the absence of rapid chemical exchange between Lewis acid–base complexed and physisorbed TMP at both loading levels; i.e., distinct peaks are observed, especially for the higher loading sample. Hence, the change in the position of the peak maximum and the apparent peak width in the single-pulse spectra of TMP on γ-alumina must originate in both the distribution of chemical shifts within the Lewis complex manifold and the changing ratio of Lewis complexed to physisorbed phosphines in this system.

Only the Brønsted acid sites of silica–alumina can be assayed quantitatively in the present study, and this result of the Brønsted site assay is dependent upon the choice of probe molecule. A more extensive array of more rigid probes might allow a mapping of the steric constraints of these sites. This would require detailed consideration of the variation in Brønsted binding constants arising from the changes in phosphine substituents and the range of topographical environments on the surface. A more direct approach to characterizing both Brønsted and Lewis sites would be to search for a probe with greater differences in chemical shift and binding constants among the three types of surface species. Preliminary studies indicate that phosphine oxides may offer such advantages.[74]

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Registry No. TMP, 594-09-2; TEP, 554-70-1; TBP, 998-40-3; SiO_2, 7631-86-9; Al_2O_3, 1344-28-1.

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Orientation and Mobility of a Copper Square-Planar Complex in a Lipid Bilayer†

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Abstract: The copper complex 3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato)copper(II), CuKTSM2, a derivative of a potent antitumor drug, has been found to partition favorably into dimyristoylphosphatidylcholine (DMPC) vesicles. An indirect electron spin resonance (ESR) method to study partitioning was developed; by using the spin-label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl), which partitions similarly between the hydrophobic and hydrophillic phases, it is found that CuKTSM2 broadens only the TEMPO hydrophobic signal thereby establishing the partition of the complex. ESR spectra of oriented membranes show that the complex is well-oriented with the plane of the complex normal to the bilayer surface. At 1 mol %, CuKTSM2 shifts the fluid/gel phase transition temperature by 1.4 °C. By using saturation recovery ESR techniques to measure the effect of bimolecular collisions between Cu complexes and stearic acid spin labels on the spin–lattice relaxation time of the nitroxide moiety, it was found that the translational diffusion constant of the complex lies between 6.0 and 9.5 $\times 10^{-7}$ cm^2/s in the fluid phase. This value is ten times greater than that of the lipids. The saturation recovery method shows that the complex tends to be distributed throughout the bilayer.

The chemical basis for the pharmacological activity of metallo drugs has been a subject of increasing research in recent years.[2,3] Bis(thiosemicarbazonato)copper complexes constitute one group of copper complexes for which the relationship between structure and biological activity has been examined. They form tetradentate chelates with Cu^{2+} involving two nitrogen and two sulfur donor atoms (Figure 1). The copper complex of 3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazone) (R_3, R_4 = H), CuKTS, has potent antitumor properties in animals.[4] The substitution of methyl groups for hydrogens at R_3 and R_4 greatly decreases the toxicity of the complex toward cells.[5] This is thought to be due to the decrease in rate and extent of reductive dissociation of CuKTSM2 relative to CuKTS which occurs when these structures react with cells.[5-7] Replacement of hydrogens by methyl groups lowers the $E_{1/2}$ of the Cu(II) complexes from −178 to −283 mV and results in a decrease in the first-order rate constant for reductive dissociation of the bis(thiosemicarbazonato)copper(II) from 8×10^{-3} s^{-1} for CuKTS to 3×10^{-5} s^{-1} for CuKTSM2. The methylated structure also has a much larger 1-octanol/H_2O partition coefficient.[7]

The relative stability of CuKTSM2 in Ehrlich tumor cells is seen in the retention of a red hue by the cells, characteristic of

† Abbreviations used: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; DSPC, L-α-distearoylphosphatidylcholine; TEMPO, 2,2,6,6-tetramethylpiperidine-1-oxyl; 5-SASL, 5-doxyl stearic acid spin label; 16-SASL, 16-doxyl stearic acid spin label; CuKTSM2, [3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonato)]copper(II); CuKTS, [3-ethoxy-2-oxobutyraldehyde bis(thiosemicarbazonato)]copper(II).

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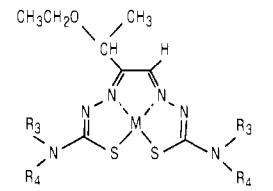

	R_3	R_4
H_2KTS	H	H
H_2KTSM	H	CH_3
H_2KTSM2	CH_3	CH_3

Figure 1. Structure of bis(thiosemicarbazonato)copper(II) complexes and derivatives of the ligand.

the presence of the complex in a nonpolar environment, presumably membrane.[7] Its cupric ESR spectrum is also undiminished over time.[8] However, it is found in cells that CuKTSM2 is essentially immobilized at ambient temperature. Either CuKTSM2 is bound to a substance with a large molecular weight or the environment of the complex slows down its motion. As the concentration of CuKTSM2 is raised, an apparent endpoint is reached. The chelate becomes cytotoxic to cells at the same concentration at which mobile CuKTSM2 is detected in the ESR spectrum. Thus, it becomes important to know what factors influence the motion of this complex in membranes. This report describes a study of the behavior of CuKTSM2 in simple membrane bilayers where it is possible to determine its rotational and translational motion and orientation.

Results

Localization of CuKTSM2 in Liposome Suspension. A direct ESR determination of the partition coefficient of CuKTSM2 is problematical for the ESR spectra for CuKTSM2 with fast motion and for slow motion overlap (data not shown). Since the major contribution is from the slow motion spectrum and the slow motion spectrum is difficult to simulate, spectral subtractions to determine the signal for CuKTSM2 with fast motion are not yet feasible. That CuKTSM2 is indeed localized in the lipid bilayer is evident from the interaction of CuKTSM2 with the spin label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) in DMPC (dimyristoylphosphatidylcholine) liposomes (Figure 2). In the absence of CuKTSM2, the two components of the well-resolved high field ESR line can be used as a measure of the amount of spin label in the hydrophobic, lipid bilayer and the polar solvent, H_2O (peak heights of these components are indicated by I^{Hy} and I^P respectively in Figure 2).[9] The solubility of TEMPO in the bilayer decreases abruptly at the main phase transition temperature of the membrane, and TEMPO is excluded from the lipid bilayer into the H_2O phase (Figure 3).

