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A Water-Soluble Spin Probe Newly Developed for Liposomal Studies

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A water-soluble spin probe, disodium 4-[(4,8-sulfonatooxy-2-naphthyl)thioureylene]-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-T) was newly synthesized and characterized comparing with several different TEMPO derivatives from the view point of a possibility of utilization in the liposomal studies. Of TEMPO derivatives studied, TEMPO-T was the best probe because of the high water solubility, the less lipophilicity, the less leakiness from liposome, and the reliable stability to both temperature and pH changes.

Electron spin resonance (ESR) is extremely useful in the studies of membrane dynamics and microenvironment of the reaction field such as microviscosity or micropolarity. Typically, stable paramagnetic labels containing a nitroxide radical such as 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) are used. Different from other methodologies such as fluorescence and absorption spectrometries, in addition, the spin probe method is relatively convenient in the biological studies because transparency of the sample is not basic requirement and the technique is very sensitive, typically requiring only about 10^{-6} M (1 M=1 mol dm^{-3}) spins in a very small amount of the sample. Therefore, many informations about membrane dynamics such as fusion,[1] fluidity,[2] lateral diffusion,[3] or flip-flop[4] of lipid membranes have been drawn using spin probes which are derivatives of fatty acids or phospholipids. Nevertheless, there are few examples in which water-soluble spin probes are employed in the biological studies.[5–7] McConnell has investigated the phase transition of lipid membrane based on the principle that the partition of TEMPO to lipid membrane from aqueous phase increases in the liquid crystal phase of the membrane.[5] Morse and his co-workers studied the microviscosity of cytosol of erythrocyte using 4-amino-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-N).[6,7] Unfortunately, however, TEMPO and its several derivatives are somewhat amphiphilic and they sometimes distribute into lipid membrane due to the microenvironment around the probe.[5] In order to overcome this problem, in this work, we newly synthesized a water soluble spin probe, disodium 4-[(4,8-sulfonatooxy-2-naphthyl)thioureylene]-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-T), and characterized it comparing with several different TEMPO derivatives from the viewpoint of a possibility of utilization in the liposomal studies.

As expected, of six spin probes, TEMPO, TEMPO-OH (4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxyl), TEMPO-N, TEMPO-P (disodium 4-phosphonatooxy-2,2,6,6-tetramethyl-1-piperidinyloxyl), TEMPO-S (sodium 4-sulfonatooxy-2,2,6,6-tetramethyl-1-piperidinyloxyl), and TEMPO-T, employed in this work (Fig. 1), TEMPO-T was the best because of the high water solubility, the less lipophilicity, the less leakiness from liposome, and the reliable stability to both temperature and pH changes.

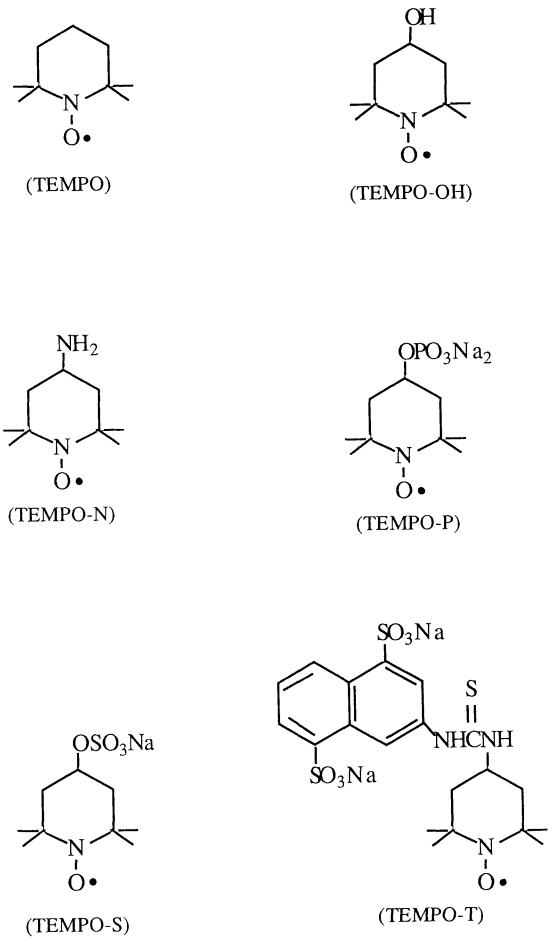

Fig. 1. Structures of TEMPO and TEMPO derivatives employed in this work.

