rhodopsin has been well studied, and at least some aspects appear subject to modification by the lipid environment. Nuclear magnetic resonance (nmr) spectra of good quality and resolution can be obtained from photoreceptor membrane preparations, so that their phospholipid organization and dynamic properties can be investigated in a manner quite analogous to nmr studies of simpler model lipid bilayer systems. In addition, nmr is a useful analytical tool for characterizing the isolated ROS membrane components. The following contribution summarizes the current status of nmr studies of native retinal ROS disk membranes. A brief discussion of ex-

12 M. F. Brown, Ph.D. Dissertation, University of California, Santa Cruz (1975).
Preparation of Retinal ROS Membranes and Extracted Phospholipids for Nmr Spectroscopy

During the preparation of retinal ROS membranes, and particularly when working with the native extracted ROS phospholipids, precautions against oxidative damage are essential. The retinal phospholipids are highly polyunsaturated and are protected against oxidation in vivo by the presence of various antioxidants such as α-tocopherol (vitamin E). Denaturation of iron sulfur proteins and reagent impurities, however, introduce iron ions during tissue disruption and membrane purification, which results in catalysis of lipid peroxidative degradation. The native retinal disk membranes can be largely protected against iron catalyzed lipid peroxidation by the addition of 0.1 mM Ca-EDTA to all buffer solutions. In addition, working under an argon atmosphere, when possible, is to be recommended. On extraction into organic solvents, the highly polyunsaturated ROS phospholipids are extremely sensitive to air oxidation. Therefore, it is necessary to maintain the lipids under an inert atmosphere at all times. Also, the addition of an antioxidant such as butylated hydroxytoluene (BHT) to the organic solvents is strongly recommended.

Highly purified bovine ROS membranes are prepared for nmr spectroscopy according to established procedures, followed by osmotic shock and washing by centrifugation in low ionic strength buffer or water containing 0.15 mM CaEDTA. The retinal phospholipids are extracted from the pelleted ROS disk membranes using a modification of the Folch et al. procedure, in which 50 μg/ml of BHT is added to the organic solvents. Prior to extraction, the ROS suspension is mixed with a large molar excess of hydroxylamine (pH 7, 100:1, hydroxylamine to retinal) and exposed to room light for 30 min to convert retinal to retinaloxime. The total extracted ROS lipids are then applied to a column of silic acid in chloroform (0.3 g silic acid/mg applied lipids). The neutral lipids (retinal pigments, free fatty acids, diacylglycerols, cholesterol, vitamin E, and BHT) are eluted with several column volumes of chloroform/methanol (9:1, v/v), followed by elution of the phospholipids with methanol.

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Fig. 1. (a) Apparatus used for silicic acid column chromatography of ROS lipids. Eluting solvents contain BHT (~1 BHT/1000 phospholipids) and are slowly bubbled with argon gas throughout the purification procedure. The eluant, collected into a round-bottomed flask equipped with a sidearm, is also slowly bubbled with argon. (b) Apparatus used for LH-20 column chromatography of ROS phospholipids. In this case a fraction collector enclosed in a plastic glove bag inflated with argon gas is employed.

Alternatively, the ROS phospholipids can be purified on a column of Sephadex LH-20 (3.5 × 85 cm, 1 g/mg applied lipids). The column is filled with LH-20 slurried in 95% ethanol and washed with several column volumes of the same solvent. The total ROS lipids are applied in 95% ethanol and the phospholipids are eluted near the column void volume, followed by the various yellow pigments and neutral lipids.

During column purification it is recommended that the elutant be continually purged with argon and the fractions collected under an argon atmosphere, as illustrated in Fig. 1. After column purification, the ROS phospholipid concentration is determined using phosphate analysis and BHT is added to adjust the molar ratio of BHT to phospholipid to about 1:1000. It is very important that the composition of the total extracted lipids

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and purified ROS phospholipids be characterized by quantitative thin-layer chromatography and gas-liquid chromatography. The final, purified ROS phospholipids are then dried to a thin film by rotary evaporation, hydrated by addition of buffer, and dispersed by swirling with several glass beads. Visually, the hydrated ROS phospholipids appear as a milky white suspension, similar to dispersions of other phospholipids such as egg phosphatidylcholine.

