THE FRACTION OF THE LIPID IN A BIOLOGICAL MEMBRANE THAT IS IN A FLUID STATE: A SPIN LABEL ASSAY

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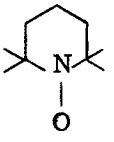
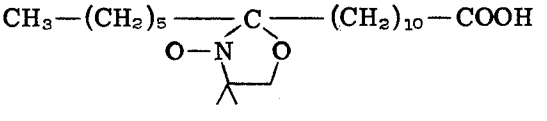
Summary

The solubility of the spin label TEMPO (I) in membranes depends on the fluidity of the membrane. An empirical relationship has been established between TEMPO solubility and fluidity, measured by the order parameter S_{10}. This relationship is employed to determine the fraction of the lipid in membranes of rabbit muscle sarcoplasmic reticulum that is in a fluid state. We find that 84% of the lipid in this membrane is in a fluid state at 25° C.

Many biological membranes exhibit the fluid hydrophobic properties of pure phospholipid bilayers.[1,2] The importance of fluid regions in membranes is demonstrated by the temperature dependence of transport in bacterial membranes, which can be correlated with physical properties of the isolated lipids.[3-6] It is of great interest to know what fraction of the lipid in a biological membrane is in a fluid state. The small spin label molecule TEMPO (I) partitions between aqueous and hydrophobic regions in model and biological membranes much as it does between hexane and water.[7]



(I)



(II)

We describe here a convenient assay, based on this solubility of TEMPO in membranes, for determining the fraction of the lipid that is in a fluid state in a biological membrane.

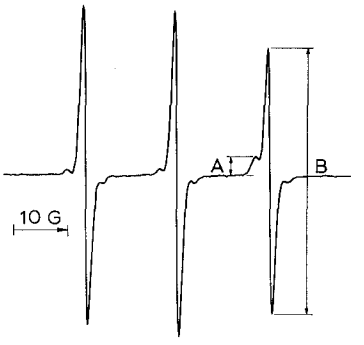


Figure 1. A resonance spectrum of TEMPO (5×10^{-5} M) in an aqueous suspension of cholesterol and lipids extracted from rabbit sarcoplasmic reticulum (10.5% cholesterol w/w). The spectrum was recorded at 25° C and the total lipid concentration was 28.77 mg/ml. A is the height of the resonance line (above the baseline) arising from TEMPO in a fluid hydrophobic region. B is the peak-to-peak height of the derivative line due to TEMPO in aqueous solution.

Our assay for membrane fluidity involves three steps. The first is to obtain the resonance spectrum of TEMPO in the membrane of interest. Figure 1 gives an illustrative spectrum. The high-field line is split, the inner signal (with height A) arising from TEMPO tumbling rapidly in a fluid hydrophobic environment and the outer signal (with height B) arising

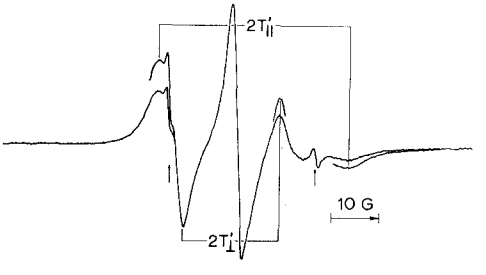


Figure 2. A resonance spectrum of the fatty acid spin label (II) in an aqueous suspension of cholesterol and lipids extracted from sarcoplasmic reticulum (10.5% cholesterol w/w). The spectrum was recorded at 25° and the total lipid concentration was ~20 mg/ml. For order parameter, S_{10}, calculations, $T_{\parallel}'$ and $T_{\perp}'$ are measured as indicated in the figure and values of T_{xx}, T_{yy} and T_{zz} (5.8, 5.8, 31.9 g) are those of Hubbell and McConnell.[1] Vertical arrows indicate signals due to fatty acid label (II) free in solution in equilibrium with bound fatty acid.

from TEMPO in water.[7] The relative intensity of A and B depends on the concentration of membrane lipid present and on the partition coefficient of TEMPO between aqueous solvent and membrane. As shown below, the partition coefficient of TEMPO in bilayer model membranes depends inversely on the order parameter. Therefore, the second step is to determine the order parameter of the membrane lipid using a convenient spin label fatty acid such as (II). A typical spectrum of (II) in membrane lipids is shown in Figure 2. The third step employs an empirical relationship between order parameter and TEMPO partition coefficient to determine the fraction of lipid in the biological membrane that is in a fluid state.

