# $^{23}$Na NMR Study of the Effect of Organic Osmolytes on DNA Counterion Atmosphere

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ABSTRACT The effect of different organic osmolytes on the DNA counterion condensation layer has been investigated by $^{23}$Na NMR relaxation measurements . The zwitterionic compounds glycine, β-alanine, 4-aminobutyric acid, and 6-aminocaproic acid have shown an increasing capacity to decrease the amount of sodium ions in the vicinity of the macromolecule. The experimental data have been correlated with the dielectric constant increase in their corresponding solutions and have been compared with the prediction of counterion condensation theory. Polyols (sorbitol and mannitol) did not display the same effect. These compounds largely increase the relaxation rate of sodium ions in the proximity of DNA, unlike the zwitterionic compounds. This probably results from a perturbation of the water dynamic around the macromolecule, of the primary or secondary hydration shell of the sodium nuclei involved, or both.

## INTRODUCTION

Euryhaline aquatic invertebrates are poikilosmotic species living in environments of fluctuating salinities. As their possibilities for blood osmoregulation are generally weak, their cells have to cope with large changes in blood osmolality in the course of their acclimatation to media of different salinities. In comparison, the possibility for survival of mammalian species in anisosmotic media are far more limited. One of the problems met by these cells during an osmotic stress is the modification of intracellular chromatin structures as a result of large changes in the intracellular concentration of inorganic ions. The presence of large amounts of amino acids and polyols (glycine, taurine, proline, mannitol, sorbitol, etc.), called osmotic effectors, organic osmolytes, or compensatory solutes, in the intracellular media of euryhaline invertebrates seems to prevent such structural modifications (Delpire et al., 1985; Gilles, 1988).

Previous studies (Buche et al., 1989, 1990, 1993) have confirmed this protection effect. Indeed, these compounds hinder chromatin precipitation induced by NaCl, KCl, $CaCl_2$, or $MgCl_2$ in vitro. Similar protection effects have also been observed in the study of the effect of glycine addition on DNA precipitation induced by spermine$^{4+}$, spermidine$^{3+}$, and $Tb^{3+}$ (Flock et al., 1995).

According to the counterion condensation model of Manning (1978) as well as to the most recent development of the Poisson–Boltzmann theory (Le Bret et Zimm, 1984; Sharp, 1994) and Monte Carlo simulations (Mills et al., 1986; Vorontsov-Velyaminov and Lyubartsev, 1989; Jayaram et al. 1994), the strong coulombic field that is due to polyion charges induces a large degree of inhomogeneity in the location of small ions. Counterions cluster close to the polyion surface, where co-ions are scarce. Many experimental works have confirmed this counterionic behavior. Precipitation of DNA and chromatin induced by salt addition has been attributed to the enhancement of the extent of phosphate charge neutralization (Wilson and Bloomfield, 1979; Bloomfield et al., 1980; Manning, 1989; Bloomfield, 1991; Marquet and Houssier, 1991) as a result of the increase in the amount of positive charges in the proximity of negative phosphate charges. The protection effect of these organic osmolytes could be caused by a modification of the electrostatic interactions between polyelectrolyte and counterions. This hypothesis was indirectly verified in a previous publication: Using different zwitterionic compounds (glycine, β-alanine, 4-aminobutyric acid, and 6-aminocaproic acid), we correlated the protection effect observed with a medium dielectric constant increase (Flock et al., 1996).

To verify this hypothesis directly, we studied, by $^{23}$Na NMR, the modification of DNA–ion interaction when organic osmolytes are added to the solution. This technique has frequently afforded a direct and sensitive probe of the interactions of small ligands with macromolecules (Braunlin et al., 1987; Schultz et al., 1992; Wright and Lerner, 1994). In particular, $^{23}$Na has often been utilized owing to its advantageous NMR properties from technical and theoretical viewpoints. Numerous $^{23}$Na NMR investigations of double-helical DNA have been reported (Anderson et al., 1978; Bleam et al., 1980, 1983; Nordenskiöld et al., 1984; Delville et al., 1986; van Dijk et al., 1987; Groot et al., 1994). When sodium is in the vicinity of a macromolecule, its relaxation rate is much faster than when it is free in solution. It is thus possible, by this technique, to detect a modification of the extent to which counterions are associated with DNA when organic osmolytes are added in solution.

