Biophysical Chemistry 3 (1975) 183–191


# A PROTON-RELAXATION ENHANCEMENT STUDY OF THE INTERACTION OF MANGANOUS IONS WITH PHOSPHOLIPIDS IN AQUEOUS DISPERSIONS

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Received 12 November 1974

An interaction of dipalmitoylphosphatidylcholine (PC) and phosphatidylserine (PS) with manganous ions has been investigated by measuring the effect of bound manganese upon the longitudinal relaxation rate, $1/T_1$, of the solvent water protons and evaluating the enhancement factor $\epsilon_b$. The observed enhancement values were used to determine the number of interacting sites per polar head group, $n$, and the values of association constants, $K_A$, of manganese to PC and PS. Changes in $\epsilon_b$ correlate with structural changes at the interacting site. By increasing the temperature one can see an abrupt decrease in $\epsilon_b$ within the temperature interval from 40 to 50°C indicating the thermal phase transition of PC as established by calorimetry, fluorescence and high-resolution NMR measurements. That an enhancement of $1/T_1$ of the solvent-water protons occurs at all is explained by assuming a restricted rotation of the $Mn^{2+}$–aquo complex in the bound state. In addition we suppose that the rotation of the $Mn^{2+}$–aquo complex is the mechanism which dominates the relaxation of the water protons in the bulk solvent when phospholipids are present.

## 1. Introduction

Phospholipids are a major constituent of many biological membranes. There is considerable evidence for the occurrence of lipid bilayer regions within the structure of these membranes [1,2]. The characteristics of the membrane, like stability and permeability are dependent on the mobility and structure of the fatty-acid chains and the polar head groups of the phospholipids.

The permeability to ions is one of the most important features of the biological membrane [3,4]. The binding of ions at the polar groups which are in contact with the aqueous solution of the ions is directly connected to the process of entry into the membrane. The knowledge of the degree and strength of ion binding should therefore be helpful in understanding some biological functions of the polar head groups.

Metal-ion binding has been investigated by means of the proton-relaxation enhancement (PRE) method [5]. In contrast with the standard equilibrium dialysis the PRE method requires no solutions of high ionic strength, but in its most direct application it is only useful for certain paramagnetic ions (e.g. $Mn^{2+}$, $Cu^{2+}$, $Cr^{3+}$).

The PRE technique is based on interactions between nuclei and unpaired electrons. The longitudinal nuclear relaxation rate, $1/T_1$, of the protons of water is increased by the presence of certain paramagnetic ions [6–8]. This effect is enhanced when the ions are bound to macromolecules. This phenomenon was first observed in solutions of DNA containing transition metals[☆] [5] and in solutions of $Mn^{2+}$–protein complexes [9]. The enhancements are ascribed to restricted rotation of the $Mn^{2+}$–aquo complex in the bound state which will increase the rotational correlation time $\tau_R$. Since in solutions of $Mn^{2+}$ the correlation time for the electron–nucleus dipolar interaction, $\tau_C$, is determined by $\tau_R$, the value of $\tau_C$ will also increase on binding [10]. Therefore the observed relaxation times $T_1^{*}$ [☆☆] for the water protons

☆ Strictly speaking, we are dealing with hydrated ions, for simplicity, however, we are often speaking of "metals" rather than of ions.

☆☆ In the following, the terms with asterisks (*) represent parameters in the presence of phospholipids.

carry information about the rotation of the bound $Mn^{2+}$–aquo complex and its molecular dynamics. The theoretical basis of the PRE technique and the applications to systems containing macromolecules have been presented and discussed elsewhere [5,10, 11].

In this paper we report a more detailed study of the interaction of manganous ions with phospholipids in aqueous dispersions. We have investigated two binary systems consisting of an aqueous solution containing a salt of $Mn^{2+}$ and dipalmitoylphosphatidylcholine and phosphatidylserine, respectively.

The results presented here demonstrate that PRE measurements can be used for determining association constants and numbers of binding sites per polar head group and for explaining some features of the $Mn^{2+}$–aquo complex in the bound state.

## 2. Materials and methods

1,2-dipalmitoyl-L-3-phosphatidylcholine was purchased from Fluka GmbH, Neu-Ulm, and purified by silicagel column chromatography employing the known procedures [12,13]. Phosphatidylserine ex bovine brain was obtained from Koch-Light Lab., Colnbrook, and was purified as well by chromatography.

