

Electron Paramagnetic Resonance of Copper Ion and Manganese Ion Complexes with the Ionophore A23187[†]

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ABSTRACT: The binding of Cu²⁺ and Mn²⁺ to the ionophore A23187 in chloroform, 90% ethanol, and sonicated phospholipid dispersions in aqueous mediums has been investigated with electron paramagnetic resonance (epr). The spectra indicated axial symmetry for the Cu²⁺ complexes and distorted octahedral for the Mn²⁺ complexes. The coordination between metal ion and its ligands is predominantly ionic in character. The stoichiometry, at the concentrations employed, was found to be 1:2 M²⁺/ionophore except in 90% ethanol where evidence existed for the 1:1 Cu-A23187 complex, as well. Through competition with Mn²⁺, the sequence of relative affinities in 90% ethanol was measured to

be: Mn²⁺ > La³⁺ > Cu²⁺ > Ca²⁺ \approx Mg²⁺ > Sr²⁺. The K_A of Mn-A23187 binding is $\approx 10^{10}$ M⁻². In phospholipid dispersions the spectral characteristics of the Cu complex, particularly g_{\parallel} , were observed to be a sensitive function of the hydrocarbon chain mobility. This allowed a calculation of the rotational correlation time τ of the complex to be made. In sonicated dipalmitoyllecithin τ was computed to be $\sim 10^{-9}$ sec, reflecting a local viscosity similar to that sensed by the nitroxide spin-label 2,2,6,6-tetramethylpiperidin-1-oxyl. In a (1:1) lecithin-cholesterol dispersion the complex was significantly more immobilized.

The ionophorous antibiotic A23187 has been shown to transport divalent cations into organic solvents or across biological membranes (Reed, 1972; Caswell and Pressman, 1972; Reed and Lardy, 1972; Scarpa *et al.*, 1972; Wong *et al.*, 1973). Like the other widely studied divalent cation ionophore X537A, it is a monocarboxylic acid which forms a 1:2 M²⁺-(ionophore⁻)₂ complex in nonpolar solvents. Presumably, it is these neutral complexes which pass most easily through a lipid membrane phase (Caswell and Pressman, 1972). A23187 is unique, however, among the small carrier molecules now isolated in its nearly total specificity for divalent over monovalent cations. The structure of A23187 (C₂₉H₃₇N₃O₆) includes two spiro ring ether oxygens, a pyrrole ketone, as well as the carboxyl group, available for coordination with metal ions (Chaney *et al.*, 1974).

After binding a paramagnetic divalent cation to A23187, the complex can be studied with electron paramagnetic resonance (epr). From the spectra it is possible, in principle, to obtain information on the strength and stoichiometry of binding, the crystal field symmetry around the metal, and the degree of spin delocalization onto ligand atoms through charge transfer or covalency. Reported here are epr spectra of Cu²⁺- and Mn²⁺-A23187 complexes in a nonpolar solvent (chloroform) and in a polar medium (90% ethanol) and interpretations in terms of some of the above properties. Finally, spectra of these paramagnetic complexes in sonicated phospholipid dispersions are examined. Evidence is presented for the usefulness of the Cu²⁺-A23187 complex as a probe of membrane fluidity and the rotational correlation time of the complex is calculated.

Experimental Procedure

A23187 was generously donated by R. Hamill of the Eli Lilly Co. Cations were extracted into chloroform by vortex-

ing an aqueous solution containing 20 mM Tris buffer (pH 7.4) plus 1 mM MnCl₂ or CuSO₄ with an equal volume of A23187 dissolved at varying concentration in CHCl₃ and allowing the phases to separate. Lipid dispersions were prepared by evaporating a CHCl₃ solution of the ionophore and the lipid under a N₂ stream, sonicating the residue in aqueous 20 mM Tris or Hepes¹ buffer for 20–30 min in a Branson HS-20 sonicator, adding CuSO₄ or MnCl₂ to aliquots of the suspension, and sonicating for another 10 min.