We have also used this approach to study the effect of the aminocarboxylic acids mentioned above on the exchange of

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sodium ions near DNA with hexamethonium$^{2+}$. This cationic polyamine analog has two quaternary ammonium centers [$-N(CH_3)_3^+$] separated by six methylene ($CH_2$) groups, and its competition with $Na^+$ has been studied by Padmanabhan et al. (1988), who used $^{23}Na$ and $^{14}N$ NMR relaxation techniques. During the course of the titration, the relaxation rates of $^{23}Na$ are progressively reduced as sodium ions are displaced from the vicinity of DNA, and analysis of the concentration dependence of these relaxation rates provides qualitative and quantitative information about the competitive association of the divalent cation and $Na^+$ with DNA.

The theories cited above model the electrostatic interactions between polyelectrolyte and counterions in some different ways, and different predictions are thus obtained. Among them, Manning's theory has often been used because of its simplicity and has been shown to give a relatively good description of the DNA behavior in salt solution. In this theory, the population of counterions is divided in two categories, depending on whether the ions are close to the macromolecule (contained in a condensation layer: $Na^+$ bound) or free in solution. We have used this theory in relation to $^{23}Na$ NMR experiments, considering that the sodium nuclei experiencing a faster relaxation in the proximity of DNA can be identified as the $Na^+$ bound of the theoretical model.

## MATERIALS AND METHODS

### Samples

Calf thymus DNA (Sigma Type I) was purified to a residual protein content smaller than 1%. Stock DNA solutions at high concentration ( ~10 g/l) were prepared and dialyzed against cacodylate buffer (1 mM, pH 6.5). For each DNA stock solution (~50 ml) the dialysis bag was left to equilibrate for 10 h against 5 l of buffer, and dialysis was repeated five times with fresh buffer. We used an extinction coefficient $\epsilon(260\ nm) = 6600\ M^{-1}\ cm^{-1}$ to determine their concentration in mononucleotide residues.

We estimated the ratio of phosphate over sodium concentration in the DNA solution ($C_p/C_1 = 0.99$) from the sodium concentration outside the dialysis bag, taking the Donnan effect into account. Atomic absorption analysis has confirmed that the excess of sodium ions compared with the DNA phosphate charges was not greater than 8%.

Glycine (USB), β-alanine, 4-aminobutyric acid, 6-aminocaproic acid (Fluka), taurine, proline (Sigma), sorbitol, and mannitol (Merck) were used directly in solid form to prepare the samples. We have also used NaCl (Lammers and Pleuger) and $D_2O$ (Sigma).

Titrations of DNA solution by the divalent cation hexamethonium$^{2+}$ [(*N,N,N,−N′,N′,N′,*−hexamethylhexamethylene diammonium dibromide, $(CH_3)_3N(Br)(CH_2)_6N(Br)(CH_3)_3$ (Sigma; abbreviated $Hex^{2+}$)] were carried out by addition of microliter quantities of stock solutions (250 mM) to 4-ml samples contained in NMR tubes. After each addition, the sample was thermally equilibrated before NMR measurements were performed.