The dispersions were made by dissolving 200 mg of the lipid in $CHCl_3$, evaporating the solvent under nitrogen and adding 5 ml of bidistilled water without an attempt to remove oxygen from the solutions. These crude dispersions were sonicated under nitrogen in an ice-cooled glass tube using the MSE Ultrasonic Disintegrator at 20 kHz and amplitude level 8 for ten minutes. These ultrasonicated dispersions were used to prepare solutions of different concentrations of the phospholipids during the type I titrations (see sect. 3). The experiments were performed in pure water without buffer at about pH 6.4. $Mn^{2+}$ ions were added as small volumes of aqueous solutions of $MnCl_2 \cdot 4H_2O$ to the phospholipid dispersions after sonication.

The proton relaxation times $T_1$ were measured with a pulsed nuclear magnetic-resonance spectrometer described previously [14,15] using 0.6 ml sample volumes. It operates at 20 MHz by the $90°$–$\tau$–$90°$ pulse-sequence method. The values of $T_1$ were accurate to within ± 5%. To eliminate systematic errors the value of $T_1$ of pure degassed water was checked in appropriate intervals and always found to be 3.5 sec at 25°C. The temperature was controlled to ±1°C.

## 3. Results and treatment of data

### 3.1. Comparison of PC and PS proton-relaxation enhancement data

Usually the proton-relaxation enhancement factor for a solution, $\epsilon^*$, is defined as

$$\epsilon^* \equiv \frac{1/T^*_{1p}}{1/T_{1p}} = \frac{1/T^*_1 - 1/T^*_{1(0)}}{1/T_1 - 1/T_{1(0)}}. \quad (1)$$

$T_1$ is the observed relaxation time in the presence of manganese, $T_{1(0)}$ the observed relaxation time in the absence of manganese (2.78 sec at 25°C). The value of $T_{1(0)}$ was 2.48 sec at 25°C and constant over the measured concentration range of phospholipid until $0.5 \times 10^{-1}$ M.

Two types of "titration" were performed with $\epsilon^*$ as parameter.

*Type I titration*: Variation of the phospholipid concentration at constant manganous-ion concentration.

*Type II titration*: Variation of the manganous-ion concentration at constant phospholipid concentration.

Fig. 1 shows the values of $T_1$ for the water protons as a function of the manganous-ion concentration at 25°C and pH 6.4 at a constant PC concentration ($10^{-2}$ M) and the corresponding diagram at a constant PS concentration ($10^{-3}$ M). Both titrations are of type II.

The observed enhancement factor, $\epsilon^*$, is the weighted average of $\epsilon_b$, the characteristic enhancement of the manganous ions in the bound state and $\epsilon_f$, the enhancement of free, i.e. unbound, manganous ions. The value of $\epsilon_f$ is unity on the assumption that the microviscosity of the regions surrounding the free manganous ions is identical in the solutions in which phospholipids are present or absent [10]. Hence

$$\epsilon^* = ([Mn]_b/[Mn]_t)\,\epsilon_b + [Mn]_f/[Mn]_t, \quad (2)$$

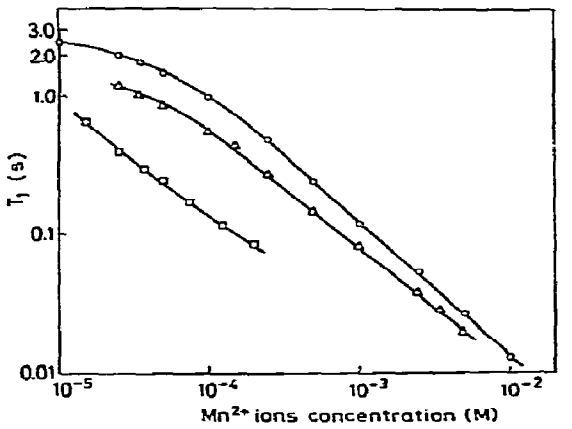



Fig. 1. Type II titration. Values of $T_1$ for the water protons as a function of the manganous ion concentration at 25°C and pH 6.4. ○ Aqueous solution of $Mn^{2+}$, △ aqueous solution of $Mn^{2+}$ at a constant PC concentration ($10^{-2}$ M), □ aqueous solution of $Mn^{2+}$ at a constant PS concentration ($10^{-3}$ M).

where $[Mn]_b$ is the concentration of metal ions bound to the phospholipid and $[Mn]_f$ the concentration of metal ions free in solution; $[Mn]_t$ represents the total ion concentration. $\epsilon_b$ corresponds physically to the situation of measuring an enhancement of $1/T_1$ of a solution in which all the manganous ions are bound. The value of $\epsilon_b$ can be obtained by a type I titration and plotting $1/\epsilon^*$ against the reciprocal of the total concentration of PC and PS, respectively.

