*Biochimica et Biophysica Acta*, 465 (1977) 466—470


BBA 77649

# MAGNETIC RESONANCE STUDY OF THE DISTRIBUTION OF 2,2,6,6,-TETRAMETHYLPIPERIDINE-*N*-OXYL IN PHOSPHATIDYLCHOLINE BILAYERS

LAUREL O. SILLERUD and RONALD E. BARNETT *

*Departments of Physiology and Chemistry, University of Minnesota, Minneapolis, Minn. 55455 (U.S.A.)*

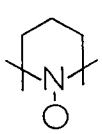
(Received August 21st, 1976)

## Summary

With the aid of paramagnetic praseodymium ions the resonances at 60 MHz of the inward and outward facing choline methyl protons of sonicated egg yolk phosphatidylcholine vesicles were resolved. The subsequent addition of 2,2,6,6,-tetramethylpiperidine-*N*-oxyl (TEMPO) to the vesicle suspension broadened the inner and outer resonances equally. TEMPO easily penetrates the egg yolk phosphatidylcholine vesicles and has free access to the entire lipid volume above the gel to liquid crystalline transition temperature. The electron spin resonance (ESR) spectrum of TEMPO in the egg yolk phosphatidylcholine suspension exhibits features indicating that TEMPO dissolves principally in the hydrocarbon portion of the egg yolk phosphatidylcholine bilayer. The egg yolk phosphatidylcholine methylene chain proton resonances are also broadened by TEMPO notably to a greater extent than the choline methyl resonances. These data indicate that TEMPO should be more sensitive to the melting behavior of the fatty acyl chains of phospholipid suspensions than to the polar head groups.

## Introduction

The stable paramagnetic molecule TEMPO (2,2,6,6,-tetramethylpiperidine-*N*-oxyl) (Scheme I) has recently found application in the study of the thermo-



Scheme I

* Current address: Department of Biochemistry and Nutrition, Virgina Polytechnic Institute and State University, Blacksburg, Virginia 24061, U.S.A.

tropic behavior of biological membranes [1]. The electron spin resonance (ESR) spectrum of TEMPO in the presence of membranes is characterized by a splitting of the high field line of the nitroxide triplet into two lines. The low field member of this doublet arises from TEMPO dissolved in the membrane, while the high field line of the pair arises from TEMPO free in the bulk aqueous phase. Shimshick and McConnell [2] and Wu and McConnell [3] have used the dramatic increase of the intensity of the membrane associated signal of TEMPO at the gel to liquid crystalline transition temperature to measure melting curve for mixed phospholipid systems. It is of interest in this regard to attempt to interpret such curves in terms of partition coefficients for TEMPO between the membranous and aqueous phases. One would like to know if TEMPO has free access to the entire lipid volume or if it senses only a limited portion of the volume occupied by the lipid [4]. It is the purpose of this paper to present evidence indicating that above the gel to liquid crystalline transition temperature TEMPO freely passes through the membrane bilayer and dissolves mainly in the hydrocarbon portion of the bilayer.

## Experimental

Egg yolk phosphatidylcholine was extracted from fresh egg yolks according to the method of Singleton et al. [5], and stored under nitrogen at −20° C. Vesicles were prepared in $^2H_2O$ (Aldrich Inc.) by sonication [6] of a coarse lipid dispersion to clarity with a Branson W185 sonifier operating at 35 watts for 45 min at 4° C. The $^2H_2O$ was previously deoxygenated by bubbling dry argon through it for 30 min prior to use for vesicle formation. Thin-layer chromatography of the egg yolk phosphatidylcholine vesicle suspension was done on Kontes-Quantum LQDF precoated silica gel plates developed with $CHCl_3$ : $CH_3OH$ : $H_2O$ : $CH_3COOH$; (65 : 40 : 2.6 : 10, v/v). The spots were visualized with iodine vapor. No lipid degradation [7] was detected with this method. The vesicle suspension was centrifuged for 20 min, at 10 000 × $g$ at 4° C to remove any small titanium particles introduced during sonication, as well as any undispersed lipid, and was used for the $^1$H-NMR and ESR experiments immediately. Appropriate amounts of praseodymium chloride (P.C.R. Inc.) in $^2H_2O$ were added to an aliquot of the egg yolk phosphatidylcholine vesicle suspension to resolve the inner and outer phosphatidylcholine choline methyl resonances. $^1$H-NMR spectra were recorded at 44° C on a Varian A60D at 60 MHz.