### $^{23}Na$ NMR experiments

$^{23}Na$ NMR Fourier-transform spectra were measured at 79.393 MHz with a Bruker AM 300 WB spectrometer locked internally on the deuterium signal. The samples were prepared by dissolution of solid organic osmolytes in solutions of DNA or NaCl containing 10% $D_2O$ by volume. NMR measurements were performed with a spectral width of 1000–2000 Hz, an acquisition time of 1–3 s, and a dead time of ±500 μs. With these parameters, an acquisition time of 2 h (corresponding to 3000–5000 acquisitions) was necessary for a satisfactory signal-to-noise ratio (>50) with 8 mM NaDNA solution at 20°C. Solutions of NaCl required much smaller acquisition times (±10 min or ± 200 acquisitions) because the line widths were smaller. Under the conditions of the present study, sodium spectra of DNA and NaCl solutions exhibited no significant deviation from Lorentzian form, and the transverse relaxation rate ($R_2$) was simply evaluated from the line widths at half-height $\Delta\nu_{1/2} = R_2/\pi$. Longitudinal relaxation times ($T_1 = 1/R_1$) were measured with the standard (180°-*t*-90°) sequence, followed by a nonlinear regression on at least nine different determinations for variable delays *t*. Temperature was controlled at ±1°C.

## RESULTS

### Sodium-23 relaxation rates in solutions of NaDNA and influence of osmotic effectors

The sodium nucleus (spin $I = 3/2$ and abundance 100%) has a moderately large quadrupole moment; it can interact with electric field gradients created by its environment. This situation is encountered with DNA, for which the spin relaxation of $Na^+$ ions directly surrounding DNA is important because of the big electric field developed by this macromolecule. Two different environments, the macromolecule and the solvent, are thus to be considered for sodium in NaDNA solution, which give rise to the respective relaxation rates $R_b$ and $R_f$ (two-state model). When the exchange between these sodium nuclei is fast in comparison with the relaxation rates of the nuclei, the observed relaxation rate ($R$) is an average value that depends on their respective fractions, $p_b$ and $p_f$ ($R = p_fR_f + p_bR_b$). Fig. 1 illustrates the importance of this process: the $^{23}Na$ line width at half-height, $\Delta\nu_{1/2}$, in the presence of DNA (8 mM) at 20°C is approximately seven times (47.9 Hz) larger than its value in the NaCl solution (6.72 Hz).

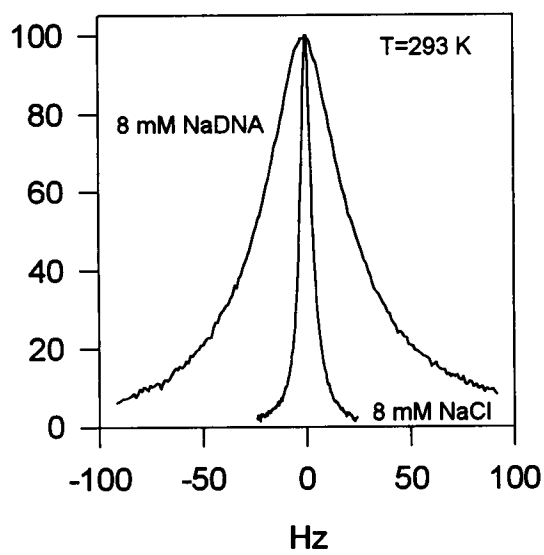


FIGURE 1 $^{23}Na$ NMR signals of solutions of NaCl (8 mM) and DNA (8 mM with $[P^-]/[Na^+] = 0.99$) at 20°C.

According to van Dijk et al. (1987), in a calf thymus solution at high field and below approximately 30°C the transverse relaxation time of sodium ions consists of two terms. In this case, the $^{23}$Na line shape is a superposition of two Lorentzian components, the fast and slow components, representing, respectively, 60% and 40% of the signal. The line widths at half-height of these two Lorentzians allow the determination of the fast and slow transverse relaxation rates of sodium nuclei ($R_{2f}$ and $R_{2s}$). In the two-state model approximation we have

$$R_{2f} = p_f R_f + p_b R_{b2f}, \quad (1)$$

$$R_{2s} = p_f R_f + p_b R_{b2s}, \quad (2)$$

where $R_{b2f}$ and $R_{b2s}$ are, respectively, the fast and the slow transverse relaxation rates of sodium ions "bound" to DNA.

