Sodium-23 Nuclear Magnetic Resonance Studies of Cation–Deoxyribonucleic Acid Interactions†

M. Louise Bleam,‡ Charles F. Anderson, and M. Thomas Record, Jr.*

ABSTRACT: The concentration and temperature dependences of sodium-23 nuclear magnetic resonance (^{23}Na NMR) line widths, $\Delta\nu_{1/2}$, have been investigated in aqueous solutions of helical NaDNA ([P] ~0.01 M) also containing $MgCl_2$ and/or NaCl. In solutions of NaDNA and NaCl where [Na]/[P] $\lesssim$ 3, the association of sodium ions with DNA produces a significant enhancement in $\Delta\nu_{1/2}$. At constant temperature, $\Delta\nu_{1/2}$ decreases with increasing [Na]/[P]; at constant [Na]/[P], $\Delta\nu_{1/2}$ decreases with increasing temperature over the range 4–60 °C, in the domain of stability of the double helix. The dependence of $\Delta\nu_{1/2}$ on [P]/[Na] has been analyzed in terms of the two-state approximation for the transverse relaxation of rapidly exchanging nuclei. This analysis indicates that the number of associated sodium ions per phosphate charge is insensitive to large changes in the concentration of NaCl. Qualitatively, this finding is consistent with the counterion condensation model but not with the predictions of the cylindrical Poisson–Boltzmann equation. When sodium is the only counterion present in a solution of helical DNA, ^{23}Na NMR measurements do not by themselves provide sufficient information to determine the extent of sodium ion association or its temperature dependence. To address these questions, titrations of NaDNA solutions with $MgCl_2$ have been carried out at different temperatures. From an analysis of the initial linear portions of these titration curves it can be concluded that, at least from 6 to 33 °C, the extent of sodium ion association is independent of temperature, as is expected for exclusively electrostatic interactions, and the pronounced decrease in ^{23}Na line widths with increasing temperature must be due to the temperature dependence of the relaxation rate characteristic of associated sodium ions. On the basis of the two-state model for ^{23}Na relaxation, the initial slopes of plots of $\Delta\nu_{1/2}$ vs. [Mg]/[P] have been analyzed and compared with the predictions of the Poisson–Boltzmann equation and with those of a modified form of the counterion condensation model. The discrepancies between experiment and theory appear to be significant, but they could result from a breakdown of the assumption that the association of magnesium ions with DNA does not affect the relaxation rate(s) of associated sodium ions.

The physical properties of nucleic acids in solutions containing added salt(s) are, in general, significantly affected by electrostatic interactions between the small electrolyte ions and the phosphate charges on the nucleic acid. Information about these interactions is therefore essential for the development of quantitative descriptions of the conformation and flexibility of nucleic acids, the stability of their secondary and tertiary structure, and their binding equilibria with charged ligands. Theoretical analyses of electrostatic effects on these physical properties have been extensively reviewed (Record et al., 1978, 1981; Manning, 1978). Near a highly charged rodlike polyion, such as helical DNA, long-range Coulombic forces cause the accumulation of a local counterion concentration that is much higher than the bulk salt concentration. Theoretical descriptions of this effect have been based on either the cylindrical Poisson–Boltzmann equation or the counterion condensation model. According to both of these treatments, the local counterion concentration close to a highly charged polyion is determined primarily by its average axial charge density. Thus, any equilibrium process that alters the polyion charge density is expected to result in the net uptake or release of counterions near the polyion. This effect is manifested in the dramatic salt dependences that have been observed in studies of the melting and ligand-binding equilibria of helical DNA. Observations such as these can be analyzed in detail if the nature and extent of counterion binding to DNA can be determined.

Sodium nuclear magnetic resonance (NMR) has provided useful information about the association of small ions with macroions in solution (Laszlo, 1978). The natural abundance of ^{23}Na (spin $I = {}^3/_2$) is 100%; its NMR sensitivity is relatively high. The sodium nucleus has a moderately large quadrupole moment. In the absence of paramagnetic species, the NMR relaxation of sodium is governed exclusively by the interactions of its nuclear quadrupole with local electric field gradients that result from fluctuating asymmetry in the charge distribution surrounding the nucleus. The fluctuations in this quadrupolar interaction can be caused by various kinds of molecular motions, usually characterized by a single correlation time, τ_c. At a given applied field, the longitudinal and transverse relaxation rates, T_1^{-1} and T_2^{-1}, are functions of τ_c and $\langle e^2q^2\rangle$, the mean square amplitude of the fluctuating field gradient. In the limit of "extreme narrowing", $\omega_{Na}\tau_c \ll 1$ (where ω_{Na} is the Larmor frequency of ^{23}Na), $T_1^{-1} = T_2^{-1} \sim q^2\tau_c$, and the Fourier-transformed sodium spectrum is a single Lorentzian peak whose width at half-height is $\Delta\nu_{1/2} = 1/(\pi T_2)$ (Dwek, 1973). Under the conditions of the present study (ω_{Na} = 26.4 MHz, corresponding to 100 MHz for protons, at temperatures in the range 4–60 °C), sodium spectra in DNA solutions exhibit no significant deviations from Lorentzian form (Bleam, 1980), and transverse relaxation rates may be calculated directly from line-width measurements. In dilute aqueous solutions of sodium salts ($\lesssim$1 M) at temperatures ~20 °C, the ^{23}Na relaxation rates $T_1^{-1} = T_2^{-1} = 17\ s^{-1}$ (Eisenstadt & Friedman, 1967). In salt solutions that also contain double-stranded DNA, if the ratio of sodium to phosphate mo-

† From the Departments of Chemistry, College of Letters and Science (M.L.B., C.F.A., and M.T.R.), and Biochemistry, College of Agricultural and Life Sciences (M.T.R.), University of Wisconsin, Madison, Wisconsin 53706. *Received May 18, 1983.* This work was supported by grants from the National Science Foundation (PCM 79-04607 and PCM 81-20031) and the National Institutes of Health (GM 23467) to M.T.R.

* Address correspondence to this author at the Department of Chemistry.

‡ Present address: Department of Chemistry, Central College, Pella, IA 50219.

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nomer ([Na]/[P]) $\lesssim 3$, then the observed relaxation rates of ^{23}Na are significantly enhanced (Reuben et al., 1975; Anderson et al., 1978). The variations in these enhancements with temperature and with the concentration of small ions (magnesium and/or sodium) have been determined in the present study for the purpose of investigating the nature and extent of counterion association with double-stranded DNA.