If the samples are to be used for $^1$H nmr spectroscopy, they are generally suspended in a deuterated inorganic buffer solution (BO$_4^{-3}$ or PO$_4^{-3}$) to minimize contributions from the interfering water and buffer proton resonances. The purified retinal ROS membranes are pelleted and allowed to soak for about 12 hr at 4°C in deuterated buffer, containing 0.1 mM EDTA, to allow $^1$H ↔ $^2$H exchange with the solvent. The membrane suspension is then pelleted (27,000 g, 60 min) and resuspended in deuterated buffer; the procedure is repeated several times. Alternatively, the ROS membranes can be frozen and lyophilized out of deuterated buffer one or more times. (On lyophilization, ROS membranes retain their native absorption spectra and are highly regenerable.) For $^1$H nmr studies, the final concentration of the samples should be about 30–50 mg of phospholipid/ml. (The concentration of ROS membrane constituents can be determined from the 500-nm absorbance using the following relations: 0.89 mg rhodopsin/ml/A$_{500}$ unit; 1.6 mg phospholipid/mg rhodopsin, after correction for the presence of opsin.)

To obtain good-quality natural abundance $^{13}$C nmr spectra of native retinal ROS membranes and extracted ROS phospholipid dispersions, the samples must be highly concentrated (~75–100 mg of phospholipid/ml). At such high concentrations the ROS membranes have the consistency of a thick paste and are difficult to manipulate, prior to sonication. The following procedure is generally used in our laboratories.

1. Suspend the ROS membranes in 5–10 ml of buffer per milliliter of final sample.
2. Sonicate (see below).
3. Centrifuge at 27,000 g for 1 hr. (After this procedure the ROS membranes are highly concentrated and, because of the sonication procedure, are also easily pipetted.)
4. Remove all supernatant.
5. Sonicate again for about 10 min.
6. Transfer sample into the nmr tube and seal under argon.

For $^{31}$P nmr studies, either sonicated or unsonicated samples (~60 mg of phospholipid/ml) can be used. Unsonicated phospholipid dispersions may contain a minor subfraction of small vesicles that exhibit relatively
sharp isotropic spectra. Any small vesicles can usually be converted into multilamellae exhibiting characteristic $^{31}$P nmr spectra by one or more freeze–thaw cycles.

Small volumes of suspensions of ROS membranes and ROS phospholipids, at a concentration of about 30–100 mg phospholipid/ml, under argon on ice, can be effectively sonicated at a power setting of about 40–50 W using a microtip, until the sample is clear and relatively nonviscous (about 20 min). If highest concentrations are desired, the ROS membrane samples are best presonicated at a lower concentration, following the procedure described previously for $^{13}$C nmr membrane studies. The resulting suspensions consist predominantly of unilamellar vesicles with an average diameter of about $10^3$ Å, as shown by electron microscopy and gel-filtration chromatography.\textsuperscript{13,15,31}

Nmr Methods

A discussion of experimental nmr techniques is beyond the scope of the present article; for a short and lucid introduction to the use of pulsed Fourier transform nmr methods, the reader is referred to the book by Farrar and Becker.\textsuperscript{32} Excellent introductions to theoretical nmr methods can be found at an intermediate level in Carrington and McLachlan\textsuperscript{33} and at a more advanced level in Slichter\textsuperscript{34} and Abragam.\textsuperscript{35} The spectra reported in this article were obtained using superconducting solenoid spectrometer systems operating at magnetic field strengths of 4.2 and 8.4 tesla (180 and 360 MHz for $^1$H). For further details the original literature should be consulted.

Proton Nmr Studies of Retinal ROS Membranes and Vesicles of Extracted Phospholipids

$^1$H Nmr spectra of native bovine ROS membranes and extracted ROS phospholipids were first reported by Brown and co-workers.\textsuperscript{12–15} Although many of the primary conclusions from this earlier work are now

supported by more recent \(^2\)H nmr\(^{26-39}\) and spin-label EPR studies,\(^{40-42}\) some of the interpretations are in need of revision, because of recent theoretical advances in understanding the \(^1\)H nmr lineshapes of vesicle systems,\(^{21,43}\) as well as more recent nmr studies. We present here an up-to-date interpretation of the \(^1\)H nmr results obtained for native ROS membranes and their implications for studies of lipid–protein interaction in general.

High-resolution \(^1\)H nmr spectra of purified bovine ROS phospholipids dissolved in chloroform:methanol (9:1) reveal distinct resonances assigned to the polyunsaturated and saturated fatty acyl chains and the phosphocholine head groups, as shown in Fig. 2a. The areas of the various resonances are in good agreement with the lipid composition of the ROS disk membrane.\(^7,8\) In particular, the amplitudes of the vinyl and doubly allylic methylene resonances (5.4 and 2.8 ppm, respectively) reveal that the polyunsaturated ROS phospholipids are highly intact and have not undergone detectable oxidative degradation during purification.

