Interactions of Cu^{2+} and VO^{2+} Ions with Phosphatidylcholine Vesicular Membranes as Studied by the ^{1}H NMR Paramagnetic Relaxation Method

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The spin-relaxation effects of the paramagnetic ions Cu^{2+} and VO^{2+} on the protons of solvent water and lipids in phosphatidylcholine unilamellar vesicles were first observed in order to elucidate the details of the interactions in the membrane surface region. VO^{2+} ions gave higher rates of the effective paramagnetic relaxation induced by binding to a membrane while Cu^{2+} ions had a larger partition coefficient from water to the membrane phase. It is concluded that VO^{2+} occupies a binding site closer to the N-methyl groups in the polar-head network and less accessible to a cation, which causes stronger motional restriction to a bound cation, compared with the site occupied by Cu^{2+}.

Copper(II) and oxovanadium(IV) (vanadyl), of which the transition metals exist in living systems as essential elements, have recently attracted much attention due to their pharmacological effects on cancer and diabetes.[1] In addition to this pharmaceutical interest, there has been a biophysical interest in utilizing the stable cations as nitroxide-alternative ESR spin-probes of the polar regions of lipid membranes. These interests have urged us to study the interaction of the cations with membranes since no details of this interaction have been reported. Cu^{2+} and VO^{2+} are both paramagnetic cations with one unpaired electron (spin quantum number $S=1/2$). In NMR spectroscopy, these act as relaxation reagents rather than as shift reagents, to stimulate the spin relaxation of protons in the surrounding environments much more effectively than nuclei do. The relaxation effect is generally enhanced when spin-relaxant ions are bound to macromolecules to be motionally restricted.[2] Thus, the effect has so far been used to study the interaction of the ions with DNA, enzymes, and other macromolecules.[3] This method of paramagnetic relaxation, which was applied to a membrane system in the present study, has a distinct advantage over the lanthanide-induced shift method[4] in providing not only static information on association equilibria but also dynamic information on membrane-bound cations. We report that the relaxation data determined for solvent–water and lipid protons in small unilamellar vesicles of phosphatidylcholine allowed us to elucidate the dynamic and static characters of the membrane binding-sites for Cu^{2+} and VO^{2+}.

Experimental

Small unilamellar vesicles (SUVs; 20—30 nm o.d.) of egg-yolk phosphatidylcholine (EPC) were prepared on a bath-type sonicator (Branson-220) according to an established procedure.[5] The SUV solution prepared was diluted to several concentrations between 40 and 120 mM (mmol dm^{-3}). The resultant solutions were used as standards for diamagnetic contributions to the spin-lattice relaxation time (T_1) of solvent–water protons. Samples containing paramagnetic species at 2 mM were prepared by adding a small amount of concentrated $CuCl_2$ (or $VOSO_4$) solution to the SUV solutions. All solutions were bubbled with argon gas to remove oxygen. Subsequently, 20 mm^3 of SUV solution were transferred to a 2 mm-o.d. tube which was set inside a 5 mm-o.d. tube containing $CDCl_3$ for field locking. In addition, 100 mM EPC–SUVs in the presence or absence of the paramagnetic species were prepared in D_2O and were transferred to a 5 mm-o.d. tube to observe the T_1 of the lipid protons.

T_1 measurements were carried out at 20 °C using the null-point technique of inversion recovery[6] on a JEOL FX-100 NMR spectrometer operating at 100 MHz. The NMR spectra of solvent–water protons in the samples gave a single line and the longitudinal magnetization exhibited a single exponential-decay with an interval time t in a 180°-t-90° pulse sequence, indicating a fast exchange among the different phases of water in EPC-SUVs. Lanthanide-induced shift experiments were performed by adding 25 mM Eu^{3+} (or 10 mM Pr^{3+}) extravesicularly to 100 mM EPC-SUVs in D_2O, to separate the resonance peaks of the N-methyl protons on the inner and outer surfaces of the SUVs. The integration of the respective peaks provided the proportion of outside-layered EPCs to inside-layered EPCs in the bilayer. The mean $[EPC]_{out}/[EPC]_{in}$ was 2.0($\pm$0.1).

EPC was obtained as a chloroform solution from Sigma Chemical Co. and used without further purification. Cupric chloride and oxovanadium(IV) sulfate n-hydrate from Wako Chemicals were washed with ethanol, dried well under vacuum, and used as anhydrous $CuCl_2$ and $VOSO_4\cdot 3H_2O$.[7]

$EuCl_3 \cdot 6H_2O$ was purchased from Aldrich Chemical Co. Deionized water was of 18 MΩ cm^{-1} quality and D_2O (Aldrich) was of 99.96% purity; both were used as SUV solvents.