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Materials and Methods

4-Amino-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-N) (Wako Pure Chemicals, Tokyo), 4-hydroxy-2,2,6,6-tetramethylpiperidine (Aldrich, Milwaukee), 2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO) (Aldrich, Milwaukee), 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-OH) (Aldrich, Milwaukee), 6-carboxyfluorescein (CF) (Wako Pure Chemicals, Tokyo), Fluorescein diacetate (FDA) (Funakoshi, Tokyo), disodium 3-isothiocyanato-1,5-naphthalenedisulfonate (Funakoshi, Tokyo) all were commercially available and purified by recrystallization and/or chromatographic technique prior to the use if necessary. Other organic and inorganic reagents were also commercially obtained as analytical grade and used without further purification.

Measurements. IR was measured on a JASCO IRA-100 and NMR was on a JEOL JNM-GX-400. Hydrodynamic diameter of liposomes was measured on a Photal DLS-700 (Otsuka Electronics Co., Osaka). FAB Mass spectrometry was kindly made at the Center of Instrumental Analysis, Faculty of Pharmaceutical Science, Nagasaki University.

Synthesis of Sodium 4-Sulfonatooxy-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-S). Although TEMPO-S has been first synthesized by Keith et al.,[8] they have not described the detailed procedures for preparation and its identification. They obtained TEMPO-S by direct sulfonation of TEMPO-OH using chlorosulfuric acid and did not perform satisfactory purification and identification at all.[8] In this work, therefore, we first synthesized sodium 2,2,6,6-tetramethyl-4-piperidinyl sulfate[9] and then oxidized it by hydrogen peroxide under the mild conditions[10] to give TEMPO-S.

To 4-hydroxy-2,2,6,6-tetramethylpiperidine (0.5 g, 3.1×10^{-3} mol) dissolved in 20.0 ml of chloroform in a 100 ml vol flask was added chlorosulfuric acid (0.4 g, 3.4×10^{-3}mol) within 10 min under stirring on an ice bath. After reacted for another 1 h, crystalline mass obtained was filtrated and washed by hexane and chloroform to give 2,2,6,6-tetramethyl-4-piperidinyl hydrogensulfate (**1**): Yield, 377.0 mg (32%). IR (KBr disk) ν_{SO_2}, 1210 and 1390 cm^{-1}; ^1H NMR (DMSO-d_6 with TMS) δ=4.40 (1H, m, *H*(C4)), 1.89 (2H, q, *H*(C3) and *H*(C5)), 1.08 (6H, s, C*H*$_3$(C2 and C6)), 1.01 (6H, s, C*H*$_3$(C2 and C6)), and 0.95 (2H, t, *H*(C3 and C5)).

To a 200.0 ml aqueous alkaline solution containing 350.0 mg (1.3×10^{-3} mol) of **1** and 10.0 mg of sodium tungstophosphate were added 2.0 ml of 35% aqueous H_2O_2 solution and the resulting mixture was reacted under stirring for 3 d at room temperature. After lyophilization of the reaction mixture, the solid mass obtained was dissolved in methanol. After methanol insoluble materials were eliminated by filtration, the methanolic filtrates were concentrated in vacuo up to 1.0 ml and submitted to a column chromatography on a LH-20 (ϕ 3.0×85.0 cm) developed by methanol to give yellowish crystals of TEMPO-S: Yield, 238.0 mg (27%). IR (neat) ν_{SO_2}, 1380 and 1220 cm^{-1} and ν_{NO}, 970 cm^{-1}. FAB Mass (in methanol) Found: *m/z*, 276 (M+2). Calcd for $NaC_9H_{16}O_5NS+H^+$: M, 274.289. TLC (silica gel) developed with acetic acid : 1-butanol : water=1 : 2 : 2, by vol and detected by I_2-vapor: R_f=0.6, one spot.