\(^1\)H Nmr spectra of multilamellar dispersions of ROS phospholipids suspended in excess buffer are characterized by much broader, non-Lorentzian lineshapes (Fig. 2b). The relevant lineshape theory is due to Wennerström\(^{44}\) and to Bloom and co-workers.\(^{21,45,46}\) It is reasonable to assume that lateral diffusion averages intermolecular \(^1\)H dipolar interactions to sufficiently small values such that the lineshapes are dominated by intramolecular \(^1\)H dipolar interactions. Since the phospholipid motions are axially symmetric about the bilayer normal, the residual time-averaged dipolar interactions are then described by a Hamiltonian of the form\(^{44,45}\)

\[
\langle H_D \rangle = \langle H_D \rangle (\beta = 0^\circ) P_2(\cos \beta)
\]  


Fig. 2. $^1$H Nmr spectra of (a) a C$_2$H$_3$:C$_3$H$_5$O$_2$H solution (9:1 v/v) and (b) unsonicated and sonicated $^3$H$_2$O dispersions of the total extracted and purified ROS phospholipids in 0.1 M borate containing 0.1 M KCl, pH 7. The spectra were obtained at 360 MHz in the Fourier transform mode at 20° using a spectral width of ±5 kHz (quadrature phase detection), 16 K data points, 100-μsec data acquisition delay, and a 20-sec interpulse delay. Before Fourier transformation the free induction decays were multiplied by an exponential function with a time constant corresponding to a 0.5-Hz Lorentzian line broadening. A 90° pulse was 24 μsec. For further details and additional resonance assignments see Brown et al. 15

where $P_2(\cos \beta) = \frac{1}{2}(3 \cos^2 \beta - 1)$ and $\beta$ is the angle between the bilayer normal $n$ and the applied magnetic field $B$. The result, for unsonicated bilayers, is that one obtains a logarithmic lineshape, characterized by a sharp central peak and unusually broad wings. 44,45 For sonicated membrane vesicles, where a dramatic sharpening of the lipid resonance lines is
observed (cf. Fig. 2b)\textsuperscript{47}, the \(^1\)H nmr lineshapes are given by\textsuperscript{21,43}

\[
F(\omega) = \frac{1}{\pi} \int_{-\infty}^{\infty} f(\omega') \frac{T_2(\omega')}{1 + \omega^2[T_2(\omega')^2]} \, d\omega'
\tag{2}
\]

where \(\omega = 0\) denotes the center of the resonance line.\textsuperscript{48} Equation (2) states that in membrane vesicle preparations each chemically shifted resonance line consists of a superposition of Lorentzians, with linewidth parameters \(T_2(\omega')\) corresponding to a very large number of allowed spin transitions. The appropriate weighting factors are given by the bandshape \(f(\omega')\) for bilayers oriented at \(\beta = 0^\circ\), i.e., for \(n \parallel B\). For the purpose of the present discussion, the important point is simply that the vesicle lineshapes are related to the distribution of orientational dipolar order parameters for the various proton containing groups in the sample, characterized in Eq. (2) by \(f(\omega')\). In principle, this means that the \(^1\)H lineshapes are related to the amplitude of the phospholipid molecular motions, as well as their time-averaged conformation; however, such information is not directly extractable from the \(^1\)H nmr spectra themselves and further studies, e.g., employing \(^2\)H nmr\textsuperscript{19}, may be required.

A comparison of representative \(^1\)H nmr spectra of sonicated ROS membranes and ROS phospholipids is shown in Fig. 3. In spite of the fact

\textsuperscript{47} The Hamiltonian for dipolar interactions is given in its most general form by \(H_\text{d}(t) = \langle H_\text{d}(t) \rangle + [H_\text{d}(t) - \langle H_\text{d}(t) \rangle]\). In liquids the time-averaged Hamiltonian \(\langle H_\text{d}(t) \rangle\) is zero and the second (relaxation) term dominates the resonance linewidth. In rigid solids, on the other hand, the static (first) term dominates. Membranes constitute an intermediate case, where part of the dipolar interaction is essentially static and dominates the zero-frequency spectral density, and part is time dependent because of restricted molecular motion, leading to a characteristic frequency or power spectrum for the fluctuating part of the dipolar interaction. For the case of vesicles, isotropic tumbling renders \(\langle H_\text{d}(t) \rangle\) over the (faster) local motions time dependent as well, albeit on a longer time scale. The problem, then, is to interpret the vesicle lineshapes properly in terms of \(\langle H_\text{d}(t) \rangle\) as determined for unsonicated multilamellar dispersions, together with possible relaxation contributions. In the past this has been a source of some controversy [cf. A. F. Horwitz et al., \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{69}, 590 (1972); D. Lichtenberg et al., \textit{Biochim. Biophys. Acta} \textbf{382}, 10 (1975)]. It should be noted that, a priori, large contributions to the vesicle lineshapes from relatively low-frequency/large-amplitude intravesicular motions cannot be excluded if \(\langle H_\text{d}(t) \rangle\) is small. However, it now appears that the lineshapes can be explained in terms of modulation of the time-averaged residual dipolar interactions over those motions faster than vesicle reorientation (\(\tau_c < 10^{-7}\) sec) by the tumbling of the vesicles themselves [M. Bloom et al., \textit{Biochemistry} \textbf{17}, 5750 (1978)].