Results and Discussion

The results of the T_1's observed for solvent–water protons in EPC–SUVs are summarized in the form of relaxation rates ($R_o=1/T_{1o}$) in Table 1. The R_o in the absence of paramagnetic cations, equivalent to a diamagnetic relaxation rate ($R_{d,o}$), increased with an increase in [EPC] from 0 to 120 mM. This corresponded to an increase in the fraction of the membrane-bound water[8] in equilibrium with the bulk water since the former water is motionally restricted to give a larger R_d. R_o increased significantly in the presence of 2 mM paramagnetic cations. Subtraction of $R_{d,o}$ from this rate at the respective [EPC] yielded the pure contribution of the paramagnetic relaxation ($R_{p,o}$). $R_{p,o}$, as is easily confirmed from Table 1, increased with [EPC], indicating an increased distribution of the cations to the membrane phase from the aqueous phase in the EPC–SUV solutions. This distribution as delineated by an equilibrium constant K_b was analyzed by use of the site model of an "adsorbent" membrane, as follows:

$$M^{2+} + S \rightleftarrows M^{2+}\cdot S \quad (1)$$

$$K_b = [M^{2+}]_b/([M^{2+}]_f \cdot [S]_f) \quad (2)$$

$$[M^{2+}]=[M^{2+}]_b + [M^{2+}]_f$$
$$\text{and} \quad [S]=[S]_b + [S]_f \quad (3)$$

Here, $[M^{2+}]$ and [S] denote the total concentrations of cations and binding sites, respectively, and the subscripts "b" and "f" discriminate between the bound and free states, respectively. Under the condition $[S] \gg [M^{2+}]$, which can be assumed for the present case, Eq. 2 is rewritten to:

$$K_b = [M^{2+}]_b/\{([M^{2+}] - [M^{2+}]_b)\cdot[S]\} \quad (4)$$

On the other hand, $R_{p,o}$ is expressed as:

$$R_{p,o} = R_{p,b}\cdot([M^{2+}]_b/[M^{2+}]) + R_{p,f}\cdot([M^{2+}]_f/[M^{2+}]) \quad (5)$$

using the molar fractions $[M^{2+}]_b/[M^{2+}]$ and $[M^{2+}]_f/[M^{2+}]$. Here, $R_{p,b}$ and $R_{p,f}$ are the paramagnetic relaxation rates which are assigned to be inherent for M^{2+} ions in the bound state and in the free state, respectively. Transformation of Eq. 5 gives rise to:

$$[M^{2+}]_b/[M^{2+}] = (R_{p,o} - R_{p,f})/(R_{p,b} - R_{p,f}) \quad (6)$$

and combination of Eqs. 4 and 6 results in:

$$\frac{1}{R_{p,o} - R_{p,f}} = \frac{1}{R_{p,b} - R_{p,f}}\cdot\frac{1}{K_b}\cdot\frac{1}{[S]} + \frac{1}{R_{p,b} - R_{p,f}} \quad (7)$$

Here, $R_{p,f}$ can be found from the R_o's at [EPC]=0 in Table 1 and [S] can be derived from the relation $[S]=[EPC]_{out}/n$ where $[EPC]_{out}$ is the concentration of outside-layered EPCs in the SUVs only which can participate in forming the binding site owing to membrane impermeability to metal cations and n is the number of EPC molecules constituting the site. The value of $[EPC]_{out}$ determined from Eu^{3+}-induced shift experiments on the N-methyl protons in EPC–SUVs satisfied the relation $[EPC]_{out}=(2/3)[EPC]$ in agreement with the previous results.[4] Using Eq. 7 modified by the above results, the values of $1/(R_{p,o}-R_{p,f})$ were plotted against those of $1/[EPC]_{out}$, as shown in Fig. 1. A least-squares fit allowed us to determine $R_{p,b}$ and K'_b ($=K_b/n$) independently of each other from the intercept and the slope. Here, K'_b is an "apparent" binding constant reduced to unit $[EPC]_{out}$ (not to unit [S]). The values of $R_{p,b}$ and K'_b are give in Table 2, together with those of $R_{p,f}$. Once $R_{p,b}$ was thus obtained, the mole fraction of bound cations, $[M^{2+}]_b/[M^{2+}]$, was determined from Eq. 6 and partition coefficient P for the membrane-to-aqueous phase was then determined as $[M^{2+}]_b$ per mole outer-EPC divided by $[M^{2+}]_f$ per mole water. The values of $[M^{2+}]_b/[M^{2+}]$ at [EPC]=120 mM and P taken as an average at [EPC]=40 to 120 mM are also included in Table 2.