Samples were studied in a dual cavity at 9.5 GHz on a Varian E-12 spectrometer, equipped with a variable temperature accessory or at 35 GHz where noted. Recorded in conjunction with each sample spectrum at X-band was that of a pitch standard placed in the other compartment of the dual cavity. Magnetic field was measured with a Hall probe unit calibrated over a wide range of field with nuclear magnetic resonance (nmr). The position and height of the pitch signal were used to correct for cavity Q and frequency shifts in measurements of sample signal intensity and g values, respectively.

Results

The M²⁺-A23187 Complex in Chloroform. The amount of metal ion extracted from Tris buffered aqueous phase into chloroform, following separation of the two phases, was determined by loss of epr signal from the supernatant. The spectrum being monitored was either the usual Mn(H₂O)₆²⁺ sextet (Cohn and Townsend, 1954) or the signal due to the Cu-Tris complex (Figure 4a). In either case, 50% of the signal was lost upon inclusion of an equimolar quantity of A23187 and nearly 100% when the ionophore concentration was double that of the paramagnetic ion. The same results were obtained when 8 mM of the lipophilic anion tetraphenylboron was added to the sample. Thus, we conclude, in agreement with previous reports (Reed, 1972;

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¹ Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Tempo, 2,2,6,6-tetramethylpiperidin-1-oxyl; DPL, dipalmitoyllecithin.

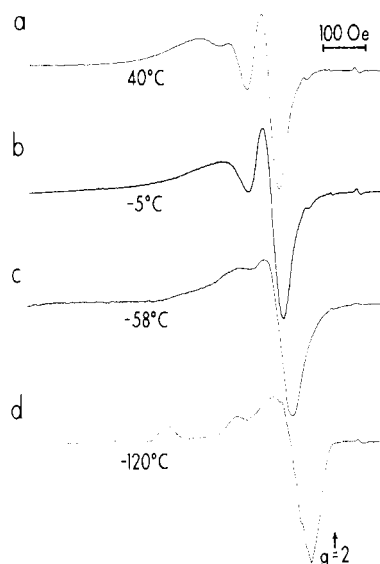


FIGURE 1: Epr spectra of the Cu^{2+} -A23187 complex in chloroform solution.

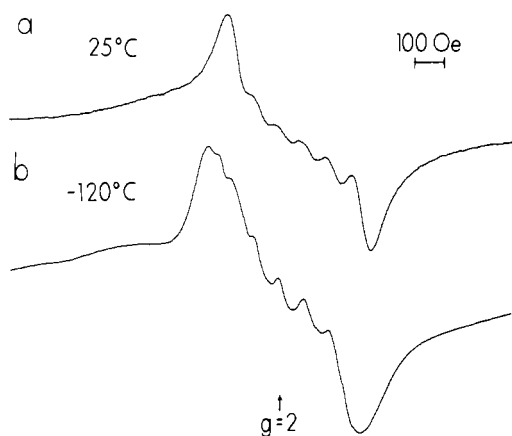


FIGURE 2: Epr spectra of the Mn^{2+} -A23187 complex in liquid (a) and frozen (b) chloroform solution.

Caswell and Pressman, 1972; Reed and Lardy, 1972; Pfeiffer *et al.*, 1974) that the neutral 1:2 M^{2+} -ionophore complex is extracted into organic solvents.

When the chloroform phases were examined with epr, the spectra shown in Figures 1 and 2 were observed. The absorption pattern of frozen Cu-A23187 complex (Figure 1d) is typical of Cu^{2+} complexes possessing nearly axial symmetry (Mälmström and Vännegård, 1960). The quartet at low (high) field are hyperfine lines deriving from complexes where the axis of symmetry is nearly parallel (perpendicular) to the external field. The g values and magnitude of the hyperfine parameters are: $g_{\perp} = 2.069 \pm 0.005$, $g_{\parallel} = 2.312 \pm 0.005$, $A_{\perp} = 12 \pm 3$ Oe, $A_{\parallel} = 150 \pm 3$ Oe. This set of values implies that the coordination between Cu and ionophore is predominantly ionic ($\sim 70\%$) in character (Mälmström and Vännegård, 1960). In the liquid, the spectra undergo line-shape changes due to rotational averaging of g value and hyperfine anisotropy along with motional narrowing. With rapid tumbling the hyperfine structure disappears while the parallel and perpendicular components of the spectra move closer together.

