Seelig, J., Gally, U. H., & Wohlgemuth, R. (1977) *Biochim. Biophys. Acta 467*, 109-119.
Senko, M. E., & Templeton, H. (1960) *Acta Crystallogr. 13*, 281-285.
Shipman, J. J., Folt, V. L., & Krimm, S. (1962) *Spectrochim. Acta 18*, 1603-1613.
Snyder, R. G., & Schachtschneider, J. H. (1969) *J. Mol. Spectrosc. 30*, 290-309.
Sugeta, H. (1975) *Spectrochim. Acta, Part A 31*, 1729-1737.
Sugeta, H., Go, A., & Miyazawa, T. (1972) *Chem. Lett.*, 83-86.
Sundaralingam, M. (1968) *Nature (London) 217*, 35-37.
Vieler, P., & Galsomias, J. (1968) *Bull. Soc. Chim. Fr.*, 461-462.
Wallach, D. F. H., & Verma, S. P. (1975) *Biochim. Biophys. Acta 382*, 542-551.
Wolfe, S. (1972) *Acc. Chem. Res. 5*, 102-111.
Yeagle, P. L., Hutton, W. C., Huang, C., & Martin, R. B. (1977) *Biochemistry 16*, 4344-4348.

Interaction of Metal Ions with Phosphatidylcholine Bilayer Membranes†

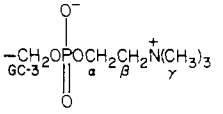
Hideo Akutsu‡ and Joachim Seelig*

ABSTRACT: The interaction of mono-, di-, and trivalent metal ions with bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) was investigated with deuterium and phosphorus magnetic resonance. With selectively deuterated lipids the measurements of the residual deuterium quadrupole splitting provided a sensitive handle to monitor directly the binding of ions, including the weak binding of Na^+ or $(CH_3)_4N^+$. For the α segment of the choline group ($-NCH_2CD_2O-$) changes in the quadrupole splitting of up to 9 kHz were observed. All measurements were made with nonsonicated DPPC dispersions. The ion concentrations were varied between 5 mM and 2 M, an almost 50-fold larger concentration range than accessible with nuclear magnetic resonance shift reagents. From a systematic comparison of various ions the following conclusions could be derived. (1) Addition of metal ions led to a structural change at the level of the polar groups. The glycerol backbone or the beginning of the fatty acyl chains was not affected. (2) The strength of interaction increased with the charge of the metal ion in the order $Na^+ < Ca^{2+} < La^{3+}$. However, distinct differences were also noted between ions of the same charge. Furthermore, the strongly hydrophobic tetraphenylammonium ion induced almost the same change as La^{3+}. (3) The variation of the quadrupole splittings with ion concentration exhibited a plateau value at high concentrations of La^{3+}. The titration curves of DPPC with Ca^{2+} and La^{3+} could be described in terms of a Langmuir adsorption isotherm with an interaction potential. Apparent binding constants of $K_{LaCl_3} \simeq 120\ M^{-1}$ and $K_{CaCl_2} \simeq 19\ M^{-1}$ were derived. (4) The addition of NaCl considerably enhanced the binding of Ca^{2+} and La^{3+}, apparently without affecting the plateau value of the quadrupole splitting. (5) The ion-induced conformational changes were qualitatively similar for all ions investigated. The various binding data could be summarized by plotting the quadrupole splittings of the α segment ($-OCD_2CH_2N-$) vs. those of the β position ($-OCH_2CD_2N-$). This plot yielded a straight line comprising all ions and concentrations investigated except Eu^{3+}. The quadrupole splittings of DPPC observed in the presence of chloroform or cholesterol and the variation of the quadrupole splittings with temperature could also be summarized in a linear plot that was different from that obtained for metal ion binding. This suggests the existence of at least two kinds of structural responses of the polar head groups to external perturbations.

Phosphatidylcholine is one of the predominant phospholipids in membranes, and a large fraction of most membrane surfaces is occupied by phosphocholine groups. The interactions of metal ions with the uncharged phosphatidylcholine bilayer can be expected to be relatively weak compared to those with negatively charged lipids such as phosphatidylglycerol or phosphatidylserine. Nevertheless, even small changes in the head-group orientation and flexibility could significantly alter the electrical properties of the membrane surface, producing, in turn, changes in the physiological or biochemical characteristics of the membrane. Thus, the problem of metal ion binding to phosphatidylcholine bilayers has attracted much attention, and a variety of methods have been employed [for a review, see Hauser & Phillips (1979)]. Deuterium magnetic resonance is a particularly promising method in this respect since quite large changes in the residual deuterium quadrupole splittings can be induced by the interaction with ions (Brown & Seelig, 1977). In the present study this effect has been exploited more systematically, and results on the concentration, temperature, and ion dependence are provided. A set of phosphatidylcholine molecules selectively deuterated in the choline moiety was employed in order to analyze separately the changes of the various head-group segments, including the glycerol backbone. The movement of the phosphate group was followed by phosphorus magnetic resonance.

Materials and Methods

For simplification of discussion the following nomenclature is employed for the glycerol backbone and the phosphocholine head-group segments:



1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC)[1] was

† From the Department of Biophysical Chemistry, Biocenter of the University of Basel, CH-4056 Basel, Switzerland. *Received April 20, 1981.* Supported by Swiss National Science Foundation Grant 3.409.78 and a long-term fellowship from the European Molecular Biology Organization (H.A.).

‡ Present address: Institute for Protein Research, Osaka University, Suita, Osaka 565, Japan.

0006-2960/81/0420-7366$01.25/0

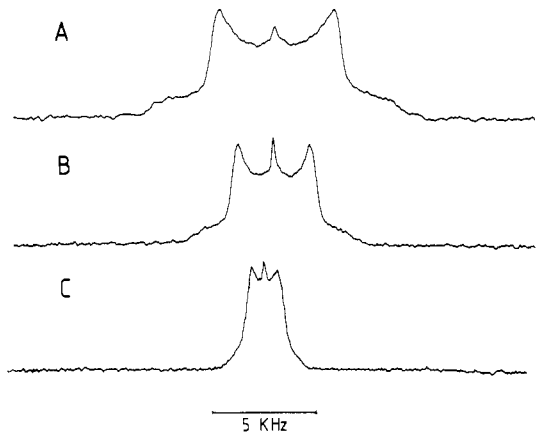


FIGURE 1: Deuterium NMR spectra (at 46.1 MHz; quadrupole echo method) of unsonicated multibilayers of DPPC-α-CD_2 ($-N^+-CH_2CD_2O$-DPPC) in the presence of $CaCl_2$ or $LaCl_3$. The absolute value of the residual quadrupole splitting $|\Delta\nu_Q|$ is given by the separation between the two largest peaks in the powder-type spectra. There is only little temperature dependence at the α position. (A) No ions, 50 °C, excess H_2O, $\Delta\nu_Q$ = 5.95 kHz; (B) 0.1 M $CaCl_2$, 1 mM EDTA, 10 mM Pipes, pH 7.0, 59 °C, $\Delta\nu_Q$ = 3.38 kHz; (C) 0.05 M $LaCl_3$, no buffer, 50 °C, $\Delta\nu_Q$ = 1.25 kHz.