Fig. 2 evidences the effect of the temperature on the $^{23}$Na line widths in our DNA solution (8–8.5 mM). At 5° and 10°C a significant deviation from Lorentzian form has been observed in sodium NMR spectra (not shown). However, this departure was not sufficient to yield with a good accuracy the two transverse relaxation rates $R_{2s}$ and $R_{2f}$ by deconvolution of the spectra. Above 10°C no departure from Lorentzian form of the $^{23}$Na peaks was found, and the line width at half-height of these peaks can be considered a direct measure of the transverse relaxation rate ($R_2 = \pi\Delta\nu_{1/2} = R_{2f} = R_{2s}$) averaged for all different magnetic environments of sodium ions (Anderson et al., 1978; Bleam et al., 1980, 1983; Braunlin et al., 1986). The line width decrease with increasing temperature shown in Fig. 2 is in good agreement with the fast-exchange hypothesis (Bleam et al., 1983; Nordenskiöld et al., 1984).

Organic osmolytes modify the line width of $^{23}$Na NMR spectra (Fig. 3). In NaCl solutions, $\Delta\nu_{1/2}$ increases with the added compound concentration. The behavior of the line width in DNA solutions is strongly dependent on the type of osmotic effector added. Indeed, the decrease in $\Delta\nu_{1/2}$ with the addition of glycine contrasts with the sharp increase in $\Delta\nu_{1/2}$ obtained with sorbitol and mannitol.

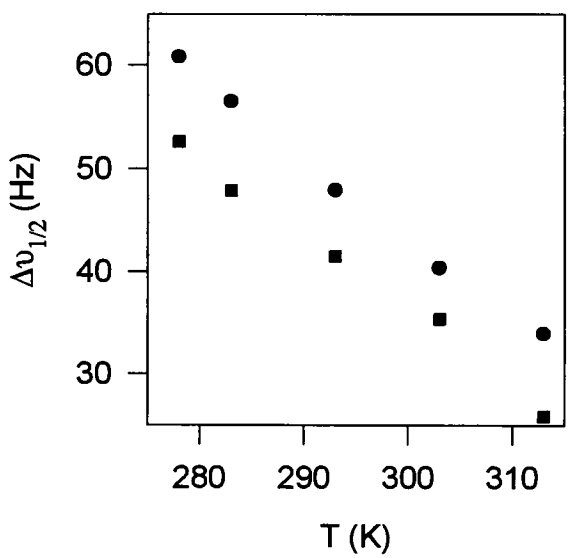


FIGURE 2 Modification of the line widths at half-height ($\Delta\nu_{1/2}$) of $^{23}$Na NMR signals with absolute temperature for DNA (8–8.5 mM) solutions in the absence (●) and presence of 1 M glycine (■).

