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FLUID LIPID FRACTION IN ROD OUTER SEGMENT MEMBRANE

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SUMMARY

Rod outer segment membrane is analyzed using the spin label technique by means of two probes. The solubility of the first label, 2,2,6,6-tetramethylpiperidin-1-oxyl, is correlated with the membrane fluidity which is measured using a stearic acid spin probe. The two values are compared to the solubility-fluidity relationship which characterizes a model system in which all lipids are in a fluid state. The analysis leads to the conclusion that only two thirds of the membrane lipids are fluid. This conclusion is reinforced by the observation that partial lipid removal leaves rigid lipids associated with the rhodopsin molecules.

INTRODUCTION

Recently, Hagins [1] introduced a hypothesis which postulated that Ca^{2+} could be involved in the transduction process occurring in rod outer segments of the visual cells. A Ca^{2+} flux seems to be induced by illumination in isolated frog rod outer segments [2, 3]. Although the functional relationship between rhodopsin illumination and Ca^{2+} movement is not yet understood, other analytical approaches of the visual excitation process have revealed that illumination of the rhodopsin molecule leads to a modification of the physical properties of the phospholipid matrix in which the visual pigment is embedded.

Chen and Hubbell [4] have reported that heterogeneous patterns become apparent under the electron microscope when rhodopsin is incorporated in artificial membranes made up from pure phospholipids. These patterns result from lateral separation between a pure solid lipid phase and a rhodopsin-containing phase. Upon illumination, a modification of the phase pattern occurs. More recently, a birefringence analysis [5] has revealed the appearance of a membrane structure change associated with rhodopsin bleaching which can be accounted for by phospholipid disorientation.

Application of the spin label technique [6, 7] has shown that light increases

Abbreviation: TEMPO, 2,2,6,6-tetramethylpiperidin-1-oxyl.

the fluidity of the disk membranes in native rod outer segments. However, it is not yet clear whether this fluidity change relates to a global and homogeneous modification of the membrane properties or, alternatively,whether it arises from a light-induced perturbation which modifies the partition of a lateral phase separation already present within the membrane in the dark-adapted stage. In the present work, an analysis of this latter possibility is carried out by means of a method described by McConnell et al. [8] which involved the use of two spin labels. By using model systems composed of egg lecithin-cholesterol liposomes and working above the phase transition temperature, an empirical relationship is established between the lipid solubility of the first label, 2,2,6,6-tetramethylpiperidin-1-oxyl, and the liposomes fluidity which is measured with a second label, a stearic acid derivative. In order to evaluate the fluid fraction of the rod outer segment membrane, the membrane fluidity is measured and correlated to the proper solubility of the first label. This latter value is compared to the solubility within a model system characterized by a same fluidity. We conclude that at 37 °C two thirds of the disk membrane lipids are in a fluid state. Results obtained with lipid depleted membranes are in agreement with this finding.

MATERIALS AND METHODS

Chemicals. 2,2,6,6-Tetramethylpiperidin-1-oxyl (TEMPO) has been synthetized by the method of Rozantsev [9]. The fatty acid spin probe, *N*-oxyl-4,4-dimethyloxazolidin derivative of 5-ketostearic acid (5-doxyl stearic acid), is a commercial product (Syva, Palo Alto). Egg lecithin (Type III-E, Sigma, St. Louis), obtained in sealed ampules, was used without further purification. Dimyristoyllecithin, dipalmytoyllecithin and cholesterol were purchased from Fluka.

Preparation of the liposomes. Egg lecithin-cholesterol liposomes were prepared by evaporating to dryness under N_2 200 μl of a hexane solution of lecithin (100 mg/ml) and redissolving the dry material in chloroform. To each sample 0–500 μl of chloroform solution of cholesterol (10 mg/ml) were added. The mixtures were evaporated under N_2 and 500 μl of 0.16 M Tris · HCl (pH 7.1) was added. The samples were mechanically agitated for 2 h at 25 °C whereupon the labels were introduced: 10 μl of TEMPO (5 mM in Tris . HCl buffer) was added to 100 μl of egg lecithin-cholesterol suspension. The fatty acid spin label was dissolved in ethanol (1 mg/ml), 100 μl samples are evaporated to dryness under N_2 and 100 μl of liposome suspension was added. The labeled samples were agitated for 15 min at 37 °C.