Paramagnetic Cu^{2+} and VO^{2+} ions in an aqueous so-

Table 1. The R_o/s^{-1} of Water Protons in EPC–SUVs with and without 2 mM M^{2+}

[EPC]/mM	0	40	50	60	90	120
EPC–SUVs	0.352	0.439	0.475	0.488	0.587	0.666
$+Cu^{2+}$	1.69	1.98	2.05	2.10	2.24	2.39
$+VO^{2+}$	1.10	1.55	1.66	1.76	2.09	2.26

All values are within an error of 2%.

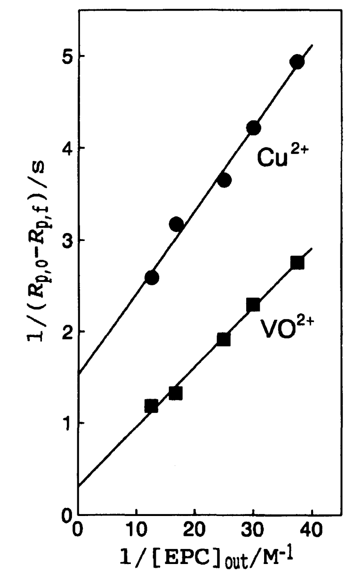

Fig. 1. Plots of $1/(R_{p,o}-R_{p,f})$ against $1/[EPC]_{out}$. Correlation coefficients to a straight line are 0.988 for Cu^{2+} and 0.994 for VO^{2+}.

Table 2. Parameters[a)] for Cu^{2+} and VO^{2+} in EPC–SUVs as Determined by Paramagnetic Spin Relaxation of Water Protons

	$R_{p,b}$ / s^{-1}	$R_{p,f}$ / s^{-1}	K'_b / M^{-1}	P	$[M^{2+}]_b/[M^{2+}]$[b)]
Cu^{2+}	1.99	1.34	17.0	950	0.59
VO^{2+}	4.05	0.75	4.7	260	0.26

a) Determined at 2 mM M^{2+}. b) Mole fraction in 120 mM EPC–SUVs.

lution are coordinated by six and five water molecules,[9)] respectively, to form octahedral aqua-complexes. The fast proton-relaxation of coordinated water exchanging for surrounding water is the origin of R_p for overall-water protons.[2)] The inherent R'_ps, i.e. $R_{p,b}$ and $R_{p,f}$, depend on the correlation time τ_c for the electron–nuclear dipolar interaction, which is contributed by the terms of the rotational correlation time, τ_r, the electron spin relaxation time, τ_s, and the residence time, τ_h, of coordinated water. Of these terms, τ_r is regarded[2)] as dominant in the aqua-complexes. Further, the binding to "macromolecules" such as SUVs prolongs the rotational correlation time of hydrated cations and hence enhances the R_p observed in a fast-motional regime.[10)] The values of $R_{p,b}/R_{p,f}$, i.e., 1.5 for Cu^{2+} and 5.4 for VO^{2+}, therefore, indicate that, upon binding to the membrane, VO^{2+} ions undergo stronger motional restriction than Cu^{2+} ions do. This suggests the existence of higher sterical-hindrance around VO^{2+} ions and/or stronger interaction of VO^{2+} ions with the phosphate groups located on the polar-head region of EPC membranes. Supporting evidence was presented from T_1 measurements of lipid protons in 100 mM EPC–SUVs. As seen in Table 3, the presence of 2 mM paramagnetic cations in the SUVs induces an increase in R_o for N-methyls on the polar-head region, but induces no change in R_o's for the methylenes[11)] and C-methyls of acyl chains located in the membrane interior. In addition, the increment, i.e., $R_{p,o}$, for the N-methyls is 6-fold larger in VO^{2+} than in Cu^{2+}, which means a 12-fold "effective" relaxation by VO^{2+} if the populations of bound cations at [EPC]=100 mM are taken into account (refer to Table 2). These facts let us infer[10)] that both cations partitioned to the membrane are certainly localized near the polar-head region and VO^{2+} ions, relative to Cu^{2+} ions, are in close proximity to the lipid N-methyl groups and hence to the phosphate groups.