Spectra of Mn-A23187 in CHCl_3 at room temperature and at -140° are shown in Figure 2. Over the whole range of temperatures from the melting to the boiling point of

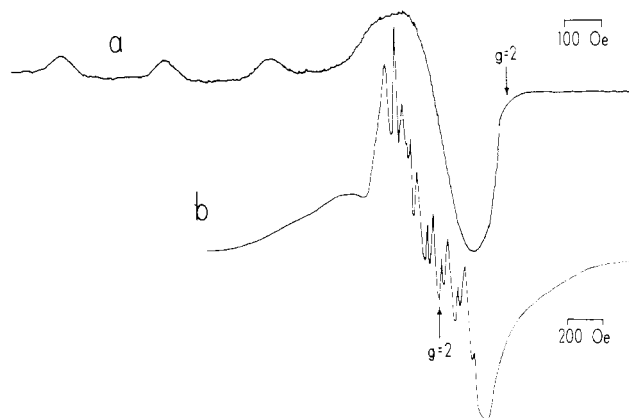


FIGURE 3: (a) 1:1 Cu^{2+} -A23187 complex in frozen 90% ethanol near 77°K . The sample contained 20 mM Hepes (pH 7.4) and 5×10^{-4} M CuSO_4 and A23187. (b) Mn^{2+} -A23187 in frozen 90% ethanol (-160°). The sample contained 25 mM Hepes (pH 7.4), 330 μM A23187, and 80 μM MnCl_2 .

CHCl_3 , the spectrum of the complex changed only slightly. It could be approximated by a computer simulation of six Lorentzian line shapes with the usual ~ 95 Oe hyperfine splitting and with a width of ~ 125 Oe plus a broad anisotropic component. At Q band (20°) the lines were narrowed to 21 Oe peak to peak and the width of the entire hyperfine envelope was observed to be 460 Oe. The hyperfine splitting of 90–95 Oe means that the component of covalency in the Mn-ionophore bonds is $\lesssim 8\%$ (Schlaak and Weiss, 1972). The spectra of Figure 2 show some anisotropy, particularly at low temperature, indicating that the complex is distorted from octahedral symmetry. The broad absorption at low field (high g) is consistent with a point group symmetry no higher than orthorhombic giving both D and E terms in the effective spin Hamiltonian. The strengths of the zero field splitting terms are between 300 and 500 Oe (Griscom and Griscom, 1967).

Spectra in Polar Medium. In order to study binding in a polar environment, concentrated MnCl_2 or CuSO_4 was added in a small volume to a 90% ethanol–water mixture containing 10 mM Hepes (pH 7.4) and 5×10^{-4} M A23187. Samples to which Mn were added showed only a very broad epr signal until the $[\text{Mn}]$ exceeded 2.5×10^{-4} M, after which a hyperfine sextet (width ~ 50 Oe) typical of the $\text{Mn}(\text{H}_2\text{O})_6^{2+}$ complex in 90% ethanol appeared, growing linearly in intensity with additional Mn. Comparing the observed signal heights with that of a control containing only 0.25 mM MnCl_2 in 90% ethanol, it was easy to see that the Mn was nearly all bound until its concentration exceeded half the A23187 concentration, after which all further Mn was free. Based on these observations and the limits on epr sensitivity and precision in these experiments, we can say that at these concentrations Mn also forms a tight 1:2 complex with A23187 in a polar medium and that the association constant is greater than 10^{10} M^{-2} . This stoichiometry and binding affinity are consistent with the findings of Pfeiffer *et al.* (1974).