\textsuperscript{48} For the lineshape formula given by Eq. (2) to be valid, the motional narrowing condition \(M_2 \tau_c^2 \ll 1\) must be satisfied, where \(M_2\) is the residual second moment of the unsonicated lineshape and \(\tau_c\) is the vesicle reorientational correlation time. At present, these quantities can only be crudely estimated, and thus further studies are required, in particular measurement of \(M_2\) and the degree of polydispersity of \(\tau_c\) for the ROS membrane and ROS phospholipid vesicle suspensions.
that about 20% of the ROS phospholipids are estimated to be in contact with rhodopsin at any given instant, the observed lineshapes are quite similar in both cases. As we have previously emphasized, the observed sharp components correspond to only a fraction of the total intensity (40–50%), with a broad underlying background observed in 1H correlation nmr studies [M. F. Brown et al., Biochemistry 16, 2640 (1977)]. Although originally attributed by us to a population of more ordered phospholipids in slow exchange with fluid lipids giving rise to the sharp components, a more plausible explanation at present is that the relatively large diameter of the sonicated ROS phospholipid vesicles (~10 Å), together with their possible size polydispersity, may result in incomplete motional averaging of residual static 1H dipolar interactions, characterized by the residual second moment $M_2$. If this is the case, then overlapping of the tails of the various broad resonance components could account for the underlying background intensity. Nevertheless, our proposal of a broad phase transition centered near 5–10° based on 1H nmr studies appears to be correct and is now confirmed by both differential scanning calorimetry [G. P. Miljanich et al., Biophys. J. 21, 135a (1978)] and parinaric acid fluorescence measurements [L. A. Sklar et al., J. Biol. Chem. 254, 9583 (1979)].
are due almost exclusively to the ROS phospholipids; distinct protein components are not seen, e.g., in the 6–8 ppm aromatic spectral region. Thus, the resonances from rhodopsin, which constitutes 36 wt.% of the membrane, appear to be broad and largely unresolved under the spectral acquisition conditions employed; that is, the line narrowing induced by sonication affects predominantly the lipid components of the spectra, and thus broad line nmr studies together with moment analysis may be necessary to resolve the contribution from rhodopsin. The observation of sharp, well-resolved resonance lines for sonicated vesicles of both the retinal ROS membranes and ROS phospholipids is consistent with the contention that the intermolecular \( ^1 \H \) dipolar interactions are modulated by rapid lateral diffusion of the ROS phospholipids in both cases, with essentially zero time average as assumed in the lineshape analysis described earlier. We have previously interpreted such data to infer that the lipids in contact with rhodopsin are in relatively rapid exchange with bulk membrane lipids, and that rhodopsin does not greatly perturb the ordering of either the saturated or unsaturated acyl chains. The validity of this analysis is dependent on the assumption that the percentage of the total lipid signal observed is similar in the absence and presence of rhodopsin. Previous experiments indicate that this is the case, however, a more complete study is in progress. These qualitative conclusions have been supported by more recent spin-label EPR and \( ^2 \H \) nmr studies employing a variety of membrane systems. A rather similar model for lipid–protein interaction has been proposed subsequently by Kang et al.

Information on the dynamic properties of membraneous phospholipids can be provided by spin-lattice \( (T_1) \) relaxation time measurements, which are sensitive to molecular fluctuations in the MHz frequency range. Although it is relatively easy to measure the \( ^1 \H \ T_1 \) relaxation times of membrane vesicles to a high degree of precision using modern