In the presence of CuKTSM2, the line marked I^{Hy}(CuKTSM2) for TEMPO in Figure 3 is greatly reduced with respect to the line marked I^{Hy} in the absence of CuKTSM2. The signal amplitude for spin label is presumed to be reduced in the lipid bilayer because of line broadening via exchange and dipole–dipole interactions between the paramagnetic species CuKTSM2 and TEMPO. The signal intensity of TEMPO in the polar solvent, H_2O, is about the same in the presence and absence of CuKTSM2. Since the total concentration of TEMPO in bilayer and water is the same in the presence and absence of the complex, CuKTSM2 does not appear to affect the partition of spin label between the two phases for temperatures far away from the main phase transition (below 14 °C and above 26 °C, Figure 3). Estimates of the partition coefficient of the copper chelate between the membrane and water phase above and below the main transition temperature were made based on measurements of the reduction

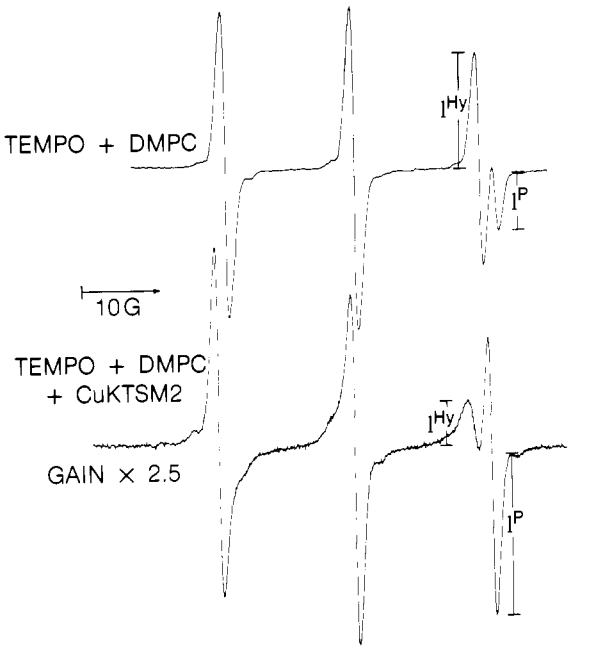

Figure 2. ESR spectra for the spin probe TEMPO (2,2,6,6-tetramethylpiperidin-1-oxyl spin label) in DMPC (L-α-dimyristoylphosphatidylcholine) liposomes at 40 °C in the absence (top spectrum) and presence (bottom spectrum) of CuKTSM2. The mole ratio of $CuKTM_2$/DMPC is 1/100. Note the high field line is split into two components for which P is related to the amount of TEMPO in the polar phase and Hy is related to the amount of TEMPO in the hydrophobic lipid bilayer.

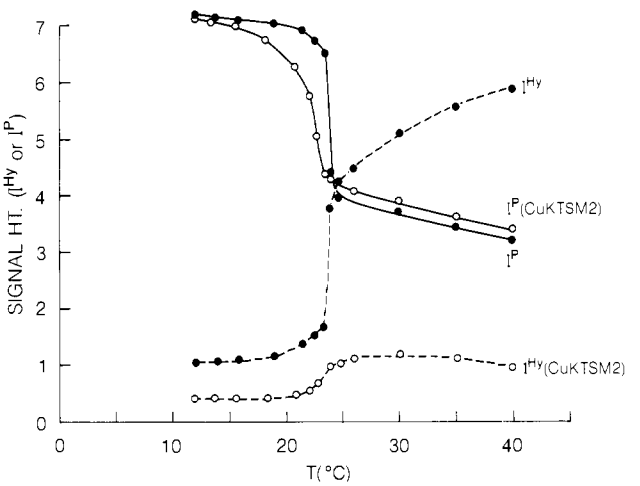

Figure 3. ESR signal height (either Hy or P defined in Figure 2) vs. temperature for TEMPO in the hydrophobic phase in the absence (Hy) and presence (Hy(CuKTSM2)) of CuKTSM2 and in the polar phase in the absence (P) and presence (P(CuKTSM2)) of CuKTSM2.

of the signal height of TEMPO and its broadened line width in the membrane phase (see Methods: partition coefficient). Values much greater than 50 for the lipid/water coefficient were obtained, consistent with the coefficient previously measured for octanol/H_2O.[7] Note that the main phase transition for the membrane in the presence of CuKTSM2 (CuKTSM2/lipid is 1/100) has broadened and shifted from 23.6 to 22.2 °C.

Rotational Motion of CuKTSM2 in the Lipid Bilayer. ESR spectra for CuKTSM2 in the fluid phase of DMPC liposomes above the main phase transition temperature and in the gel-phase below the main phase transition temperature are similar to those obtained for immobilized copper complexes (Figure 4). The motion of CuKTSM2 must be slow because spectral intensity exists in the $g_\parallel$ and $g_\perp$ region. Averaging of these ESR parameters into isotropic values would indicate fast rotational motion. CuKTSM2 is not completely immobilized either above or below the main phase

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Table I. Results of T_1 Measurements for the Central Line of the ESR Spectra of 16-SASL and 5-SASL in DMPC Liposomes in the Absence and Presence of CuKTSM2[a]

spin label	temp (°C)	T_1 (no CuKTSM2) (μs)	T_1 (CuKTSM2) (μs)	T_1^{-1} (CuKTSM2) − T_1^{-1} (no CuKTSM2) (μs^{-1})
16-SASL	25	2.42	0.49	1.63
	33	2.02	0.30	2.84
	45	1.71	0.26	3.26
5-SASL	25	4.75	0.87	0.94
	33	4.33	0.48	1.85
	45	3.2	0.33	2.72

[a] Molar ratio of CuKTSM2/DMPC = 1/50.