The spectrum of the MnA_2 complex in 90% ethanol is shown in Figure 3b. More structure can be seen than in frozen CHCl_3 solution, probably because of a slower spin–lattice relaxation rate in 90% ethanol. The spectrum of Figure 3a can be decomposed into at least three components: (1) a very sharp sextet centered at $g = 2$ with a hyperfine splitting of ~ 95 Oe, (2) a somewhat broader set of six lines offset by ~ 40 Oe toward lower field (there is also some evi-

TABLE I: Mn²⁺/M²⁺⁽³⁺⁾ Competition.^a

Competing Ion/Mn ²⁺	% Mn ²⁺ Displaced
Mg ²⁺ , 25 × Mn ²⁺	27
Ca ²⁺ , 25 × Mn ²⁺	38
Sr ²⁺ , 50 × Mn ²⁺	18
La ³⁺ , 1.5 × Mn ²⁺	37
Cu ²⁺ , 3 × Mn ²⁺	30

^a Each sample contained 0.25 mM MnCl₂, 0.50 mM A23187, and 20 mM Hepes (pH 7.4) in addition to a given amount of competing ion listed above as a ratio to total Mn²⁺. The fraction of Mn²⁺ displaced by the competing ion was determined from the intensity of the free signal.

dence for a weaker sextet shifted towards high field), (3) a broad asymmetric absorption with wings very similar to those seen in frozen CHCl₃. When the ethanol-water mixture was melted, the hyperfine structure was washed out leaving a spectrum similar in appearance to that of Figure 2a. The spectra in frozen 90% ethanol and CHCl₃ may differ either because of conformational differences in the ionophore or the substitution of solvent ligands in the inner coordination sphere of the Mn²⁺.

When Cu²⁺ was added to the ionophore in 90% ethanol, an epr signal similar to that of Figure 1a appeared. The signal intensity increased with increasing [Cu²⁺] until the concentrations of Cu²⁺ and ionophore were equal after which no further change occurred until the onset of copper hydroxide precipitation. The nonbinding buffer Hepes (pH 7.4) was used in the experiments performed in 90% ethanol so as to avoid competition between the ionophore and Tris for Cu²⁺.

The spectrum of the Cu complex in frozen 90% ethanol was identical with that in frozen chloroform (Figure 1d) so long as the Cu-A23187 ratio was less than 1:2. When [Cu] and [ionophore] were equal, however, the spectrum of Figure 3a was seen with parameters $g_{\parallel} = 2.283 \pm 0.005$, $g_{\perp} = 2.070 \pm 0.005$, and $|A_{\parallel}| = 152 \pm 3$ Oe, while the free Cu species in frozen 90% ethanol did not visibly contribute to the epr spectrum. These results imply the existence of two distinct Cu-A23187 complexes in 90% ethanol, quite probably the 1:2 complex when excess ionophore is available and 1:1 otherwise. Pfeiffer *et al.* (1974) have reported a similar phenomenon with La³⁺ binding to A23187 in ethanol. The lower g_{\parallel} suggests that the covalency of the 1:1 Cu complex is probably somewhat larger, approximately 35% according to the calculations of Malmstrom and Vännegård (1960).

Relative Binding Affinities. Through competition with a paramagnetic ion it is possible with epr to estimate the binding affinities of diamagnetic cations. Samples containing Mn²⁺ and just enough ionophore (double the [Mn]) to bind all the Mn were prepared in 90% ethanol. Then a competing ion was added in a small volume and the displaced Mn determined by an epr measurement of free Mn. The results are summarized in Table I.