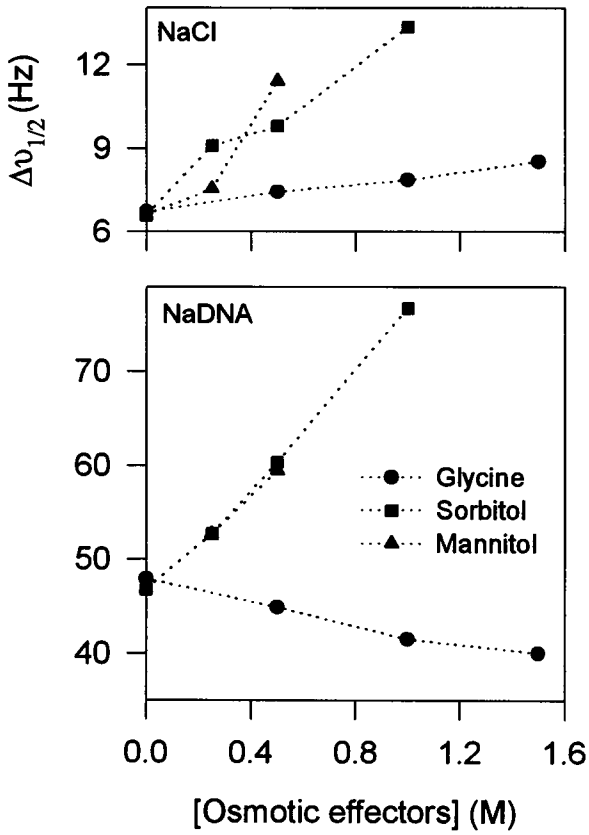


FIGURE 3 Modification of the line width at half-height ($\Delta\nu_{1/2}$) of $^{23}$Na NMR signals of NaCl (8–8.5 mM) and DNA (8–8.5 mM) solutions at 20°C as a function of osmotic effector concentrations.

## Influence of organic osmolytes on the relaxation rate of sodium ions bound to DNA

The theoretical background of the analysis of quadrupolar ion NMR relaxation data in polyelectrolyte solutions is not trivial. Although considerable progress has been achieved in the past few years (Groot et al., 1994; Schultz et al., 1994; van Dijk et al., 1987), the origin and mechanism of $^{23}$Na relaxation in DNA solutions are not yet fully understood. It is not our aim to deal with this problem here, but we must nevertheless consider the possibility that osmotic effectors change the relaxation process of sodium ions "bound" to DNA. To address this possibility, we conducted some experiments at 5°C and with a higher DNA concentration (17 mM).

As we saw above, at 5°C some departures from the Lorentzian form have been found in the $^{23}$Na spectra. The determination of the fast and slow transverse relaxation rates of sodium ions would have allowed us to consider the above hypothesis with more confidence than with a single

transverse relaxation rate. Unfortunately, these slight departures were not sufficient to permit us to determine the two transverse relaxation rates with a good degree of accuracy. To solve this problem we determined an "average" transversal relaxation rate by measuring the line width at half-height of the pseudo-Lorentzian signals and, using an inversion recovery method, also determined the longitudinal relaxation rates of sodium ions in the same conditions. These transversal and longitudinal relaxation rates are listed in Table 1 and obey the following relationships:

$$R_2 = p_f R_f + p_b R_{2b}, \quad (3)$$

$$R_1 = p_f R_f + p_b R_{1b}, \quad (4)$$

where the subscript 1 or 2 indicates a longitudinal or a transverse relaxation rate, respectively.

As has been observed for glycine at 20°C, addition of zwitterionic compounds to a DNA solution decreases the transverse relaxation rate of sodium ions. The longitudinal relaxation rate follows the same rule. Addition of polyols to DNA solutions induces the opposite variation.

According to Eqs. 3 and 4 it is necessary to know $p_b$ to calculate $R_{2b}$ and $R_{1b}$ from the experimental data. According to Manning's theoretical predictions, the fraction of DNA phosphate charges neutralized by sodium ions ($\theta$) is given by the following equation (Manning, 1978):

$$\ln\left(\frac{\theta}{V(C_1 - \theta C_p)}\right) + 1 = -2(1-\theta)\xi \ln(1 - e^{-\kappa b}), \quad (5)$$

where $\xi$ is the axial charge density parameter given by $\xi = q^2/(4\pi\epsilon_0\epsilon_r kTb)$, with $q$ the protonic charge, $b$ the distance between two charge groups (1.7 Å for native DNA), $\epsilon_0$ the vacuum permittivity, $\epsilon_r$ the medium's dielectric constant ($\epsilon_r$ = 80 for water at 20°C), $k$ the Boltzmann constant, and $T$ the absolute temperature. $C_1$ and $C_p$ are, respectively, the total concentrations of $Na^+$ and DNA; $V$, the volume per mole of mononucleotide of the region surrounding the polynucleotide within which $Na^+$ are said to be bound, is given by ($V$ is in units of $dm^3$/mol of mononucleotide if $b$ is expressed in meters) and $\kappa$, the Debye screening parameter, is given by $\kappa^2 = 2 \times 10^3 \times N_{av}(q^2/\epsilon_0\epsilon_r kT)I$, where $N_{av}$ is Avogadro's number and $I$ is the ionic strength computed from the salt concentrations.