Synthesis of Disodium 4-Phosphonatooxy-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-P).[11] 2-Cyanoethyl dihydrogenphosphate was obtained by cation exchange of barium 2-cyanoethyl phosphate (1.4 g, 4.0 mmol) with 50.0 ml of swelled Dowex 50W-X8 resin under stirring at room temperature for few minutes. After separation of the ion-exchange resin by filtration, the filtrates and washings were combined and lyophilized. To the oily materials so obtained were added 4.0 ml of dry pyridine and 172.0 mg (1.0 mmol) of TEMPO-OH. The reaction mixture was then evaporated in vacuo. In order to completely remove water from the reaction mixture, the same procedure was repeated four times. After the final addition of 5.0 ml of dry pyridine, 1.5 g of dicyclohexylcarbodiimide (DCC) was added to it and the resulting mixture was kept for 3 d at room temperature. Urea precipitated was separated by filtration 1 h after the addition of 2.0 ml of water in order to terminate the reaction. Sodium hydroxide was added to make the filtrates alkaline (to be approximately 0.4 M NaOH) and the alkaline solution was refluxed for 1 h in order to completely hydrolyze the 2-cyanoethyl moiety. After evaporation of the solvent, yellowish reaction products were dissolved in methanol, filtrated to remove a small amount of insoluble materials, concentrated up to 1.0 ml, and submitted to a LH-20 column chromatography (ϕ 3.0×85.0 cm). From the effluent yellowish crystals of TEMPO-P were obtained: Yield, 190.0 mg (62%). TLC (silica gel) developed with acetic acid : 1-butanol : water=1 : 2 : 2 by vol and detected by I_2-vapor: R_f=0.65. IR (neat): $\nu_{P=O}$, 1130 and 1070 cm^{-1}.

Synthesis of Disodium 4-[(4,8-Sulfonatooxy-2-naphthyl)-thioureylene]-2,2,6,6-tetramethyl-1-piperidinyloxyl (TEMPO-T). A mixture of 100.0 mg (0.6 mmol) of TEMPO-N and 468.0 mg (1.2 mmol) of disodium 3-isothiocyanato-1,5-naphthalenedisulfonate dissolved in 50.0 ml of methanol in a 100 ml vol flask equipped with a reflux condenser was refluxed for 3 h.[12] After removing methanol in vacuo using a rotary evaporator, yellowish crude products obtained were submitted to a LH-20 column chromatography to give pure TEMPO-T: Yield, 80.0 mg (23%). TLC (silica gel) developed with 25% aqueous ammonia : ethanol=23 : 77 (by vol) and detected by I_2-vapor: R_f=0.8. IR (neat): $\nu_{S=O}$, 1200 and 1040 cm^{-1}. FAB Mass (in water) Found: *m/z* 518 (M+2). Calcd for $C_{20}H_{25}O_7N_3S_3+H^+$: M, 516.093.