Before evaluating this type I titration we may describe the interaction of manganous ions with phospholipids by the equilibrium

$$Mn_f + jP_f \rightleftharpoons MnP_j, \tag{3}$$

where $Mn_f$ and $P_f$ represent free metal ions and free phospholipid molecules, respectively, and $j$ a stoichoimetric factor. We define the effective association constant, $K_A$, as:

$$K_A \equiv K^{(j)} = [MnP_j]/[Mn]_f[P]_f = [Mn]_b/[Mn]_f[P]_f, \tag{4}$$

where $[P]_f$ is the concentration of free binding sites. $[P]_f$ is equal to the concentration of total sites $n[P]_t$ minus $[Mn]_b$, where $[P]_t$ is the total lipid concentration in the outer monolayer (see below) and $n \equiv 1/j$ the number of binding sites per polar group. Therefore we can write eq. (4) as

$$K_A = [Mn]_b/(n[P]_t - [Mn]_b)[Mn]_f. \tag{5}$$

Now we are able to evaluate the type I titration. Combining eq. (2) and the following equation:

$$[Mn]_t = [Mn]_b + [Mn]_f, \tag{6}$$

we get expressions for $[Mn]_b$ and $[Mn]_f$:

$$[Mn]_b = \{(\epsilon^* - 1)/(\epsilon_b - 1)\}[Mn]_t, \tag{7}$$

and

$$[Mn]_f = \{(\epsilon_b - \epsilon^*)/(\epsilon_b - 1)\}[Mn]_t. \tag{8}$$

Putting these expressions in eq. (5) we obtain for high phospholipid concentrations – the experimental conditions in performing the type I titration were chosen in such a manner – where $n[P]_t \gg [Mn]_b$ and $\epsilon_b n[P]_t \gg 1/K_A$:

$$1/\epsilon^* = 1/\epsilon_b + 1/\epsilon_b nK_A[P]_t, \tag{9}$$

i.e. the reciprocal of the observed enhancement factor is a linear function of the reciprocal of the total phospholipid concentration. The extrapolation of $1/[P]_t \rightarrow 0$ allows $\epsilon_b$ to be obtained and the slope will yield $nK_A$.

In this connection it should be mentioned that the synthetic bilayer membrane is impermeable to the $Mn^{2+}$ ions [16]. So the total accessible phospholipid concentration depends on the size of the vesicle. Our own high-resolution NMR measurements ($^1H$ and $^{13}C$, unpublished results) have shown that 64.5% of the total phospholipid molecules are in the outer monolayer which is accessible to the $Mn^{2+}$ ions. On the assumption that there is a spherical double layer of 50 Å thickness we obtain an average vesicle size of 386 Å in diameter under the experimental conditions mentioned above. In the case of PS we assume a similar size. Therefore, in eqs. (5,9,10), $[P]_t$ is defined as the accessible phospholipid concentration in the outer monolayer.

Figs. 2 and 3 show the values of $1/\epsilon^*$ as a function of the reciprocal of the total PC and PS concentration, respectively, at a constant $Mn^{2+}$ ion concentration ($0.25 \times 10^{-4}$ M). According to eq. (9) we obtain a straight line at high phospholipid concentrations. Table 1 summarizes the experimental results. The margin of error is estimated to be ± 7%.

It should be mentioned that the concentration of

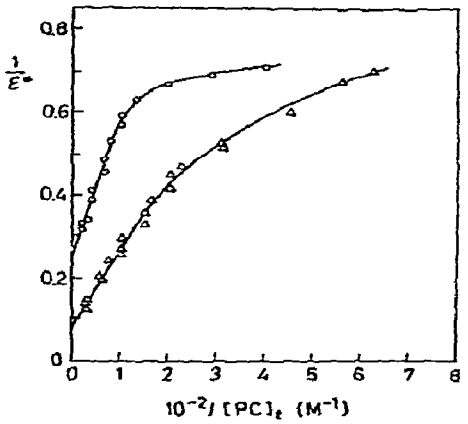



Fig. 2. Type I titration. Double reciprocal plot of $\epsilon^*$ versus total PC concentration. The manganous-ion concentration is held constant ($0.25 \times 10^{-4}$). ○ At 54°C, △ at 25°C.

free $Mn^{2+}$ ions can directly be measured by performing an ESR titration with the same dispersions as used in the type I titration. Combining ESR and PRE data by means of eq. (8) one obtains a set of determinations for $\epsilon_b$. In this work we may confine to the double reciprocal plot in determining $\epsilon_b$.