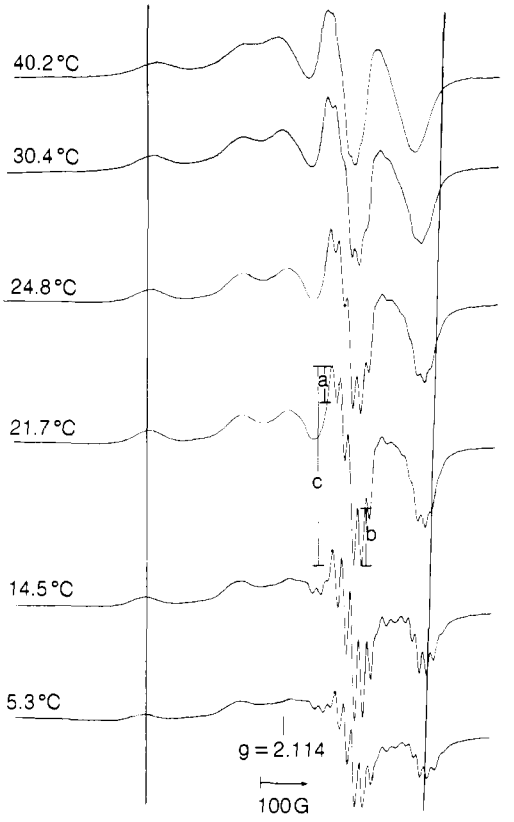

Figure 4. ESR spectra for CuKTSM2 in DMPC liposomes (mole ratio of CuKTSM2/DMPC is 1/100) as the temperature varies above and below the main phase transition temperature. The dashed lines show how the high and low field lines move with temperature.

transition temperature for the liposome membrane as indicated by the shift of the high and low field lines as the temperature decreases (vertical lines in Figure 4).

Another interesting feature of the ESR spectra for CuKTSM2 in the liposome membranes is the improved resolution of the hyperfine structure of the most intense peaks with decreasing temperature (Figure 4). Analysis of the $g_\perp$ region is complicated because of "overshoot lines" and because the magnitude of the copper hyperfine splitting is similar to the nitrogen hyperfine splitting. This complex pattern can be simulated for CuKTSM2 with two nitrogen and two sulfur donor atoms.[8] A Cu motion parameter defined in the legend of Figure 4 is used to measure the resolution of the superhyperfine structure. This Cu motion parameter is sensitive to the rate of slow tumbling of the square-planar copper complex in the lipid bilayer, i.e., the rotational correlation time(s). Abrupt changes at the main phase transition temperature of the bilayer are observed for the motion parameter vs. temperature for CuKTSM2 incorporated into DMPC, DPPC (dipalmitoylphosphatidylcholine), and DSPC (distearoylphosphatidylcholine) liposomes (Figure 5). CuKTSM2 incorporated into EYPC (egg yolk phosphatidylcholine) liposomes shows only a monotonic change for the Cu motion parameter, because all data are taken in the fluid phase for this liposome. The main phase transition temperature monitored by CuKTSM2

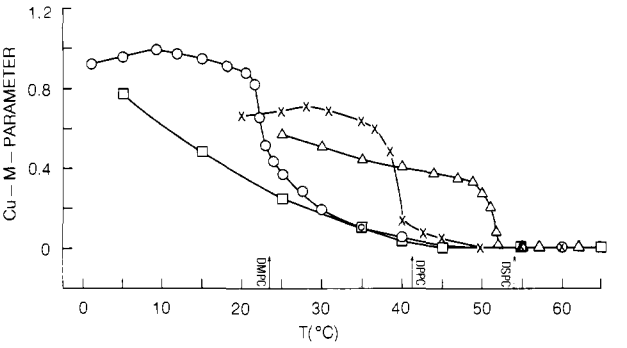

Figure 5. Plot of Cu motion parameter vs. temperature for CuKTSM2 in lipid bilayers: L-α-dimyristoylphosphatidylcholine, DMPC, (○); L-α-dipalmitoylphosphatidylcholine, DPPC (×), L-α-distearoylphosphatidylcholine, DSPC, (△), and egg yolk phosphatidylcholine, EYPC (□). (Molar ratio of CuKTSM2/lipid is 1/100). Arrows indicate main phase transitions in absence of CuKTSM2.

is broader and about 1.2 °C lower than the phase transition temperature in the absence of the copper complex.[9,10]

The Diffusion-Concentration Product for CuKTSM2 within the Bilayer. Heisenberg exchange between paramagnetic copper and spin label not only results in a broadening of ESR spin label lines (see Methods: partition coefficient) but also results in a shortening of the effective spin–lattice relaxation time (T_1) of the spin label.[11] In this work, measurements of T_1 for the stearic acid spin label 16-SASL (16-doxyl stearic acid spin label) with the nitroxide moiety at the 16 position of the hydrocarbon chain close to the center of the bilayer and 5-SASL (5-doxyl stearic acid spin label) nitroxide moiety close to the surface in the fluid phase of DMPC liposomes were made by using the saturation recovery technique. All measurements of T_1 were made on the central line ($M_1 = 0$) above the main phase transition temperature in the presence and absence of CuKTSM2 (molar ratio of CuKTSM2/DMPC is 1:50). The following points are drawn from the T_1 data in Table I for 5-SASL and 16-SASL. First, T_1's for 16-SASL are shorter than T_1's for 5-SASL. Next, the large changes in T_1 in the presence of CuKTSM2 are due to the translational-diffusion-concentration product for CuKTSM2 at a given depth in the membrane, D_{CuKTSM2}[CuKTSM2]. Finally, [T_1^{-1} (CuKTSM2) − T_1^{-1} (no CuKTSM2)], i.e., the CuKTSM2 transport parameter[12] at the 16-SASL position, is about 50% larger than the transport parameter at the 5-SASL position. Since [T_1^{-1} (CuKTSM2) − T_1^{-1} (no CuKTSM2)] is proportional to the translational-diffusion constant times the concentration of CuKTSM2, the diffusion-concentration product of CuKTSM2 at the center of the lipid bilayer is 50% higher than the diffusion-concentration product for CuKTSM2 near the bilayer surface. The terms in the diffusion-concentration product cannot be separated by using our methods, but it is reasonable to assume that both the diffusion and the concentration of CuKTSM2 are higher in the center of the bilayer.