Preparation of Liposomes (SUV, MLV, and REV). Egg phosphatidylcholine (egg PC) (15.0 mg, 19.5 μmol) or a mixture of dimyristoylphosphatidylcholine (DMPC) (23.3 mg, 34.5 μmol) and cholesterol (6.7 mg, 17.3 μmol) was dissolved in 2.0 ml of chloroform in a 50 ml vol round-bottomed flask. After evaporation of chloroform in vacuo using a rotary evaporator, the thin lipid film formed in the flask was kept overnight in a vaccum desiccator. To the thin film were added 1.0—5.0 mM of a spin probe dissolved in 2.0 ml of 20 mM Tris-HCl buffer containing 200.0 mM NaCl (pH 7.4). The mixture was shaked on a Vortex mixer and then sonified using a probe-type sonifier (Tomy UD-201, Tokyo) for 10 min at 40 W and temperature 10.0 °C higher than the phase-transition temperature of the lipid employed. A liposomal suspension so obtained was gel-filtrated on a Sepharose 4B column (ϕ 1.8×30.0 cm) using the same buffer and multilamellar vesicles (MLV) and small unilamellar vesicles (SUV) were separated from untrapped spin probe. Large oligolamellar vesicles (REV) were prepared by reversed—phase evaporation method.[13] For example, a mixture of 30.0 mg (38.9 μmol) of egg PC or 23.3 mg (34.5 μmol) of DMPC and 6.7 mg (17.3 μmol) of cholesterol was placed in a 50 ml vol round bottom flask and dissolved in 2.0 ml of chloroform. A thin lipid film formed by removal of the solvent in vacuo using a rotary

evaporator was kept overnight in a vaccum desiccator. To this thin film were added 3.0 ml of diethyl ether and 1.0 ml of 5.0 mM of a spin probe in a 20 mM Tris-HCl buffered solution. The resulting mixture was sonified for 20 min at 40.0 °C on a bath-type sonifier (Branson 2200) and diethyl ether was removed under 350 mmHg (1 mmHg=133.322 Pa) using a rotary evaporator. Then, another 3.0 ml of the same buffer solution was added to it and diethyl ether was further removed under 700 mmHg. Finally, the rest of diethyl ether was completely removed off by bubbling gaseous nitrogen. Untrapped free spin probes were separated by centrifugation (30000×g). The concentration of liposome was determined as lipid concentration using an Assay Kit Clinical (Phospholipid-Test Wako, Wako Pure Chemicals, Tokyo). Particle sizes of liposomes were measured by dynamic light scattering method.

The method for studies of spin probe released from liposome was the following. For example, a lipid thin film formed from egg PC (30.0 mg) according to the established method (vide supra) was swelled by 4.0 ml of 1.0 mM spin probe in 20.0 mM Tris-HCl buffer containing 200.0 mM NaCl (pH 7.4) and further sonified at 0 °C for 15 min under nitrogen atmosphere to give a liposomal suspension. To 500.0 μl of the liposomal suspension so obtained were added 200.0 μl of 0.35 M sodium ascorbate in the same Tris-HCl buffer and submitted to ESR measurement at 37.0 °C (vide infra). In this method, the separation of free spin probes uncapsulated in liposome was not necessary because free spin probes being at exterior of liposome were completely quenched by ascorbate anion added.

ESR Measurements. Electron spin resonanse (ESR) was measured on a JEOL JES-FX1XG equipped with a thermo control unit, JEOL ES-DVT using a 1.0 mm thickness flat cell (JEOL ES-LC11). When A-tensor of di-t-butyl nitroxide radical[14] is temporarily employed instead of that of TEMPO, because the A-tensor of TEMPO has not been reported yet, data obtained are analyzed by following equations:[15]

$$\tau_c^{(m^2)} = 7.1\times10^{-10}\,\delta H_{(0)}[R_{(-1)}+R_{(+1)}-2], \quad (1)$$

$$R_{(m)}=\delta H_{(m)}/\delta H_{(0)}=\{I_{(0)}/I_{(m)}\}^{1/2} \quad (2)$$

$m=+1, 0, -1$ (nucleus spin quantum number of nitrogen atom),

$$a^N=1/3(A_{\parallel}+2A_{\perp}), \quad (3)$$

where $\delta H_{(m)}$ and $I_{(m)}$ stand for line widths and intensities of the triplet absorption of the nitroxide radical at the lowest (m=+1), the central (m=0), and the highest magnetic fields (m=−1). The hyperfine coupling constant (hfc), a^N, is obtained using Eq. 3 from observed $A_{\parallel}$ and $A_{\perp}$.