Dimyristoyllecithin, dipalmitoyllecithin and mixed lipid liposomes were formed and labeled in a similar way.

Preparation of rod outer segment membrane suspension. Rod outer segment membranes were prepared according to the method of de Grip et al. [10]. The material was lyophilized and kept at −20 °C. 1 mg of material dissolved in 1 ml emulphogene solution (1 % w/w in Tris·HCl buffer) is characterized by a difference in absorbance at 500 nm of 0.29 absorbance units before and after illumination (1 cm light path). The purity of the preparation was assessed by means of the A_{280}:A_{500} and A_{400}:A_{500} ratios which were 2.8 and 0.27, respectively. The lyophilized material was taken up in Tris buffer and the suspension (20–90 mg/ml) agitated in

darkness for 2 h. The labels were incorporated as explained above in the case of the lecithin-cholesterol liposomes.

Lipid removal. Total lipid extraction was carried out by treating the membranes with 2:1 (v/v) chloroform/methanol mixture [11]. The residue was separated by centrifugation, the procedure being repeated three times. The final residue was dried under N_2 and resuspended in buffer by vigorous shaking. Partial lipid removal was performed in a similar manner by treating either lyophilized membranes with hexane [11], or dried Triton X-100 extracts (2% w/w) with toluene [12].

Electron spin resonance measurements. Electron spin resonance (ESR) spectra were recorded on a Varian E3 X-band spectrometer equipped with a temperature control unit. The temperature was regulated within 0.5 °C. An aliquot of the sample was introduced in a glass capillary (2 cm length, 0.8 mm internal diameter) and the spectra immediately recorded.

RESULTS

Location of 5-doxyl stearic acid in mixed liposomes

The chain flexibility of stearic acid label molecules incorporated in lipid bilayers can be characterized by the order parameter S which is derived from the spectral data by the relationship:

$$S = \frac{T_{\parallel} - T_{\perp}}{26.3}$$

where $2T_{\perp}$ and $2T_{\parallel}$ represent the inner and the outer hyperfine splittings. The ESR spectra obtained with egg lecithin liposomes at 37 °C and with dimyristoyllecithin at 28 °C are shown in Figs 1A and 1B. They have similar features and are characteristic of highly fluid lipids ($S = 0.586$ and 0.618 respectively). When stearic acid labels are incorporated in dipalmitoyllecithin liposomes at 28°C, this temperature being below the lipid transition temperature (41 °C), the widths of the peaks are broader (Fig. 1C) and the order parameter is much larger ($S = 0.729$).

The method proposed by McConnell et al. [8] for evaluating the fluid fraction of a biological membrane is based on the fundamental assumption that stearic acid labels locate preferentially in the more fluid regions. By studying mixed lipid systems, Butler et al. [13] have shown recently that although this assumption is not entirely correct, it constitutes a good approximation when 12-doxyl stearic acid labels are used. We measured by the same method [13] that 5-doxyl stearic acid is 15–20 times more soluble in dimyristoyllecithin than in dipalmitoyllecithin liposomes at 28°C. The experimental temperature was chosen so that a mixed system containing 36 % dipalmitoyl and 64 % dimyristoyllecithin could be characterized according to the phase diagram reported by Shimshick and McConnell [14] by about 50% fluidity at 28 °C. The corresponding spectrum (Fig. 1D) reveals that most of the label molecules probe a fluid environment ($S = 0.619$).