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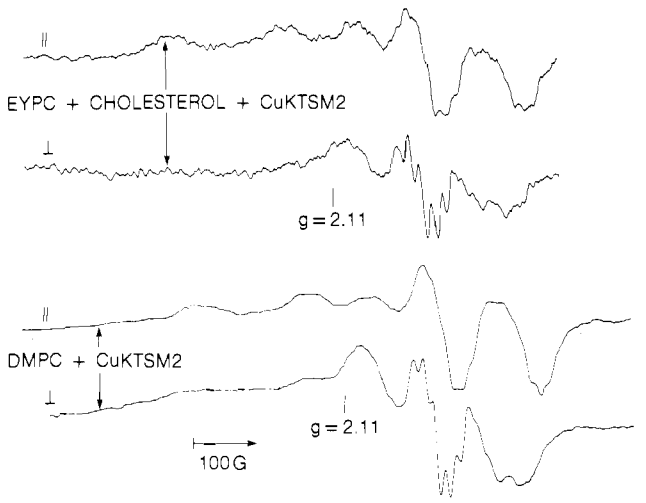

Figure 6. ESR spectra of EYPC–30% cholesterol and DMPC–0% cholesterol multibilayer films containing CuKTSM2 (molar ratio of CuKTSM2/lipid is 1/25). ESR spectra were recorded at 25 °C with the surface of the film parallel (||) and perpendicular (⊥) to the applied magnetic field.

In order to estimate the translational-diffusion constant for CuKTSM2, the following assumptions are made: all CuKTSM2 is located in the membranes (as was previously indicated by the favorable partition coefficient), and CuKTSM2 is uniformly distributed within the bilayer (for details see Methods: saturation recovery studies). With these assumptions, the translational-diffusion constant for CuKTSM2 in DMPC bilayers at 33 °C near the membrane surface is 6×10^{-7} cm^2/s; near the center of the bilayer it is 9.5×10^{-7} cm^2/s. Translational diffusion of CuKTSM2 in fluid phase bilayers is more than an order of magnitude faster than lateral diffusion of lipid molecules.[13]

Orientation of CuKTSM2 in the Lipid Bilayers. ESR spectra of CuKTSM2 dissolved in oriented multibilayer films of DMPC and also of EYPC–cholesterol (see Experimental Section) obtained at 25 °C with the magnetic field either parallel or perpendicular to the bilayer plane are shown in Figure 6. These spectra can be compared with those of Figure 4 obtained by using the DMPC system with a random distribution of bilayer orientations. It is apparent that CuKTSM2 is preferentially oriented in the bilayers.

Dr. S. Schreier (private communication) recommended the use of the EYPC + cholesterol system because it tends to form more uniformly oriented bilayers than DMPC. There are other differences. DMPC above the phase transition temperature exhibits a monotonic increase in fluidity from the surface to the center of the bilayer;[14] EYPC + cholesterol is very rigid to a depth of 12 carbons, corresponding to dimensions and location of cholesterol, and exhibits considerable fluidity between the 12th and 18th carbon.[15]

The absence of any detectable signal in the $g_{\parallel}$ region for the magnetic field perpendicular to the EYPC bilayer surface seen in Figure 6 is consistent with intercalation of the plane of the complex into the bilayer parallel to the acyl chains, Figure 7. In this model, when the magnetic field is parallel to the bilayer surface, all orientations should be equally weighted, and the spectrum should resemble one of those shown in Figure 4 for randomly oriented DMPC bilayers above the main phase transition temperature. The EYPC + cholesterol spectrum with the magnetic field parallel to the bilayer (Figure 4) does indeed resemble the spectrum from randomly oriented DMPC obtained at 30.4 °C.

Although the model of Figure 7 must generally be true, there is a residual problem in interpretation of the spectra of Figure

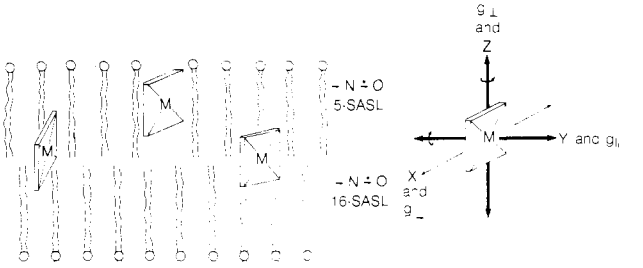

Figure 7. Schematic drawing of a lipid bilayer region containing oriented CuKTSM2 complex. Laboratory x, y, and z axes and g-tensor axes $g_{\parallel}$ and $g_{\perp}$ for the square planar copper complex are indicated. Arrows show the possible rotational motion. Positions of the nitroxide moieties of 5-SASL and 16-SASL relative to the bilayer surface are also indicated.

6. For the magnetic field perpendicular to the bilayer plane, the model predicts a signal crystal-like $g_{\perp}$ spectrum, which in principle can only extend over a spectral width of about 60 G instead of the 300 G that is observed. It seems possible that, if the complex is randomly oriented about its unique axis and if the minor elements of the g-tensor were not the same, one would expect a powder pattern over the range spanned by the differences in the minor elements. It is also suggested that the spectrum arises from a superposition of signals from CuKTSM2 near the bilayer where it is well-oriented and rigid and CuKTSM2 in the center of the bilayer where it undergoes considerable anisotropic motion. The well-resolved structure seen on the central line of the EYPC + cholesterol spectrum in the perpendicular orientation arises from a superposition of nitrogen superhyperfine lines and Cu $g_{\perp}$ hyperfine lines associated with immobilized well-oriented CuKTSM2 constituents.

