RETINAL ROD OUTER SEGMENT LIPIDS FORM BILAYERS IN THE PRESENCE AND ABSENCE OF RHODOPSIN: A ^{31}P NMR STUDY

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1. Introduction

The molecular organization of the retinal rod outer segment (ROS) disc membrane has occupied the attention of many investigators recently. Studies have focussed on the protein organization [1–5], the phospholipid composition and transmembrane asymmetry [6–10], rhodopsin–lipid interactions [11–17], and the possible functional role of the highly polyunsaturated ROS phospholipids in visual phototransduction [18]. One area of current interest involves studies of the influence of the various phospholipid head groups, acyl chain unsaturation, and rhodopsin on the phase polymorphism of the retinal photoreceptor membrane phospholipids. Since these ROS phospholipids are extremely polyunsaturated, with docosahexenoic acid (C22:6ω3) constituting nearly 50 mol% of the total membrane fatty acids [19], great care must be taken to avoid lipid oxidative damage [20]. NMR [13–15], differential scanning calorimetry [21], and parinaric acid fluorescence studies [22] demonstrated a broad, endothermic phase transition in dispersions of bovine ROS membranes and their total extracted phospholipids, with a midpoint near 4–6°C [21]. The observed thermal behavior is believed due to a gel–liquid crystalline transition of a fraction of the ROS phospholipids [14,22]. Over 15–50°C, no further distinct calorimetric transitions are detected in either preparation, although fluorescence methods suggest the onset of lateral phase separation in the ROS membranes at $\leq$25°C [23].

In [24] it was proposed on the basis of ^{31}P NMR studies that, although the native photoreceptor disc membrane exists largely in the lamellar phase, aqueous dispersions of its extracted phospholipids do, in fact, favor the hexagonal phase near physiological temperature. Thus, according to these authors, rhodopsin would play a major structural role in the ROS disc membrane, in addition to its functional role, by stabilizing the lamellar phase of the ROS phospholipids. If correct, these conclusions would have important implications for studies of lipid–protein interactions, in general. Furthermore, these conclusions would negate the assumption made in [13–15,17,25] that the ROS lipid dispersions are an appropriate reference system for studies of the effect of rhodopsin on phospholipid structural dynamics [26,27]. Consequently, we have carried out ^{31}P NMR experiments employing ROS lipids and ROS disc membranes under conditions similar to those used in [13–15]. We find, under these conditions, that the ^{31}P NMR spectra are indicative of the lamellar (L_α) phase for both the ROS membrane and ROS lipid dispersions. In this letter, we report these preliminary ^{31}P NMR findings and briefly discuss their implications for studies for rhodopsin–lipid interactions.

2. Experimental

Cattle eyes were obtained locally and transferred to dark containers on ice in $\leq$10–15 min after slaughter. Retinal ROS disc membranes were purified as in [19,28]. CaEDTA (0.1 mM) was added to all buffers used for ROS membrane purification, all solutions

Abbreviations: ROS, rod outer segment; NMR, nuclear magnetic resonance; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; SM, sphingomyelin; BHT, butylated hydroxytoluene; PUFA, polyunsaturated fatty acid; TLC, thin-layer chromatography

were argon-purged immediately before use, and all experimental manipulations were performed under an argon atmosphere to prevent lipid oxidative damage [19]. The ROS membrane samples used for these studies had A_{280}/A_{500} ratios in the range 2.2–2.6, before regeneration [28], and were <15% bleached.