The relative affinities deduced from these data, under the assumption that the stoichiometry for each ion is 1:2, are: Mn²⁺ (250) > La³⁺ (46) > Cu²⁺ (12) > Ca²⁺ (2.3) > Mg²⁺ (1.0) > Sr²⁺ (0.18). This assumption is questionable in view of the apparent stoichiometries of Cu²⁺ and La³⁺ binding described in the last section of the observations of Caswell and Pressman (1972) indicating that the Ca²⁺-

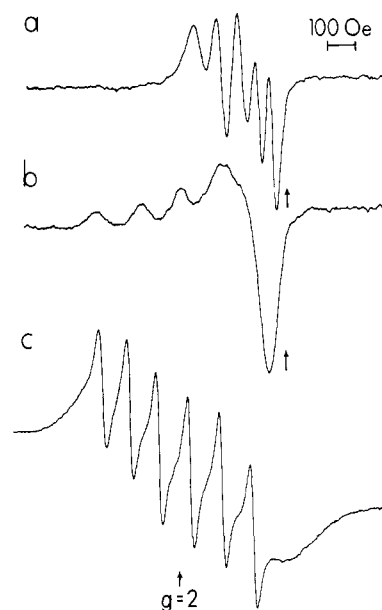


FIGURE 4: (a) Epr spectrum of Cu²⁺ binding in Tris-buffered lecithin dispersion. The dispersion in 20 mM Tris buffer (pH 7.4) contained 10 mg of dipalmitoyl- α -lecithin + 100 nmol of CuSO₄ in 0.6 ml total volume. (b) Epr spectrum of Cu²⁺-A23187 complex in sonicated lecithin. The sample contained 270 nmol of A23187 in addition to the contents above. (c) Epr spectrum of Mn²⁺-A23187 complex in lecithin dispersions. The sample contained 15 mg of lecithin, 150 nmol of MnCl₂, and 380 nmol of A23187 in 0.4 ml of 25 mM Tris buffer (pH 7.4).

and Mg²⁺-A23187 complexes may be 1:1 in polar media. However, the latter has been disputed by both Case *et al.* (1974) and Pfeiffer *et al.* (1974) who claim that the Ca²⁺ and Mg²⁺ complexes are mainly 1:2 in ethanol. The series above should, however, be regarded cautiously as a qualitative index of relative binding strengths. In this respect there is good agreement with measurements on extraction of cations into organic solvents (Pfeiffer *et al.*, 1974) where the series Mn²⁺ >> Ca²⁺ > Mg²⁺ >> Sr²⁺ was found, and on (fluorescence) measurements of binding in 80% ethanol where it was found (Caswell and Pressman, 1972) that Ca²⁺ \approx Mg²⁺ > Sr²⁺. If, on the other hand, one assumes in accord with the results of the last section that the Mn complex is 1:2, but takes the Ca²⁺ or Mg²⁺ binding to be 1:1 with an affinity of ~ 30 mM⁻¹ (Caswell and Pressman, 1972), the affinity of Mn²⁺ binding is computed from Table I to be $K_A \sim 1.5$ – 5 μ M⁻².

The M²⁺-A23187 Complex in Sonicated Lecithin Dispersions. Figure 4a shows a spectrum from a sample in which Cu²⁺ was sonicated with dipalmitoyllecithin (DPL) in Tris buffer. No trace of the free hydrated Cu²⁺ spectrum, a line at $g = 2.2$ with a width of ~ 145 Oe, is observed due to Cu²⁺ association with Tris. When the ionophore A23187 was included during sonication in a molar ratio to Cu²⁺ of >2:1, the spectrum appeared as in Figure 4b. The lack of any detectable contribution to the spectrum from the Cu²⁺-Tris complex (Figure 4a) indicates that nearly all of the Cu²⁺ was bound to the ionophore. Titrations showed saturation of Cu²⁺ binding to the ionophore in sonicated DPL at a molar ratio of one Cu²⁺ ion to two A23187 molecules. On the other hand, the value of g_{\parallel} determined from Figure 4b is closer to that obtained for the 1:1 complex in frozen 90% ethanol than for the 1:2 complexes in either frozen 90% ethanol or chloroform solutions.