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51 A lower limit to the rate of phospholipid lateral diffusion can be estimated from the intermolecular contribution to the second moment of hexagonally packed saturated hydrocarbon chains rotating about their long molecular axes [E. R. Andrew, J. Chem. Phys. 18, 607 (1950)]. For motional averaging of the intermolecular part of the rotationally averaged second moment to occur via lateral diffusion, the lateral jump lifetime must be such that \( M_2 \text{ (inter)} \tau_{LD}^2 \leq 1 \). From the value of \( M_2 \text{ (inter)} \approx 2 \tau G^2 = 1.5 \times 10^9 \text{ (rad/sec)}^2 \), an upper limit of \( \tau_{LD} < 10^{-5} \text{ sec} \) is calculated, and, from the relation \( \langle r^2 \rangle = 4D_T \tau_{LD} \), a lower limit to the translational diffusion coefficient of \( D_T > 10^{-10} \text{ cm}^2/\text{sec} \) is obtained. Thus it is viewed likely that the phospholipid exchange rate in rhodopsin-containing membranes \( (v_{ex} \approx 4 \times 10^7 \text{ Hz}) \) approaches that in pure bilayers \( (v_{ex} \approx 10^7 \text{ Hz}) \), although a precise determination is not yet available. Analogous conclusions have been drawn from \( ^2 \H \) nmr studies [A. Seelig and J. Seelig, Hoppe-Seyler's Z. Physiol. Chem. 359, 1747 (1978)].
pulsed Fourier techniques\textsuperscript{52}, their detailed interpretation is complex because of the possibility of spin diffusion along the phospholipid molecules, as well as the presence of \textit{intra-} and \textit{intermolecular} fluctuating dipolar interactions that can contribute to the $T_1$ relaxation\textsuperscript{14}. In our previous $^1$H $T_1$ studies of bovine ROS membranes, we observed markedly nonexponential $T_1$ relaxation behavior, in contrast to the exponential $T_1$ relaxation observed for the ROS phospholipids. Similar observations of biexponential relaxation behavior have been subsequently made for frog photoreceptor membranes\textsuperscript{52} and native and reconstituted sarcoplasmic reticulum vesicles.\textsuperscript{53} However, it now appears, in contrast to our original interpretation,\textsuperscript{13,14} that the presence of a slow $^1$H $T_1$ relaxation component in these membrane samples may be due to the presence of higher levels of residual water protons compared to the suspensions of ROS phospholipid vesicles prepared from dry films. Recently, we have prepared samples with very low residual water proton content from lyophilized ROS membranes suspended in deuterated buffer, and we find, in this case, that the $T_1$ relaxation of both the ROS membranes and ROS phospholipids is indeed exponential, within experimental error (Fig. 4; see table). If $^1$H$_2$O is added back to the ROS membrane samples, the $T_1$ relaxation becomes nonexponential, as originally observed.\textsuperscript{13,14,54} Although further work is required, it appears that the slower $^1$H $T_1$ relaxation component may be due either to cross-relaxation (spin-diffusion) between the phospholipid and aqueous spin systems or to an unresolved broad water resonance. An important conclusion from these studies is that the initial slope of the $^1$H inversion recovery plots should be used in evaluating the phospholipid dynamic properties of membranes in the presence of substantial residual water protons. It is, however, preferable to use data from the entire $T_1$ decay plot, which can be done if the water proton levels are made very low (e.g., by lyophilization) or if the residual water signal is effectively suppressed by selective irradiation. When initial slopes are compared\textsuperscript{12–14} or residual water is reduced by lyophilization, the $^1$H $T_1$'s of the ROS membranes and ROS phospholipids are found to be quite similar, as shown in Fig. 4 and the table. However, a small but significant decrease in the $T_1$ relaxation times of the ROS membrane phospholipids, compared to those of the ROS phospholipid vesicles, is observed (see the table) and corresponds to a reduction in the average rate of the phospholipid segmental motions in

\textsuperscript{52} G. P. Miljanich and A. J. Deese, unpublished.


\textsuperscript{54} These results are further supported by the observation that in samples of ROS membranes or sarcoplasmic reticulum membranes exhibiting biexponential relaxation behavior, saturation of the water proton impurity resonance results in disappearance of the slow relaxation component, i.e., the relaxation becomes exponential [A. J. Deese \textit{et al.}, \textit{Biophys. J.}, in press].
Fig. 4. Three hundred sixty-megahertz \(^1\text{H}\) spin-lattice \(T_1\) relaxation of lyophilized, dark-adapted ROS membrane vesicles in 0.068 \(M\) phosphate buffer, pH 7, at 20° (cf. Fig. 2). The \(T_1\) relaxation times were measured using the inversion recovery pulse method (180°-\(\tau\)-90°). The data are fitted to first-order plots of \(\ln(M_0 - M_x)\) vs. \(\tau\), where \(M_x\) is the resonance amplitude after a time interval \(\tau\) between the inverting (180°) and sampling (90°) pulses; \(M_0\) is the corresponding amplitude for long pulse intervals (see text). The data acquisition parameters are given in Fig. 1.

the ROS membranes,\(^{22,23}\) due, presumably, to the influence of rhodopsin. Since the correlation times for phospholipid segmental motions are typically in the range 0.01–0.1 nsec,\(^{22,23}\) whereas lateral self-diffusion is a much slower process \((\tau_c \approx 100\) nsec\(), the ROS phospholipids can be viewed as translationally fluid, yet hindered somewhat in the rate of their segmental motions by contact with rhodopsin, as previously concluded.\(^{13-15}\) The preceding conclusions are also supported by \(^{13}\)C nmr studies (next section).