We obtain in the case of the DNA solution used in this study, namely, 17 mM DNA with $[P^-]/[Na^+] = 0.99$, $\theta = 0.712$, which corresponds to a $p_b$ value of 0.705 ($p_b = \theta C_p/C_1$); the transverse and longitudinal relaxation rates of sodium ions bound to DNA are thus estimated to be $R_{2b} = 259\ s^{-1}$ and $R_{1b} = 155\ s^{-1}$, respectively.

According to Eqs. 3 and 4, the decrease in the sodium relaxation rate experimentally observed when glycine is added to the DNA solution can be explained only by a modification of the relaxation rates of sodium ions bound to DNA ($R_{1b}$ and $R_{2b}$), by a change in the fraction of sodium ions bound ($p_b$), or by both. Unfortunately, these parameters are closely correlated in Eqs. 3 and 4, and we must resort to additional information to determine their respective variation. For example, it is well known that the addition of glycine increases the medium dielectric constant (Cohn and Edsall, 1943). We can thus argue that the fraction of sodium ions bound to DNA must decrease in the presence of this zwitterionic compound. Indeed, the interaction between $Na^+$ and negative phosphate charges is governed by electrostatic forces whose strength is decreased when the dielectric constant increases. Calculation on the basis of Eqs. 3 and 4, with the $R_{1b}$ and $R_{2b}$ values reported above for DNA solution, gives $p_b$ values of 0.586 and 0.585, respectively, for a DNA solution containing 1 M glycine. This corresponds to an ejection of 17% of the sodium ions bound to DNA. The remarkable agreement between these two values of $p_b$ indicates that the addition of glycine to the DNA solution does not greatly modify the relaxation rates of sodium ions bound to DNA. This result is in accord with those from UV spectroscopy, circular dichroism, and electric linear dichroism, which have shown only very small perturbations of DNA structure in the presence of glycine in sodium salt solution. Moreover, such sodium ejection would explain the protection effect of glycine in the DNA precipitation experiments (Flock et al., 1995). However, these results do not prove that the addition of glycine to the DNA solution does not modify $R_{1b}$ and $R_{2b}$. As for DNA alone, the observed temperature dependence of $\Delta\nu_{1/2}$ for a DNA solution in the presence of 1 M glycine (Fig. 2) validates the fast exchange hypotheses. Similar results were obtained with taurine (Table 1).

In the presence of sorbitol, the same reasoning gives a $p_b$ value of 1.196 with Eq. 3 and 1.442 with Eq. 4. These

**TABLE 1 Longitudinal and transversal relaxation rates measured in different NaDNA (17 mM) and NaCl (17 mM) solutions containing osmotic effectors**

| NaCl (17 mM) | $R_f$ ($s^{-1}$) | DNA (17 mM) | $R_2$ ($s^{-1}$) | $R_1$ ($s^{-1}$) | $p_b$ | $R_{2b}$ ($s^{-1}$) | $R_{1b}$ ($s^{-1}$) |
|---|---|---|---|---|---|---|---|
| – | 29 | – | 191 | 118 | 0.705* | 259# | 155# |
| Glycine 1M | 32 | Glycine 1M | 165 | 104 | 0.585 ± 0.001¶ | | |
| Taurine 0.5M | 31 | Taurine 0.5M | 164 | 101 | 0.574 ± 0.009¶ | | |
| Sorbitol 1M | 60 | Sorbitol 1M | 298 | 197 | | See Fig. 4 | |
| Mannitol 0.5M | 54 | Mannitol 0.5M | 232 | 145 | | See Fig. 4 | |

*Calculated according to Eq. 5.
#Calculated with Eqs. 3 and 4.
¶Calculated according to Eqs. 3 and 4, with $R_{2b} = 259\ s^{-1}$ and $R_{1b} = 155\ s^{-1}$.