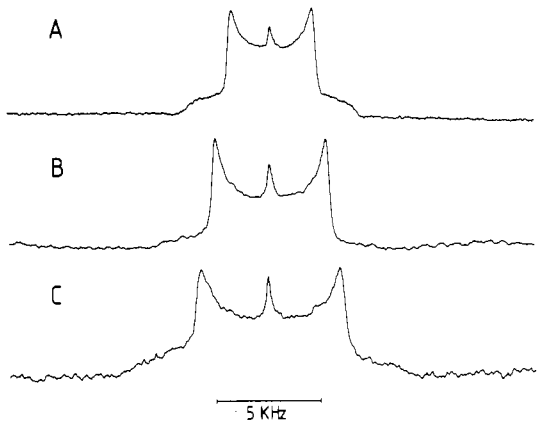


FIGURE 2: Deuterium NMR spectra (at 46.1 MHz; quadrupole echo method) of unsonicated multibilayers of DPPC-β-CD_2 ($-N^+-CD_2CH_2O$-DPPC) in the presence of $CaCl_2$ and $LaCl_3$. (A) No ion, 1 mM EDTA, 10 mM Pipes, pH 7.0, 59 °C, $\Delta\nu_Q$ = 4.22 kHz; (B) 0.1 M $CaCl_2$, 1 mM EDTA, 10 mM Pipes, pH 7.0, 59 °C, $\Delta\nu_Q$ = 5.28 kHz; (C) 0.5 M $LaCl_3$, 1 mM EDTA, 10 mM Pipes, pH 6.0, 59 °C, $\Delta\nu_Q$ = 6.56 kHz.

selectively deuterated at the segments indicated above as described by Gally et al. (1975). Nondeuterated DPPC was obtained from Fluka, Switzerland. All other chemicals were AR grade. Lipid dispersions (coarse liposomes) were prepared by thoroughly mixing DPPC at 50 °C with buffer solutions containing various metal ions. The buffer composition was normally 10 mM Pipes and 1 mM EDTA, pH 7.0; for $LaCl_3$ and $EuCl_3$ the pH was lowered to pH 6.0. The concentration of the inorganic salts (employed as chlorides) was varied between 5 mM and 2 M. At low concentrations of metal ions (<20 mM) EDTA was omitted. For the NMR experiments two types of samples were prepared. In one set of samples the lipid (~30 mg) was dispersed in ~30 mL of buffer at 50 °C and centrifuged to form a pellet. The pellet was transferred to a NMR sample tube and measured in the presence of excess buffer. In a second series of experiments the lipid concentration was kept constant at 70 mM. Within experimental error no differences were detected in the quadrupole splittings of the two types of samples. Deuterium NMR spectra were recorded at 13.8 and 61.4 MHz with the single-pulse mode and at 46 MHz with the quadrupole echo technique (Davis et al., 1976). The experimental conditions were the same as described earlier (Seelig et al., 1981). Phosphorus NMR measurements were made at 36.4 MHz under proton-decoupling conditions [cf. Gally et al. (1975)]. Deuterium T_1 relaxation times were measured at 46.1 MHz by the conventional 180°–τ–90° sequence modified to include the quadrupole echo.

Results

Typical ^2H NMR spectra of unsonicated bilayers of DPPC-α- and -β-CD_2 above the phase transition are shown in Figures 1 and 2, respectively. The spectra were recorded for DPPC in buffer (A), in 0.1 M $CaCl_2$ (B), and in 0.05 or 0.5 M $LaCl_3$ (C). All systems gave rise to typical bilayer spectra as anticipated for coarse dispersions of liposomes [cf. Seelig (1977)]. Since only one type of signal was observed, it was not possible to distinguish between ion-free and ion-complexed phospholipid head groups. Indeed these data suggest that these species are not long-lived on the ^2H NMR time scale. The influence of ions was reflected in the size of the quadrupole splittings. Thus the addition of metal ions always *decreased* the quadrupole splitting of the α-CD_2 group (Figure 1). In the absence of ions the residual quadrupole splitting was 5.95 kHz [Figure 1A; cf. also Gally et al. (1975)] and was reduced to 3.45 and 1.25 kHz in the presence of 0.1 M $CaCl_2$ and 0.05 M $LaCl_3$, respectively. The β-CD_2 segment exhibited the opposite tendency: in the absence of ions the quadrupole splitting was 4.2 kHz and *increased* to 5.3 and 6.6 kHz upon addition of 0.1 M $CaCl_2$ and 0.5 M $LaCl_3$, re-

Table I: Deuterium Quadrupole Splittings $\Delta\nu_Q$ (kHz) of DPPC-α-CD_2 and DPPC-β-CD_2[a]

	temp (°C)					
	50		59		69	
concn	none	+1 M NaCl	none	+1 M NaCl	none	+1 M NaCl
	DPPC-α-CD_2					
$LaCl_3$						
0 mM	5.95	5.2	5.95	5.2	5.95	5.2
5 mM	3.96	0.5	4.02	0.81	3.87	0.94
10 mM	3.37	0.23	3.32	0.38	3.14	0.50
20 mM	2.56	0.0	2.45	0.1	2.39	0.23
50 mM	1.25	−0.20	1.25	−0.16	1.16	−0.14
150 mM	0.0	−0.45	0.0	−0.26	0.0	−0.18
500 mM	−0.85		−0.59		−0.41	
1 M	−1.71		−1.27		−0.64	
2 M					−2.83	
$CaCl_2$						
10 mM	4.85	3.11	4.83	3.19	4.82	3.17
100 mM	3.33	1.12	3.38	1.53		1.59
350 mM	1.62		1.69		1.75	
1 M	−0.33		−0.16			
	DPPC-β-CD_2					
$LaCl_3$						
0 mM	4.81		4.22			
20 mM	6.32		5.69			
0.45 M	7.53		6.84			
$CaCl_2$						
10 mM			4.43			
0.1 M			5.30			
0.35 M	7.07		6.35			
1.0 M	7.49		6.64			
NaCl						
1.05 M	5.06		4.48			

[a] Only the relative signs of the quadrupole splittings can be determined.

[1] Abbreviations used: NMR, nuclear magnetic resonance; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; EDTA, ethylenediaminetetraacetic acid; Pipes, 1,4-piperazinediethanesulfonic acid.