Before extraction of the ROS lipids, the membrane suspensions were mixed with a 100:1 molar excess of hydroxylamine to retinal and bleached to convert retinal to retinaloxime [9,29]. The ROS membranes were then pelleted and the total lipids extracted using a modification of the procedure in [30], in which 50 μg/ml of the antioxidant butylated hydroxytoluene (BHT) were added to all organic solvents [13]. (If BHT is omitted from the organic solvents used for lipid extraction and purification, substantial peroxidation of the highly polyunsaturated ROS phospholipids is the result, in our experience.) The ROS membrane phospholipids were purified by column chromatography using either of two methods. In the first method [13], the total extracted ROS lipids were applied to a column of silicic acid in chloroform (0.3 g silic acid/mg applied lipids) and the neutral lipids (retinal pigments plus free fatty acids, diacylgycerols, cholesterol, α-tocopherol, and BHT) were first eluted with several column volumes of chloroform:methanol (9:1 v/v), followed by elution of the ROS phospholipids with methanol. In the second procedure, the ROS phospholipids were purified by chromatography on a column of Sephadex LH-20 in ethanol (3.5 × 85 cm; 1 g/mg applied lipids). The total ROS lipids were applied in 95% ethanol, containing 50 μg BHT/ml, and the phospholipids were eluted near the column void volume, followed by the various retinal pigments and other neutral lipids. During column purification, the elutant was continually purged with argon and the fractions were collected under an argon atmosphere [13]. After purification, the BHT concentration of the purified retinal phospholipids was immediately increased to ~1 BHT/1000 phospholipids. The purified ROS phospholipids were dried to a thin film by rotary evaporation, followed by exposure to high vacuum (typically 5 μm of Hg) for several hours at room temperature. The ROS phospholipids were then hydrated in a round bottom flask by addition of, in most cases, 0.02 M Hepes buffer (pH 7) and dispersed by gently swirling the suspension with several glass beads. In some cases, the samples were stored frozen prior to measuring their NMR spectra.

For each ROS phospholipid sample studied, the head group composition was determined by quantitative thin-layer chromatography on silica gel plates in chloroform:methanol:water (65:25:5; containing 50 μg/ml BHT). Spots were visualized by exposure to I_2 vapor and the areas corresponding to the various phospholipid species were scraped into test tubes and analyzed for phosphorus using a modification [29] of the procedure in [31]. The fatty acyl chain composition of the ROS phospholipid samples was determined by gas–liquid chromatography. The fatty acid methyl esters were prepared from the extracted membrane lipids by addition of 30% boron trichloride in methanol and chromatographed in an all glass system on 10% SP-2330 (Supelco). Further details are in [9,29].

^{31}P NMR spectra were obtained in the Fourier transform mode as in [32,33].

3. Results

Fig.1 shows ^{31}P NMR spectra of bovine retinal ROS phospholipids dissolved in chloroform:methanol (9:1 v/v). The upper spectrum, fig.1a, was obtained from a sample of total ROS lipids extracted by a modification of the procedure in [30], designated as ROSLIP-1, which was not further purified. The lower spectrum, fig.1b, was obtained from a sample of ROS phospholipids which were Folch extracted, followed by column purification on Sephadex LH-20, designated as ROSLIP-2. The spectra depicted were obtained under conditions of continuous (bilevel) ^{1}H decoupling; essentially identical spectra were obtained using gated ^{1}H decoupling [34], which eliminates the ^{31}P [^{1}H] nuclear Overhauser effect (NOE) [35]. Thus differential NOEs for the resolved resonances, which would alter their relative intensities, are not observed.

The major downfield peak (arbitrarily assigned a chemical shift of 0 ppm in fig.1) is assigned to the ROS phosphatidylethanolamine (PE), while the major upfield peak (at 1.2 and 1.4 ppm in fig.1a and 1b, respectively) is assigned to the ROS phosphatidylcholine (PC). The spectral assignments are based on comparison to ^{31}P NMR spectra of egg PC (not shown) and various other phospholipids in organic solvents [36]. The small differences in the chemical shifts between the two samples in fig.1 are not viewed as significant, since the ^{31}P chemical shifts of phospholipids in organic solvents are quite sensitive to metal ions and the methanol content, and the samples were prepared and studied under somewhat different con-