Our tentative explanation for the DMPC spectra, Figure 6, lower, is essentially the same. The bilayer is much thinner, the fluidity gradient is more gradual, and the degree of orientation is thought to be not as good. These factors, together, are thought to give rise to the differences between DMPC and EYPC that are seen in the spectra.

Discussion

ESR studies with nitroxide spin-labels have been extensively used to characterize the partitioning, concentration times mobility, and reorientation effects of liposomes.[16,17] To the best of our knowledge, this is the first ESR study of a copper complex dissolved in a lipid bilayer. The methodology employed here can be summarized as follows.

1. The spin probe–spin label method used to study copper complex partitioning: This technique in which the known partitioning of TEMPO is used to establish the unknown partitioning of CuKTSM2 under conditions in which CuKTSM2 partitions favorably into one phase appears to be new and of value in a wide variety of situations.

2. The spin probe–spin label saturation recovery method used to study copper complex translational diffusion: In our laboratory, spin label oximetric methods have been extensively developed. Bimolecular collisions of molecular oxygen with spin-labels change the effective spin–lattice relaxation time. These changes can be monitored by using the saturation recovery technique. Conceptually the role of oxygen has been replaced by CuKTSM2 in the work described here by straightforward analogy.

3. Rotational diffusion of CuKTSM2: Motional effects are evident in the ESR spectra of CuKTSM2. Interpretation can be argued qualitatively. However, as yet no study, either experimental or theoretical, of copper spectra under slow tumbling conditions has been published.

4. Oriented lipid bilayers: This is a powerful approach, developed to a considerable degree by S. Schreier. Our study highlights some of the difficulties in interpretation, however.

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Computer based spectral simulations in which various motional and orientational models are tested is an obvious extension.

Membrane chemistry is an important field onto itself. It has historically been advanced by a variety of extrinsic probe techniques including fluorescent probes, spin label probes, and isotopically substituted NMR probes. Our introduction of copper complexes as probes of membrane properties constitute an additional physical approach to the study of lipid bilayers. It is apparent that a large number of experiments become possible varying the lipid composition, the structure of the copper complexes, the structure of spin label probes, the temperature, lipid organization, the host media composition including pH and ionic strength, etc.

In pharmacology, transport of metallo drugs is an important subject. For example, Kilkuskie et al.[18] have hypothesized the existence of metal complex transport pathways between plasma membranes and cell nuclear membranes for metallo chemotherapeutic drugs. Also, Sun and Crane have hypothesized that the mode of action of copper bleomycin involves interaction with membrane bound enzymes.[19]

We have shown that CuKTSM2 partitions very favorably into lipid bilayers and interacts close to the surface of the bilayer with the spin-label 5-SASL and predominates in the center of the bilayer with the 16-SASL spin-label. It is well-oriented in the bilayer with the planes of the bilayer and of the complex orthogonal. Rotational diffusion of CuKTSM2 is slow but has not yet been determined quantitatively. Translational diffusion is ten times more rapid than lipid diffusion, perhaps a surprising result. The main phase transition temperature of DMPC is broadened and decreased by 1.4 °C by the introduction of 1 mol % CuKTSM2. Signal heights may vary by about 10% by removing and reinserting the sample. The differences in Figure 3 from one sample to another far above and below the main phase transition are within experimental error, but differences are significant at the main phase transition temperature for the bilayer in the presence and absence of copper. Similar plots are obtained for the ratio of I^{Hy}/I^{P} (unpublished) and for the Cu motion parameter (Figure 3). The ratio of the peak heights for these plots avoids the necessity of an internal standard to measure absolute peak heights. This somewhat bulky complex is accommodated well and does not unduly perturb fluidity far above and below the main phase transition temperature (Figure 3). Far above the main phase transition temperature, $2T'$ is not altered in the presence and absence of CuKTSM2 (unpublished data), again suggesting that membrane fluidity is not unduly perturbed.

Experimental Section

Materials. CuKTSM2 (3-ethoxy-2-oxobutyraldehyde bis(N^4,N^4-dimethylthiosemicarbazonatocopper(II)) was generously supplied by Dr. Harold G. Petering. All phospholipids were purchased from Sigma (St. Louis, MO), cholesterol (crystallized) from Boehringer, Mannheim (Indianapolis, IN), and spin labels from Aldrich (Milwaukee, WI). For experiments with 5-SASL and 16-SASL the buffer was 0.1 M borate at pH 9.5. This rather high pH was chosen to ensure that all carboxyl groups of the spin labels were ionized in phosphatidylcholine membranes.[20-22] Otherwise, phosphoric acid (0.2 M) at pH 7 in the presence and absence of TEMPO (5×10^{-4} M) was used. The phase transition temperature,[23] electrostatic properties,[24] and structure[22] of phosphatidylcholine membranes are unchanged from pH 4.5 to 9.5.

Sample Preparations. Liposomes used in this work were multilamellar dispersions of lipids. A mixture of lipid (1.0×10^{-5} mol), CuKTSM2 (1.0×10^{-7} mol), and in some experiments spin-labels (5-SASL or 16-SASL, 1.0×10^{-7} mol) in chloroform was dried with a stream of nitrogen and further dried under a reduced pressure (~0.1 mmHg) for at least 12 h. Buffer (0.1 mL) was added to dried lipid at about 20 °C above the phase transition of the phospholipid membranes and vortexed vigorously. This liposome suspension was used for experiments with TEMPO spin label. For other experiments, the lipid dispersion was centrifuged briefly at 4 °C, and a portion of the fluffy pellet was then transferred to a Pasteur pipette or to a capillary made of gas permeable methylpentene polymer TPX.[25,26] This plastic is permeable to nitrogen, oxygen, and carbon dioxide and is substantially impermeable to water. A TPX sample tube was placed inside the ESR dewar insert and equilibrated with nitrogen gas that was used for temperature control. The sample was thoroughly deoxygenated to obtain the correct ESR line shape and spin–lattice relaxation time for the nitroxide spin-label. Presence of oxygen can lead to an increase in line width[27] and shortening of spin–lattice relaxation time[12] of spin-labels.