Results and Discussion

Temperature Effect on Radical Stability. For all the spin probes used in this work, the stability of the nitroxide radical was investigated at 37.0 and 95.0 °C. No decay of the peak intensity was observed even after heating an aqueous sample solution up to 95.0 °C. All the spin probes employed were very stable even at the higher temperature.

pH Effect on Radical Stability. Since nitroxide radical is known to be usually quenched by a strong acid,[16] the radical stability was investigated over a wide range of pH. TEMPO-T was rather stable over a range of pH 3.0—13.0 and quenched approximately 50% at pH 2.0 and almost quantitatively at pH 1.0. For other spin probes, they showed a very similar trend in the stability. They were rather stable over a range of pH 2.0—12.0 and approximately 25% of the radicals were destroyed at pH 1.0 and 20% at pH 13.0.

Effect of Microviscosity around Spin Probe. Nitroxide radical is sensitive to the viscosity of microenvironment around the probe.[15] For TEMPO and its five derivatives, the sensitivity to microviscosity around the probes was investigated by measuring the rotational correlation time (τ_c) of 0.2 mM spin probe in 0—20% (by wt) aqueous glycerol solutions: Namely, as a function of viscosity of the media: $\tau_c=4\pi r^3\eta/3\kappa T$. All the spin probes used showed a reliable linear correlation between τ_c and the solvent viscosity, η, (Fig. 2).

TEMPO-T was most sensitive to the viscosity change (Fig. 2) and seemed to be a better probe for measurement of the microviscosity.

Effect of Solvent Polarity on Hyperfine Coupling Constant (hfc). Unpaired electron density on nitrogen atom increases in a polar solvent, because of an increase in the polarization of the N-O bond: $>$:N–O$\cdot \longleftrightarrow >\cdot N^+$–O:$^-$.[17] This leads to an increase in the hfc (a^N). The hfc-values (hfc/mT) of the spin probes (0.2 mM) in various acetone-water solutions were measured. For all the spin probes studied, the hfc-values linearly increased with an increase in the solvent polarity as indicated by simple acetone content. The result of TEMPO-T is shown in Fig. 3 as an example. Other

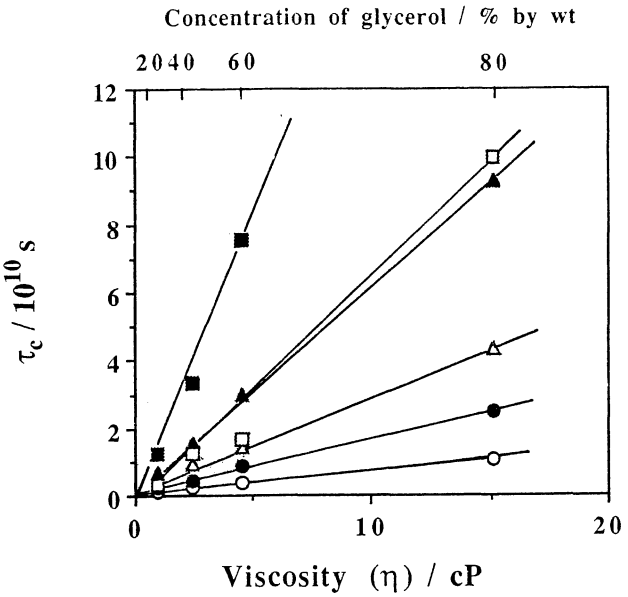

Fig. 2. Correlation between microviscosity (η) and the rotational correlation time (τ_c) of the spin probes (0.2 mM) in an aqueous glycerol solution at 37.0 °C.
—○—; TEMPO, —●—; TEMPO-OH, —△—; TEMPO-N, —▲—; TEMPO-P, —□—; TEMPO-S, and —■—; TEMPO-T.