These facts can be explained as follows. The low field hf structure in the DPL dispersion is due to the neutral

TABLE II: Cu²⁺-A23187 Complex in Sonicated Lipid Dispersions.^a

Dispersion	Temp (°C)	$g_{\parallel} - 2$	$g_{\perp} - 2$	$ A_{\parallel} $
DPL	15	0.290 ± 0.005	0.080 ± 0.005	147 ± 3
	30	0.283	0.080	147
	45	0.275	0.084	145
	65	0.275	0.090	138
	85	0.263	0.093	132
Egg lecithin	15	0.283	0.081	145
	30	0.275	0.081	144
	45	0.269	0.082	139
	65	0.259	0.089	137
	85	0.095		
DPL-cholesterol	25	0.315	0.073	150

^a Each sample contained 15 mg of lipid, 0.225 mg of A23187, 150 nmol of CuSO₄ in 1 ml of Tris-Cl buffer (15 mM, pH 7.4).

TABLE III: Cu²⁺-A23187 in Frozen Solutions.

Medium (°K)	Cu ²⁺ -A23187	$g_{\parallel} - 2$	$g_{\perp} - 2$	$ A_{\parallel} $
90% ethanol (77)	<1/2	0.314 ± 0.005	0.070 ± 0.005	150 ± 3
90% ethanol (77)	1/1	0.283	0.071	147
Chloroform (120)	1/2	0.312	0.069	151

Cu²⁺(ionophore⁻)₂ but the g value anisotropy is partially averaged by tumbling of the complex. The rotation cannot be too rapid or the hyperfine structure would be totally averaged (Figure 1a-c), thus the complex must be at least partially buried in the interior of the lipid phase. The validity of this interpretation is strongly supported by the data in Table II where the splitting parameters are listed for the Cu-ionophore complex in phospholipid dispersions. As one would predict with rotational averaging, g_{\parallel} decreases, g_{\perp} increases, and A_{\parallel} decreases with increasing alkyl chain fluidity. This is reflected both in the trends observed with temperature variation and the small but consistent differences between the more fluid egg lecithin and DPL. Moreover, consistent with the picture of cholesterol as a stiffener of membranes, the DPL-cholesterol dispersion had g values and hyperfine splitting nearly identical with those obtained from the 1:2 complex in frozen CHCl₃ or 90% ethanol (Table III).

The spectrum of Mn added in a substoichiometric amount to a sonicated DPL-ionophore dispersion was a broad signal at room temperature with anisotropy like that seen in CHCl₃. Titration again showed that in this system two molecules of A23187 binds a single Mn²⁺ ion. The detail of the central portion of the spectrum (Figure 4c) was obscured by the narrow sextet lines arising from a few per cent free Mn²⁺ in the sample.

Discussion

Titration of binding to A23187 monitored by fluorescence indicated the stoichiometries of Ca²⁺-, Mg²⁺-, and Sr²⁺-ionophore complexes to be 1:2 in nonpolar media but 1:1 in alcohol-water mixtures (Caswell and Pressman, 1972). On the basis of this observation, the authors suggested that the process of transport involves first the formation of a 1:1 complex at the polar exterior on one side of a membrane, followed by the binding of a second A23187 mole-

cule, "extraction" of the complex into the nonpolar interior, and reversal of the process on the other side.

The results presented here are not inconsistent with this view. Only 1:2 complexes were detectable in CHCl₃ or phospholipid dispersions while in polar solvent evidence was found for the 1:2 Mn complex and both the 1:1 and 1:2 Cu-ionophore complexes. The apparent discrepancy in the stoichiometry of the M²⁺-A23187 complex in ethanol-water (Caswell and Pressman, 1972) is not serious. The stoichiometry observed in a particular case will depend on the magnitudes of the first and second association constants plus the concentrations of the reagents. The differences then between the epr results and those obtained with fluorescence may reflect either the generally stronger affinity of the transition ions or lower concentrations employed with the more sensitive fluorescence technique or both. Factors of this kind must be kept in mind whenever evaluating discrepancies between reported stoichiometries. For example, Case *et al.* (1974) found evidence for the 1:1 Ca-A23187 complex in CHCl₃, but the ionophore concentration was much lower and the metal ion concentration higher than employed here. Again the same authors reported that the M²⁺-A23187 binding is predominantly 1:1 in mitochondrial and sarcoplasmic reticulum membranes, which may appear to conflict with our observations in the DPL "membrane" system. But it should be noted that the concentration of ionophore, whether measured relative to total sample volume or to membrane dry weight, was 2 or 3 orders of magnitude lower than employed here. This would naturally tend to favor the 1:1 species.