Table 3. The R_o/s^{-1} of EPC Protons in 100 mM EPC–SUVs with and without 2 mM M^{2+}

	N-Methyls	Methylenes[a)]	C-Methyls
EPC–SUVs	4.3	3.2	2.0
$+Cu^{2+}$	5.0	3.2	2.0
$+VO^{2+}$	8.7	3.2	2.0

Errors estimated are 2% for the N-methyls and 5% for the methylenes and C-methyls. a) Most methylenes of EPC acyl-chains except for those near both terminals.[11)]

The dynamic behavior of cations as viewed from the $R_{p,b}$ value appears to be different from the picture expected from the K'_b (or P) value which is larger for Cu^{2+} than for VO^{2+} (Table 2). Such an apparent conflict, which has sometimes been found for other molecular interactions,[12)] could be reconciled by considering the specificity of the binding sites for Cu^{2+} and VO^{2+}. A lanthanide-induced shift study[4)] has suggested two possible binding sets in the interaction, i.e., cation : phosphate=1 : 1 and 1 : 2, of which the latter means the chelation of a cation by two lipid-phosphate groups neighboring each other. The respective sets correspond to n=1 and 2 in the present site-model. It seems reasonable to assign the sites of n=1 and 2 to Cu^{2+} and VO^{2+}, respectively, as schematically depicted in Fig. 2. In the figure, Cu^{2+} ions preferentially occupy a site present outside the polar-head region but still within the bound-water region while VO^{2+} ions select a site present inside the polar-head network. The n=2 site provides an environment less accessible to a cation, closer to the polar N-methyl groups, and motionally more restricted for cations, compared with that provided by the n=1 site. Thus, the specification of the respective sites dissolves the above conflict and also reveals the "true" binding constants per unit [S]: i.e., K_b ($=n \cdot K'_b$)=17 M^{-1} for Cu^{2+} and 9.4 M^{-1} for VO^{2+}, although the reason why Cu^{2+} and VO^{2+} prefer their respective sites remains as a future problem.

The dynamic behavior found with ^1H NMR relaxation was supported by a stochastic-Liouville lineshape analysis[13)] on the ESR spectra of Cu^{2+} and VO^{2+} in dipalmitoylphosphatidylcholine (DPPC) dispersions hy-

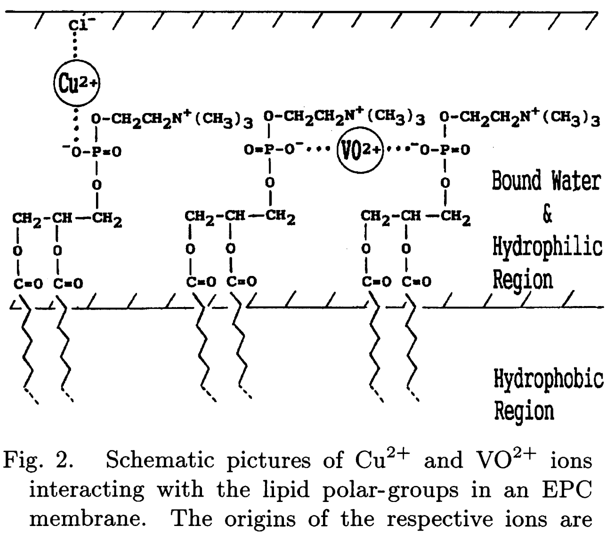

Fig. 2. Schematic pictures of Cu^{2+} and VO^{2+} ions interacting with the lipid polar-groups in an EPC membrane. The origins of the respective ions are $CuCl_2$ and $VOSO_4$. The axis connecting the phosphate and N-methyl groups is largely inclined to the membrane bilayer and the bound water reaches the C2-carbon of the acyl chains according to the most recent study (see Ref. 8).

drated to 50 wt%: the data[14] at 25 °C showed averaged rotational correlation times, $\tau_{c,av}$, of 2.1 ns for Cu^{2+} and 5.6 ns for VO^{2+} with anisotropies, $\tau_{c,xy}/\tau_{c,zz}$, near 1 and 100, respectively. The results confirm that membrane-bound VO^{2+} ions are strongly restricted and highly anisotropic in molecular rotation in comparison to Cu^{2+} ions. Eventually, it should be emphasized that Cu^{2+} is likely to probe the bound water and VO^{2+} to probe the lipid polar-head itself. Further, the present findings will aid in understanding the metal ion-induced pharmacological effects[1] which appear in the coexistence of Cu^{2+} with major drugs and on the administration of $VOSO_4$ and its related compounds. Finally, we could say that the ^1H NMR paramagnetic relaxation method demonstrates considerable power in the investigation of molecular interactions between spin-relaxant ions and vesicular membranes.

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10) In this case, the inherent R_p is dependent on the term τ_c/r^6 where r is an averaged distance between a paramagnetic center and the concerned protons. For water-protons R_p, τ_c and r refer to the rotation and the paramagnetic center-ligand protons distance of an aqua-complex, while for lipid-protons R_p, they refer to the motion and the length of the axis connecting the paramagnetic center and the lipid protons, respectively. Thus, if the value of effective relaxation for the N-methyl protons is attributed entirely to the $1/r^6$ term, VO^{2+} is then positioned 1.5-fold closer to the protons than Cu^{2+} is. See Ref. 2.

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