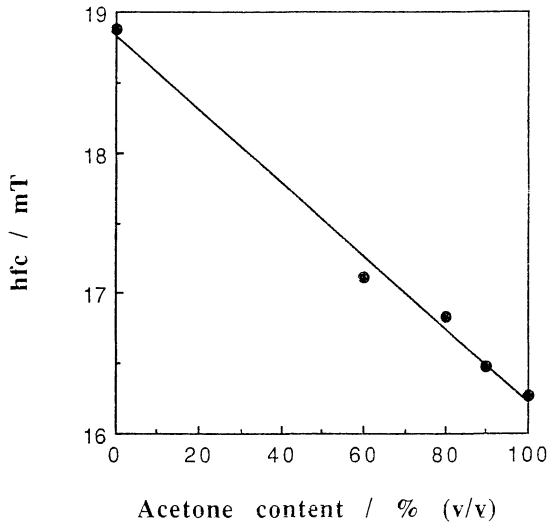

Fig. 3. The hfc-values (hfc/mT, 1 gauss=0.1 mT) of the TEMPO-T (0.2 mM) in various acetone-water solutions at 37.0 °C.

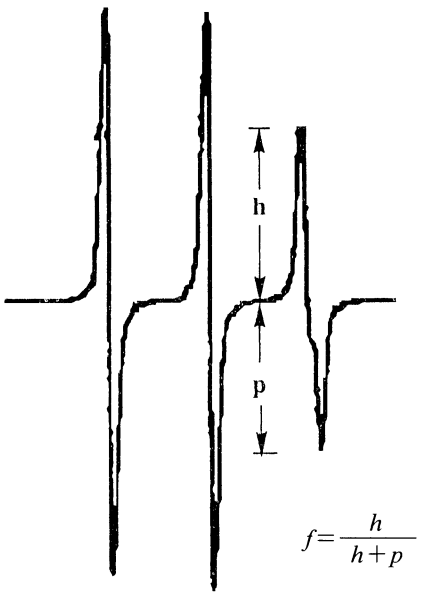

Fig. 4. ESR spectrum of TEMPO (0.5 mM) in the presence of egg PC liposome (details, see text).

spin probes also gave almost similar results and lie on the same line (though data are not shown). However, all other spin probes always gave smaller hfc-values than TEMPO-T. This means that, in the case of TEMPO-T, the N–O bond is more largely polarized and the odd electron density on nitrogen atom of the piperidine ring is higher compared with the cases of other spin probes.

Distribution of Spin Probes into 1-Octanol and Egg PC Liposomal Bilayer. TEMPO itself is somewhat amphiphilic and distributes into liposomal membrane to some extent.[5] This occasionally gives a confusion when one wishes to draw information about microenvironment in a microscopically heterogeneous system. In order to understand the amphiphilicity of these spin probes, the partition of these probes to 1-octanol was investigated and compared with that to egg PC liposomal membrane. 1-Octanol (2.0 ml) was mixed with the same volume of 0.2 mM spin probe in 20 mM Tris-HCl buffer under stirring. From 1-octanol layer, 1.0 ml of a sample solution was pipetted out and submitted to ESR in order to determine the spin concentration distributed into organic layer. Relative partition coefficients of the probes to that of TEMPO as control are summarized in Table 1 along with results in the partition of these probes into lipid bilayer membrane (vide infra).

In an aqueous medium, nitroxide radical gives larger hfc-value (a^N) and smaller g-value, while in a lipid membrane the radical gives smaller hfc- and larger g-values. Therefore, ESR spectra of the two species of the same probe which is partitioned in two different environments, an aqueous phase and lipid bilayer, are superimposed, and the partition coefficient (f) of the probe in lipid bilayer can be roughly estimated from the intensity ratio of the two peaks appearing at the highest magnetic field (Fig. 4): $f=h/(h+p)$, where the intensity "h" corresponds to the spin concentration as distributed to lipid bilayer, while the intensity "p" is the spin concentration as remained in an aqueous medium.[5,18] Restrictively, "f" does not reflect the real molar ratio of the two lipid species because the molecular motion of the species are different in the different environment.[19]