Aliquots of a 50 mM solution of TEMPO in $^2H_2O$ were added to the praseodymium treated egg yolk phosphatidylcholine vesicle suspension and the $^1$H-NMR spectrum of the vesicles was examined for paramagnetic line broadening. The ESR spectrum of TEMPO in the egg yolk phosphatidylcholine vesicle suspension was recorded at 35° C on a Varian EM500 ESR spectrometer operating at 9.5 GHz. TEMPO was synthesized in this laboratory according to the method of Briere et al. [8].

## Results and Discussion

The ESR spectrum of 3 mM TEMPO in the presence of the egg yolk phosphatidylcholine vesicles at a concentration of 180 mg/ml at 35° C showed char-

acteristic splitting of the high field line into two components, labelled H and P. The low field member H of this doublet with a hyperfine splitting of 16.6 ± 0.3 Gauss arises from TEMPO dissolved in the egg yolk phosphatidylcholine while the other line P a higher field with a hyperfine splitting of 18.2 ± 0.3 Gauss arises from TEMPO free in the bulk aqueous ($^2H_2O$) phase. The spectrum of TEMPO in the egg yolk phosphatidylcholine vesicle suspension closely resembles previously published spectra [9]. The hyperfine splitting of H the bilayer associated TEMPO signal is smaller than the splitting for the free solution TEMPO signal P. The hyperfine splitting of nitroxides is known to be sensitive to the polarity of the local environment of the nitroxide moiety (see ref. 4), with a smaller splitting seen in hydrocarbon solvents than in water. This suggests that the membrane TEMPO signal arises from a portion of the bilayer structure that significantly excludes water, i.e., the fatty acyl chain portion of the bilayer. The H signal of TEMPO is only slightly broader than the P signal, implying that the mobility of TEMPO in the egg yolk phosphatidylcholine is only slightly restricted, compared to the aqueous phase.

As reported by Bystrov et al. [10], the addition of paramagnetic lanthanide ions to a phosphatidylcholine bilayer vesicle suspension shifts the resonance of the choline methyl protons that face the outside of the vesicle away from the resonance of the interior facing choline methyl protons. In this study praseodymium was the chosen shift reagent.

One sees in Fig. 1 that the outer resonance was shifted downfield with respect to the relatively unshifted inner resonance at $\delta$ = 3.3 ppm. The titration of shift of the outer resonance downfield from the inner is shown in Fig. 2. The shifts agree with those reported by Andrews et al. [11]. The appearance of two distinct choline methyl resonances of the egg yolk phosphatidylcholine vesicles in the presence of $Pr^{3+}$ gives evidence of the impermeability of the vesicles to ions. The addition of $Pr^{3+}$ to the egg yolk phosphatidylcholine vesicle suspension broadened the outer choline resonances more than the inner resonances.

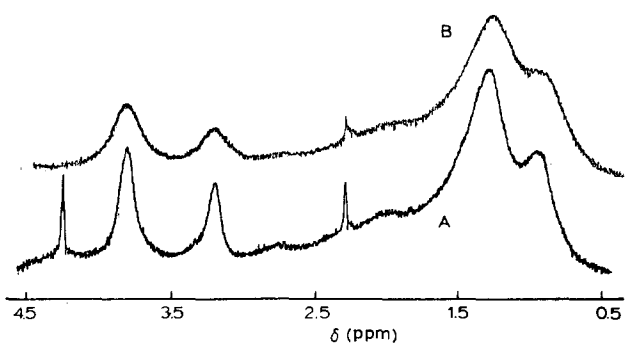
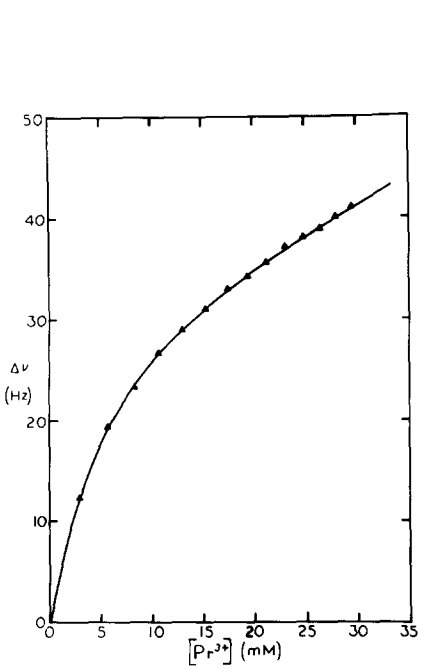