Experimental Procedures

Calf thymus DNA was purchased from Worthington Co. To remove protein contaminants, the DNA was dissolved to give a concentration of 0.4 mg/mL in a solution containing 0.1 M NaCl and 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (pH 8) and extracted with phenol at room temperature at least 4 times. This procedure was followed by extraction with ether and precipitation with ethanol. Phage T7 was prepared by innoculating at a multiplicity of 3 a 60-L culture of *Escherichia coli* C grown to $OD_{500} = 0.6$ in LB broth (Maniatis et al., 1982). After lysis, cell debris was removed by flow centrifugation, and the supernatant was made 1% in NaCl, 10% in poly(ethylene glycol), and 0.1% in sodium dextran sulfate (percentages on a weight basis). The phage was recovered in a volume of 2 L from the dextran sulfate (bottom) phase. This volume was then reduced by centrifugation to less than 100 mL. After a 10-fold dilution, the dextran sulfate was precipitated by addition of solid KCl to ~1 M. The phage was further purified by banding on CsCl step density gradients. T7 DNA was prepared from T7 phage by phenol extraction as described above. To reduce the viscosity at the concentration levels required for accurate NMR measurements, it was necessary to reduce the molecular weight of the calf thymus and T7 DNA. Samples of DNA were partially redissolved to a monomer concentration of 20 mM with the same buffering solution mentioned above and were sonicated in 15–20-mL volumes. The sonication sequence was 20 bursts of 15-s duration interspersed with 15-s pauses to avoid overheating the solution. After sonication, average molecular weights were approximately 2×10^6, as determined by boundary sedimentation velocity with a Beckman Model E ultracentrifuge.

Stock solutions of DNA were prepared by a sequence of dialyses at 4 °C lasting 8–12 h each. The first dialysis was against a solution of 0.01 M trisodium ethylenediaminetetraacetic acid (Na_3EDTA) (pH 8) to remove polyvalent cations. The DNA then was dialyzed against two solutions of 0.1 M NaCl and 0.01 M Tris buffer (pH 8) and finally against five changes of a solution containing 0.01 M NaCl but no buffer. If the pH decreased below 7 in the unbuffered dialysis solutions, it was increased by adding small amounts of NaOH. If the DNA solution appeared cloudy after dialysis, it was clarified by centrifugation at 5000 rpm for 5 min. After the dialysis sequence, the DNA monomer concentrations were determined by measuring the UV absorbance at 260 nm with a Gilford spectrophotometer. The extinction coefficient was taken to be 48.3 (μg/mL)/OD, and the monomer molecular weight 337 g/mol. To check for denaturation, the percent hyperchromicity of each DNA stock solution was measured by alkaline denaturation (pH 12) and shown to be in an acceptable range (28–30%). Total sodium concentrations in the DNA stock solutions were determined by neutron-activation analysis, performed by the Nuclear Reactor Laboratory at the University of Wisconsin. Accurately weighed samples containing 0.4 mL of undiluted DNA were irradiated for 30 min, and the resulting γ radiation was later counted at two wavelengths for 30 min. Duplicate determinations were reproducible to ±5%.

In order to conserve the DNA samples and to obtain a uniform sample temperature during the NMR measurement (see below), the sample volumes used in the 12-mm NMR tubes were only about 3 mL at the beginning of the titrations. Of this volume, 0.5 mL was ^2H_2O. Titrations were carried out by the addition of microliter quantities of stock solutions containing NaCl or $MgCl_2$. The magnesium stock solutions (approximately 0.3 M) were made up by a careful weighing of $MgCl_2 \cdot 6H_2O$ that had been dried for several days in a vacuum desiccator at room temperature. After each titration was completed, the pH of the DNA sample solution was measured. Final pH values were in the range 4.6–6.2. To verify that no denaturation had occurred during the course of titration, the hyperchromicities or melting curves of representative DNA samples were redetermined after completion of the NMR experiments.

The NMR measurements were performed on a Varian XL-100/15 spectrometer with a Varian 620/L computer-based Fourier-transform data system and a Sykes cassette. The ^{23}Na spectra were obtained with the Varian Gyro Observe system, using the ^2H_2O resonance for the lock signal. The 90° pulse width was approximately 40 μs. Acquisition times were at least $8T_2$ and never shorter than 0.06 s. Assigned spectral widths were such that a minimum of 900 data points was acquired during each acquisition time.

The temperature was carefully maintained within a 0.3 °C range with a Varian variable-temperature controller. A constant flow of dry nitrogen was passed through an ice bath for temperatures higher than 20 °C or through crushed dry ice for temperatures at or below 20 °C. The cold nitrogen gas was then heated at a rate controlled by heat sensors located near the probe in the magnet. The desired temperature could be maintained by adjusting either the flow rate of the nitrogen or the temperature dial on the temperature controller. The probe temperature was measured with a thermocouple placed about 1 cm from the bottom of a capillary, which was inside an NMR tube partially filled with liquid. There could be a small systematic error in sample temperatures determined by this method, but it does ensure a reproducible probe temperature with minimal fluctuations. Water baths at the appropriate temperatures were used to equilibrate the samples before placing them in the probe. Then, an additional 20 min or more was allowed in order for thermal equilibrium with the probe to be established. Temperatures were usually measured before and after each set of spectra were taken. If the temperature had drifted more than ±0.15 °C, it was then readjusted, and another set of spectra were taken. During times when the temperature was particularly stable, spectra corresponding to two points on the titration curve were taken before measuring the temperature again. A total of two to five spectra were taken for each addition of salt.

Numerical calculations with the cylindrical Poisson–Boltzmann equation were performed on a Harris/7 computer. Computational methods are described elsewhere (Bleam, 1980).

Results and Discussion

Sodium-23 Relaxation Rates in Solutions of NaDNA: Variation with Temperature and with Sodium Concentration. Figure 1 demonstrates the pronounced temperature dependence of ^{23}Na line widths ($\Delta\nu_{1/2}$) for a solution of double-stranded calf thymus NaDNA and for a solution of T7 NaDNA in native and denatured forms. The much weaker temperature dependence of $\Delta\nu_{1/2}$ in a representative blank solution containing only 0.1 M NaCl is also shown for comparison. The small displacement in the curves for calf thymus

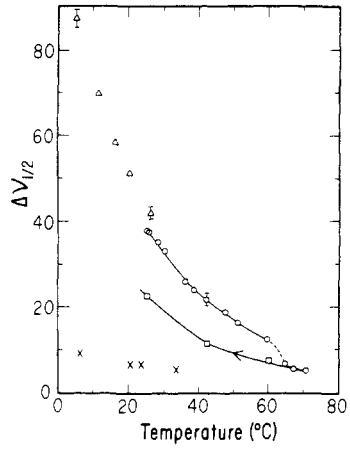

FIGURE 1: Sodium-23 NMR line widths, $\Delta\nu_{1/2}$ (Hz), vs. temperature: 0.013 M calf thymus NaDNA with 0.020 M NaCl (△); 0.015 M T7 NaDNA with 0.022 M NaCl (○); 0.015 M T7 NaDNA with 0.022 M NaCl after denaturation (□); 0.1 M NaCl (×).