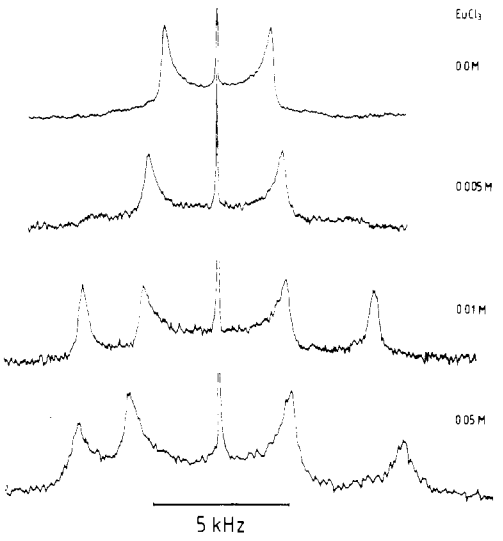


FIGURE 3: Deuterium NMR spectra (at 61.4 MHz; single pulse mode) of DPPC-β-CD_2 ($-N^+CD_2CH_2O$-DPPC) multibilayers dispersed in various concentrations of $EuCl_3$, pH 6.0, temperature 59 °C.

spectively. Representative quadrupole splittings for DPPC-α- and -β-CD_2 at different temperatures and ion concentrations are summarized in Table I.

Under proton-decoupling conditions two very closely spaced quadrupole splittings could be discerned for DPPC-α-CD_2 in the absence of ions (Gally et al., 1975). This small motional inequivalence of the two deuterons (difference of the two quadrupole splittings ~300 Hz) was generally not detectable in the presence of ions except for Cd^{2+} and Eu^{3+}. With Cd^{2+} the intrinsic line width was smaller and the two signals could be resolved even without proton decoupling. The effect of Eu^{3+} on the ^2H NMR spectra was found to deviate characteristically from that of all other ions investigated in this study. This is illustrated in Figure 3 for bilayers of DPPC-β-CD_2. At low concentrations of Eu^{3+} ($c_{Eu^{3+}} < 5$ mM) the spectra were similar to those shown in Figure 2 whereas at higher concentrations a second signal of about equal intensity appeared. The difference between the two quadrupole splittings increased with increasing Eu^{3+} concentration; simultaneously, the centers of the two signals moved apart. Similar effects were observed for DPPC-α-CD_2, and a more detailed account will be given elsewhere. Due to instrumental limitations the larger of the two quadrupole splittings was not detected in our earlier study (Brown & Seelig, 1977).

Adsorption isotherms (at 59 °C) for NaCl, $CaCl_2$, and $LaCl_3$ are shown in Figure 4. The figure reveals that (1) the ion binding affinity increases in the order $Na^+ < Ca^{2+} < La^{3+}$, (2) the maximum change in $\Delta\nu_Q$ is about twice as large for the α segment as for the β segment, and (3) a limiting value of $\Delta\nu_Q$ is apparently reached at high concentrations of trivalent ions. The influence of NaCl on the quadrupole splitting was small but well beyond experimental error. Other monovalent ions such as $N(CH_3)_4Br$ and tetraphenylammonium bromide induced changes in the quadrupole splittings that were quantitatively different from those observed with NaCl. This is illustrated in more detail in Table II. In spite of the fact that the binding of Na^+ was relatively weak, the presence of NaCl significantly enhanced the binding of $CaCl_2$ and $LaCl_3$ (Table III) and generated steeper adsorption isotherms for these ligands (Figure 4). It is important to note that the limiting value of the quadrupole splitting is apparently not changed by addition of 1 M NaCl.

The temperature dependence of the quadrupole splittings in the presence of various cations is summarized in Figures

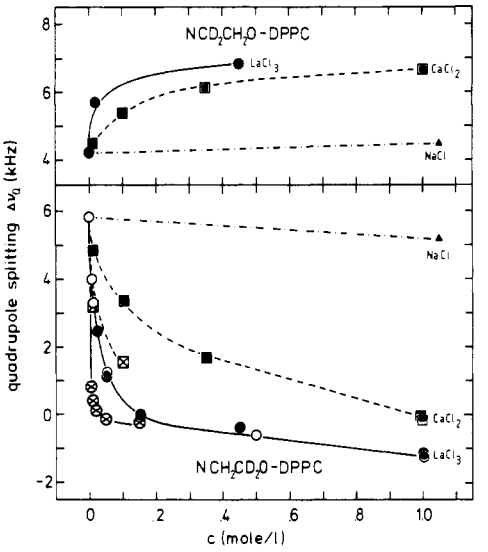


FIGURE 4: Adsorption isotherm of DPPC multibilayers for different types of cations at 59 °C. The quadrupole splittings of DPPC-α- and -β-CD_2 are plotted as a function of ion concentration. Only the size, but not the sign, of the quadrupole splitting can be measured. (Open symbols) 30 mg of lipid was dispersed in 30 mL of buffer and centrifuged to form a pellet; no buffer was used for $LaCl_3$ dispersions (pH ~5); (closed symbols) constant lipid concentration of 70 mM DPPC in buffer (cf. Materials and Methods); (crossed symbols) in addition to $CaCl_2$ and $LaCl_3$ the dispersion contained 1 M NaCl.

Table II: Deuterium Quadrupole Splittings $\Delta\nu_Q$ (kHz) of DPPC Deuterated at the α- and β-Choline Segments: Interaction with Monovalent Ions[a]

temp (°C)	1.05 M $N(CH_3)_4Br$		no ion		1.05 M NaCl		1.05 M $N(Ph)_4Br$	
	$\Delta\nu_\alpha$	$\Delta\nu_\beta$	$\Delta\nu_\alpha$	$\Delta\nu_\beta$	$\Delta\nu_\alpha$	$\Delta\nu_\beta$	$\Delta\nu_\alpha$	$\Delta\nu_\beta$
45	6.4	4.7	6.0	5.2	5.2	5.4	−1.4	7.3
50	6.4	4.4	5.95	4.8	5.2	5.1	−1.2	7.2
60	6.4	3.8	5.95	4.2	5.2	4.4	−0.6	6.9
70	6.4	3.1	5.95	3.5	5.2	3.8		

[a] Data interpolated, in part. Only the relative sign of $\Delta\nu_Q$ can be determined.

Table III: Effect of NaCl on the Deuterium Quadrupole Splittings $\Delta\nu_Q$ (kHz)[a] of DPPC-α-CD_2 in the Presence of 5 mM $LaCl_3$

NaCl concn (M)	temp (°C)		
	50	59	69
no NaCl	3.96	4.02	3.87
0.01	3.66	3.62	3.48
0.1	2.4	2.36	2.36
1	0.50	0.81	0.94

[a] Only the relative signs of the quadrupole splittings can be determined.

5 and 6. The α segment was found to be almost insensitive to temperature except at very high concentrations of $LaCl_3$ (Figure 5). However, the β segment exhibited a pronounced decrease of $\Delta\nu_Q$ with temperature (Figure 6). Almost the same slope was observed for the various ions employed. The interaction of ions with the phosphocholine terminal methyl groups (γ segment) induced relatively small changes as illustrated in Figure 6 for 0.35 M $CaCl_2$. This can be explained by the fact that the quadrupole splitting of the terminal methyl group is always very small due to essentially free rotation around both the N–C_γ and the C_β–N bonds [cf. Gally et al. (1975)].