Once determined, $\epsilon_b$ can be employed for calculating the value of $[Mn]_b$ by means of eq. (7) on the assumption that there is only one class of binding sites and therefore only one value of $\epsilon_b$.

Now we are able to use the type II titration for

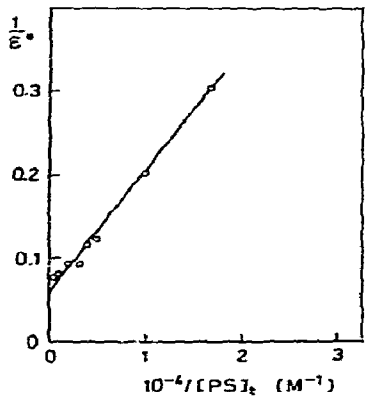



Fig. 3. Type I titration. Double reciprocal plot of $\epsilon^*$ versus total PS concentration at 54°C. The manganous-ion concentration is held constant ($0.25 \times 10^{-4}$).

Table 1
Comparison of the enhancement factors, $\epsilon_b$, the values of $nK_A$, the numbers of interacting sites per polar group $n$, and the association constants, $K_A$, for dipalmitoylphosphatidylcholine and phosphatidylserine, respectively [a].

| PRE parameter | Phospholipid | |
|---|---|---|
| | PC | PS |
| $\epsilon_b$ | 4.0 | 16.7 |
| $10^{-2} \times nK_A$ $(M^{-1})$ (type I titration) | 0.66 | 41.0 |
| $10^{-2} \times nK_A$ $(M^{-1})$ (type II titration) | 0.75 | 54.0 |
| $10^2 n$ | 0.47 | 6.8 |
| $10^{-4}K_A$ $(M^{-1})$ (type II titration) | 1.4 | 8.0 |
| $10^{-4}\bar{K}_A$ $(M^{-1})$ [eq. (6)] | 1.6 | 9.9 |

[a] The type I and II titrations were performed at 54°C; at this temperature both phospholipids are above the phase transition temperature.

determining the values of $K_A$ and $n$ by means of eq. (5) after rearrangement:

$$[Mn]_b/[P]_t[Mn]_f = nK_A - ([Mn]_b/[P]_t)K_A \,, \qquad (10)$$

i.e. the type II titration data are plotted according to the methods of Scatchard and Black [17]. With $\bar{\nu} \equiv [Mn]_b/[P]_t$ and $\bar{\nu}' \equiv \bar{\nu}/[Mn]_f$ we get:

$$\bar{\nu}' = nK_A - \bar{\nu}K_A \,. \qquad (11)$$

Plots of $\bar{\nu}'$ versus $\bar{\nu}$ then allow $K_A$ and $n$ to be obtained. Eq. (11) is only valid if there is one class of equivalent independent sites [11]. Generally, if there is more than one class of binding sites because $\epsilon_b$ and $n$ vary during the titration, the curves will be complex. The intersection of the tangent to the curve at low values of $\bar{\nu}$ with the abcissa will yield a value of $n$ which is the number of sites having an enhancement factor as used for evaluating eq. (7).

Figs. 4 and 5 show the "Scatchard and Black" plots for the interaction of $Mn^{2+}$ with PC and PS, respectively. Using the value 4.0 for $\epsilon_b$ in the case of PC and 16.7 in the case of PS one obtains values of $n$, $nK_A$ and $K_A$ which are also summarized in table 1. These values are less precise than the values of the type I titration and are estimated to be about ± 20%.