and T7 DNA can be attributed to slight differences in the sodium to phosphate ratio in the two samples. For T7 DNA, the variation in ^{23}Na line width with temperature is reversible without hysteresis between 25 and 50 °C and thus does not result from any irreversible change in the DNA such as denaturation or degradation. Above 60 °C, the break in the curve for T7 DNA indicates the onset of denaturation, which appears to occur over the range 60–67 °C. A higher range, 66–73 °C, was determined spectrophotometrically for the melting of a dilute sample of the same DNA containing the same NaCl concentration (0.022 M) as in the NMR experiments. The relatively high DNA concentrations required for accurate ^{23}Na line-width measurements lower the effective electrolyte activity and thus could lower the temperature range of the melting transition. The breadth of the transition can be ascribed to the polydispersity of sonicated DNA molecules. Subsequent cooling from this transition region permits observation of the effect of denatured DNA on ^{23}Na line widths, since at these low salt concentrations renaturation is kinetically blocked (Studier, 1969). In Figure 1, the line widths that were measured within a few hours after the initial melting of the DNA are significantly smaller than the line widths that were measured over the corresponding temperature range (25–59 °C) prior to denaturation. However, in solutions containing denatured DNA, $\Delta\nu_{1/2}$ is still significantly enhanced. Results similar to those in Figure 1 have recently been reported (Levij et al., 1980; Mariam & Wilson, 1983).

From the measurements represented in Figure 1, it is evident that at temperatures below approximately 50 °C, the presence of DNA, especially in its native form, can substantially broaden the NMR line width of ^{23}Na. To account for the observed increase in $R = T_2^{-1} = \pi\Delta\nu_{1/2}$ for ^{23}Na in solutions containing nucleic acids, the simplest approach is to postulate the "two-state" model: all nuclei contributing to the NMR signal either are close enough to a polyion to be considered "bound" or are "free" in solution. The observed relaxation rate is the population-weighted average of the relaxation rates R_B and R_F, characteristic of the bound and free states, respectively:

$$R = p_B R_B + p_F R_F \quad (1)$$

where p_B and p_F are the fractions of nuclei in the two states; $p_B + p_F = 1$. Equation 1 can be valid only if the rate of chemical exchange between the two states is much greater than R_B. [Further details about the underlying theory can be found in the book by Dwek (1973).] The "fast-exchange" condition is fulfilled under the conditions of the experiments reported here, because the line shapes are Lorentzian and the line widths decrease with increasing temperature (see Figure 1). (According to eq 1, the decrease in R with increasing temperature could be due to the temperature dependence of p_B and/or $R_B - R_F$; however, in the following section it is demonstrated that p_B has no temperature dependence.)

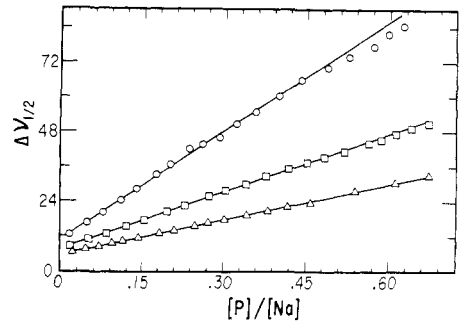

FIGURE 2: Sodium-23 NMR line widths, $\Delta\nu_{1/2}$ (Hz), vs. [P]/[Na] (the ratio of DNA phosphate to total sodium): 0.013 M calf thymus DNA at 6.2 (○), 20.3 (□), and 33.3 °C (△). Straight lines are linear least-squares fits to data points for which [P]/[Na] ≤ 0.45.

Under the condition of "extreme narrowing" ($\omega\tau_c \ll 1$), the relaxation rate of a quadrupolar nucleus in solution has the general theoretical form $R \sim q^2\tau_c$. At present, there is no accurate theoretical means of calculating the mean square electric field gradient, $\langle e^2q^2\rangle$, or the correlation time, τ_c, of a nucleus associated with a polyion. It is known, nevertheless, that contributions from the polyion charges to $\langle e^2q^2\rangle$ must be short range, and it is likely that polyelectrolyte effects on τ_c extend only to nuclei that are either localized at definite sites on the polyion or delocalized within a small volume of the immediately surrounding solution. Thus, the association of a sodium ion with DNA in either mode (site-binding or radial localization) would cause the relaxation rate of ^{23}Na to be enhanced, whereas the relaxation rates of nuclei more than a few ångströms from the DNA surface would be essentially unaffected. It follows from eq 1 that the average relaxation rates calculated from ^{23}Na line widths can be expressed as a simple linear function of p_B, the extent of sodium association with DNA, *provided that* R_B and R_F are independent of solution composition. Then the NMR measurements directly monitor changes in p_B, which can be compared with the predictions of molecular theories of counterion association with polyions in solution.

In Figure 2 the dependence of ^{23}Na line widths on the concentration of added NaCl is given for solutions of double-stranded calf thymus DNA at three representative temperatures. The abscissa [P]/[Na] was chosen for these plots in order to focus on r, the extent of association of Na^+, defined by the relation $p_B \equiv r[P]/[Na]$. With this definition, eq 1 can be written

$$R = R_F + (R_B - R_F)r[P]/[Na] \quad (2)$$

The straight lines in Figure 2 were fitted by a least-squares analysis of data for which [P]/[Na] < 0.45. At higher values of this ratio, the downward deviations in the titration curves at 6 and 20 °C may be due to slight departures from the limit of extreme narrowing, which, if present, are expected to be more pronounced at the lower temperature. [At ω_{Na} = 52.9 MHz (200 MHz for protons) the limit of extreme narrowing is definitely violated in solutions of calf thymus DNA (~10 mM) in the range 4–20 °C (D. K. Chang and L. Nordenskiold, unpublished data).] Figure 2 illustrates the significant temperature dependence of ^{23}Na NMR line widths in DNA solutions, which is expected on the basis of Figure 1. Since

R_F is not a strong function of temperature (see Figure 1), it follows from eq 2 that the observed temperature dependence is due primarily to the product $r(R_B - R_F)$. The extent to which either r or R_B contributes to this temperature dependence cannot be isolated without a means of determining these quantities separately. Another significant feature of the titrations illustrated in Figure 2 is the linearity of $\Delta\nu_{1/2}$ (hence, R) over a broad range of [P]/[Na]. In a previous ^{23}Na NMR study of counterion association with helical DNA, it was demonstrated that the slope of linear plots of R vs. [P]/[Na] does not change with the absolute DNA concentration over the range 0.0025 M < [P] < 0.015 M [Bleam et al., 1980; see also Anderson et al. (1978)]. [However, it has been determined that at higher magnetic fields, where ^{23}Na spectra begin to show deviations from Lorentzian form, the sodium relaxation rates vary as the sample is diluted at constant [P]/[Na] (L. Nordenskiold, unpublished data).]