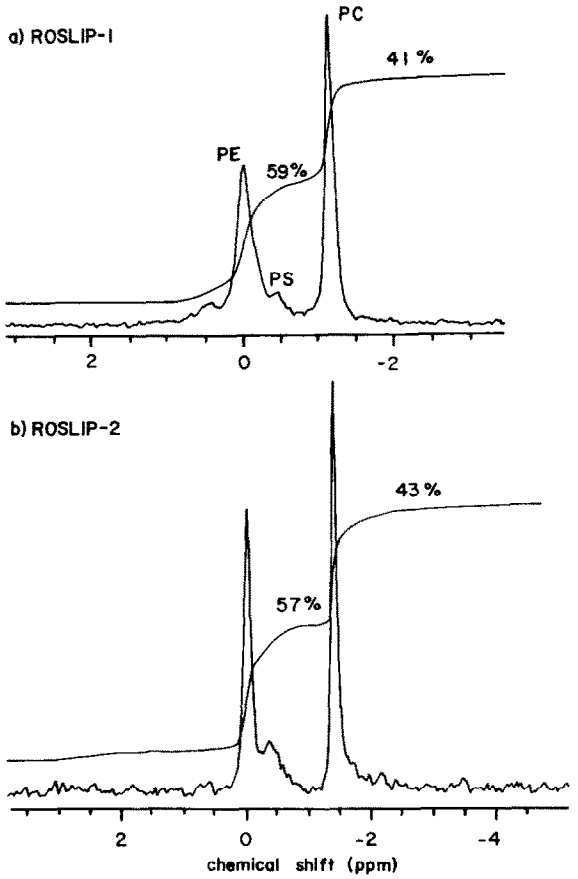

Fig.1. ^{31}P NMR spectra of two different samples of total extracted ROS phospholipids (~60 mg) in chloroform:methanol (9:1, v/v) at 20°C. The two samples are designated ROSLIP-1 (a) and ROSLIP-2 (b) and were extracted and purified as described in the text. The ^{31}P NMR spectra were obtained at 73 MHz using continuous (bilevel) ^{1}H decoupling, in the Fourier transform mode with quadrature phase detection, accumulating 200 scans with a 5 s interpulse delay. The spectral integrals are indicated in the figure, together with the resonance assignments. The zero point of the chemical shift scale is arbitrary.

ditions. The minor resonance upfield of PE (at 0.4 ppm) is tentatively assigned to the ROS phosphatidylserine (PS) [36]. The small downfield peak in fig.1a (at −0.5 ppm) is at present unidentified.

The integrated areas of the resolved head group resonances are indicated in fig.1 and in table 1, together with the head group composition of the same NMR samples as determined by quantitative TLC. These values are consistent with more extensive studies of the head group composition of the ROS disc membrane phospholipids [9,29,37]. The good agreement

Table 1
Head group composition of bovine ROS phospholipid NMR samples determined using thin-layer chromatography and ^{31}P NMR

	ROSLIP-1[a]		ROSLIP-2[b]	
	TLC	^{31}P NMR	TLC	^{31}P NMR
% PC	39.6	41	40.0	43
% PE	45.8	59	45.6	57
% PS	14.6		14.4	
(+PI, SM)[c]				

[a] Data refer to an unpurified Folch extract of total ROS membrane phospholipids

[b] Data refer to Folch extracted ROS phospholipids purified by column chromatography on Sephadex LH-20

[c] The spots corresponding to these lipid species were scraped from the TLC plates and analyzed together for total phosphorus. Although not resolved in the present experiment, PS, PI and SM have been found to constitute 14.3%, 1.9% and 1.3% of the total bovine ROS phospholipids, respectively [29]

of the ^{31}P NMR, TLC, and established head group content demonstrates the intactness and reproducibility of the ROS phospholipid preparations employed for these studies. The ROS phospholipid samples were further characterized by gas–liquid chromatography, as indicated in table 2. The high content of polyunsaturated fatty acids (PUFAs) clearly indicates the lack of oxidation of the samples; if ROS membranes are permitted to oxidize by exposure to air in the absence of antioxidants, their PUFA content can be dramatically reduced [20].

Table 2
Fatty acid composition of bovine ROS phospholipid NMR samples

Fatty acid	Mole percentage	
	ROSLIP-1	ROSLIP-2
16:0	13.5	14.2
16:1	0.2	0.2
18:0	19.1	19.5
18:1	4.8	4.9
18:2	0.7	0.6
20:4	4.7	4.8
22:4	0.4	0.3
22:5ω6	2.0	2.0
22:5ω3	1.0	0.8
22:6	49.8	48.5
24:4	2.6	2.7
24:5	0.5	0.3