physically impossible values ($p_b$ cannot be greater than 1, and similar values must be obtained by Eq. 3 and 4) prove that in the presence of sorbitol there is a modification of the transverse and longitudinal relaxation rates of sodium ions bound to DNA. To check the accuracy of this modification we calculated from Eqs. 3 and 4 the values of $R_{1b}$ and $R_{2b}$ corresponding to physically acceptable values of $p_b$ (ranging from 0.1 to 0.9; Fig. 4). In all cases the values of $R_{1b}$ and $R_{2b}$ are much greater than 155 and 259 $s^{-1}$, respectively. Similar results are obtained with mannitol (Fig. 4). This indicates that the environment of sodium nuclei in the vicinity of DNA is considerably modified in the presence of these polyols, which could result in a change in their primary or secondary hydration shells.

## Dielectric constant effect on sodium condensation layer around DNA

In an earlier paper (Flock et al., 1996) we showed a strong correlation between the dielectric constant of the medium and the solubility of DNA in the presence of multivalent cations. We have thus conducted $^{23}$Na NMR experiments on DNA in solutions of increasing dielectric constant obtained by addition of various concentrations of the aminocarboxylic acids glycine, β-alanine, 4-aminobutyric acid, or 6-aminocaproic acid, whose molar dielectric constant increments in water are, respectively, 22.6, 34.6, 51, and 77.5 at 25°C (Cohn and Edsall, 1943). Fig. 5 shows the variation of the $^{23}$Na line width at half-height in NaCl and NaDNA (8 mM) solutions as a function of the dielectric constant in these aminocarboxylic acid solutions. Correlation between the line width and the dielectric constant is evident. In NaCl solutions, $\Delta\nu_{1/2}$ increases slightly with the dielectric constant. In NaDNA solutions the line width decreases with this parameter. By considering that the transverse relaxation rate

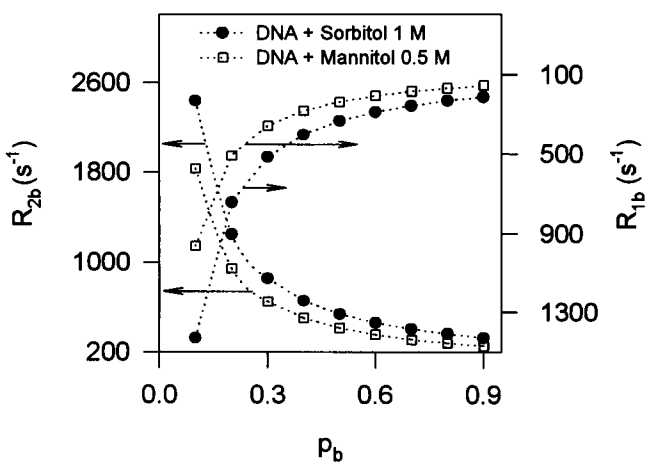


FIGURE 4 Longitudinal ($R_{1b}$) and transverse ($R_{2b}$) relaxation rates of sodium ions "bound" to DNA calculated for different values of $p_b$ according to Eqs. 3 and 4 and experimental relaxation rates shown in Table 1 for NaCl and DNA (17 mM) solutions containing 1 M sorbitol and 0.5 M mannitol.