Although eq 2 does not imply that R must be a linear function of [P]/[Na], it does require that $R \rightarrow R_F$ as [P]/[Na] $\rightarrow$ 0. If the linear fits to the titration data in Figure 2 are extrapolated to [P]/[Na] = 0, the resulting intercepts only slightly exceed the ^{23}Na line widths measured at corresponding temperatures in NaCl solutions containing no DNA. This finding demonstrates the short range of the effect of DNA on the ^{23}Na relaxation rate. The linearity of R vs. [P]/[Na] also indicates that the product $r(R_B - R_F)$ is independent of solution composition. Moreover, r and R_B must be individually constant, unless their variations are exactly compensated over a considerable range of concentrations. Coincidental compensation cannot be disproved without an independent means of determining r or R_B. However, it is known that in simple electrolyte solutions the observed relaxation rates of quadrupolar nuclei increase with increasing salt concentration (Eisenstadt & Friedman, 1967). Thus, any increase in r, the number of associated counterions per polyion charge, might be expected to produce an *increase* in R_B. Consequently, the simplest interpretation of the constancy of $r(R_B - R_F)$ is that the extent of association of sodium with double-stranded DNA does not vary with added salt.

The apparent constancy of R_B and R_F indicates that the two-state model, eq 1, is *effectively* valid but does *not* prove that all sodium nuclei that are associated with DNA have exactly the same relaxation rate. The quantity R_B could itself be an average of different relaxation rates characteristic of various subpopulations of associated nuclei. If the relative numbers of nuclei in these subpopulations do not vary much as the total number of sodium ions in solution is changed, then the average value of R_B could appear to be independent of [P]/[Na]. The constancy of $r(R_B - R_F)$ implied by the linearity of the plots in Figure 2 is not a feature common to all nucleic acids. For sodium titrations of single-stranded poly(rA), whose local conformation probably varies with salt concentration (Dewey & Turner, 1979), plots of $\Delta\nu_{1/2}$ vs. [P]/[Na] show definite curvature (Bleam, 1980).

In solutions of helical DNA having sodium as the only counterion, the observed insensitivity of r to large changes in salt concentration is in qualitative accord with Manning's molecular theory of counterion condensation (Manning, 1977, 1979). In a solution containing a highly charged rodlike polyion and a large excess of some 1:1 electrolyte, the number of condensed (bound) counterions per polyion charge is predicted to be virtually independent of salt concentration, until it approaches 1 M. This mode of counterion association is solely due to the attractive electrostatic field resulting from all the polyion charges; localization at particular sites on the polyion is not assumed, although this effect could be incorporated. In the formulation of the condensation model, associated univalent counterions are assumed to be uniformly distributed within a small annular volume of solution adjacent to the polyion. For helical DNA, this volume is predicted to extend about 7 Å from the (unhydrated) polyion surface. The influence of DNA charges on the relaxation rates of sodium nuclei is probably not significant at distances greater than 7 Å. Thus, r in eq 2 can be identified with the number of condensed counterions per polyion charge predicted by Manning's theory (Anderson et al., 1978). However, the theoretical extent of counterion condensation, $r = 0.76$ for double stranded DNA, cannot be checked by analyzing titrations of DNA solutions in which sodium is the only counterion, because r cannot be separated from the product $r(R_B - R_F)$ if R is a linear function of [P]/[Na].

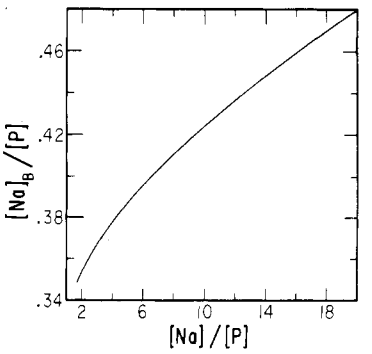

FIGURE 3: Poisson–Boltzmann prediction of extent of association of sodium with helical DNA, $[Na]_B/[P]$, as a function of total sodium, [Na]/[P]. $[Na]_B/[P]$ was computed by numerical integration of the Poisson–Boltzmann counterion radial distribution function over the distance 3 Å from the surface of the model DNA cylinder. The range of the abscissa corresponds to the data given in Figure 2.

A well-known alternative theoretical description of the counterion distribution surrounding a rodlike polyion is based on the cylindrical Poisson–Boltzmann equation. [Comparisons of predictions derived from this equation with those of the condensation model have recently been reviewed (Anderson & Record, 1982).] To evaluate r in eq 2, the Poisson–Boltzmann counterion radial distribution function can be integrated numerically (Bleam, 1980). The inner limit for this integration, a, is the distance of closest approach of the counterion to the polyion axis. The choice of the outer limit, $a + c$, depends on how far from the surface of the polyion its effect on the ^{23}Na relaxation rate is assumed to extend. Since there is no evidence that the association of sodium with double-stranded DNA is accompanied by dehydration (Manning, 1979), reasonable lower bounds on a and c are 13 and 3 Å, respectively. Accordingly, the Poisson–Boltzmann counterion radial distribution function for the system NaDNA + NaCl has been integrated numerically from 13 to 16 Å to obtain $[Na]_B/[P] \equiv r$, as a function of [Na]/[P]. The result of this computation is plotted in Figure 3, for a range of sodium concentrations corresponding to the titrations in Figure 2.

Data points could not be plotted in Figure 3 without making an arbitrary choice of R_B. For *any* value of R_B, experimental values of $[Na]_B/[P]$ inferred from the line-width data in Figure 2 have no systematic dependence on [Na]/[P] and thus would appear as a horizontal band on Figure 3. The width of this band, determined by scatter in the data, does not exceed 0.02 unit; thus it is significantly less than the variation in $[Na]_B/[P]$ predicted by the Poisson–Boltzmann (PB) equation. If larger values of a or c are assumed in the integration of the counterion radial distribution function, then the extent of sodium binding is predicted to show an even stronger dependence on salt

concentration. Conversely, assignment of smaller values to c and (especially) to a promotes closer agreement between experiment and the radial distribution computed from the PB equation (Gunnarsson & Gustavsson, 1982). Similarly, it has been shown that treating a as an adjustable parameter, having a value less than the known structural radius of a rodlike polyion, can lead to congruence between the theoretical predictions of the PB equation and those of the condensation model (Manning, 1975; Wilson et al., 1980). However, if the quantity a is required to have a structural significance ($a >$ 10 Å for helical DNA), then the PB computations of $[Na]_B/[P]$ presented in Figure 3 are not in agreement with the ^{23}Na NMR data in Figure 2. In contrast, the constancy of $r = [Na]_B/[P]$ that can be inferred from the linearity of these line-width data is a crucial feature of the molecular condensation model (Manning, 1977, 1979). However, to test the quantitative accuracy of either theoretical approach, titrations of NaDNA with NaCl do not provide sufficient information.