More recently, \(^1\text{H}\) nmr studies of phospholipid vesicles containing rhodopsin have been reported by O'Brien and co-workers.\(^{55,56}\) Although

<table>
<thead>
<tr>
<th>Group</th>
<th>$^1$H $T_1 \pm SD$ (sec)</th>
<th>$^{13}$C $T_1 \pm SD$ (sec)</th>
<th>$^{13}$C NOE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Membranes</td>
<td>Lipids</td>
<td>Membranes</td>
</tr>
<tr>
<td>CH=CH</td>
<td>0.69 ± 0.01</td>
<td>0.98 ± 0.06</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>N(CH$_3$)$_3$</td>
<td>0.52 ± 0.01</td>
<td>0.85 ± 0.01</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>CH=CHCH$_2$CH=CH</td>
<td>0.64 ± 0.01</td>
<td>0.71 ± 0.02</td>
<td>0.48 ± 0.04</td>
</tr>
<tr>
<td>CH=CHCH$_2$</td>
<td>0.64 ± 0.02</td>
<td>0.73 ± 0.02</td>
<td>—</td>
</tr>
<tr>
<td>(CH$_3$)$_n$</td>
<td>0.64 ± 0.01</td>
<td>0.71 ± 0.02</td>
<td>0.48 ± 0.03</td>
</tr>
<tr>
<td>CH$_3$</td>
<td>0.76 ± 0.01</td>
<td>0.68 ± 0.01</td>
<td>2.09 ± 0.08</td>
</tr>
</tbody>
</table>

$^a$ $T_1$ measurements at 8.4 tesla (360 MHz $^1$H; 90 MHz $^{13}$C).

$^b$ Data from Brown et al.  

Spin-lattice ($T_1$) relaxation times and $^{13}$C–$^1$H nuclear Overhauser effects (NOE) of ROS membrane and ROS phospholipid vesicles at 20°C.

PHYSICAL STUDIES ON RETINAL PHOTORECEPTORS
Zumbulyadis and O’Brien⁵⁶ also conclude that the bulk of the ROS membrane phospholipids are in fast exchange with those phospholipids in contact with rhodopsin,⁵⁷ their results are based on an analysis of linewidth changes due to the presence of rhodopsin in the recombinant vesicles. A possible alternative interpretation of the lineshape data is that the observed changes are due to a progressive increase in vesicle size with increasing rhodopsin content, thereby resulting in a decrease in the rate of vesicle tumbling and broader spectral lines.²¹

Carbon-13 Nmr Studies

The first ¹³C nmr studies of retinal ROS membranes were reported by Millet et al.,⁵⁸ who were able to obtain high-resolution natural-abundance ¹³C nmr spectra of ROS disk membranes and to measure their spin-lattice (T₁) relaxation times. More recently, Zumbulyadis and O’Brien⁵⁶ have published ¹³C nmr spectra of sonicated ROS membranes and ROS phospholipid dispersions, as well as recombinant membrane vesicles containing rhodopsin.

Representative ¹³C nmr spectra from our own work⁵⁹ are shown in Fig. 5. As is the case for ¹H nmr studies (preceding section), distinct resonances are observed from the polyunsaturated and saturated fatty acyl chains and the phosphocholine head groups, with very similar spectra obtained for both the ROS membranes and ROS phospholipid vesicles, i.e., little contribution from rhodopsin is resolved.⁶⁰ In contrast to the results of Zumbulyadis and O’Brien,⁵⁶ we do not observe a large differential broadening of the saturated (CH₂)n chain resonance near 30 ppm in the ROS membranes versus the ROS phospholipid vesicles, nor are we able to detect a substantial differential broadening of the glycerol and other resonances in the 60–75 ppm region (Fig. 5). In the ¹³C nmr spectra of

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⁵⁷ These authors have misinterpreted the results of our earlier T₁ analysis [M. F. Brown et al., Proc. Natl. Acad. Sci. U.S.A. 77, 1978 (1977)] to mean that exchange between “boundary” and “nonboundary” lipids is slow compared to T₁, i.e., on the order of seconds or more. The observation of two relaxation components only implies the existence of two weakly interacting phospholipid spin systems, within which fast lateral diffusive exchange would be possible to account for motional averaging of intermolecular dipolar interactions. Clearly, the association of a given T₁ component exclusively with “boundary” or “nonboundary” lipids is not a warranted conclusion; however, as discussed in the text, any slow T₁ components may represent water relaxation and not lipid dynamics as originally suggested.