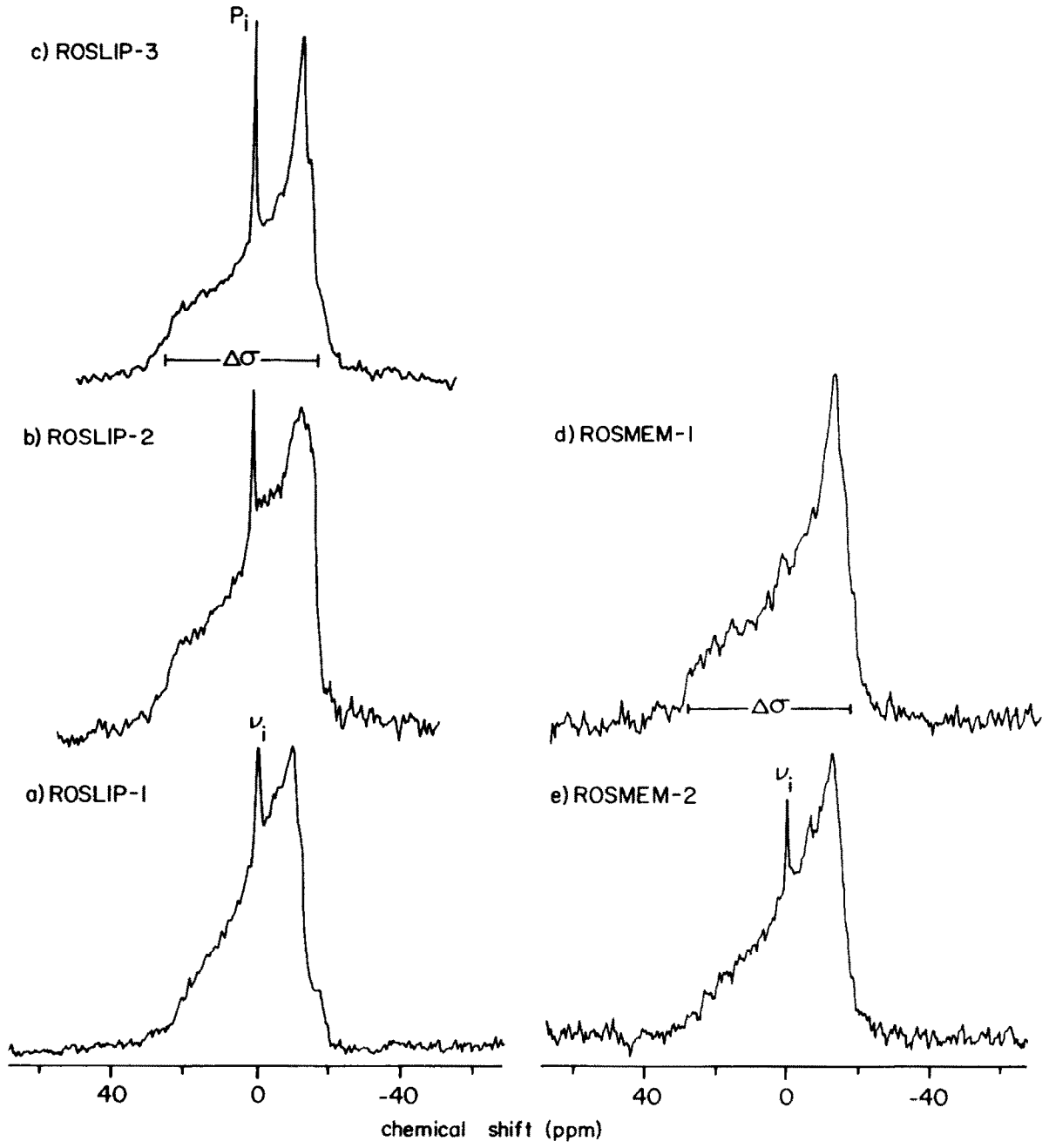

Fig.2. Proton-decoupled ^{31}P NMR spectra of three different samples of total extracted bovine ROS phospholipids and two different samples of osmotically shocked, dark-adapted bovine ROS disc membranes. The ROS phospholipid samples are designated as ROSLIP-1, ROSLIP-2, and ROSLIP-3 (a–c, respectively), and were extracted and purified as described in the text. The ROS membrane samples are designated ROSMEM-1 and ROSMEM-2 (d,e). The preparations each contained 60–80 mg phospholipid and were dispersed in 0.02 M Hepes buffer (pH 6.8) with the exception of sample ROSLIP-3, which was originally prepared in 0.067 M phosphate buffer (pH 7.0) and subsequently exchanged by centrifugation into 0.1 M borate (pH 7.5) containing 0.1 M KCl. The sharp peak near 0 ppm in (c) is due to residual P_i. The ^{31}P NMR spectra were obtained at 15°C as in [32,33], accumulating 2000–3000 scans, with ~5 W of ^1H decoupling power during data acquisition. The residual phosphorus chemical shielding anisotropy is indicated in the figure by $\Delta\sigma$ and the corresponding (average) isotropic chemical shift by ν_i (determined from ^{31}P NMR spectra of the corresponding sonicated vesicles; not shown). The sharp peaks at ν_i in (a,e) are believed due to contributions from small vesicles.