Fig. 1. $^1$H-NMR spectra at 60 MHz of the egg yolk phosphatidylcholine vesicles (170 mg/ml) suspension at 44°C (A) in the presence of 25 mM $PrCl_3$ (B) with 1.0 mM TEMPO added to a sample in (A). The assignments of the peaks as measured from tetramethylsilane are: $\delta$ = 0.9 ppm, the acyl fatty chain terminal methyl group; $\delta$ = 1.3 ppm, the $CH_2$ groups of the fatty acyl chains; $\delta$ = 3.3 ppm, the choline methyl groups on the inside surface of the phosphatidylcholine vesicles (no $Pr^{3+}$ bound); and $\delta$ = 3.8 ppm, the choline methyl groups on the outside surface of the lecithin vesicles ($Pr^{3+}$ bound). The sharp peak at $\delta$ = 2.3 ppm is due to a small amount of contaminating acetone, which was used in purifying the phosphatidylcholine.



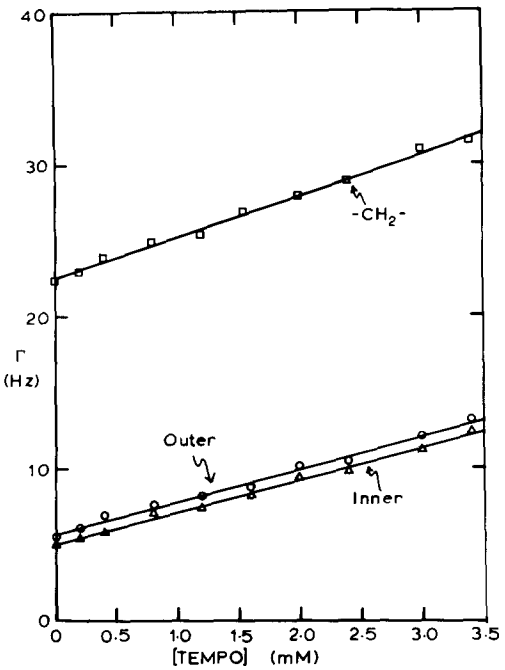




Fig. 2. The $^1$H-NMR chemical shift difference between inner and outer choline methyl resonances of egg yolk phosphatidylcholine vesicles (200 mg/ml) as a function of praseodymium concentration at 60 MHz.

Fig. 3. The $^1$-H-NMR linewidth of egg yolk phosphatidylcholine vesicles (174 mg/ml) in the presence of 13.0 mM praseodymium chloride. —△—, inner choline methyl; —○—, outer choline methyl; —□—, methylene chain; as a function of TEMPO concentration.

This extends the permeability measurements of Blok et al. [12], who found that vesicles formed from synthetic phosphatidylcholine with saturated fatty acyl were impermeable to ions. The egg yolk phosphatidylcholine in this study possessed at least some unsaturated fatty acyl chains as shown by the existence of resonances in the $^1$H-NMR spectrum assignable to olefinic protons.

Integration of the peaks arising from the inner and outer choline methyl resonances gave a ratio $r = 0/I$ of outer to inner cholines of $r = 1.81 \pm 0.03$, corresponding to 64.4% outer and 35.6% inner facing cholines. If one assumes a bilayer thickness of 3.5 nm [13], then these measurements are consistent with a vesicle radius of 14.5 nm.

To study the effects of TEMPO on the $^1$H-NMR spectrum of the egg yolk phosphatidylcholine vesicles $Pr^{3+}$ was added to an aliquot of the egg yolk phosphatidylcholine vesicle suspension to gave a final $Pr^{3+}$ concentration of 13.0 mM. This resolved the inner and outer signals of the egg yolk phosphatidylcholine choline methyl groups and enabled one to examine the effect of TEMPO on each class of choline methyl as well as on the other egg yolk phosphatidylcholine resonances. It can be noted from Fig. 1 that TEMPO broadens all the egg yolk phosphatidylcholine resonances.