Phosphorus-31 NMR spectra were routinely recorded for all samples prepared for ^2H NMR and served as a control for

Table IV: Influence of Ions on Deuterium T_1 Relaxation Times (at 46.1 MHz)

deuterated compd	conditions	T_1 (ms) at temp (°C) 30	50	59	69	E_a (kcal/mol)
DPPC-α-CD_2	H_2O		29.7	40.3	57.8	7.5
	150 mM $LaCl_3$, pH 6.0, 1 mM EDTA, 10 mM Pipes		25.8	30.1	34.1	3.2
	150 mM $LaCl_3$, 1 M NaCl		22.8	24.7		
	1 M $CaCl_2$, pH 7.0, 1 mM EDTA, 10 mM Pipes		20.9	26.1	31.4	4.6
	100 mM $CaCl_2$, pH 7.0, 1 mM EDTA, 10 mM Pipes		27.2	37.1		
DPPC-β-CD_2	no ion, pH 7.0, 1 mM EDTA, 10 mM Pipes		38.4	52.8	67.3	6.5
	20 mM $LaCl_3$, pH 6.0, 10 mM Pipes		34.5	44.3		
D_2O	1% D_2O, pH 7.0, 1 mM EDTA, 10 mM Pipes	528				
	1% D_2O, pH 6.0, 10 mM Pipes, 150 mM $LaCl_3$, 1 mM EDTA	546				
	1% D_2O, pH 7.0, 1 M $CaCl_2$, 1 mM EDTA, 10 mM Pipes	450				

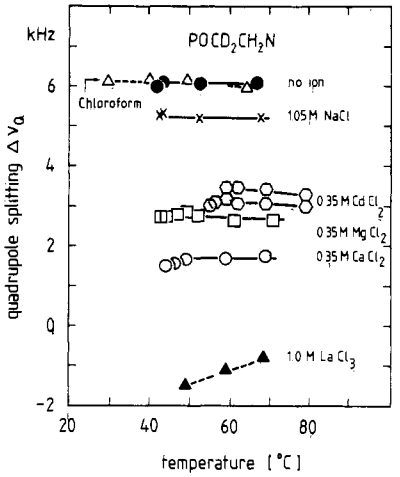


FIGURE 5: Temperature dependence of the residual quadrupole splitting of DPPC-α-CD_2.

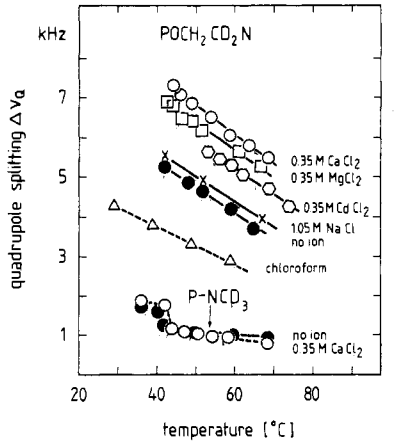


FIGURE 6: Temperature dependence of the residual quadrupole splitting of DPPC-β-CD_2 and DPPC-γ-CD_3.

the phase behavior of the system. Indeed, independent of the concentration and charge of the cations studied, the proton-decoupled ^{31}P NMR spectra always conformed to the theoretical line shape anticipated for a random dispersion of the liquid-crystalline bilayers [cf. Seelig (1978)], even when the ^2H NMR spectrum showed a singlet. The characteristic parameter of ^{31}P NMR spectra is the chemical shielding anisotropy, $\Delta\sigma$, which measures the motional restrictions experienced by the phosphate segment. Figure 7 compares the

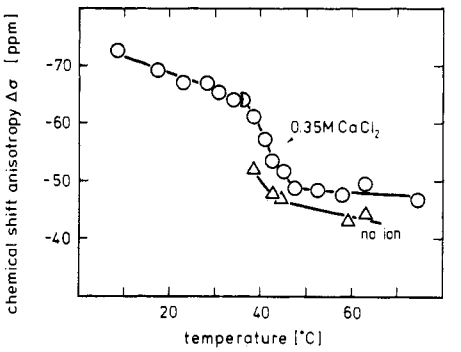


FIGURE 7: Variation of the phosphorus chemical shielding anisotropy with temperature.

temperature dependence of $\Delta\sigma$ of pure DPPC and DPPC in 0.35 M $CaCl_2$. The phase transition of pure DPPC at 41 °C was not abolished in the presence of $CaCl_2$, as indicated by the large change of $\Delta\sigma$ at this temperature. This is in agreement with calorimetric measurements that could detect only small changes in the thermodynamic properties of DPPC in the presence of various ions (Chapman et al., 1977). Figure 7 further demonstrates that the chemical shielding anisotropy decreased from −45 ppm for pure DPPC to −49 ppm upon addition of 0.35 M $CaCl_2$. Similar changes were found for other divalent and trivalent ions. Though numerically small, these differences are significant enough to indicate a structural change at the level of the phosphate group. By contrast, the addition of cholesterol to DPPC bilayers had no effect on $\Delta\sigma$, even at equimolar concentrations (Brown & Seelig, 1978).

The influence of ions on the glycerol backbone and on the fatty acyl chain region was also investigated by ^2H NMR. For backbone-labeled (GC-3) DPPC the quadrupole splitting was 27.3 kHz in the absence of ions [average of two closely spaced splittings; cf. Gally et al. (1975)] and 26.9 kHz in the presence of 0.35 M $CaCl_2$ (at 59 °C). This reduction of $\Delta\nu_Q$ by 1–2% is insignificant compared to the pronounced changes observed for the α and β segment and the phosphate group. Similarly, no effect of ions could be detected on DPPC bilayers labeled at the C-2 segments of both fatty acyl chains.

Measurements of T_1 relaxation times provide insight into the dynamic aspects of head-group motions, and the results of a few deuterium T_1 relaxation studies are summarized in Table IV. For all systems studied the T_1 relaxation times increased with increasing temperature, indicating that the molecular motions governing the T_1 relaxation process were

in the so-called fast correlation time regime where $\omega_0\tau_c \ll 1$ (ω_0 is the deuterium resonance frequency in rads per second; τ_c is the correlation time in seconds). Table IV reveals that the T_1 relaxation times in the presence of Ca^{2+} and La^{3+} were shorter and had a smaller activation energy than in the absence of these ions. For 1 M $CaCl_2$ part of the observed relaxation enhancement could be explained by a viscosity increase of the bulk aqueous phase, as suggested by control experiments using D_2O solutions (cf. Table IV). The T_1 relaxation time of D_2O (1% in H_2O) was only slightly changed upon addition of 150 mM $LaCl_3$ but decreased by about 20% in the presence of 1 M $CaCl_2$. The latter effect was most probably caused by the increased viscosity of the 1 M $CaCl_2$ solution. It can be estimated that the viscosity of a 1 M $CaCl_2$ solution is 1.285 times larger than that of pure water [cf. Robinson & Stokes (1959)]. Since $T_1 \propto 1/\eta$ (in the fast correlation time limit), this viscosity increase predicts a reduction of the T_1 relaxation time from 530 ms in dilute buffer to about 440 ms in 1 M $CaCl_2$, in good agreement with the experimental observation.