Sodium-23 Relaxation Rates in Solutions Containing NaDNA, NaCl, and $MgCl_2$: Variation with Magnesium Concentration and with Temperature. In the preceding section it was demonstrated that the extent of association of sodium with DNA cannot be quantified by analyzing titration data for a solution in which sodium is the only counterion. In principle, R_B could be measured directly if it were possible to achieve experimental conditions under which all the sodium ions in the system were associated with DNA. For example, the limit $p_B \rightarrow 1$ would be attainable in a DNA solution containing a small amount of sodium and a second type of counterion whose affinity for DNA is much lower. Among the counterions most likely to have a low affinity relative to sodium are the bulky tetraalkylammonium ions. Sodium titrations of (n-Bu_4N)DNA (double stranded) were carried out for the purpose of determining R_B and thereby r (Anderson et al., 1978). Unfortunately, for this system the direct measurement of R_B was not possible, because the binding affinity of tetrabutylammonium for DNA is not sufficiently less than that of sodium. [Moreover, the accuracy of line widths measured at low values of $[Na]/[P]$ was poor, even at the relatively high DNA concentrations ($[P] \gtrsim 0.01$ M) used in that study.] Nevertheless, by fitting the entire sodium titration curve to an expression derived on the basis of some additional assumptions about the sodium vs. tetrabutylammonium exchange equilibrium, it was possible to estimate the *total* extent of univalent counterion association: $0.65 < r < 0.85$. In a subsequent ^{23}Na NMR study of the competitive association of sodium and tetrabutylammonium with DNA, cationic buffering agents were eliminated, and the sample temperature was more strictly controlled (Bleam, 1980). When the observed variations of $\Delta\nu_{1/2}$ with additions of sodium or tetrabutylammonium were fitted to the theoretical expression derived by Anderson et al. (1978), a higher extent of counterion binding was determined: $0.9 < r < 1.0$. (The uncertainty in this estimate arises from a significant correlation between two of the parameters used in the fitting.) Thus, it appears that there is no counterion whose affinity for DNA is sufficiently less than that of sodium to permit a precise measurement of r. Displacing bound sodium ions with counterions whose affinity for DNA is much greater could provide a more definitive way of quantifying the extent of counterion association with DNA and of investigating the effect of temperature on r.

Previously, it was reported by us (Bleam et al., 1980) that the ^{23}Na NMR line width in a solution containing double-stranded DNA and NaCl can be decreased substantially by

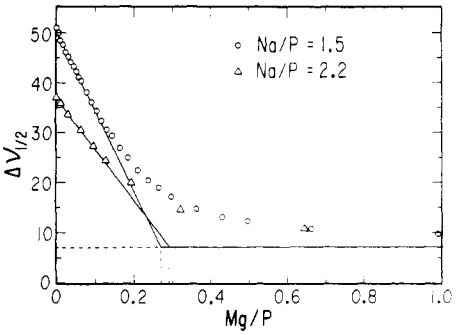

FIGURE 4: Sodium-23 line widths, $\Delta\nu_{1/2}$ (Hz), vs. $[Mg]/[P]$ at constant $[Na]/[P]$: 0.013 M calf thymus DNA at 20.3 °C with $[Na]/[P] = 1.5$ (○) or $[Na]/[P] = 2.2$ (△). Linear least-squares fits of data for which $[Mg]/[P] \leq 0.07$ are represented by straight lines. The broken horizontal line has an ordinate equal to the intercept at $[P]/[Na] = 0$ of a plot of $\Delta\nu_{1/2}$ vs. $[P]/[Na]$ for a comparable titration of NaDNA with NaCl.

the addition of $MgCl_2$ (if $[Na]/[P] \lesssim 3$). Thus, the displacement by magnesium of sodium associated with DNA can be monitored by ^{23}Na NMR spectroscopy. Figure 4 presents line-width data for two titrations of NaDNA ($[P] = 0.013$ M) with $MgCl_2$ at 20.3 °C. For the upper curve, $[Na]/[P] = 1.5$; for the lower one, $[Na]/[P] = 2.2$. Significantly lower values of this ratio cannot be achieved, because of the Donnan effect on small-ion distributions at dialysis equilibrium in the preparation of DNA samples (refer to Experimental Procedures). Higher levels of $[Na]/[P]$ are not informative, because the initial enhancement of the sodium line width would be too small to permit an accurate determination of the response to additions of $MgCl_2$. Both titration curves in Figure 4 appear linear over the range $0 \leq [Mg]/[P] \leq 0.1$. Since the intercepts and slopes of these linear portions can be ascertained with relatively high accuracy, they may provide reliable information about the extent of sodium association with helical DNA.

To derive an expression for the ^{23}Na transverse relaxation rate, $R = \pi\Delta\nu_{1/2}$, as a function of $[Mg]/[P]$, it is useful to introduce the definition

$$n \equiv (r^0[P] - [Na]_B)/[Mg]_B \quad (3)$$

where the subscript B refers to ions associated with DNA and r^0 is the value of $[Na]_B/[P]$ prior to any addition of $MgCl_2$. The quantity n is the ratio of the total number of sodium ions that have been displaced by magnesium to the total number of associated magnesium ions at any point in the titration. In general, n would be expected to vary during the titration. On the assumption that associated magnesium ions do not affect the (average) relaxation rate of associated sodium ions, eq 3 can be introduced into eq 2 to give

$$R = R_F + r^0(R_B - R_F)[P]/[Na] - np_M(R_B - R_F)([P]/[Na])([Mg]/[P]) \quad (4)$$

where $p_M \equiv [Mg]_B/[Mg]$. From this equation, it can be deduced that the product $np_M(R_B - R_F)$ does not vary over the initial, linear portions of the titration curves in Figure 4. The eventual upward curvature in $\Delta\nu_{1/2}$ with increasing $[Mg]$ is an expected result of decreases in n and/or p_M.