Stearic acid spin label in native and lipid depleted membranes

The ESR spectrum observed at 37° C with native rod outer segment membranes labeled with 5-doxyl stearic acid is shown in Fig. 2A and compares favorably to that obtained with egg lecithin liposomes (Fig. 1A). However, before attemp-

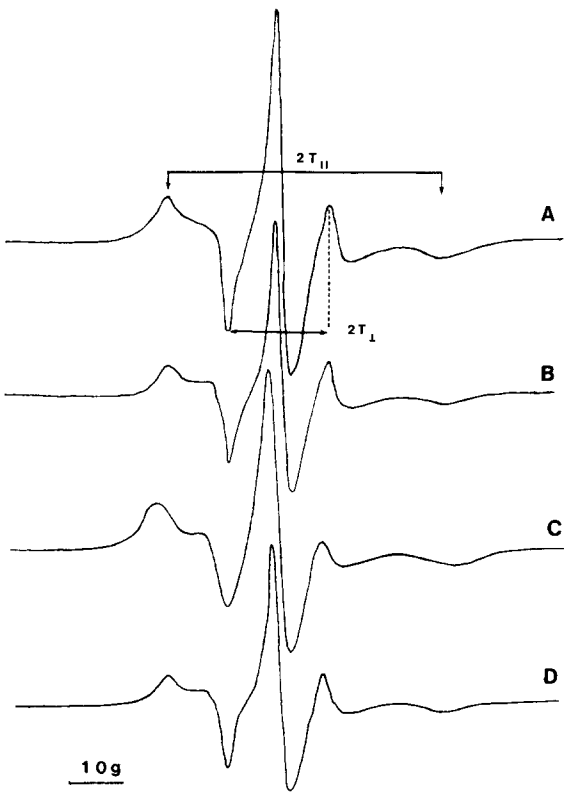

Fig. 1. Electron spin resonance spectra of 5-doxyl stearic acid in: (A) Egg lecithin liposomes (36.4 mg/ml), 37 °C, $S = 0.586$. (B) Dimyristoyllecithin liposomes (55.3 mg/ml), 28 °C, $S = 0.618$. (C) Dipalmitoyllecithin liposomes (53.6 mg/ml), 28 °C, $S = 0.729$. (D) 64 % dimyristoyllecithin, 36 % dipalmitoyllecithin liposomes (38.6 mg/ml), 28 °C, $S = 0.619$. Microwave power: 4 mW. Modulation amplitude: 1 gauss.

ting to evaluate the fluid fraction of these membranes, one must verify that probe binding to rhodopsin molecules is not susceptible of influencing the conclusion. An ideal check would be to analyze the binding capacity of an undenatured lipid depleted protein. Unfortunately, removal of all the phospholipids associated with rhodopsin produces its denaturation [12]. The protein influence was however evaluated as follows: in a first experiment, rhodopsin is completely lipid depleted and the residue is spin labeled. The corresponding spectrum shown in Fig. 2B reveals that the label chain flexibility is highly inhibited; probably because of strong hydrophobic interactions occurring between hydrocarbon chains and apolar amino acids. Because the outer hyperfine splitting is so large, it can be seen that such highly immobilized component is absent in the spectrum of Fig. 2A. In a second kind of experiment, partial lipid removal is carried out. The toluene extraction procedure leaves 20–30 mol phospholipid/mol rhodopsin [12] while hexane extraction leaves about 45 mol phospholipid/mol rhodopsin [11]. The corresponding ESR spectra are shown in Fig. 2C and 2D, respectively. They have very similar features and resemble those which characterize strongly immobilized labels (Fig.