Discussion

The interaction of cations with phosphatidylcholine bilayers has been investigated extensively by means of high-resolution ^1H and ^{31}P NMR [cf. Hauser et al. (1975, 1976, 1977, 1978); Hutton et al. (1977); McLaughlin et al. (1978); Grasdalen et al. (1977); Westman & Eriksson (1979)]. For such studies, cations with specific magnetic properties are required which shift the resonances of the complexed lipids. Furthermore, the measurements must be performed with single-walled vesicles of small diameter in order to obtain high-resolution NMR spectra since nonsonicated lipid dispersions (coarse liposomes) generally yield rather broad resonance lines, thus obscuring the small changes induced by paramagnetic ions.

In contrast to NMR studies using shift reagents, ^2H NMR relies on internal probes, namely, on the deuterons covalently bound to the phospholipid molecules. The magnetic properties of the ions employed are immaterial, and the studies can be performed equally well with diamagnetic ions. Thus, there are no limitations with respect to the type of ions to be used, and even the weak interaction of monovalent ions such as Na^+ or $(CH_3)_4N^+$ can be monitored with high sensitivity over a wide concentration range. Strong interactions as encountered, for example, with La^{3+}, Eu^{3+}, or tetraphenylammonium, may cause changes in the quadrupole splitting of as large as 9 kHz. Another advantage of ^2H NMR is the fact that the experiments can be performed with coarse phospholipid dispersions. Indeed, sonication of the lipid dispersions in order to produce small bilayer vesicles would only be detrimental since the quadrupole splittings would be averaged out by the vesicle tumbling [cf. Stockton et al. (1976); for reviews on ^2H NMR, see Seelig (1977); Mantsch et al. (1977)].

Quantitative Analysis of Adsorption Isotherms. The experimental data (Figure 4 and Table I) suggest a limiting value for the quadrupole splittings at high ionic concentrations, equivalent to a complete saturation of the bilayer surface with ions. This saturation behavior was not observed in earlier studies using shift reagents because the measurements were restricted to concentrations of less than 30 mM [cf. Hauser et al. (1977); Westman & Eriksson (1979), Figure 2]. At higher concentrations of lanthanide ions the single-shelled vesicles were apparently no longer stable and formed aggregates. The present work extends the accessible concentration range by almost 2 orders of magnitude.

A comparison of the $LaCl_3$ adsorption isotherm with and without 1 M NaCl furthermore suggests that the quadrupole splitting at the saturation level is independent of the ionic composition of the bulk solution. With this assumption it is possible to operationally define the degree of saturation, θ, as

$$\theta = \frac{n}{n_{total}} = \frac{\nu_{obsd} - \nu_0}{\nu_{max} - \nu_0} \quad (1)$$

where n and n_{total} are the numbers of occupied and total binding sites, respectively. In this approach the binding sites have only a formal meaning and are not identical with the number of phospholipid molecules in the bilayer surface. ν_0 is the quadrupole splitting in the absence of ions whereas ν_{max} denotes the limiting splitting at maximum loading of the bilayer surface. ν_{obsd} is the observed quadrupole splitting at a given ion concentration. For DPPC-α-CD_2 the residual quadrupole splitting in the absence of metal ions was ν_0 = 5.95 kHz (temperature independent between 45 and 70 °C; cf. Figure 5). The limiting value measured in the presence of 2 M $LaCl_3$ was −2.83 kHz, and it is assumed therefore that $\nu_{max} \approx -3.0$ kHz. The calculated θ values for $LaCl_3$ and $CaCl_2$ are summarized in Table V.

Table V: Degree of Saturation (θ) and Activity Coefficients (γ) for $LaCl_3$ and $CaCl_2$ Binding to DPPC-α-CD_2 at 59 °C

concn	θ	$\gamma_{25\,°C}$[a]	concn	θ	$\gamma_{25\,°C}$[a]
$LaCl_3$			$CaCl_2$		
0 mM	0		10 mM	0.13	0.89
5 mM	0.22	0.636	0.1 M	0.29	0.518
10 mM	0.29	0.56	0.35 M	0.48	0.45
20 mM	0.39	0.483	1.0 M	0.68	0.5
50 mM	0.53	0.388			
0.15 M	0.66	0.31			
0.5 M	0.73	0.279			
1.0 M	0.81	0.36			

[a] Data taken from Robinson & Stokes (1949) and Spedding et al. (1976).

It can be expected that the interaction of metal ions with the bilayer surface is dependent on the degree of saturation, θ; the larger θ, the more difficult is the binding of further ions due to charge repulsion. As an empirical approximation the binding data may be represented in the form of a Langmuir adsorption isotherm with interaction potential V [cf. Aveyard & Haydon (1973)]:

$$\frac{\theta}{1-\theta}\frac{1}{\gamma c}\exp\left(\frac{V\theta}{RT}\right) = K \quad (2)$$

Here c is the equilibrium ion concentration, γ the activity coefficient, and K the equilibrium constant. A plot of $\ln [[\theta/(1-\theta)][1/(\gamma c)]]$ vs. θ should result in a straight line with the intercept $\ln K$ and the slope $-[V/(RT)]$. Figure 8 shows such plots for the $CaCl_2$ and $LaCl_3$ adsorption isotherms. The activity coefficients were taken from Robinson & Stokes (1949) and Spedding et al. (1976) (at 25 °C) and are listed in Table V. To a good approximation straight lines are obtained for $LaCl_3$ up to 0.15 M and for $CaCl_2$ up to 1 M. From a linear regression analysis one calculates apparent binding constants of $K_{LaCl_3} \simeq 120\ M^{-1}$ and $K_{CaCl_2} \simeq 19\ M^{-1}$ as well as interaction potentials of $V_{LaCl_3} \simeq 4.3$ kJ/mol and $V_{CaCl_2} \simeq 6.5$ kJ/mol. This simple analysis demonstrates that the binding of La^{3+} is about 5 times stronger than that of $CaCl_2$, which is in qualitative agreement with earlier studies using shift reagents (Hauser et al., 1977; Hauser & Phillips, 1979; McLaughlin et al., 1978; Grasdalen et al., 1977; Westman & Eriksson, 1979). Unfortunately, a more detailed quantitative comparison of our results with the earlier data is not possible since rather specific models with well-defined phospholipid to metal ion stoichiometries have been used in the earlier interpretations.

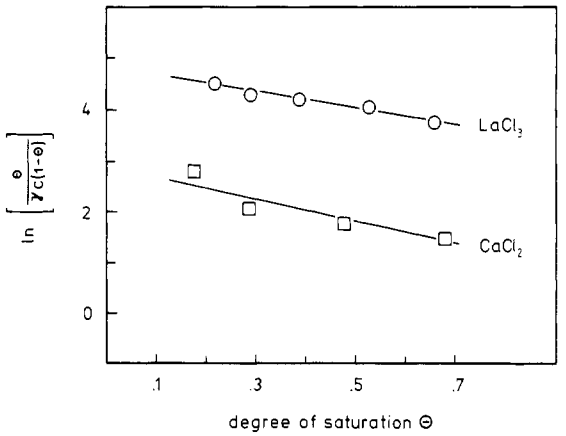


FIGURE 8: Binding of $CaCl_2$ and $LaCl_3$ to DPPC-α-CD_2 (Figure 4) represented in terms of an adsorption isotherm with interaction potential. The measuring temperature is 59 °C. θ = degree of saturation; c = concentration of metal ion; γ = activity coefficient (cf. text).