Table 1. Partition of Spin Probes to 1-Octanol and Egg PC Liposomal Membrane

Probe	pH	Relative partition coefficient	
		1-Octanol	Egg PC
TEMPO	7.4	1.000	1.000
TEMPO-OH	7.4	0.890	0.163
TEMPO-S	7.4	0.006	0.020
TEMPO-N	7.4	0.087	0.086
TEMPO-N	11.0	0.721	0.131
TEMPO-P	3.0	0.023	0.024
TEMPO-P	7.4	0.002	0.006
TEMPO-T	7.4	0.006	0.024

Lipid thin film, which was prepared from 75.0 mg of egg PC according to the method as described in Materials and Methods, was swelled with 1.0 ml of 0.5 mM spin probe in 20.0 mM Tris-HCl buffer containing 200.0 mM NaCl. The liposomal suspension so obtained was incubated for 10 min at 37.0 °C prior to the ESR measurement. Results are listed in Table 1 as relative partition coefficient to that of TEMPO (1.0).

Compared with TEMPO, all the water soluble TEMPO derivatives showed much less partition to lipid bilayer. This trend was almost comparable to that to 1-octanol. The solubilities of TEMPO-N and -P were affected by pH of the medium. As the result, the substitution of sulfonate or sulfate group leads to a significant decrease in the partition of the probe into lipid bilayer membrane.

Spin-Spin Exchange Broadening and Narrowing of Nitroxide Radical. A sharp triplet signal of nitroxide radical having free molecular motion at the lower concentration is broadened by spin-spin exchange broadening mechanisms at the higher concentration or in an environment where the molecular motion of the radical is restricted. In a solid state, a rapid spin-spin exchange occurs and gives a narrow absorption peak with a Lorentzian curve by the exchange narrowing mechanisms. In order to know what is the lowest concentraion to give "motional narrowing" for TEMPO-T, ESR was measured in 0.5 mM MES (sodium 2-morpholinoethanesulfonate) buffer containing 200.0 mM NaCl over a concentration range of 0.2—400.0 mM. For TEMPO-T, the exchange broadening of the triplet was observed at concentrations above 20.0 mM and the peak completely became a broadened singlet with Lorentzian mode at 400.0 mM. For TEMPO-T, the desirable concentration to escape the spin-exchange broadening was below 5.0 mM (Fig. 5-B). In the case of TEMPO-S, the lowest concentration to escape the spin exchange broadening was below 2.0 mM and the highest concentration to give a singlet due to spin-spin exchange narrowing was approximately 200.0 mM (Fig. 5-A).

Encapsulation in and Release from Liposome. In order to study the leakiness of these spin probes from liposome, the probes were encapsulated in several kinds of liposomes and their release was followed at 37.0 °C by monitoring quench of the released nitroxide radical by a radical scavenger, ascorbate anion, existing at exterior of liposome. It was certified in advance that no ascorbate anion permeates into liposome under the conditions employed. The reaction rate between nitroxide radical and ascorbate anion also was determined in advance using a rapid flow ESR technique and found to be k=0.825 s^{-1} and $t_{1/2}$=0.84 s. These values suggest us that the rate of radical quench is much faster than that of the spin probe release and the spin probe released from liposome is instantaneously quenched by the quencher at exterior of liposome. Regarding egg PC liposome, for the spin probes besides TEMPO-T, more than 50% of the encapsulated probe spontaneously leaked out within 15 min from the liposome. Even in the case of DPPC (dipalmitoylphosphatidylcholine) liposome, which is considered to be more rigid and less permeable than egg PC liposome, 50% of TEMPO-S leaked within 55 min under the same conditions. The sequence of leakiness was TEMPO>TEMPO-OH ≫TEMPO-N>TEMPO-S≈TEMPO-P. Different from these probes, TEMPO-T did not leak out at all under the same conditions even from egg PC liposome as well as DPPC liposome. Nevertheless the extent of partitioning of TEMPO-S and -P to 1-octanol and egg PC liposomal membrane was almost comparable to or less than that of TEMPO-T (Table 1), TEMPO-T was less leaky from liposomes compared with both TEMPO-S and -P. This less leakiness of TEMPO-T from liposome should be ascribed to its larger molecular size compared with the other two probes.