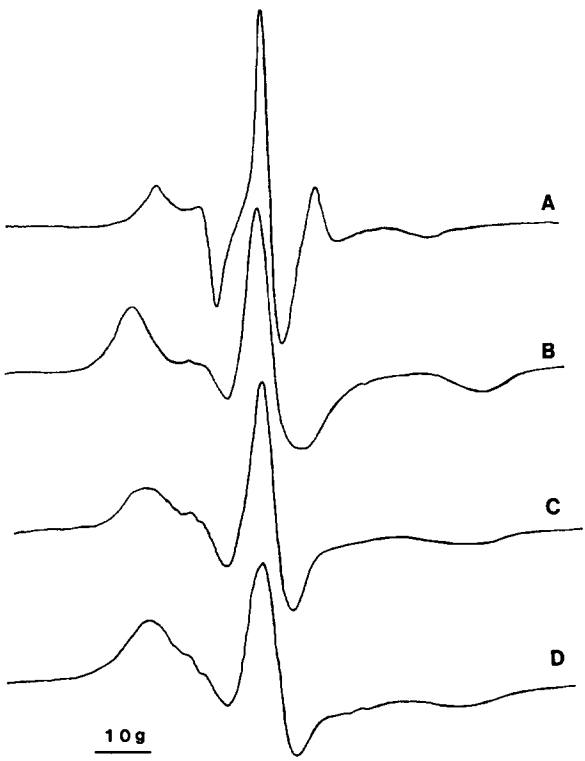

Fig. 2. Electron spin resonance spectra of 5-doxyl stearic acid in: (A) Rod outer segment membrane suspension (45 mg/ml), 37 °C, $S = 0.612$. (B) Chloroform/methanol extracted membranes (16 mg/ml), 37 °C, $S = 0.883$ (estimated value). (C) Toluene extracted membranes (13.7 mg/ml), 37 °C, $S = 0.778$. (D) Hexane extracted membranes (22.7 mg/ml), 37 °C, $S = 0.756$. Microwave power: 4 mW. Modulation amplitude: 1 gauss.

1C). Therefore, although the precise location of the label is difficult to ascertain, it is tempting to suggest from these results that rigid lipids coat rhodopsin molecules.

Relation between fluidity and solubility in lecithin-cholesterol liposomes

The ESR spectrum of TEMPO in an aqueous suspension of lecithin-cholesterol liposomes is composed of two triplets of which only the high field lines are separated. The largest hyperfine splitting corresponding to the most polar solvent, the B component of the spectrum (Fig. 3) is associated with nitroxide molecules in aqueous solution, while the A peak corresponds to free radicals in a hydrophobic environment. Since the widths of the A and B components are different, their amplitudes cannot be taken as a straightforward measurement of the amounts of label in the two types of environment. However, variations of the A/B ratio do reflect modifications occurring in the solubility of TEMPO within the aqueous and lipidic phases. For example, results presented in Fig. 3 show the influence of the lipid concentration on the A/B ratio. The different lines correspond to various lecithin-cholesterol mixtures with cholesterol concentrations ranging from 0 to 18.9% (w/w).

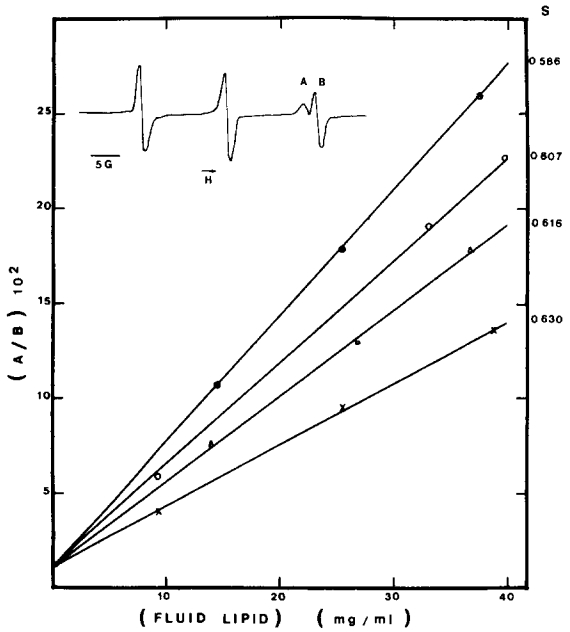

Fig. 3. Plot of the A/B amplitude ratio as a function of the total lipid concentration for various lecithin-chleosterol mixtures. Measurements were performed at 37 °C. Pure egg lecithin $S = 0.586$, 9.1 % cholesterol $S = 0.607$, 13.0 % cholesterol $S = 0.616$, 18.9 % cholesterol $S = 0.630$. Insert: electron spin resonance spectrum of TEMPO in cholesterol-lecithin liposomes. The A and B peaks represent label molecules in hydrophobic and hydrophilic environments, respectively.