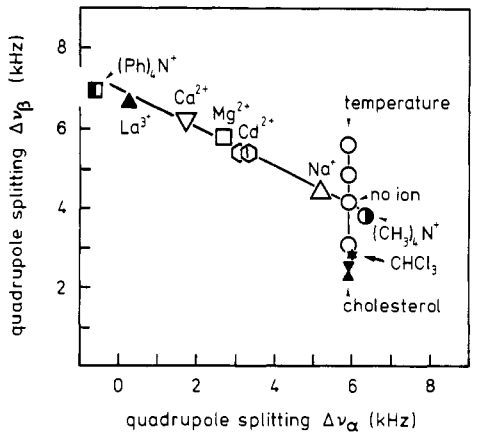


FIGURE 9: Quadrupole splitting of the β-CD_2 group plotted vs. the corresponding splitting of the α-CD_2 group. The measuring temperature is 59 °C. Data are compared at constant ionic strength $I = (1/2)\sum_i z_i^2 C_i = 1.05$ M. Actual concentrations: monovalent ions, 1.05 M; divalent ions, 0.35 M; $LaCl_3$, 0.175 M.

The physical meaning of the interaction potential V is illustrated by rewriting eq 2 in the form $\theta/(1-\theta) = K\gamma c \exp[-V\theta/(RT)]$. The adsorption of metal ions produces a positive electrostatic potential at the bilayer surface. As the "loading" increases, the effective metal ion concentration $c_{eff} = c \exp[-V\theta/(RT)]$ at the membrane surface decreases due to charge repulsion. At full loading ($\theta = 1$) the surface potential would be

$$\Psi^0 = V/(z_+F_0) \quad (3)$$

(z_+ = electric charge number of cation; F_0 = Faraday constant), yielding $\Psi^0_{LaCl} \simeq 15$ mV and $\Psi^0_{CaCl_2} \simeq 34$ mV. At intermediate loading the surface potential would be $\Psi(\theta) = \Psi^0\theta$. For comparative purposes let us calculate the surface potential of DPPC in the presence of 50 mM $CaCl_2$. According to Figure 4 the quadrupole splitting of DPPC-α-CD_2 at 50 mM $CaCl_2$ is 3.95 kHz, yielding $\theta = 0.22$; hence, $\Psi_{CaCl_2} = 34 \times 0.22 \simeq 8$ mV. This is in reasonable agreement with the experimentally determined ζ potential at this ionic concentration ($\sim$10 mV) as well as the estimated Ψ potential from conductance measurements ($\sim$10 mV) (McLaughlin et al., 1978). In the presence of high concentrations of NaCl the binding of La^{3+} and Ca^{2+} is much enhanced as evidenced by Tables I and III. This effect has been noted earlier under different experimental conditions [cf. Hauser et al. (1977); Westman & Eriksson (1979)] and is probably caused by a screening of the positive charges by Cl^- and/or a binding of Cl^- to the membrane surface. The enhanced binding of polyvalent cations in the presence of monovalent ions could play an important role in regulating biomembrane activities.

Ion-Induced Conformational Changes. The addition of metal ions has practically no effect on the quadrupole splittings of the glycerol backbone or the fatty acyl chain segments, suggesting that the interaction with the lipid bilayer is limited to the polar region. Here the most notable spectroscopic changes are observed for the α- and β-CD_2 segment. Since the absolute values of $\Delta\nu_\beta$ *increase* in the presence of metal ions (as does $|\Delta\sigma|$ of the phosphate group), this excludes the possibility of a more random movement of the phosphocholine dipole and provides evidence for a conformational change of the choline head group. In Figure 9 the quadrupole splitting of the β segment, $\Delta\nu_\beta$, is plotted vs. that of the α segment, $\Delta\nu_\alpha$, for various types of ions and ion concentrations. To a good approximation all data fall on a straight line with

$$\Delta\nu_\beta = -0.43\Delta\nu_\alpha + 6.7 \text{ kHz} \quad (4)$$

at 59 °C. Exactly the same linear relationship is obtained if the adsorption isotherms for $CaCl_2$ and $LaCl_3$ are represented in this type of plot. Also included in Figure 9 are results on the temperature dependence of the α- and β-CD_2 splittings in the absence of ions (Gally et al., 1975) and on the effects of cholesterol (Brown & Seelig, 1978) and chloroform (this study). The latter perturbations gave rise to the vertical line in Figure 9 since they induced changes in the β-CD_2 segment only and left the α-CD_2 segment unaffected. The following conclusions are therefore suggested by Figure 9. (1) A strictly linear relationship (eq 4) exists between the ion-induced spectroscopic changes at the α- and β-CD_2 segment, independent of the type and concentration of the ions employed. This result lends further support to the above assumption of a common limiting quadrupole splitting at saturation. (2) The strength of interaction and the concomitant conformational changes are not determined exclusively by electrostatic forces. This is clear from a comparison of ions of the same charge such as Ca^{2+}, Mg^{2+}, and Cd^{2+}. Under identical experimental conditions the induced conformational change increased in the order $Cd^{2+} < Mg^{2+} < Ca^{2+}$. A particularly large effect was noted for $(Ph)_4N^+$, which can be rationalized by an increased membrane affinity of this ion due to its strongly hydrophobic phenyl groups. (3) The conformational change described by eq 4 is unique for metal ions. A second type of conformational response is induced by changes in temperature or by the addition of cholesterol or chloroform. These perturbants affect the quadrupole splitting of the β-CD_2 segment only, which suggests that the structural reorganization is limited to a change in the torsion angle of the C_α–C_β bond. The common feature of these perturbants is probably to increase the distance between the polar groups, thereby weakening the intermolecular interactions and changing the torsion angle of the C_α–C_β bond.