Experimental

TEMPO was synthesized by the method of Rozantsev.[8] For measurements with TEMPO, 8μl of the label in water (5×10^{-3}M) was added to 0.8 ml of lipid (25 mg/ml) to give a final TEMPO concentration of approximately 5×10^{-5}M. The fatty acid spin label (II) was prepared as described elsewhere from 12-ketostearic acid.[9] 12-Ketostearic acid was prepared by oxidation of 12-hydroxystearic acid with nickel-aluminum alloy.[10] Membranes were labeled with (II) by exchange from bovine serum albumin. Membranes (9-10 μmoles phospholipid) added to 0.13 ml of a 2.5% solution of the bovine serum albumin - spin label fatty acid complex (2:1) were incubated for 30 min. at 22° C. The membranes were separated from excess bovine serum albumin complex by three centrifugations from buffer at 120,000 x g for 60 min. Membranes from the sarcoplasmic reticulum of rabbit muscle were prepared[11] and suspended at concentrations of 5 mg to 10 mg of lipid/ml in 0.1N KCl and 0.05N histidine (pH 7.0). Concentrations of lipid were calculated from phosphate analyses[12] and the reported lipid composition of sarcoplasmic reticulum.[13] All resonance spectra were recorded at 25° C.

Results and Discussion

Quantitative analysis of a paramagnetic resonance signal having two components requires knowledge of the heights and widths of the individual lines. However, when the lines are overlapping, as in Figure 1, it is sometimes convenient to calibrate the changes in some spectral parameter as a function of the experimental variables of interest. In our case,

these variables are the concentration of fluid lipid and hydrocarbon chain flexibility.[1, 14] The order parameter, S_{10}, as determined with the spin label (II), is a quantitative measure of chain flexibility, or the amplitude of anisotropic motion in lipid bilayers.[1, 14] This order parameter is defined by the equation,

$$S_{10} = \frac{T_{\parallel}' - T_{\perp}'}{T_{zz} - .5(T_{xx}+T_{yy})} = \frac{T_{\parallel}' - T_{\perp}'}{26.1} \quad (1)$$

where the variables are given in Figure 2 and in the caption.

The amount of TEMPO dissolved in fluid membrane lipid is measured using the "TEMPO parameter," (A/B) x 10^2, as determined from the amplitudes A and B in Figure 1. For calibration of this TEMPO parameter, we have used egg lecithin as well as lipids extracted[15] from sarcoplasmic reticulum in mixtures of varying proportion with cholesterol. The effect of cholesterol is to decrease the amplitude of motion of lipid acyl chains and to increase the order parameter.[16, 17] Plots relating the TEMPO parameter to concentration of fluid lipid, [FLUID LIPID], are shown in Figure 3. For pure lipid bilayers, above the transition temperature of

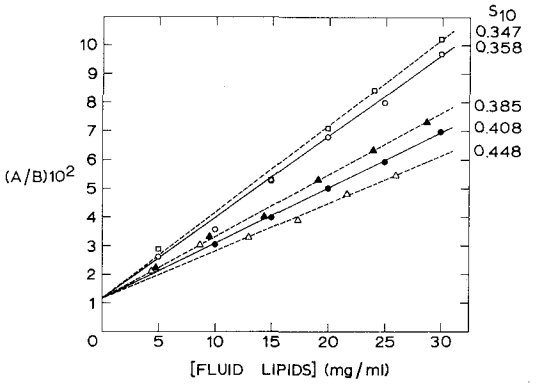


Figure 3. Plots of TEMPO parameter, (A/B) x 10^2 vs. the concentration of total lipid (mg/ml) for representative values of S_{10}. The solid lines correspond to mixtures of cholesterol and egg lecithin. The dashed lines correspond to mixtures of cholesterol and extracted lipids of rabbit muscle sarcoplasmic reticulum. The plots are for cholesterol concentrations (w/w) of 20% (●—●), 16.4% (O—O), 20.9% (Δ---Δ), 10.5% (▲---▲), and 3.0% (□---□).

the lipid, the concentration of fluid lipid is equal to the concentration of total lipid. Each plot in Figure 3 corresponds to a single sample with a characteristic order parameter. The y-intercept of the plots is not zero because the signal of TEMPO in lipid regions overlaps the ^{13}C hyperfine satellite of TEMPO in the aqueous phase. Clearly, there is a linear dependence of TEMPO parameter, $(A/B) \times 10^2$, on concentration of fluid lipid. The slope, "specific TEMPO parameter", (ρ), of each plot is a function of the order parameter and has the simple form

$$\rho = \frac{(A/B)' \times 10^2}{[\text{FLUID LIPID}]} \qquad (2)$$

where $(A/B)' = (A/B)-(A/B)_0$. Here $(A/B)_0$ is the ratio of the two peak heights in the absence of lipid.