Oriented multibilayers were obtained according to a method described by Dr. Schreier et al.[28] A mixture of lipids (phosphatidylcholine or egg yold phosphatidylcholine and cholesterol: 1.0×10^{-6} mol and CuKTSM2 4.0×10^{-8} mol in chloroform (total volume about 0.2 mL)) was placed inside a quartz ESR flat cell. Chloroform was evaporated with a stream of wet nitrogen, forming films on the inner surface of the flat cell. This preparation was placed under vacuum for more than 12 h to remove residual solvent. Hydration with the appropriate buffer was accomplished by letting the films equilibrate with the aqueous phase in the flat cell. ESR spectra were recorded after equilibrium had been reached (about 30 min after adding buffer). Draining off the excess aqueous phase was not necessary.

ESR Measurements. ESR spectra were obtained with a Varian E-109 X-band spectrometer with Varian temperature control accessories and an E-231 Varian multipurpose cavity (rectangular TE_{102} mode). ESR spectra of oriented multibilayers were recorded with the quartz flat cell parallel to the applied magnetic field and again after a 90° rotation of the flat cell.

Partition Coefficient. A number of reports have indicated that the partition coefficients of small molecules in anisotropic media such as lipid bilayers differ from that for isotropic media such as octanol.[29,30] It is known that the partition coefficient for CuKTSM2 among the hydrophobic solvent, octanol, and water is greater than 100,[7] but there is no direct support for using this data with lipid bilayers. Therefore an indirect method utilizing the nitroxide radical spin-label, TEMPO, was used to estimate the partition coefficient for CuKTSM2 between the hydrophobic region of the lipid bilayer of DMPC liposomes and water.

ESR spectra of TEMPO in Figure 2 originate from a superposition of two spectra, one from the spin-label in the water environment and the other from the spin-label in the hydrophobic region of the lipid bilayers. In either environment, the ESR spectrum consists of three sharp lines with two different isotropic g values and different hyperfine coupling constants. Only the high field hyperfine line is split into two components indicative of the two environments (Figure 2). The peak height indicated by I^{Hy} is proportional to the amount of spin-label in the hydrophobic region of the membrane, and the peak height, I^{P}, is proportional to the amount in the polar water environment (Figure 2). The relative intensities of I^{P} and I^{Hy} depend on the partition coefficient of the spin-label between the lipid and water phases, the relative amount of lipid and water, and the line widths in the two environments. The change in height of I^{Hy} and the increase in line width for the hydrophobic line in TEMPO is due to the presence of CuKTSM2 (Figures 2 and 3). It is assumed that CuKTSM2 does not change the TEMPO partition coefficient but only broadens the lines of the ESR spectra of TEMPO via a Heisenberg exchange interaction during bimolecular collision of spin-label with CuKTSM2. The line width for the Lorentzian line of TEMPO broadened due to collision with CuKTSM2 is described by the following equation

$$\Delta H = \Delta H_0 + \left(\frac{2}{\sqrt{3}\gamma_e}\right)p \qquad (1)$$

where ΔH_0 is the line width in the absence of CuKTSM2, γ_e the gyro

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magnetogyric ratio for the electron, and p the probability that a spectroscopically observed event occurs when a collision occurs.[31,32] The frequency of collision of spin-label with the CuKTSM2 molecules, ω, according the Smoluchowski equation equals

$$\omega = 4\pi R(D_{SL} + D_{CuKTSM2})[CuKTSM2] \quad (2)$$

where R is the interaction distance, D_{SL} and $D_{CuKTSM2}$ are the spin label and copper complex translational-diffusion constants, and [CuKTSM2] is expressed in molecules per unit volume. This equation can be written for both the polar and the hydrophobic part of the spectra. Inequalities for diffusion constants of the spin-label TEMPO and CuKTSM2 in water (less viscous, P) and hydrophobic bilayer (more viscous, H) environments are as follows

$$D^{P}_{SL} > D^{H}_{SL} \text{ where } \Delta H^{P}_0 < \Delta H^{H}_0 \text{ and } D^{P}_{CuKTSM2} > D^{H}_{CuKTSM2} \quad (3)$$

The lipid bilayer/water partition coefficient for CuKTSM2 is equal to

$$K_{CuKTSM2} = \frac{[CuKTSM2]^{Hy}}{[CuKTSM2]^{P}} \quad (4)$$

By using eq 1 and 2

$$K_{CuKTSM2} = \frac{(\Delta H^{Hy} - \Delta H^{Hy}_0)}{(\Delta H^{P} - \Delta H^{P}_0)} \cdot \frac{(D^{P}_{SL} + D^{P}_{CuKTSM2})}{(D^{Hy}_{SL} + D^{Hy}_{CuKTSM2})} \quad (5)$$

where $\Delta H^{Hy} - \Delta H^{Hy}_0$ and $\Delta H^{P} - \Delta H^{P}_0$ measure line broadening of hydrophobic and polar components. A change of peak height for either hydrophobic or polar components is due only to the line broadening effect of CuKTSM2. Then

$$I^{Hy}_0(\Delta H^{Hy}_0)^2 = I^{Hy}(\Delta H^{Hy})^2 \text{ and } I^{P}_0(\Delta H^{P}_0)^2 = I^{P}(\Delta H^{P}_0)^2 \quad (6a)$$

or

$$\left(\frac{\Delta H^{Hy}}{\Delta H^{Hy}_0}\right)^2 = \frac{I^{Hy}_0}{I^{Hy}} \text{ and } \left(\frac{\Delta H^{P}}{\Delta H^{P}_0}\right)^2 = \frac{I^{P}_0}{I^{P}} \quad (7)$$