A more detailed insight into the nature of the ion-induced conformational change is provided by a quantitative analysis of the α-CD_2 group. The experimentally observed quadrupole splitting, $\Delta\nu_Q$, can be related to the deuterium order parameter, S_{CD}, according to [cf. Seelig (1977)]

$$\Delta\nu_Q = (3/4)(e^2qQ/h)S_{CD} \quad (5)$$

The static quadrupole splitting constant $(e^2qQ/h) = 170$ kHz. S_{CD} depends on two different parameters, namely, (1) the average orientation of the C–D bond vector with respect to the axis of rotational symmetry (angle θ) and (2) the amplitude of fluctuations around this average orientation. S_{CD} is therefore composed of two factors

$$S_{CD} = S_{geom}S_{fluct} \quad (6)$$

Here

$$S_{geom} = (1/2)(3\cos^2\theta - 1) \quad (7)$$

describes the mean orientation whereas S_{fluct} accounts for the amplitudes of the time-averaged fluctuations. Inspection of Figure 9 reveals that for a particular ion concentration $\Delta\nu_\alpha = 0$ kHz, whereas the corresponding $\Delta\nu_\beta = 6.7$ kHz. The nonvanishing quadrupole splitting of the β segment together with the large chemical shielding anisotropy of the phosphate group ($\Delta\sigma \simeq -49$ ppm) excludes the possibility of a completely random movement of the α-CD_2 segment with $S_{fluct} = 0$. Thus, the observed collapse of the quadrupole splitting can only be explained by $S_{geom} = 0$ for both deuterons. Above the phase transition temperature the axis of motional averaging is given by the normal to the bilayer surface. Since it is unlikely that there is an additional internal molecular axis of rotational symmetry, it follows from eq 1 that both deuterons must be inclined at the "magic angle" ($\theta = 54.74°$) with respect to the bilayer normal. The amplitudes of the fluctuations around this average position are of course unknown. Next, a right-handed Cartesian coordinate system is attached to the α-CD_2 group in such a way that the ζ axis is in the plane spanned by the two C–D vectors and bisects the angle between the two bonds whereas the ρ axis is perpendicular to this plane. The boundary condition $\Delta\nu_\alpha = 0$ kHz for both deuterons then allows only two orientations of the α-CD_2 segment with respect to the bilayer normal, namely, (1) the bilayer normal is collinear with the ζ direction or (2) the bilayer normal is in the ξ–ρ plane and exactly bisects the angle between the ξ and ρ axes. This follows from the relation [cf. Seelig (1977), eq 66]:

$$S_{geom} = \mp 0.71 \sin(2\beta')\cos\alpha' + 0.5\sin^2\beta'\cos(2\alpha') \quad (8)$$

(The different signs distinguish the two deuterons of the α-CD_2 segment; α' is the Eularian rotation angle for rotation around ζ; β' is the angle between the ζ axis and the bilayer normal.) A relatively small change in the orientation of the α-CD_2 group will produce a significant change in the quadrupole splittings. For example, if the ζ axis is originally parallel to the bilayer normal ($\beta' = 0°$, α' is arbitrary, $\Delta\nu_\alpha = 0$ kHz), a tilt angle of $\beta' = 5°$ will produce quadrupole splittings of $0.4 < |\Delta\nu_\alpha| < 16.2$ kHz, depending on the value of α' (molecular fluctuations are neglected). In this tilted orientation the quadrupole splittings of the two deuterons will have opposite signs, but for a broad range of angles α' they are numerically identical and thus are experimentally indistinguishable. Without resorting to molecular models this simple analysis demonstrates rigorously that (1) only two orientations of the α-CD_2 segment are possible if $\Delta\nu_\alpha = 0$ kHz and (2) the smooth conformational transition from the ion-bound state with $\Delta\nu_\alpha = 0$ kHz to the ion-free state with $|\Delta\nu_\alpha| = 5.95$ is accomplished by only a small change in the average orientation of the choline group.

These results can be incorporated into a more detailed molecular model for the phosphocholine group [cf. Seelig et al. (1977); Skarjune & Oldfield (1979); H. Akutsu and J. Seelig (unpublished results)]. Due to the large number of free parameters, i.e., the torsion angles around the individual bonds, such molecular calculations are based on judicial assumptions for the head-group conformation. Adopting the model of two enantiomeric head-group conformations (Seelig et al., 1977), it is possible to show that for $\Delta\nu_\alpha = 0$ kHz the ζ axis of the α-CD_2 group must be collinear with the bilayer normal. The other possible orientation mentioned above is not consistent with the entire set of results.

General Conclusions

For all ions investigated only one type of ^2H NMR signal was observed (except Eu^{3+}), indicating a highly dynamic binding equilibrium. The maximum difference in the quadrupole splittings with and without ions was about 10 kHz, which yields an upper limit of 10^{-4} s as the longest possible residence time of the ions at the polar groups. The dynamic nature of the lipid–ion complex is further supported by the observation of a single deuterium T_1 relaxation time. Since the T_1 relaxation times were only slightly shorter in the presence of ions, it can be concluded that the fast segmental motions which dominate the T_1 relaxation process are only little slowed down by the metal ions.

The present data allow no conclusions about the detailed mechanism of lipid–ion interaction. However, the linearity in the conformational change as shown in Figure 9 suggests that the mode of binding is qualitatively similar for all ions investigated and that the observed quantitative differences simply reflect differences in the binding affinity.

The degree of saturation as defined above contains no assumptions about the stoichiometry of the cation–phospholipid interaction. Thus, the question remains open of how many phospholipid molecules constitute a "binding site" as defined by eq 1. Estimates based on the shift-reagent method propose stoichiometries of 1:1, 1:2, or 1:3 (metal ion:phospholipid) [cf. Hauser et al. (1977); McLaughlin et al. (1978); Westman & Eriksson (1979)]. These numbers were obtained, in part, by applying the Gouy–Chapman theory to the ion–phospholipid equilibrium. However, attempts to interpret the present data in terms of the Gouy–Chapman theory remained unsuccessful, presumably because the measurements span too large a concentration range.

Neutron diffraction studies together with ^2H and ^{31}P NMR have demonstrated that the mean orientation of the phosphocholine dipole in DPPC bilayers is parallel to the bilayer surface (Seelig et al., 1977; Büldt et al., 1978, 1979). The present results then indicate that this average orientation is not changed dramatically upon addition of metal ions.[2] However, even though the variations in the quadrupole splittings can be explained by quite small changes in the torsion angles, two types of structural responses can clearly be differentiated. (1) The binding of metal ions affects the torsion angles of the phosphate segment and of both choline methylene groups. (2) The addition of cholesterol or chloroform or a change in temperature changes only the torsion angle of the C_α–C_β bond.

Deuterated head-group derivatives are now available for phosphatidylethanolamine (Seelig & Gally, 1976), phosphatidylserine (Browning & Seelig, 1980), and phosphatidylglycerol (Wohlgemuth et al., 1980; Gally et al., 1981), and it will be interesting to compare the present results with those obtained for other head groups.

Acknowledgments

We thank P. Ganz for the competent synthesis of the deuterated DPPC derivatives.

References

Aveyard, R., & Haydon, D. A. (1973) *An Introduction to the Principles of Surface Chemistry*, Cambridge University Press, New York.

Brown, M. F., & Seelig, J. (1977) *Nature (London) 269*, 721–723.

[2] Contradictory conclusions have been reached with high-resolution NMR [cf. Hutton et al. (1977); Hauser et al. (1978)].