Fig.2(a–c) show ^{31}P NMR spectra of aqueous unsonicated dispersions of 3 different ROS phospholipid preparations, obtained under conditions of low power broadband ^{1}H decoupling: ROSLIP-1 and ROSLIP-2, for which composition data are presented in tables 1,2, as well as a third sample, designated ROSLIP-3, which was purified by column chromatography on silicic acid and whose phospholipid composition was indistinguishable from those of the first two samples. In each case, axially symmetric powder-type ^{31}P NMR spectra are obtained. The residual chemical shielding anisotropy ($\Delta\sigma$) of the ROS phospholipid dispersions [38] is about –42 to –43 ppm at 15°C. This value is consistent with those obtained for other PC and PE containing bilayers in the L_α phase [33,38]. (Sample ROSLIP-1 yielded a slightly reduced $\Delta\sigma$ compared to samples ROSLIP-2 and ROSLIP-3; however, any inconsistencies can be explained by possible effects due to slow tumbling [39] of any relatively small liposomes and incomplete ^{1}H decoupling.) Fig.2(d–e) show proton-decoupled ^{31}P NMR spectra of two different samples of unsonicated, osmotically shocked bovine ROS disc membranes, designated ROSMEM-1 and ROSMEM-2. As a consequence of washing the membranes by centrifugation in hypotonic buffer, phosphate-containing metabolites are lost from the ROS cytosol and the spectra are due exclusively to the membraneous ROS phospholipids. In both cases, characteristic bilayer-type ^{31}P NMR spectra are obtained, consistent with previous ^{31}P NMR results [24] and with X-ray [40] and electron microscopy studies [41]. Within the present experimental error (~ ±3 ppm), little or no difference is observed in the chemical shielding anisotropy of the ROS membrane and ROS phospholipid dispersions. No spectral components with increased $\Delta\sigma$, characteristic of the gel state [33], are detected.

Representative ^{31}P NMR spectra of sample ROSLIP-1 as a function of temperature are shown in fig.3. We have obtained similar results for each of the preparations indicated in fig.2 over 5–45°C (not shown). In some of the samples at the higher temperatures, minor, non-lamellar spectral components were occasionally observed, generally near the isotropic resonance frequency ν_i, but in each case the ^{31}P NMR spectra were predominantly characteristic of the lamellar phase. The possibility of additional phase polymorphism at significantly higher temperatures was not investigated.

4. Discussion

As shown in the preceding results section, bilayer-type ^{31}P NMR spectra can be obtained for both the