Because TEMPO-T was found to be a good candidate of the probes to use for liposomal studies, we tried to determine captured volume[22] of several liposomes using TEMPO-T. In order to completely quench nitroxide radical uncapsulated, 50.0 μl of 0.1 M aqueous ascorbate solution were added to a 1.0 ml liposomal suspension. By determining the spin concentration of

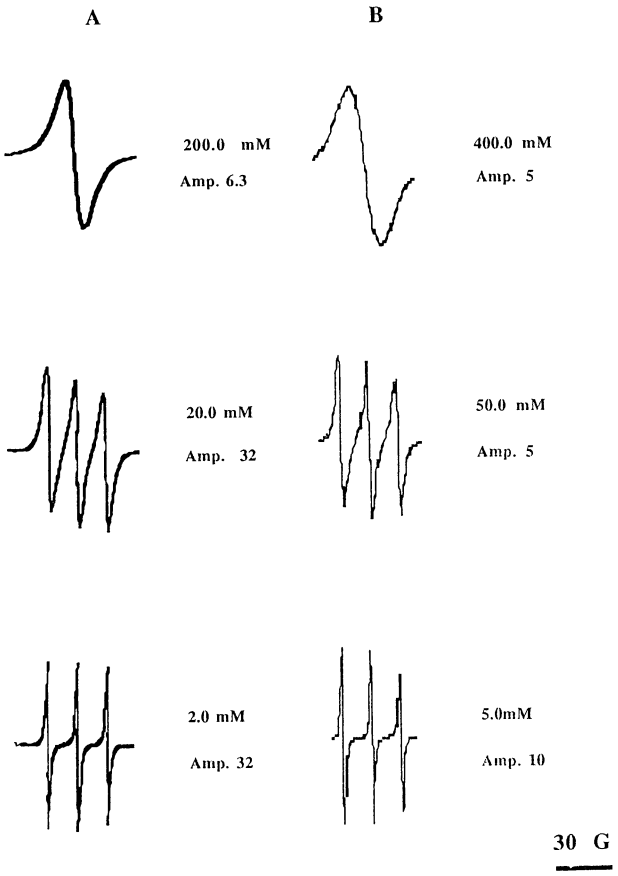

Fig. 5. ESR spectra of various concentrations of TEMPO-S (A) and TEMPO-T (B) at 37.0 °C.

Table 2. Captured Volume Determined by Spin Probe Method Using TEMPO-T

Liposome	Captured volme/μl/μg		Particle size
	Obsd	Calcd[a] (Number of layer)	nm
Egg PC[b]	1.02	1.03 (5) 1.02 (6)	117.8
Egg PC[b]	1.47		176.1
Egg PC REV	7.80	9.24 (2) 6.05 (3)	452.7
DMPC/Chol[b]	3.35	3.96 (1) 3.06 (2)	109.6
DMPC/Chol[b]	1.73		126.5
DMPC/Chol REV	5.99	7.35 (2) 4.94 (3)	367.8

a) Calculated captured-volume was obtained using particle size observed. b) Different fraction in gel chromatograpy for the same liposome preparation (details, see text).

TEMPO-T, the spin trap efficiency (namely, the captured volume of liposome) was calculated.[22] Results obtained are listed in Table 2. Both particle size and captured volume of liposomes observed using TEMPO-T were reasonable.

Conclusion

TEMPO-T which was newly synthesized in this work was a good spin probe as expected. The water solubility and lipophilicity of TEMPO-T were almost comparable to TEMPO-S, but the leakiness from liposomes was much less than TEMPO-S. This could be ascribed to the bulkiness of the molecule. TEMPO-T was, in addition, very stable over a wide range of pH 3.0—13.0 and even in boiling water. Using these advantages of TEMPO-T, captured volume of several liposomes could be determined by spin probe method. For some cellular systems, TEMPO-T may be useful probe for monitoring microscopic viscosity and/or polarity of cytosol and/or endosome.

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