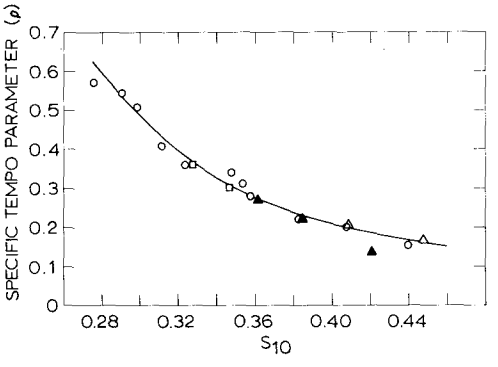


Figure 4. A plot of the specific TEMPO parameter, ρ, vs. the order parameter, S_{10}, for mixtures of cholesterol with egg lecithin (O) and with lipids extracted from sarcoplasmic reticulum (Δ, ▲, and □).

In Figure 4, the specific TEMPO parameter, ρ, is plotted versus the order parameter, S_{10}. The figure includes points for mixtures of cholesterol with lecithin and with extracted lipids of sarcoplasmic reticulum. The curve through these points is the standard curve from which the fluid fraction of a biological membrane may be calculated. By dividing the concentration of fluid lipid, [FLUID LIPID], from Eq. 2, by the concentration of total lipid, [TOTAL LIPID], one obtains

$$\%\ \text{FLUID LIPID} = \frac{(A/B)' \times 10^2}{\rho \times [\text{TOTAL LIPID}]} \cdot 100 \qquad (3)$$

TABLE I

Calculation of the % FLUID LIPID in Membranes of Sarcoplasmic Reticulum

Membrane Preparation	[TOTAL LIPID] (mg/ml)	$(A/B)\cdot 10^2$	$(A/B)'\cdot 10^2$	S_{10}	ρ	[FLUID LIPID] (mg/ml)	%FLUID LIPID
1	26.85	10.05	8.89	.322	.390	22.79	84.9
2	24.99	7.53	6.37	.352	.295	21.59	86.4
3	28.99	9.33	8.17	.331	.356	22.95	79.2
4*	15.74	5.35	4.19	.347	.310	13.52	85.9

* This vesicle preparation actively accumulated calcium. Calcium uptake was measured by a method similar to that of Meissner and Fleischer.[13]

We have applied this technique to membranes of sarcoplasmic reticulum of rabbit muscle. Table I shows the data and calculated % FLUID LIPID for preparations of this membrane. We find an average % FLUID LIPID equal to 84% of the total membrane lipid.

The spectra of (II) show no evidence of heterogeneity with respect to order parameter in membranes of sarcoplasmic reticulum or in pure bilayer lipid membranes. Therefore, we conclude that the lipids that are in a fluid state have a homogeneous fluidity (chain flexibility). This conclusion is consistent with the known rapid lateral diffusion of phospholipids in lecithin bilayers.[18, 19, 20] Our procedure assumes that the solubility of TEMPO in a lipid phase, at a given temperature, depends only on the order parameter of the lipid phase, and not explicitly on lipid composition nor on specific protein-lipid interactions that also affect order parameter.[21] In our opinion, the largest uncertainty in the method arises from experiments involving systems containing a high concentration of cholesterol, since the method assumes that the binding capacity of a homogeneous fluid phase of a given order parameter is proportional to the total weight of lipid, irrespective of the ratio of cholesterol to phospholipid. Since the molecular weight of lecithin is approximately twice that of cholesterol, this approximation involves the assumption that in a homogeneous fluid lipid phase two cholesterol molecules provide roughly the same binding capacity for TEMPO as does one lecithin molecule. A contrary (and to us unreasonable) assumption is that the cholesterol molecules provide no binding capacity for TEMPO in a fluid bilayer. With this second assumption, our above estimate of the fraction of lipid that is in a fluid state in membranes of sarcoplasmic reticulum is high by approximately 10%. The conformity of all the data in Figure 4 to a single curve provides direct support for the assumption that the solubility of TEMPO does depend on the order parameter for lipid mixtures either with or without cholesterol and does not depend strongly on lipid composition. This is particularly true for two data points near $S_{10} = .326$ where one lipid phase (from sarcoplasmic reticulum) contains no cholesterol and the other lipid phase (egg lecithin) contains 12 weight percent of cholesterol.

If a membrane were heterogeneous and had two distinct fluid lipid

phases, the presence of these phases should be revealed by a corresponding heterogeneity in the spectra of the bound fatty acid (II). In this case, a more elaborate analysis would be required. If on the other hand a membrane has two distinct phases, one fluid and the other "frozen", the present method provides a reliable assay for the fraction of lipids in a fluid state, as described in the present work. Preliminary experiments involving egg lecithin and dipalmitoyl lecithin, below the phase-transition temperature of the latter, support the validity of the present approach to heterogeneous systems of this type. Indeed, the present assay method might be used to obtain information about the phase diagrams of lipid mixtures, and offers the possibility of detailed study of phase separations in biological membranes.

Acknowledgements

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