Neither the physical explanation for the high specificity of A23187 for divalent cations, especially Mn²⁺, nor its biological significance is clear. The high affinity for Mn²⁺, ionic radius 0.80 Å, as compared to Mg²⁺ (0.65 Å) and Ca²⁺ (0.98 Å) is unlikely to be purely an ionic size effect. Possibly the 3d orbitals of a transition ion can coordinate

with a ring nitrogen on the ionophore, stabilizing the complex. However, the observed hf splitting in the epr spectra implies that there is no appreciable covalent or charge transfer component stabilizing the Mn complex, nor is there evidence of superhyperfine splitting. Another possibility is that stability is enhanced by distortion of the electron cloud away from spherical symmetry. The zero field splittings in the epr spectra would support this view.

Titration of Mn and Cu binding to A23187 in sonicated DPL, along with the spectral changes in the Cu²⁺-ionophore complex correlated with chain mobility, suggest that the epr spectrum in this case arises, in large part, from the Cu²⁺(ionophore)₂ complex sequestered in the hydrocarbon region of the dispersion. The magnitude of these *g* value variations can be used to estimate the tumbling rate and to provide a further check of this hypothesis.

According to the general theory of epr spectral averaging by time-dependent processes (Anderson, 1954), the splitting in energy units between the parallel and perpendicular components of the spectrum is given approximately by: $[(\Delta g)\beta H/2]^2 \sim [(\Delta g_0)\beta H/2]^2 - (h\nu)^2$; $\Delta g_0\beta H > h\nu$, where ν is the fluctuation rate, presumably due to tumbling of the complex, H is the center of field, $\Delta g = g_{\parallel} - g_{\perp}$ and Δg_0 is $g_{\parallel} - g_{\perp}$ for the frozen 1:2 complex. From 15 to 70° in DPL or egg lecithin (Table II) $\Delta g \sim 0.8-0.9\Delta g_0$, leading to a value for the correlation time: $\tau = 1/\nu \sim 1-2 \times 10^{-9}$ sec.

The correlation time of the nitroxide spin-label Tempo in DPL dispersions was measured to be $\sim 10^{-10}$ sec (Hubbell and McConnell, 1968). The Stokes relation for a sphere of radius r in a medium viscosity η , $\tau = 4\pi\eta r^3/3kT$, implies that in the same medium the correlation time should be roughly proportional to the molecular weight of the probe. Using this simplified model the viscosities sensed by Tempo and Cu-A23187 are in excellent agreement. The estimate of correlation time is similar to that obtained through fluorescence depolarization (Case *et al.*, 1974) on A23187 in EDTA treated mitochondrial membranes. On the other hand, A23187 was significantly more immobilized in mitochondrial membranes containing Ca²⁺ and Mg²⁺ or in sarcoplasmic reticulum membranes. Such differences in this regard between biological membranes and phospholipid dispersions are not surprising since the nonphospholipid constituents can be expected to affect local viscosity. Immobilization of the Cu²⁺-A23187 complex was observed with epr

in DPL-cholesterol dispersions.

Nmr data (Scheetz and Chan, 1972) indicate a much higher viscosity in the hydrocarbon region of phospholipid dispersions than is sensed by Tempo. Apparently impurities like Tempo or M²⁺-A23187 can disorder their local environments, rendering them more fluid. This should permit more rapid diffusion of such substances across a membrane. Conversely, cholesterol endows its environment with more rigidity. The results here indicate that cholesterol can exert this influence even in a region around the M²⁺-A23187 complex. Thus, cholesterol would tend to lower the permeability of lipid membranes to these complexes.

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