where I^{Hy}_0, I^{P}_0, I^{H}, and I^{P} are the peak-to-peak intensities for the ESR spin label signal in the presence (I) and the absence (I_0) of CuKTSM2 and in the hydrophobic (Hy) and the polar (P) environment. The ratio of line widths was calculated from the ratio of peak heights in the hydrophobic environment before and after adding CuKTSM2 (data from Figure 3); according to equation 6a: $\Delta H^{Hy} = 2.7\Delta H_0$ for 40 °C, $\Delta H^{Hy} = 2.3\Delta H^{Hy}_0$ for 24 °C and $\Delta H^{Hy} = 2.2\Delta H^{Hy}_0$ for 12 °C. Since there is very little change in the peak height of the polar component of TEMPO in the ESR spectrum after addition of CuKTSM2, only the estimated difference of I^{P}_0 and I^{P} could be obtained. Thus a decrease in polar line intensity due to line broadening by CuKTSM2 dissolved in the water is estimated to be about or less than 0.2 units on Figure 3. Calculated ratios of line widths give the inequalities

$$\Delta H^{P} < 1.03\Delta H^{P}_0 \text{ for 40 °C; } \Delta H^{P} < 1.02\Delta H^{P}_0 \text{ for 24 °C; and } \Delta H^{P} < 1.02\Delta H^{P}_0 \text{ for 12 °C} \quad (8)$$

Combining eq 3, 5, 7, and 8 yields

$$K_{CuKTSM2} >> 56 \text{ for 40 °C; } K_{CuKTSM2} >> 65 \text{ for 24 °C; and } K_{CuKTSM2} >> 86 \text{ for 12 °C} \quad (9)$$

Thus, for all conditions above and below the main phase transition temperature, practically all CuKTSM2 is located in the hydrophobic region in the lipid bilayer. Note, the correction for the contribution of the ^{13}C satellite line for the polar peak (P), which lies almost exactly under the hydrophobic peak (Hy), was not made. This correction can only increase the estimated partition coefficient for CuKTSM2. Also, since the solubility of CuKTSM2 is limited in H_2O (about 10 μM), a saturated solution of CuKTSM2 in the water phase will not cause any detectable broadening of the ESR line of TEMPO in the water phase. Therefore, the assumption that CuKTSM2 does not change the partition coefficient of TEMPO is valid because the polar peak height of TEMPO doesn't change after addition of CuKTSM2. Whereas almost all CuKTSM2 is in the bilayer, the molar ratio of CuKTSM2 to lipids is used to describe the amount of CuKTSM2 in the samples.

Saturation-Recovery Studies. Spin–lattice relaxation times were measured at X-band by using the saturation-recovery technique. In this method, the spin system is first saturated with an intense and long duration saturating pulse which tends to equilize the populations of electrons on energy levels. The recovery (return) to equilibrium is then observed with a weak observing power. The method has been reviewed by Hyde.[33] Under appropriate conditions the recovery is characterized by an exponential decay, with the time constant for the decay given by the electron spin–lattice relaxation time, T_1.

The apparatus used here was based in part on the design of Huisjen and Hyde[34] and Percival and Hyde.[35] The three-arm microwave bridge was combined with the Loop–Gap resonator.[36,37] A particular advantage of the Loop–Gap resonator is the good separation of electric and magnetic microwave fields, which permits experiments on aqueous samples with good sensitivity. Data accumulation was obtained with an adjustable aperture and digital signal averaged in 16–512 channels.[38] Approximately 2×10^7 accumulations were obtained.

It is assumed that during the bimolecular collision the dominant magnetic interaction between copper complexes and spin labels is Heisenberg exchange.[11] Because of a very short value for the copper ion spin–lattice relaxation time ($\sim 10^{-8}$ s) with respect to the spin–lattice relaxation time for steric acid spin labels ($>2 \times 10^{-6}$ s), there is immediate thermal contact of the slow relaxer to the lattice thereby shortening T_1 of the spin label.[11,12,25] The value of the copper ion spin–lattice relaxation time is longer than the duration of the collision between the copper complex and the spin label in the membrane ($10^{-9} - 10^{-10}$ s). Thus, every exchange event between a nitroxide and a copper complex is a spin–lattice relaxation event for nitroxide.[11] Also it seems likely that the exchange process is a strong encounter type and every collision contributes to the change of T_1 for the nitroxide. Then, the electron relaxation probability of spin label is

$$W_e \text{ (CuKTSM2)} = W_e \text{ (no CuKTSM2)} + p\omega \quad (10a)$$

$$T_1^{-1} \text{ (CuKTSM2)} = T_1^{-1} \text{ (no CuKTSM2)} + 2p\omega \quad (10b)$$

where ω is the frequency of collisions described by eq 2. A factor of p is introduced to allow for the possibility that some collisions might be ineffective either for steric reasons or because collisions are not of the strong encounter type.

The saturation recovery technique is a method to observe T_1 directly for spin-labels located in the membrane in the absence and in the presence of CuKTSM2. Contributions to the effective T_1 of the spin label are governed by the bimolecular collision rate with the copper complex. By analogy for the oxygen transport parameter,[12] a copper complex transport parameter is defined as

$$W \text{ (CuKTSM2)} = T_1^{-1} \text{ (CuKTSM2)} - T_1^{-1} \text{ (no CuKTSM2)} = 4\pi pR(D_{SL} + D_{CuKTSM2})[CuKTSM2] \sim 4\pi RD_{CuKTSM2}[CuKTSM2] \quad (11)$$