In Figure 4 the solid lines passing through the initial sections of the titration curves are linear least-squares fits of line-width data for the range $0 \leq [Mg]/[P] \leq 0.07$. The ordinate of the horizontal straight line is $\Delta\nu_F$, which is assumed to equal the intercept at $[P]/[Na] = 0$ of a plot of $\Delta\nu_{1/2}$ vs. $[P]/[Na]$ for a NaCl titration of NaDNA at the same concentration (0.013 M) and temperature (20.3 °C). (This titration curve is represented in Figure 2.) If the linear fits to the initial portions

Table I: Analysis of Titrations of NaDNA with $MgCl_2$ at Different Temperatures

temp (°C)	[Na]/[P]	R_F (Hz)	$r^0/(np_M)$	r^0 [a]	R_B [a] (Hz)
6.2	2.7 [b]	10.6	0.32 ± 0.02	0.64 ± 0.08	190 ± 10
20.3	1.5 [c]	7.2	0.27 ± 0.02	0.55 ± 0.07	130 ± 10
20.3	2.2 [c]	7.2	0.29 ± 0.02	0.59 ± 0.08	120 ± 10
20.3	1.6 [b]	6.9	0.27 ± 0.02	0.55 ± 0.07	130 ± 10
33.3	2.2 [c]	5.4	0.30 ± 0.02	0.60 ± 0.08	71 ± 5

[a] Calculated by assigning np_M the upper bound value of 2. [b] From the same calf thymus DNA stock solution ([P] = 0.013 M). [c] From the same calf thymus DNA stock solution ([P] = 0.013 M).

of the magnesium titration curves are extended to intersect the horizontal line $\Delta\nu_{1/2} = \Delta\nu_F$, then it follows from eq 4 that the abscissa of the point of intersection is $r^0/(np_M)$. Abscissas determined from the lines in Figure 4 are given in Table I. The uncertainty in $r^0/(np_M)$ (±0.02) is estimated from titrations conducted with different stock solutions. With the same stock solution, significantly higher precision is obtained. Since the same stock solution was used for the two titrations shown in Figure 4, the small difference in the values of $r^0/(np_M)$ may be real. The reproducibility of the ^{23}Na NMR measurements has been extensively checked (Bleam, 1980); some of the results from different DNA stock solutions are reported in Table I. One identifiable source of error in the values reported for $r^0/(np_M)$ results from the ~5% uncertainty in the extinction coefficient for helical DNA, which is required to determine [P]. Some additional error may arise from the choice of $\Delta\nu_F$, especially for the data at the higher sodium concentration.

The preceding analysis demonstrates a means of separating quantities dependent on sodium NMR relaxation (R_B, R_F) from quantities dependent on ion distributions (r^0, n, p_M). Therefore, magnesium titrations carried out at different temperatures should provide information sufficient to determine the relative importance of the contributions of r^0 and R_B to the pronounced temperature dependence of the titration curves in Figure 2. For this purpose, titrations of NaDNA with $MgCl_2$ were performed at 6 and 33 °C to supplement the data in Figure 4 (at 20 °C). The line-width data at these three temperatures are collected in Figure 5, where the ordinate, $(\Delta\nu - \Delta\nu_F)[Na]/[P]$, has been normalized in accordance with eq 4 to permit ready comparison of titrations at different values of [P]/[Na]. To account for the nonnegligible temperature dependence of $\Delta\nu_F$ in the ordinate, the free line width was determined at each temperature from the intercept of a plot of $\Delta\nu_{1/2}$ vs. [P]/[Na] for a NaCl titration of NaDNA (refer to the discussion of Figure 2). As in Figure 4, the initial sections of the titration curves in Figure 5 were fitted to least-squares lines over the range $0 \leq [Mg]/[P] \leq 0.07$, and these lines were extrapolated to intersect the x axis. The resulting intercepts are listed in Table I. These values of $r^0/(np_M)$ show a small systematic dependence on [Na]/[P] but not on temperature. It follows that the pronounced temperature variation of the titration curves in Figure 2 must be attributed to R_B rather than to r^0. A thorough analysis of the temperature dependence of R_B will require determination of the correlation time for the relaxation process. Experiments for this purpose are currently in progress (D. K. Chang and L. Nordenskiold, unpublished data).

If the association of sodium and magnesium ions with DNA is governed exclusively by electrostatic interactions, then none of the quantities r^0, p_M, and n are expected to have significant temperature dependence. According to either the PB equation

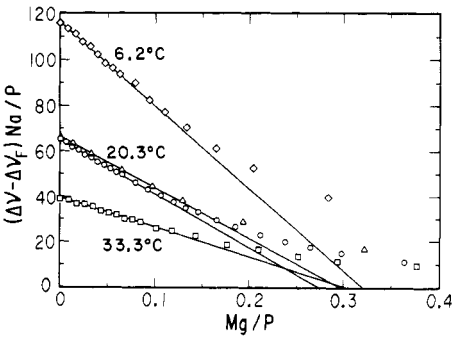

FIGURE 5: Normalized excess ^{23}Na NMR line widths, $(\Delta\nu_{1/2} - \Delta\nu_F)[Na]/[P]$, vs. [Mg]/[P] at three representative temperatures: 6.2 (◇), 20.3 (△, ○), and 33.3 °C (□). Further information about these DNA solutions is given in Table I. The straight lines are linear least-squares fits to data for which $[Mg]/[P] \leq 0.07$.

or the condensation model, counterion association with a rodlike polyion depends on temperature only through ϵT. This product of the solvent dielectric constant with temperature is itself almost constant over the temperature range of interest here. The data in Figure 5 indicate that the association of sodium with helical DNA does not vary with temperature and, hence, are consistent with an exclusively electrostatic interaction.

The extent of sodium ion association with helical DNA in the absence of magnesium, r^0, can be determined from the preceding analysis of the linear portions of magnesium titration curves only if a value can be assigned to np_M. This product cannot be evaluated from the experiments reported here, but an upper bound, $np_M \leq 2$, can be deduced from elementary considerations, since $p_M \leq 1$ and $n \leq 2$. (Values of n greater than two can be excluded because additions of $MgCl_2$ should not cause a net reduction of positive charge in the close vicinity of the DNA.) Corresponding upper bounds for r^0 and lower bounds for R_B are given in the last two columns of Table I. These upper bounds on r^0 are significantly lower than the range of values determined previously from ^{23}Na NMR studies of (n-Bu_4N)DNA (Anderson et al., 1978; Bleam, 1980).