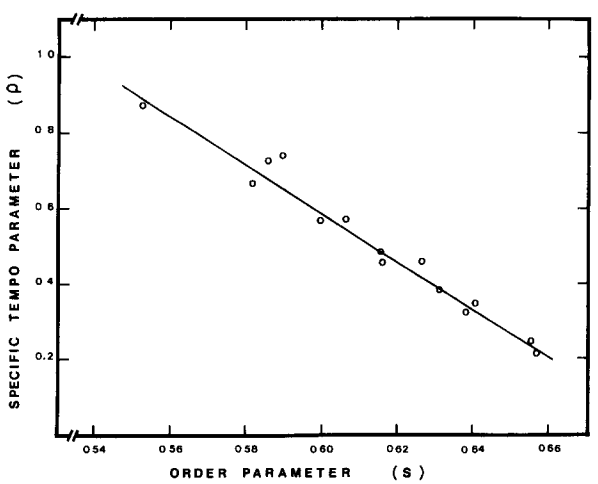

Fig. 4. Plot of the specific TEMPO parameter (ρ) versus the order parameter (S) measured with 5-doxyl stearic acid label in lecithin-cholesterol liposomes. All measurements were performed at 37 °C.

These data have all been obtained at 37 °C, a temperature significantly higher than the phase transition temperature of egg lecithin. The lines do not pass through the origin because of the underlying component due to ^{13}C hyperfine satellite of the peak corresponding to TEMPO in the aqueous phase. In this graph, the slopes of the lines characterize TEMPO solubility. The slope value (ρ) is referred to as the specific TEMPO parameter [8].

By measuring S and ρ at a given temperature, in different egg lecithin-cholesterol mixtures, one can establish a correlation between TEMPO solubility and lipid chain flexibility. The plot in Fig. 4 shows that within the variation range studied, this relationship is virtually linear at 37°C. Similar experiments performed with 12-doxyl stearic acid reveal a hyperbolic relationship between solubility and fluidity (our data and ref. 8). The difference could be related to the variation of the nitroxide position with respect to the lipid polar heads and/or due to the structural arrangement of the phospholipid-cholesterol complex.

Fluid fraction of rod outer segment membranes

Above the phase transition temperature the relationship between S and ρ is at the first approximation independent of the lipid nature [8]. The fluid fraction of a membrane can, therefore, be determined in three steps: (1) by measuring the order parameter S of the membrane, (2) by using the relationship of Fig. 4 in order to deduce a ρ value corresponding to 100% fluidity and (3) by comparing this latter value with the proper solubility of TEMPO in the biological membrane (ρ'). The fluid fraction is then given by the equation:

$$\text{Percent of fluid lipid} = \frac{\rho'}{\rho} \times 100 = \frac{(A/B)' \times 10^2}{(\text{total lipid})' \times \rho} \times 100$$

where (A/B)′ and (total lipid)′ refer to biological membrane data.

Table I lists the results obtained at 37°C with five different preparations. In order to compute the total lipid concentration, we assume that lipids account for 50% of the membrane dry weight (our data and ref. 11). By measuring the (A/B)′ ratio the corresponding ρ' values are obtained. The specific TEMPO parameter which corresponds to 100% fluidity is deduced from the graph shown in Fig. 4. It follows from our measurements that about 66% of the membrane is in a fluid state.

Experiments are performed at 37 °C because at such a temperature the spec-

TABLE I

FLUID FRACTION OF LIPIDS IN ROD OUTER SEGMENT MEMBRANES

Membrane preparation	Membrane concentration (mg/ml)	$(A/B)' \times 10^2$	ρ'	S	Percent fluidity
No. 1	86.4	17.0	0.368	0.615	74
No. 2	40.9	7.7	0.316	0.615	64
No. 3	57.3	10.3	0.317	0.618	66
No. 4	53.9	9.4	0.303	0.614	62
No. 5	23.6	5.9	0.396	0.592	62
					mean: 66±5