The copper transport parameter is a function of both concentration and translational diffusion of the copper complex in the membrane. By measuring the copper transport parameter close to the membrane surface (5-SASL) or near the middle of the bilayer (16-SASL), the ratio of the diffusion concentration product of the copper complex for these two locations is obtained (Table I). In unpublished experiments, it is shown that T_1 varies linearly with the concentration of CuKTSM2 (0.5–2.0 mol % of CuKTSM2 in lipids) at both 25 and 45 °C with either 5-SASL or 16-SASL (1:100) in the lipid bilayer. The product for $D_{CuKTSM2}$·[CuKTSM2] can be determined by measuring $W_{(CuKTSM2)}$ and making a reasonable assignment of p and R. It is assumed that $p = 1$ and $R = 6$ Å (sum of the van der Waal's radius for the Cu complex and the spin-label). Others have used 5 Å for the distance of minimum approach for Ni radical spin exchange to occur.[16] The upper limit for the distance of minimum approach for a square-planar complex appears to be close to 6 Å. It is also assumed that all of the CuKTSM2 is equally distributed throughout the bilayer and diffuses isotropically as in a three-dimensional

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system. For $D_{SL} << D_{CuKTSM2}$ the lower limit for the translational diffusion coefficient for CuKTSM2 is equal to $(60 - 95) \times 10^{-8}$ cm^2/s. Because the translational diffusion constant for 16-SASL at that temperature in DMPC is about 5×10^{-8} cm^2/s,[39] the assumption that $D_{SL} << D_{CuKTSM2}$ is appropriate.

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EXAFS Studies on the Origin of Highly Catalytic Activity in Nickel Y Zeolite

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Abstract: Nickel species contained in Y-type zeolite have been characterized by an EXAFS technique at each stage of catalyst preparation. When nickel ions were incorporated into the zeolite by aqueous ion exchange, a kind of "solution-like" hydrated state with Ni–O = 2.06 Å was suggested from the EXAFS analysis. After calcination, the nickel ions, surrounded by an average of 3.6 oxygen atoms with Ni–O = 2.05 Å, scattered around the exchangeable sites with unsaturated coordination. According to the EXAFS and ESCA (electron spectroscopy for chemical analysis) results, the nickel ions in this state could not be reduced completely by hydrogen. On the contrary, a different behavior of hydrogen reduction was observed after treatment of the hydrated-nickel zeolite with an aqueous sodium hydroxide solution. A new nickel hydroxide oligomer with Ni–O = 2.06 Å was formed in the supercage by this treatment. Moreover, calcination of this alkali-treated zeolite under oxygen atmosphere gave another new product containing nickel atoms with 3.5 oxygen neighbors at 2.08 Å and three second-nearest nickel neighbors at 2.99 Å. These oligomeric nickel–oxide clusters are certainly responsible for the high catalytic activity in CO oxidation. Hydrogen reduction of the small oxide clusters gave the zeolite catalyst which has an excellent activity for benzene hydrogenation. This material was confirmed to contain finely dispersed metallic nickel by the radial distribution function derived from the EXAFS spectrum.

Zeolites are crystalline aluminosilicates consisting of three-dimensional arrays of SiO_4 and AlO_4 tetrahedra. The void space enclosed within the unit is called the sodalite cage,[1] whereas a larger void space, called the supercage, is formed by linking sodalite units by hexagonal prisms. Windows 7.4 Å in diameter are formed in the supercage. This is schematically shown in Figure 1. The windows and cavities are large enough to allow small molecules to enter into the crystallite. Zeolite structures lead to many practical applications as adsorbents and catalysts. Zeolites also contain exchangeable cations, which play an important role in their catalytic activity. Zeolites containing transition metals are especially important in hydrogenation, hydrocracking, and hydroisomerization.[1] In spite of efforts to finely disperse metals in zeolites, preparation of highly active metal-containing zeolites is generally difficult.

Recently, Suzuki et al.[2] found that the catalytic activities of a nickel-exchanged Y zeolite were remarkably improved by soaking the zeolite in an aqueous NaOH solution (e.g., the activity for CO oxidation was enhanced from 4–5% conversion for the Ni Y zeolite up to more than 90% for the NaOH treated zeolite). They ascribed the enhanced activity to small nickel oxide aggregates in the zeolite, formed with the NaOH treatment. Investigation of the structural changes of the incorporated nickel and perhaps the elucidation of the mechanism of enhanced activity is undoubtedly valuable for developing methods of catalyst preparation.

EXAFS spectroscopy is used to determine the local structure around a specific atom in any phase.[3] This spectroscopic technique is well suited for tracing the structural changes during the processes of catalyst preparation. The main purpose of this work is to find out the structural change around the nickel atom induced by the treatment with a sodium hydroxide solution and to correlate the catalytic activity with the structure of active species, elucidated by use of EXAFS spectroscopy.

Experimental Section and Data Analysis

Sample Preparation. The sodium form of Linde Y-type zeolite (S-K-40) was ion exchanged with 0.1 M $Ni(NO_3)$ aqueous solution at 343 K. After filtration, the solid material was washed with distilled water to remove excess ions and then dried at 383 K (abbreviated hereafter as NiY-1). The NiY-1 sample was treated with aqueous NaOH solution of pH 10.5 for 1 h at 293 K, followed by washing and drying at 383 K in air (NiYOH-1). The amounts of nickel ions in both NiY-1 and NiYOH-1 were demonstrated by flame photometry as 8.97 and 8.55 wt %, respectively. Both samples (NiY-1 and NiYOH-1) were gradually heated up to 643 K and maintained for 3 h in the flow of 10% O_2/He (9 L/h). The products (NiY-2 and NiYOH-2) were cooled slowly down to room temperature and transferred to an EXAFS cell in the same atmosphere. Reduction in a flow of hydrogen (9 L/h) was performed at 643 K for 6 h (NiY-3 and NiYOH-3), followed by transfer in hydrogen to the EXAFS cell.

ESCA Measurement. The X-ray photoelectron spectra were recorded under 3×10^{-7} Torr (1 Torr = 133.3 Pa) or below by using a JEOL Model JESCA-3A spectrometer with aluminum $K\alpha_{1,2}$ radiation (1486.6 eV) as the X-ray excitation source. The electron binding energies were referenced to the Au $4f_{7/2}$ (83.8 eV) peak of the gold sputtered onto the sample.

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