Theoretical predictions for $r^0/(np_M)$ can be calculated from either the condensation model or the Poisson–Boltzmann equation. The two-variable condensation model for the competitive association of uni- and divalent counterions with a rodlike polyion (Manning, 1978) can be rederived to allow for low ratios of added salt(s) to polyion monomer (C. F. Anderson, unpublished data). For the concentration range of the magnesium titrations reported here, the generalized condensation model predicts that $r^0/(np_M) = 0.42$, approximately 40% larger than the experimental values given in Table I. Agreement between these and the values of $r^0/(np_M)$ estimated from the Poisson–Boltzmann equation has been tested by integrating the sodium ion radial distribution function from 13 to 16 Å for concentrations of NaCl and $MgCl_2$ corresponding to the experimental range (Bleam, 1980). This comparison is predicated on the assumption that the polyelectrolyte effect(s) on the relaxation rate of associated sodium nuclei extend(s) at least 3 Å from the surface of DNA. Smaller distances are improbable in the absence of any indication that associated sodium ions are significantly dehydrated. A typical plot of the Poisson–Boltzmann prediction for $[Na]_B/[P]$ vs. [Mg]/[P] is given in Figure 6, together with experimental points calculated from the ^{23}Na line-width data for the $MgCl_2$ titration of 0.013 M calf thymus NaDNA at 20.3 °C with [Na]/[P] = 1.5 (the upper curve in Figure 4). By assumption of an appropriate value for $\Delta\nu_B$, the data point at [Mg]/[P] = 0 was made to coincide with the theoretical curve. Ex-

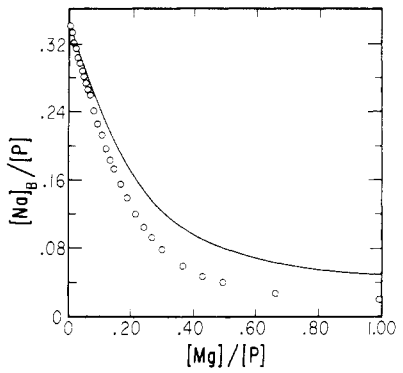

FIGURE 6: Extent of association of sodium, $[Na]_B/[P]$, as a function of [Mg]/[P] for 0.013 M calf thymus DNA at 20.3 °C, [Na]/[P] = 1.5. The (solid) theoretical curve was obtained by numerical integration from 13 to 16 Å of the sodium ion radial distribution function computed from the cylindrical Poisson–Boltzmann equation for mixed uni- and divalent counterions. Experimental points were calculated from the line-width data of Figure 4 by using the expression $[Na]_B/[P] = (\Delta\nu_{1/2} - \Delta\nu_F)[Na]/[(\Delta\nu_B - \Delta\nu_F)[P]]$. The quantity $\Delta\nu_B$ was chosen to force coincidence of the data point at [Mg]/[P] = 0 with the intercept of the theoretical curve.

trapolation of the initial slope of this curve to the x axis gives 0.34 as the Poisson–Boltzmann prediction for $r^0/(np_M)$. This value is significantly larger than the corresponding experimental intercept, 0.27 ± 0.02. [The larger values of $r^0/(np_M)$ given in Table I were determined from DNA solutions containing more NaCl and are in no better agreement with the ion distributions predicted by the Poisson–Boltzmann equation.] In comparison to the counterion condensation model, the Poisson–Boltzmann equation provides better agreement with the initial portion of the magnesium titration curve; however, the theoretical curve in Figure 6 consistently overestimates the experimental data, especially at higher values of [Mg]/[P]. The overestimate is increased if $[Na]_B/[P]$ is calculated by integrating the sodium radial distribution function over a larger distance from the DNA surface.

The predictions for $r^0/(np_M)$ calculated from either the condensation model or the Poisson–Boltzmann equation lie outside the experimental uncertainty of the values reported in Table I. Moreover, the upper bound on r^0 inferred from the magnesium titration data lies substantially below the range of values for r^0 inferred from previous ^{23}Na NMR studies (Anderson et al., 1978; Bleam, 1980). In view of these discrepancies, it is appropriate to reexamine a basic assumption implied by the use of eq 4 to analyze the linear portions of the magnesium titration curves: that the association of magnesium with DNA does not alter R_B, the average relaxation rate of the sodium nuclei remaining associated. The linearity of the initial portions of the magnesium titration curves is also consistent with a linear dependence of R_B on [Mg]/[P]:

$$R_B = R_B^0 - S[Mg]/[P] \quad (5)$$

where S is a positive constant. If eq 4 were modified to incorporate this functional form of R_B, the predicted form of the *initial* slopes of the magnesium titration curves becomes $-[np_M(R_B^0 - R_F) + r^0S][P]/[Na]$. Hence, the theoretical expression for the intercepts in Figures 4 and 5 is predicted to be $r^0[R_B^0 - R_F]/[np_M(R_B^0 - R_F) + r^0S]$ rather than $r^0/(np_M)$. Neglect of the term $r^0S/(R_B^0 - R_F)$ clearly would lead to an underestimate of $r^0/(np_M)$. Thus, the lack of agreement between the tabulated values of this quantity and the predictions of either theory could result from an unrecognized dependence of R_B on [Mg]/[P].

The decreasing linear dependence of R_B on [Mg]/[P] specified in eq 5 can be derived from a simple two-state model for the relaxation of *associated* sodium nuclei (C. F. Anderson, unpublished data). It suffices to postulate that one class of associated nuclei has a significantly faster relaxation rate and that associated magnesium ions preferentially displace this class of nuclei during the initial stages of the titration. The surface of helical DNA is heterogeneous and its charge per unit area relatively low. Consequently, in view of the short-range character of the quadrupolar interactions causing ^{23}Na relaxation, a distribution of relaxation rates is plausible. Heterogeneity in relaxation rates need not imply different classes of sodium binding "sites". A sodium nucleus close to, but not bound to, a phosphate charge may well experience a faster relaxation rate than a nucleus equally close to the DNA surface but not next to a phosphate charge. A preferential affinity of associated divalent ions for regions of the DNA surface where sodium relaxation is the most efficient would lead to the linear decrease in R_B predicted by eq 5. Alternative theoretical grounds for an equation of this form are also possible. To decide whether eq 5 is valid and, if so, to evaluate the parameter S will require experiments of a different type from those reported here. Determinations of T_1^{-1} and T_2^{-1} under conditions such that the ^{23}Na relaxation is outside the limit of extreme narrowing should be especially informative (L. Nordenskiold and D. K. Chang, unpublished data).

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Registry No. Na, 7440-23-5; Mg, 7439-95-4.