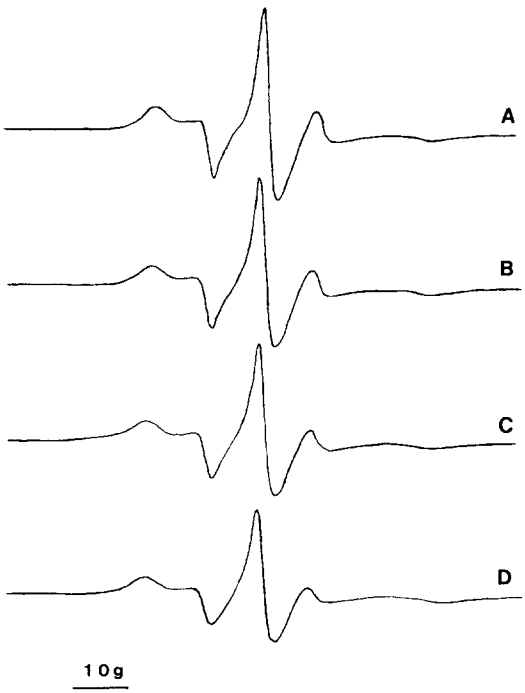

Fig. 5. Electron spin resonance of label 5-doxyl stearic acid in rod outer segment membranes (27 mg/ml): (A) 37 °C, $S = 0.612$; (B) 34 °C, $S = 0.631$; (C) 30 °C, $S = 0.652$; (D) 25 °C, $S = 0.682$. Microwave power: 4 mW. Modulation amplitude: 1 gauss.

trum closely resembles the one of the model system (Fig. 1A). At room temperature (Fig. 5), the lines are broader and it was felt that this broadening could be due to an increase of the label solubility in rigid lipid regions.

DISCUSSION

Apart from the assumptions discussed above, the method used in the present investigation implies that the binding capacity of TEMPO is proportional to the total weight of lipids, irrespective of the lipid nature and thus of the cholesterol-phospholipid ratio. It is assumed that two cholesterol molecules provide roughly the same binding capacity for TEMPO as one lecithin molecule does since the molecular weight ratio of cholesterol and phospholipid is about two [8]. This assumption leads to the conclusion that 66% of the membrane is fluid. However, if it is assumed that cholesterol does not provide a binding site for TEMPO, the reference curve in Fig. 4 has to be shifted upwards and it follows that only 55% of the membrane are in a fluid state.

On the other hand, the method does not take specifically into account lipid-protein interaction. Hong and Hubbell [15] have shown, however, that incorporation of rhodopsin in phospholipid bilayers inhibits the motion of the hydrocarbon chains. Our observations on lipid depleted membranes are in agreement with Hong and Hubbell results.

Although the present method is empirical and implies various assumptions, it gives at least a qualitative approximation. It seems thus reasonable to conclude that the rod outer segment membrane cannot be viewed as a homogeneous highly fluid phase. It has to be stressed that such conclusion can never be reached by using merely one fatty acid spin label since the probe solubilizes preferentially in the most fluid part of the membrane [13]. On the contrary, recent careful investigations tend to generalize the concept of a heterogeneous structure in biological membranes. For example, in mitochondrial membranes, cytochrome oxidase molecules are surrounded by a boundary of immobilized lipids [16]. Stier and Sackman [17] have proposed a model in which the cytochrome *P* 450-cytochrome *P* 450 reductase system is embedded in a quasi-crystalline entity surrounded by a rather fluid environment. Finally, McConnell et al. [8], using the same method as has been used in the present investigation, conclude that 84% of the lipids in rabbit muscle sarcoplasmic reticulum membranes are in a fluid state. This finding is probably related to recent observations which establish that Ca-ATPase in sarcoplasmic reticulum preparations retain their activity only down to 30 lipid molecules per ATPase molecule [18, 19].

A model can be derived from the experimental data presently available concerning the rhodopsin organization within the disks. It is well known that only part of the membrane phospholipids can be extracted with apolar solvents [11]. This already suggests a partition between loosely-bound and strongly-bound lipids. On the other hand, Chen and Hubbell [4] have reported that freeze-fracture studies of rod outer segment membranes reveal the presence of asymmetric 120 Å diameter particles present only on one face of the disks. Since the rhodopsin molecule diameter is estimated to 42 Å [20], the particles observed under the electron microscope may represent lipid-protein aggregates with immobilized lipids constituting a boundary around the rhodopsin molecules. This coating of the membrane protein by rigid lipids gives a satisfactory qualitative interpretation of our experimental results. Moreover, changes which are known to occur in the lipid-protein interaction upon illumination [5, 21], could be responsible for the increase of the membrane fluidity associated with rhodopsin bleaching [6, 7].

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