A numerical analysis of the values of $\epsilon^*$ can improve on the values obtained for $K_A$ graphically by

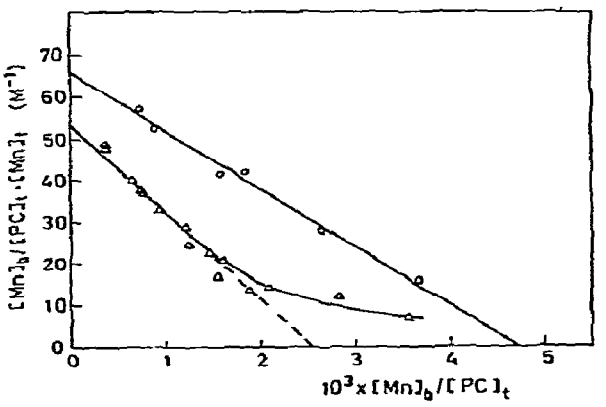



Fig. 4. PRE titration of PC with $Mn^{2+}$. "Scatchard and Black" plot of $\bar{\nu}' \equiv [Mn]_b/[PC]_t[Mn]_f$ versus $\bar{\nu} \equiv [Mn]_b/[PC]_t$. ○ At 54°C, △ at 25°C.

the "Scatchard and Black" plots. Putting the corresponding values of $[Mn]_b$ and $[Mn]_f$ [see eqs. (7,8)] in eq. (5) one obtains a set of determinations for $K_A$. The averages of these association constants, $\bar{K}_A$, are also summarized in table 1. They do agree with the values obtained by the "Scatchard and Black" plots.

For the explanation of the curvature of the plot at high values of $\bar{\nu}$ in figs. 4 and 5 see discussion below.

It should be noted that two PRE parameters for

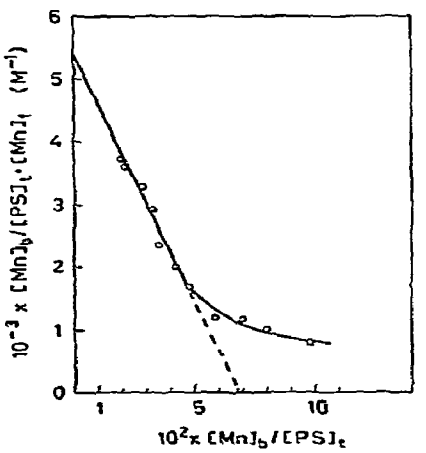



Fig. 5. PRE titration of PS with $Mn^{2+}$. "Scatchard and Black" plot of $\bar{\nu}' \equiv [Mn]_b/[PS]_t[Mn]_f$ versus $\bar{\nu} \equiv [Mn]_b/[PS]_t$ at 54°C.

dipalmitoylphosphatidylcholine are mentioned in the paper of Radda [18]. The value for $\epsilon_b$ is 30.0 and the one for the dissociation constant $K_D$ is 3.2 mM. These two values do not agree with our data (see tables 1 and 2). The experimental conditions, however, like temperature and preparation of the dispersions are not given by that author so that a direct comparison of the data is not possible.

### *3.2. Comparison of PC proton-relaxation enhancement data at different temperatures*

Fig. 6 shows the values of $\epsilon^*$ of a type I titration at pH 6.4 as a function of temperature. The different curves refer to different ratios of $[PC]_t/[Mn]_t$. Curve (a) represents the values for $\epsilon^*$ at a ratio of $2 \times 10^3$, curve (b) at a ratio of $0.6 \times 10^3$ and curve (c) at a ratio of $0.4 \times 10^3$. In the special case of the upper curve where the ratio $[PC]_t/[Mn]_t \to \infty$, $\epsilon^*$ is identical with $\epsilon_b$.

One can see at all ratios an abrupt decrease in $\epsilon^*$ within the temperature interval from approximately 40 to 50°C. This effect is most clearly seen in the case of the upper curve, i.e. by plotting $\epsilon_b$ versus temperature because all the manganous ions are bound.

For our present purpose we interpret these observations by assuming that the bound $Mn^{2+}$–aquo

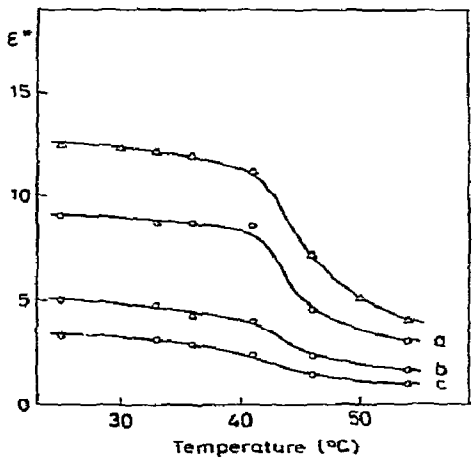



Fig. 6. Type I titration. Values of $\epsilon^*$ for PC as a function of temperature. The different curves refer to different ratios of $[PC]_t/[Mn]_t$ (see subsect. 3.2). The upper curve represents the values of $\epsilon_b$ as a function of temperature.