References

Anderson, C. F., & Record, M. T., Jr. (1982) *Annu. Rev. Phys. Chem. 33*, 191–222.
Anderson, C. F., Record, M. T., Jr., & Hart, P. A. (1978) *Biophys. Chem. 7*, 301–316.
Bleam, M. L. (1980) Ph.D. Thesis, University of Wisconsin, Madison.
Bleam, M. L., Anderson, C. F., & Record, M. T., Jr. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 3085–3089.
Dewey, T. G., & Turner, D. H. (1979) *Biochemistry 18*, 5757–5762.
Dwek, R. A. (1973) *Nuclear Magnetic Resonance in Biochemistry*, pp 37–46, 328–335, Clarendon, Oxford.
Eisenstadt, M., & Friedman, H. L. (1967) *J. Chem. Phys. 46*, 2182–2187.
Gunnarsson, G., & Gustavsson, H. (1982) *J. Chem. Soc., Faraday Trans. 1 78*, 2901–2910.
Laszlo, P. (1978) *Angew. Chem., Int. Ed. Engl. 17*, 254–266.
Levij, M., deBleijser, J., & Leyte, J. C. (1980) *Bull. Magn. Reson. 2*, 388.
Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual*, p 68, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Manning, G. S. (1975) *J. Chem. Phys. 62*, 748.
Manning, G. S. (1977) *Biophys. Chem. 7*, 95–102.
Manning, G. S (1978) *Q. Rev. Biophys. 11*, 179–246.
Manning, G. S. (1979) *Acc. Chem. Res. 12*, 443–449.
Mariam, Y. H., & Wilson, W. D. (1983) *J. Am. Chem. Soc. 105*, 627–628.

Record, M. T., Jr., Anderson, C. F., & Lohman, T. M. (1978) *Q. Rev. Biophys. 11*, 103–178.
Record, M. T., Jr., Mazur, S. J., Melançon, P., Roe, J.-H., Shaner, S. L., & Unger, L. (1981) *Annu. Rev. Biochem. 50*, 997–1024.
Reuben, J., Shporer, M., & Gabbay, E. J. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 245–247.
Studier, F. W. (1969) *J. Mol. Biol. 41*, 199–209.
Wilson, R. W., Rau, D. C., & Bloomfield, V. A. (1980) *Biophys. J. 30*, 317–324.

Investigation of the Metastable Phase Behavior of Phosphatidylglycerol with Divalent Cations by Calorimetry and Manganese Ion Binding Measurements†

Joan M. Boggs* and Godha Rangaraj

ABSTRACT: Divalent cations induce a stable, dehydrated phase in phosphatidylglycerol only at a bound cation to lipid mole ratio equal to or greater than 0.5:1, suggesting that all of the lipid must be neutralized by the cation. However, after melting of the stable phase at high temperature, it refreezes into a metastable phase if cooled rapidly. The transition temperature of the metastable state, both on cooling and on reheating, is not quite as high as that of the lipid in its protonated state, indicating that the lipid is no longer completely neutralized. This suggested that a portion of the divalent cation dissociates from the liquid-crystalline phase and does not rebind to the gel phase of the metastable state. The transition from the metastable to the stable state is exothermic and occurs on heating. Its rate increases with increase in divalent cation concentration and is decreased by Na^+, suggesting that the dissociated divalent cation rebinds during the endothermic or exothermic transitions of the metastable phase, when the lateral motion of the lipid increases. Rebinding of the cation allows more complete neutralization of the lipid head groups, close approach of opposing bilayers, and formation of the stable dehydrated state. Measurement of Mn^{2+} binding at different temperatures from the electron paramagnetic resonance spectrum of Mn^{2+} confirmed this hypothesis. These results indicate that if the rate of lateral motion is low, the affinity of acidic lipids for divalent cations decreases as the available free lipid becomes diluted by cation-bound lipid or other membrane constituents.

Complexation of divalent cations with acidic lipids results in dehydrated bilayers arranged in the form of cochleate cylinders or lamellar sheets, which melt at very high temperatures, above 100 °C in the case of phosphatidylserine (Ca^{2+} only) and phosphatidic acid but below 100 °C in the case of phosphatidylglycerol, phosphatidylethanolamine (at high pH), and dimyristoylphosphatidylserine (Mg^{2+} only) (Verkleij et al., 1974; Ververgaert et al., 1975; van Dijck et al., 1975, 1978; Jacobson & Papahadjopoulos, 1975; Papahadjopoulos et al., 1975; Harlos & Eibl, 1980a,b; Liao & Prestegard, 1981; Hauser & Shipley, 1983). Besides being the cause of isothermal phase transitions, this behavior of divalent cations may also be involved in fusion (Papahadjopoulos et al., 1977).

The complex of synthetic forms of phosphatidylglycerol (PG)[1] with divalent cations undergoes metastable phase behavior at cation to lipid mole ratios greater than 0.5:1 (Ververgaert et al., 1975; van Dijck et al., 1975; Sacré et al., 1979; Fleming & Keough, 1983). The metastable phase melts at a temperature near that of the protonated form of the lipid. It then undergoes an exothermic transition to a stable state which melts with a greater enthalpy at 65–97 °C, depending on the cation and chain length. The accessibility of the phase transition of the stable state of PG below 100 °C makes this a useful lipid with which to study the structure of the metastable and stable states and the mechanism of conversion beween them. The stable state has been shown to be dehydrated and crystalline-like while the metastable phase is in the form of hydrated bilayers by X-ray diffraction (Harlos & Eibl, 1980a) and ^{31}P NMR spectroscopy (Cullis & de Kruijff, 1976; Farren & Cullis, 1980).

In their study of the effect of divalent cations on bovine brain phosphatidylserine (PS), Portis et al. (1979) suggested that the stable dehydrated state of the Ca^{2+} complex of PS is due to formation of a "trans" complex in which lipids in adjacent bilayers are bridged while complexes melting at a lower temperature (such as Mg^{2+}–PS) are due to a "cis" complex in which only adjacent molecules in the same bilayer are bridged. However, PS has not been observed to undergo metastable phase behavior. Copeland & Andersen (1981, 1982) have developed a statistical mechanical theory which attempts to account for the effects of divalent cations on lipid phase transitions including the metastable phase behavior of the PG complex. Making use of experimental data in the literature, they invoke cis-type bridging in the stable state but not the metastable or liquid-crystalline states to account for the metastability and the transition temperatures of the metastable and stable states. However, they did not take into account the close contact of bilayers in the stable state which appears to be necessary to explain the X-ray diffraction, freeze–fracture, and ^{31}P NMR results and cannot explain the fact that the stable phase only occurs at bound ratios of cation to lipid of 0.5:1. Furthermore, certain other features of the

† From the Research Institute, The Hospital for Sick Children, and The Department of Clinical Biochemistry, University of Toronto, Toronto, Ontario, Canada M5G 1X8. *Received March 29, 1983.* This investigation was supported by the Medical Research Council of Canada and by a Career Development Award from the Multiple Sclerosis Society of Canada to J.M.B.

[1] Abbreviations: DLPG, dilauroylphosphatidylglycerol; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DSPG, distearoylphosphatidylglycerol; DTPG, ditetradecylphosphatidylglycerol; PG, phosphatidylglycerol; PS, phosphatidylserine; DSC, differential scanning calorimetry; EPR, electron paramagnetic resonance; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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