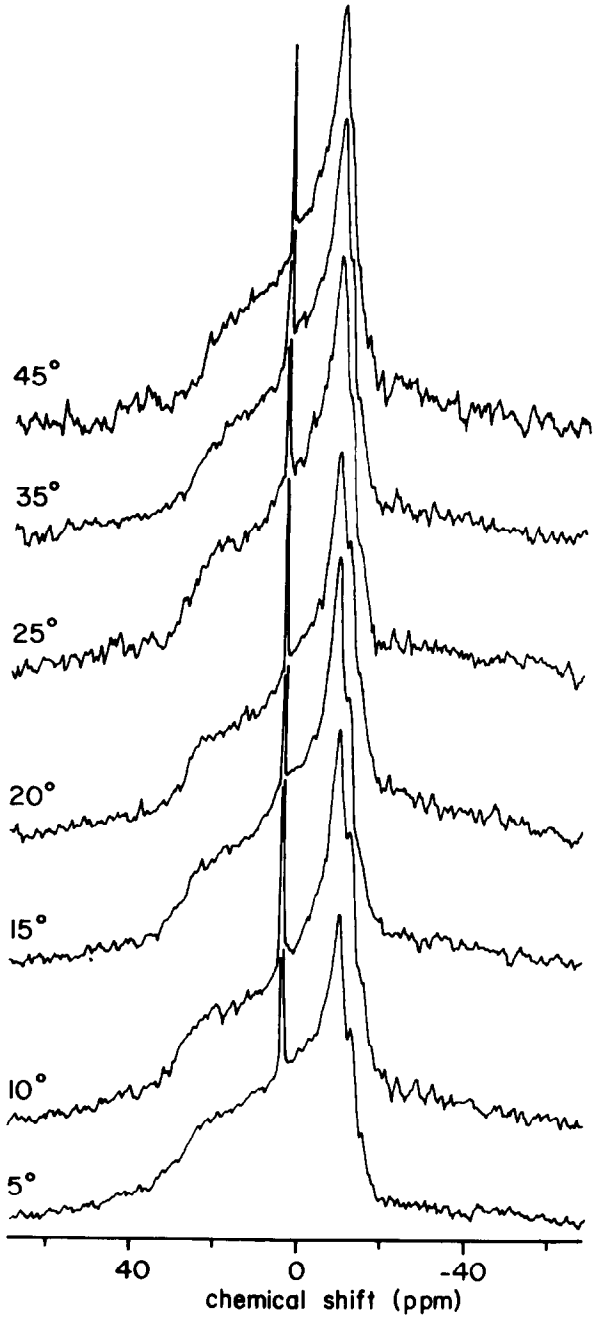

Fig.3. Proton-decoupled ^{31}P NMR spectra of sample ROSLIP-3 as a function of temperature (conditions as in fig.2). The slight baseline roll is instrumental in origin.

native bovine retinal ROS membranes, which contain the visual pigment rhodopsin as a major protein component (~36 wt% of the membrane), and aqueous dispersions of the extracted ROS phospholipids, which do not contain rhodopsin. Since the chemical shielding anisotropy of the ROS membranes and ROS phospholipid dispersions is similar, we can conclude that

(i) Both preparations exist largely in the lamellar (L_α) phase under the conditions employed;
(ii) Rhodopsin does not greatly influence the degree of ordering of the ROS phospholipid head groups, i.e., their time-averaged conformation and motional amplitude.

These conclusions are in agreement with ^{31}P NMR studies of sarcoplasmic reticulum ATPase [42] and cytochrome oxidase containing bilayers [43]. No immobilized or ordered 'boundary lipid' is detected, consistent with previous arguments against the presence of rigid boundary lipids in membranes [14,43]. The above results also support studies which show that, upon sonication, aqueous dispersions of ROS membranes and ROS phospholipids form relatively large, unilamellar vesicles [13,14].

The ^{31}P NMR results presented here disagree with those in [24] on retinal ROS disc membranes and their extracted lipids (cf. fig.2 of [24] to our fig.2). Thus, De Grip et al. [24] state that

'So far, we have not found conditions where the major part of the extracted rod outer segment lipids adopts only the bilayer configuration'.

As a result, they have proposed that:

(i) 'The extracted lipids cannot function as a model system for the lipids of the photoreceptor membrane in view of their completely different configuration and behaviour'; and
(ii) 'Rhodopsin, being the predominant membrane protein (>85%, w/w), must play a decisive role in organizing the lipids into a bilayer'.

In view of the different results obtained here, as well as in [13–15,21], the above conclusions must be regarded as unsubstantiated at present. Although we cannot presently account for the discrepancy between our results and those in [24], several points are worth mentioning.

(i) As shown for sphingomyelin bilayers [44], a number of factors can influence the shape of the ^{31}P NMR spectra. Therefore, in some cases, it may not be adequate to rely on NMR data alone to deduce the existence of nonlamellar phases, and it may be desirable to obtain corroborative X-ray results. For example, tumbling of any relatively small liposomes present in the samples ($r \lesssim 1500$–2000 Å) could lead to slow motional effects [39], which would reduce the chemical shielding anisotropy of the ^{31}P NMR spectra and make them more isotropic in appearance*.
(ii) Lipid and protein transbilayer asymmetry [6–8, 10,29] may influence the lipid phase behavior.
(iii) We have shown that the ROS phospholipids are quite sensitive to oxidation [13,20], and it is not clear from [24] sufficient precautions in this regard have been taken.

The detailed characterization of ROS lipid samples employed for biophysical studies is extremely important [9,13,46]. We have observed that unless care is taken, some purification conditions can raise the mole fraction of PE (e.g., non-quantitative elution of PC during silicic acid chromatography), resulting in nonlamellar ^{31}P NMR spectra suggestive of hexagonal phase formation (unpublished). Thus, the ROS phospholipid composition may be such that it is close to a lamellar–hexagonal phase boundary, in which case the organization could be easily perturbed by slight variations in composition and ionic strength. Additional studies are required before more definite conclusions can be reached. In general, we find that the ^{31}P NMR results are consistent with previous ^{1}H and ^{13}C NMR studies [46].

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* It should be emphasized that unoriented lipid dispersions are not completely analogous to solid powder samples in that motional effects spanning a wide range of frequencies can influence the interpretation of their NMR parameters [45]

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