complex is much more mobile when the dipalmitoylphosphatidylcholine has undergone the crystalline → liquid–crystalline phase transition at 41°C [19]. This explanation implies a lateral expansion of the polar head-group arrangement which has directly been shown e.g. by dilatometric measurements [20]. Therefore $\epsilon_b$ is a characteristic parameter of the $Mn^{2+}$ binding sites and can be used to detect structural changes at these sites. Because of the wide temperature interval the PRE method is less suitable for determining the exact transition temperature than the methods mentioned above [19,21,22]. However, the data obtained for $\epsilon^*$ as a function of temperature give information on the concentration of the manganous ions at the binding site.

Fig. 2 shows the type I titration plots for PC at 25 and 54°C, respectively.

Fig. 4 shows the "Scatchard and Black" plots for the interaction of $Mn^{2+}$ ions with PC at these two temperatures.

Summarizing the results we obtain table 2, which also includes the values for $\bar{K}_A$ obtained by numerical analysis.

In the same way as the values of $\epsilon_b$ the values of $n$ – at 54°C approximately twice the value of the one at the lower temperature – reflect the higher mobility of the polar groups in the temperature region above the transition temperature. The comparison of the values of $K_A$ and $\bar{K}_A$ at 25 and 54°C, respectively, shows that the equilibrium (3) is slightly shifted to the left side with increasing temperature.

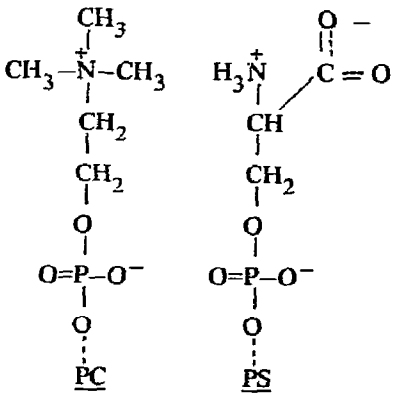
For the explanation of the curvature of the plot at high values of $\bar{\nu}$ in fig. 4, see sect. 4.

Table 2
Comparison of the enhancement factors, $\epsilon_b$, the values of $nK_A$, the numbers of interacting sites per polar group, $n$, and the association constants, $K_A$, for dipalmitoylphosphatidylcholine at 25 and 54°C, respectively

| PRE parameter | Dipalmitoylphosphatidylcholine | |
|---|---|---|
| | Temperature (°C) | |
| | 25 | 54 |
| $\epsilon_b$ | 12.5 | 4.0 |
| $nK_A$ ($M^{-1}$) (type I titration) | 47.0 | 66.0 |
| $nK_A$ $M^{-1}$) (type II titration) | 53.0 | 75.0 |
| $10^2 n$ | 0.25 | 0.47 |
| $10^{-2}K_A$ ($M^{-1}$) (type II titration) | 2.1 | 1.4 |
| $10^{-2}\bar{K}_A$ ($M^{-1}$) [eq. (6)]* | 2.0 | 1.6 |

## 4. Discussion

Before discussing the PRE data of PC and PS we may compare the chemical structure of the polar head groups of these phospholipids:




The phosphatidylcholine is a zwitter-ionic phospholipid while the phosphatidylserine has an extra negative charge.

In order to understand why an enhancement of $1/T_1$ occurs at all, it is of course necessary to have the explicit forms of $1/T_{1p}^*$ and $1/T_{1p}$ [see eq. (1)] which are the paramagnetic contributions to the observed rates $1/T_1^*$ and $1/T_1$. By Luz and Meiboom [8] $1/T_{1p}$ is shown to be:

$$1/T_{1p} = P_{Mn} q/(T_{1M} + \tau_M), \qquad (12)$$

where $P_{Mn}$ is the mole fraction of paramagnetic ion, $q$ the number of water molecules coordinated in the first hydration sphere of $Mn^{2+}$, $T_{1M}$ the relaxation time of the bound protons and $\tau_M$ the lifetime of a proton in the first hydration sphere of an $Mn^{2+}$ ion. This equation is also valid in the presence of phospholipids [parameters noted with asterisks (*)]. As derived by Dwek [10], in aqueous solutions of $Mn^{2+}$, $\epsilon_b$ becomes:

$$\epsilon_b = q^*(T_{1M})_f/q(T^*_{1M})_b = q^*(r)^6 f(\tau^*_C)/q(r^*)^6 f(\tau_C), \quad (13)$$

where $r$ is the average distance between the proton and the manganous ion. The value of $\epsilon_b$ reflects the change of $T_{1M}$ and $\tau_C$, respectively, when the manganous ions are bound to phospholipids. Prerequisite for binding is that $q^*$ becomes smaller than $q$. Since we do not know the value of $q^*$ we may say that the value for the ratio of $(T_{1M})_f/(T^*_{1M})_b$ is larger than 4.0 in the case of PC while the ratio for PS is larger than 16.7. Changing $q^*$ changes the values of $r^*$. By assuming that the Mn–$H_2O$ distance is virtually unchanged from the values in the pure aqueous complex [10], the ratios for $\tau^*_C/\tau_C$ are in the same order of magnitude as the ratios for $(T_{1M})_f/(T^*_{1M})_b$, i.e. $\tau^*_C > \tau_C$.

The expression for the correlation time is the reciprocal relationship:

$$1/\tau^*_C = 1/\tau^*_R + 1/\tau^{*\,\star}_S + 1/\tau^*_M, \quad (14)$$

i.e. the smallest $\tau^*$ dominates $\tau^*_C$. From our data it cannot be said which correlation time dominates $\tau^*_C$. For this purpose additional experiments are required. However, if $\tau_R$ makes a contribution to $\tau_C$ initially, the values of $\tau_C$ will increase on binding, i.e. it does not matter whether $1/\tau^*_R$ is important in $1/\tau^*_C$, as long as $\tau^*_R > \tau_R$, an enhancement of the relaxation rate will be observed.

Fortunately, the determinations of $\epsilon_b$ – and hence $n$ and $K_A$ – requires no quantitative knowledge of the individual correlation times so that the PRE binding data can be used in the further discussion.

The most surprising facts are the absolute values of $n$ as obtained by the "Scatchard and Black" plots (see figs. 4 and 5). For PC, $n$ is $0.47 \times 10^{-2}$, i.e. this is the ratio of one $Mn^{2+}$ ion to approximately 210 phospholipid molecules or on the average 40 ions per vesicle (about the characteristic size see subsect. 3.1). In the case of PS, one obtains a ratio of one $Mn^{2+}$ ion to approximately 15 PS molecules.

That an interaction of the manganous ions with the phospholipids really occurs has been demonstrated by several authors [16,23] by means of high-resolution NMR. It cannot be simulated by small anionic impurities eventually present, such as fatty acids. The manganous ions have an effect on the polar groups and in the case of PC we interpret our observations in the following way:

The zwitter ionic polar groups with their positive charges to the aqueous medium are able to shield the cationic $Mn^{2+}$ ions and to prevent them from forming a stoichiometric bond with the phosphate groups. The phosphate groups appear to cause only an increased probability of finding the $Mn^{2+}$ ions in the regions of the membrane surface and hence effect the magnetic properties of the $Mn^{2+}$–aquo complex in the interacting state. Therefore it seems to be better not to speak of binding, binding sites and binding constants but only of dynamic interactions. Interpretation in this sense causes $n$ and $K_A$ to be regarded as parameters which can be used to describe a measurable interaction of $Mn^{2+}$ ions with phospholipids as formal binding. The observation that all external polar groups are equivalent (own unpublished NMR results), i.e. that there is a rapid exchange of metal ions between the interaction sites which are distributed fairly uniformly over the surface of the vesicle is in good agreement with the former interpretation.

Because of this interpretation and the very small increase in $\tau_C$ the rotation of the bound $Mn^{2+}$–aquo complex seems to be the mechanism which dominates the relaxation of the water protons in the bulk solvent when PC is present.

In the case of PS one should expect a stoichiometric bond because of the carboxyl-group, i.e. a value of 0.5 for $n$. The actual value of 0.068 for $n$ is interpreted in such a manner that the $Mn^{2+}$ ions are hindered to form a stoichiometric bond because the $\overset{+}{N}H_3$ group shields the $COO^-$ group, since PS exists at a pH of 6.4 in the form of its monosodium salt [24]. The negative charge of the polar group is, however, able to cause a higher propability of finding the $Mn^{2+}$ ions on the surface of the vesicle in the case of PS than in the case of PC. This is a direct consequence of the observation that the value for $n$ is approximately one order of magnitude higher than the value for $n$ in the case of PC.