Brown, M. F., & Seelig, J. (1978) *Biochemistry 17*, 381–384.
Browning, J. L., & Seelig, J. (1980) *Biochemistry 19*, 1262–1270.
Büldt, G., Gally, H. U., Seelig, A., Seelig, J., & Zaccai, G. (1978) *Nature (London) 271*, 182–184.
Büldt, G., Gally, H. U., Seelig, J., & Zaccai, G. (1979) *J. Mol. Biol. 134*, 673–691.
Chapman, D., Peel, W. E., Kingston, B., & Lilley, T. H. (1977) *Biochim. Biophys. Acta 464*, 260–275.
Davis, J. H., Jeffrey, K. R., Bloom, M., Valic, M. I., & Higgs, T. P. (1976) *Chem. Phys. Lett. 42*, 390–394.
Gally, H. U., Niederberger, W., & Seelig, J. (1975) *Biochemistry 14*, 3647–3652.
Gally, H. U., Pluschke, G., Overath, P., & Seelig, J. (1981) *Biochemistry 20*, 1826–1831.
Grasdalen, H., Eriksson, L. E. G., Westman, J., & Ehrenberg, A. (1977) *Biochim. Biophys. Acta 469*, 151–162.
Hauser, H., & Phillips, M. C. (1979) *Prog. Surf. Membr. Sci. 13*, 297–413.
Hauser, H., Phillips, M. C., Levine, B. A., & Williams, R. J. P. (1975) *Eur. J. Biochem. 58*, 133–144.
Hauser, H., Phillips, M. C., Levine, B. A., & Williams, R. J. P. (1976) *Nature (London) 261*, 390–394.
Hauser, H., Hinckley, C. C., Krebs, J., Levine, B. A., Phillips, M. C., & Williams, R. J. P. (1977) *Biochim. Biophys. Acta 468*, 364–377.
Hauser, H., Guyer, W., Levine, B., Skrabal, P., & Williams, R. J. P. (1978) *Biochim. Biophys. Acta 508*, 450–463.
Hutton, W. C., Yeagle, P. L., & Martin, R. B. (1977) *Chem. Phys. Lipids 19*, 255–265.
Mantsch, H. H., Saito, H., & Smith, I. C. P. (1977) *Prog. Nucl. Magn. Reson. Spectrosc. 11*, 211–272.
McLaughlin, A., Grathwohl, C., & McLaughlin, S. (1978) *Biochim. Biophys. Acta 513*, 338–357.
Robinson, R. A., & Stokes, R. H. (1949) *Trans. Faraday Soc. 45*, 612–624.
Robinson, R. A., & Stokes, R. H. (1959) *Electrolyte Solutions*, 2nd ed., p 305, Butterworth, London.
Seelig, J. (1977) *Q. Rev. Biophys. 10*, 353–418.
Seelig, J. (1978) *Biochim. Biophys. Acta 515*, 105–140.
Seelig, J., & Gally, H. (1976) *Biochemistry 15*, 5199–5204.
Seelig, J., Gally, H., & Wohlgemuth, R. (1977) *Biochim. Biophys. Acta 467*, 109–119.
Seelig, J., Tamm, L., Hymel, L., & Fleischer, S. (1981) *Biochemistry 20*, 3922–3932.
Skarjune, R., & Oldfield, E. (1979) *Biochemistry 18*, 5903–5909.
Spedding, F. H., Weber, H. O., Saeger, V. W., Petheram, H. H., Rard, J. A., & Habenschuss, S. (1976) *J. Chem. Eng. Data 21*, 341–360.
Stockton, G. W., Polnaszek, C. F., Tulloch, A. P., Hasan, F., & Smith, I. C. P. (1976) *Biochemistry 15*, 954–966.
Westman, J., & Eriksson, L. E. G. (1979) *Biochim. Biophys. Acta 557*, 62–68.
Wohlgemuth, R., Waespe-Šarčević, N., & Seelig, J. (1980) *Biochemistry 19*, 3315–3321.

Radiolabeled α-Bungarotoxin Derivatives: Kinetic Interaction with Nicotinic Acetylcholine Receptors†

Ronald J. Lukas,‡ Hiromi Morimoto, Michael R. Hanley,§ and Edward L. Bennett*

ABSTRACT: The binding interactions of purified tritiated [^{3}H]-α-Bgt and monoiodinated and diiodinated derivatives of α-bungarotoxin with membrane-bound nicotinic acetylcholine receptors (nAChR) from *Torpedo californica* electroplax and rat brain have been characterized by several kinetic and equilibrium techniques. By all criteria, [^{3}H]-α-Bgt and ^{125}I-labeled monoiodinated α-Bgt ([^{125}I]-α-Bgt) exhibited comparable specificities and affinities for nAChR. In contrast, affinity of nAChR for ^{125}I-labeled diiodinated α-Bgt ([$^{125}I_2$]-α-Bgt) was reduced, and [$^{125}I_2$]-α-Bgt–nAChR complexes showed anomalous biphasic dissociation kinetics. [^{125}I]-α-Bgt and [$^{125}I_2$]-α-Bgt binding was inhibited most potently by native α-Bgt as opposed to iodinated toxins. [^{3}H]-α-Bgt was the radiotoxin most resistant to inhibitory influences. The use of well-characterized, chemically modified α-Bgt derivatives may identify ligand binding microheterogeneities and tissue-specific receptor subclasses.

Utilization of curaremimetic neurotoxins as specific probes for nAChR[1] has contributed to our knowledge of neurotransmitter receptor structure and function (Lee, 1971; Heidmann & Changeux, 1978). Despite the wide diversity of available toxins, and a significant literature on effects of chemical modification on their toxic activity (Tu, 1977), use of different naturally occurring or chemically modified toxins to probe receptor properties has been limited.

In an earlier communication [Lukas(iewicz) et al., 1978], we reported that column-purified native α-Bgt and tritiated, monoiodinated, and diiodinated α-Bgt derivatives exhibit characteristically different ultraviolet and circular dichroism spectra, suggesting that progressive iodination of an exposed tyrosine residue(s) leads to alterations in toxin secondary

† From the Chemical Biodynamics Division, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720. *Received April 14, 1981.* Supported in part by the Director of Energy Research, Office of Life Sciences Research and Nuclear Medicine Applications, Division of General Life Sciences of the U.S. Department of Energy, under Contract W-7405-ENG-48. A preliminary account of these results was presented at the Ninth Annual Meeting of Society for Neuroscience (Bennett et al., 1979).

‡ Permanent address: Division of Neurobiology, Barrow Neurological Institute, Phoenix, AZ 85013.
§ Present address: MRC Neurochemical Pharmacology Unit, Medical School, Cambridge CB2 2QH, England.

[1] Abbreviations used: α-Bgt, α-bungarotoxin; nAChR, nicotinic acetylcholine receptors; monoiodo-α-Bgt, monoiodinated α-Bgt; diiodo-α-Bgt, diiodinated α-Bgt; [^{3}H]-α-Bgt, tritium-labeled α-Bgt; [^{125}I]-α-Bgt, ^{125}I-labeled monoiodinated α-Bgt; [$^{125}I_2$]-α-Bgt, ^{125}I-labeled diiodinated α-Bgt.

0006-2960/81/0420-7373$01.25/0