The linewidth measured at half height ($\Gamma$) of the inner and outer choline methyl resonances as well as the width of the methylene resonance of the egg yolk phosphatidylcholine vesicles as a function of TEMPO concentration is shown in Fig. 3. The data show that the inner and outer choline methyl reso-

nances are identically broadened by the addition of TEMPO. The paramagnetic effect of TEMPO acting only on the outside of the vesicle would not be expected to broaden the inner resonances since the dipolar field of TEMPO extends only 3.0 nm [14] while the vesicle walls are about 3.5 nm thick. The slopes of the least squares lines fitted to the data are 2.16 ± 0.05 Hz/mM for the inner resonance and 2.12 ± 0.05 Hz/mM for the outer resonance. The methylene chain proton resonances are also broadened by the addition of TEMPO as shown in Fig. 3. The least squares line fitted to these data gives a slope of 2.68 ± 0.07 Hz/mM which is significantly greater than that found for either of the choline methyls.

The finding that TEMPO broadens both inner and outer choline methyl resonances equally suggests that TEMPO can easily penetrate the bilayer since the TEMPO was initially added only on the outside of the egg yolk phosphatidylcholine vesicles. The egg yolk phosphatidylcholine vesicles used in this study show a gel to liquid crystalline transition around −10 to −5°C (Miller, W.G., unpublished observations). TEMPO has free access to the entire lipid volume above the transition temperature as indicated by its broadening of the choline methyl and methylene chain resonances. Dix et al. [4] have assumed this fact in their analysis of ESR partition coefficient data for a closely related nitroxide (di-*t*-butyl-nitroxide) in dipalmitoyl phosphatidylcholine vesicles. The observations reported here support their assumption and enable one to place more confidence in partition coefficient measurements of this type. The finding that TEMPO exerts a stronger effect on the methylene chain resonances than on the choline methyl resonances is consistent with the ESR result that TEMPO preferentially partitions into the hydrocarbon portion of the egg yolk phosphatidylcholine bilayer.

## Acknowledgement

This work was supported in part by NIH Grants GM18152 and 1-P01-CA162280.

## References

1 Fox, C.F. (1975) in Biochemistry of Cell Walls and Membranes, MTP International Review of Science (Fox, C.F., ed.), Vol. 2, pp. 279—306, University Park Press, Baltimore
2 Shimshick, E.J. and McConnell, H.M. (1973) Biochemistry 12, 2351—2360
3 Wu, S.H. and McConnell, H.M. (1975) Biochemistry 14, 847—854
4 Dix, J.A., Diamond, J.M. and Kivelson, D. (1974) Proc. Natl. Acad. Sci. U.S. 71, 474—478
5 Singleton, W.S., Gray, M.S., Brown, M.L. and White, J.L. (1965) J. Am. Oil Chem. Soc. 42, 53—56
6 Huang, C. (1969) Biochemistry 8, 344—352
7 Hauser, H.O. (1971) Biophys. Biochem. Res. Commun. 45, 1049—1055
8 Briere, R., Lemare, H. and Rassat, A. (1965) Bull. Chim. Soc. Fr. 3273—3283
9 Tomkiewicz, M. and Corker, G.A. (1975) Biochim. Biophys. Acta 406, 197—205
10 Bystrov, V.F., Dubrovina, N.I., Barsulkov, L.I. and Bergelson, L.D. (1971) Chem. Phys. Lipids 3, 343—350
11 Andrews, S.B., Faller, J.W., Gilliam, J.M. and Barnett, R.J. (1973) Proc. Natl. Acad. Sci. U.S. 70, 1814—1818
12 Blok, M.C., van der Neut-Kok, E.C., van Deenan, L.L.M. and de Gier, J. (1975) Biochim. Biophys. Acta 406, 187—196
13 de Kruijff, B., Cullis, P.R. and Radda, G.K. (1975) Biochim. Biophys. Acta 406, 6—20
14 Levine, Y.K., Lee, A.G., Birdall, N.J.M., Metcalfe, J.C. and Robinson, J.D. (1973) Biochim. Biophys. Acta 291, 592—607