We interpret the observation that the value of $\epsilon_b$ for PS is approximately four times the value of $\epsilon_b$ for PC by assuming that the bound $Mn^{2+}$–aquo complex is more strongly immobilized in the case of PS than in PC due to the carboxyl-group. The same interpretation appears to be valid by regarding the val-

$^\star$ $\tau_S$ = electron spin relaxation time of $Mn^{2+}$.

Table 3
Comparison of the values for the binding sites per polar group, $n$, and the association constants, $K_A$, of dipalmitoylphosphatidylcholine for different ions. The data of ref. [25] are valid for 52°C, our data for 54°C

| Ions | $n$ | $K_A \times 10^{-4}$ ($M^{-1}$) |
|---|---|---|
| $Nd^{3+}$ | | 0.01–0.1 |
| $Eu^{3+}$ [25] | 0.03–0.13 | 0.028–0.94 |
| $UO_2^{2+}$ | | – |
| $Fe^{3+}$ [26] | ~0.01 | – |
| $Mn^{2+}$ | 0.0047 ± 0.001 | 1.5 ± 0.3 |

ues for $n$ (see table 1).

Values for $n$ and $K_A$ of dipalmitoylphosphatidylcholine dispersions for different ions are calculated from NMR-shift data by Levine et al. [25]. The authors obtain an upper and lower limit for the two binding parameters. In the same way adsorption measurements by MacDonald and Thompson [26] yield only an upper limit for the number of lecithin molecules taking part in binding. The results of these investigations are summarized together with the PRE results of this work in table 3.

While the adsorption method yields a value for $n$ for $Fe^{3+}$ ions which is in the same order of magnitude as the one for $Mn^{2+}$ ions, the values for $UO_2^{2+}$, $Eu^{3+}$ and $Nd^{3+}$ ions obtained from chemical-shift data differ at least by one order of magnitude from the former values.

The values for $K_A$ of dipalmitoylphosphatidylcholine for the trivalent cations are smaller than the corresponding value for the $Mn^{2+}$ ion. The value for the divalent $UO_2^{2+}$ ion, however, is not given by the authors, so that one may say that the affinity of the different ions for PC is in the order $Mn^{2+} \approx UO_2^{2+} > Eu^{3+} > Nd^{3+}$.

The higher affinity between the polar surface and the hydrated manganous ions in the case of PS is in so far of biological interest as in phospholipid mixtures the different phospholipids may be preferentially partitioned between the inner and outer faces of the vesicle [27]. Our own high resolution NMR measurements (unpublished results) have shown that e.g. in egg-yolk lecithin – phosphatidylserine mixtures at a pH of 8.3, where PS exists in the form of its disodium salt, n the average only 32.6% (compare subsect. 3.1) of the lecithin molecules are in the outer monolayer, i.e. it contains approximately three times more phosphatidylserine than lecithin molecules. The PRE results for PC and PS suggest as well the ability of PS to form the major constituent of that surface of the vesicle which is accessible to ions and possibly to other ligands like proteins.

Finally, let us consider the curvature of the plots of figs. 4 and 5. As mentioned above, eq. (11) is valid if there is only one class of independent sites. Although there is no evidence for different values of $\epsilon_b$ in the type I titration plots (see figs. 2 and 3) we obtain curvatures in the "Scatchard and Black" plots at high values of $\bar{\nu}$. Possibly another mechanism as discussed above may be valid at these higher electrolyte concentrations – in the range of which the values of $\epsilon^*$ are nearly unity, i.e. an enhancement is no longer observed, since the interacting sites are all "occupied" – but cannot be detected by PRE measurements. Therefore we have made an attempt to obtain further information about the interaction of paramagnetic ions with phospholipids in aqueous dispersions by means of high-resolution NMR ($^1H$, $^{13}C$, $^{31}P$). The results will be published in the near future.

## Acknowledgements

We wish to thank Dr. M.D. Zeidler, University of Karlsruhe, Fed. Rep. of Germany, for kindly submitting the pulsed NMR spectrometer and for his aid and advice in obtaining the $T_1$ data. We also thank Dipl. Chem. A. Blume for many helpful discussions and for critically reading the manuscript.

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