⁶⁰ The spectra shown in Fig. 5 represent close to 100% of the expected spectral intensity, as shown by studies in which large spectral windows and short data acquisition delays were employed to rule out the possibility of additional broad spectral components.
ROS phospholipid vesicles published by Zumbulyadis and O’Brien, both the unsaturated vinyl and doubly allylic CH2 resonances are greatly reduced in amplitude relative to that expected from the native ROS phospholipid composition, suggesting that oxidative degradation of the ROS phospholipid double bonds may account for some or all of the spectral differences vis-à-vis the ROS membrane vesicles (compare Fig. 5 of Ref. 56.}

**Fig. 5.** Representative 90-MHz natural abundance 13C nmr spectra of sonicated, dark-adapted ROS membranes and ROS phospholipid vesicles in 0.068 M phosphate, pH 7, at 20°C obtained under conditions of 1H dipolar decoupling. The spectra were obtained in the Fourier transform mode using a spectral width of ± 10 kHz (quadrature phase detection), 16 K data points, 50-µsec data acquisition delay, 10-sec interpulse delay, with 2–5 W of 1H decoupling power. Two hundred fifty-six free induction decays were accumulated, exponentially multiplied (20-Hz line broadening), and Fourier-transformed to obtain the spectra depicted. A 90° pulse was 24 µsec. (The position of the chemical shift scale is arbitrary.)
and cytochrome oxidase containing bilayers, which reveal the presence of "immobilized" spectral components associated with the presence of protein, and nmr studies, which do not, is that exchange of lipids on and off the surface of these integral membrane proteins may be slow on the EPR time scale, but fast on the nmr time scale ($\nu_{ex} > 10^5$ Hz). Thus, it is possible that a decrease in the rate of lipid segmental motion, e.g., as suggested by the results of $T_1$ studies, could lead to the presence of "immobilized" EPR spectra if the rotational correlation time of the spin label is increased to greater than $3 \times 10^{-8}$ sec, as originally suggested by Brown et al. [Biochemistry 16, 2640 (1977)].

Fig. 6. Representative $^1$H dipolar-decoupled 73-MHz $^3$P nmr spectra of unsonicated multilamellar dispersions of dark adapted ROS disk membranes and total extracted ROS phospholipids at 35°. The upper spectrum is from a sample of ROS membranes in 0.2 M HEPES, pH 7. The lower spectrum of ROS phospholipids is from a sample that was exchanged from phosphate buffer into 0.1 M borate, 0.1 M KCl, pH 7; hence the sharp peak near 5 ppm from residual inorganic phosphate. The spectra were obtained in the Fourier transform mode using the following data acquisition parameters: spectral width $\pm 5$ kHz (quadrature phase detection), 8 K data points, 80-$\mu$sec data acquisition delay, 250-msec interpulse delay, 5-W $^1$H decoupling power, and 40-Hz line broadening. A 90° pulse was 10 $\mu$sec in duration; 2000-3000 free induction decays were accumulated. (The position of the chemical shift scale is arbitrary; the residual chemical shielding anisotropy is indicated by $\Delta \sigma$.)
pholipid motions such as lateral diffusion exist as well, but further experimental studies are required in order to resolve this point.

Phosphorus-31 Nmr Studies

$^{31}$P Nmr is an attractive experimental tool for the study of phospholipid head group motions, as well as their phase polymorphism, since multilamellar dispersions, small (sonicated) vesicles, hexagonal phases, etc., give rise to characteristic spectra from which the type and amount of each form can be evaluated. Recently, $^{31}$P nmr spectra of unsonicated and sonicated preparations of ROS membranes and extracted phospholipids have been reported by De Grip et al. Although the unsonicated ROS disk membranes are found to give rise to typical powder-type bilayer $^{31}$P nmr spectra, the results obtained for the extracted phospholipid dispersions do not appear obviously characteristic of the lamellar phase. As a result, De Grip et al. have proposed a major structural role for rhodopsin in organizing the ROS phospholipids into a bilayer configuration.

We have also carried out $^{31}$P nmr studies of unsonicated preparations of highly purified ROS membranes and ROS lipids. These samples were prepared using conditions similar to those employed in our previous $^1$H and $^{13}$C nmr studies. Our results are quite different from those obtained by De Grip et al., in that we obtain $^{31}$P nmr spectra that are indicative of the lamellar phase for both the ROS lipids and the ROS membrane dispersions. As shown in Fig. 6, little difference is observed in the $^{31}$P chemical shielding anisotropy ($\Delta \sigma$) of the ROS membrane and ROS phospholipid preparations, which represents an average of the contributions from the various phosphorus-containing head groups, mainly phosphatidylethanolamine and phosphatidylcholine. No spectral components with increased $\Delta \sigma$, characteristic of the gel state, are detected.

Therefore, our results support the idea that ROS lipids exist mainly in the lamellar phase in the absence and presence of rhodopsin, under the conditions of our experiments. Also, rhodopsin does not appear to greatly influence the degree of ordering of the ROS phospholipid head groups, i.e., their time-averaged conformation and motional amplitude, and no immobilized or ordered "boundary lipid" is observed. It is un-

61 The ROS membrane $^{13}$C $T_1$ relaxation times are different from the earlier results of Millet et al. [Biochemistry 12, 3591 (1973)]; however, since these authors were apparently not successful in obtaining well-resolved $^1$H nmr spectra from their preparations, the possibility of oxidative damage to the lipid must be considered.
64 One possible explanation of the discrepancy between spin-label EPR studies of rhodopsin
FIG. 6. Representative $^1$H dipolar-decoupled 73-MHz $^{31}$P nmr spectra of unsonicated multilamellar dispersions of dark adapted ROS disk membranes and total extracted ROS phospholipids at 35°C. The upper spectrum is from a sample of ROS membranes in 0.2 M HEPES, pH 7. The lower spectrum of ROS phospholipids is from a sample that was exchanged from phosphate buffer into 0.1 M borate, 0.1 M KCl, pH 7; hence the sharp peak near 5 ppm from residual inorganic phosphate. The spectra were obtained in the Fourier transform mode using the following data acquisition parameters: spectral width $\pm$ 5 kHz (quadrature phase detection), 8 K data points, 80-μsec data acquisition delay, 250-msec interpulse delay, 5-W $^1$H decoupling power, and 40-Hz line broadening. A 90° pulse was 10 μsec in duration; 2000–3000 free induction decays were accumulated. (The position of the chemical shift scale is arbitrary; the residual chemical shielding anisotropy is indicated by $\Delta \sigma$.)

and cytochrome oxidase containing bilayers, which reveal the presence of "immobilized" spectral components associated with the presence of protein, and nmr studies, which do not, is that exchange of lipids on and off the surface of these integral membrane proteins may be slow on the EPR time scale, but fast on the nmr time scale ($\nu_{ss} > 10^8$ Hz). Thus, it is possible that a decrease in the rate of lipid segmental motion, e.g., as suggested by the results of $T_1$ studies, could lead to the presence of "immobilized" EPR spectra if the rotational correlation time of the spin label is increased to greater than $3 \times 10^{-8}$ sec, as originally suggested by Brown et al. [Biochemistry 16, 2640 (1977)].
clear at present exactly what might account for the discrepancies between our findings and those of De Grip et al.\textsuperscript{62} A brief discussion of this point and a more detailed report of our $^{31}$P nmr studies may be found in Ref. 63. Obviously, a more complete study is needed in order to obtain a better understanding of these very interesting lipids. However, our results support the use of extracted ROS lipids as a model for interpreting the physical properties of the ROS membranes\textsuperscript{13–15, 65} under the sample preparation conditions that we have used for our previous nmr studies. In general, the $^{31}$P nmr spectra reported here are consistent with the picture of ROS lipid organization and lipid-protein interaction deduced from $^1$H and $^{13}$C nmr studies.

Conclusions and Final Comments

Nuclear magnetic resonance is a useful physical method for studying rhodopsin–lipid interactions, particularly since high-quality nmr spectra can be obtained for native ROS disk membranes, as well as rhodopsin-containing recombinant membranes. Furthermore, both ordering and dynamic information can be inferred using nmr methods, without the introduction of probe molecules, which may perturb the bilayer structure. The results summarized in this article are consistent with the notion that the bulk of the ROS membrane phospholipids are highly fluid and that their ordering is not greatly perturbed by rhodopsin. The idea that integral membrane proteins such as rhodopsin drastically modify the lipid structure of membranes\textsuperscript{62,66–69} is not supported.

Acknowledgments

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\textsuperscript{68} F. W. Dahlquist, D. C. Muchmore, J. H. Davis, and M. Bloom, \textit{Proc. Natl. Acad. Sci. U.S.A.} \textbf{74}, 5435 (1977). These authors have not been able to reproduce their original two-component $^2$H nmr spectra in more recent work, and their interpretations have been subsequently revised [M. R. Paddy et al., \textit{Biochemistry} \textbf{20}, 3152 (1981)].