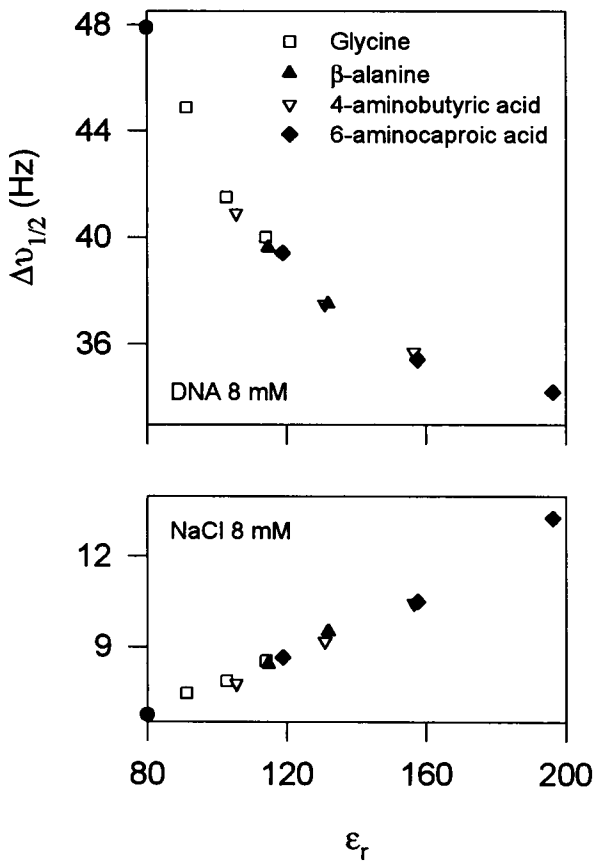


FIGURE 5 Modification of the line widths at half-height ($\Delta\nu_{1/2}$) of $^{23}$Na NMR signals of DNA (8 mM) and NaCl (8 mM) solutions at 20°C with the medium's dielectric constant increase induced by addition of aminocarboxylic acids at different concentrations.

of bound sodium ($R_{2b}$) remains constant in these different conditions, we can argue that fewer sodium ions are bound to DNA in a solution of higher dielectric constant. This is in qualitative agreement with Manning's theory and our previous precipitation experiments (Flock et al., 1996).

To obtain quantitative data we can rewrite Eq. 3, using $p_f = 1 - p_b$ and $p_b = \theta C_p/C_1$, as

$$R_2 = R_f + \theta(R_{2b} - R_f)C_p/C_1. \quad (6)$$

If $\theta$ for NaDNA is assigned the value 0.716 (obtained by identifying the bound state in NMR two-state model with the condensed state in counterion condensation theory; Eq. 5), the relaxation-rate constant $R_{2b}$ calculated according to Eq. 6 is 204 $s^{-1}$. The fractions of DNA phosphate charge neutralization calculated with this $R_{2b}$ value and Eq. 6 are shown in Fig. 6. The correlation between $\theta$ and $\epsilon_r$ is clearly shown. $R_f$ values required for these calculations are those obtained from the relaxation rates measured in equivalent NaCl solutions containing the organic osmolyte at the same concentration.

To investigate the effect of the change in the dielectric constant on the competition between cations of different valence, we measured the $^{23}$Na line width at half-height

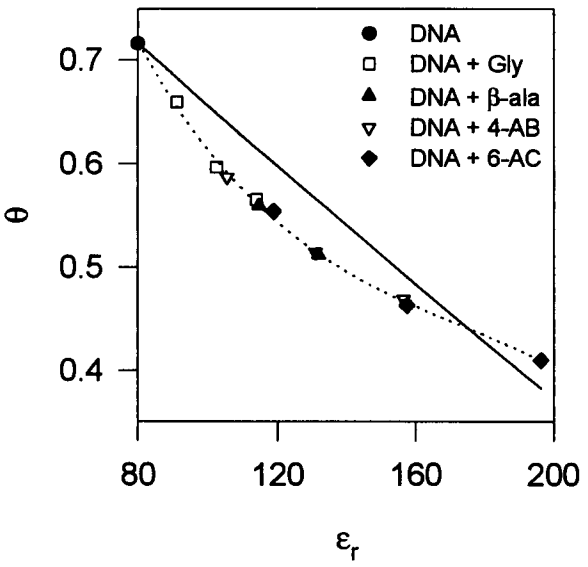


FIGURE 6 Fraction $\theta$ of DNA phosphate charge neutralized by sodium counterions in the presence of aminocarboxylic acids: glycine (*Gly*), β-alanine (*β-ala*), 4-aminobutyric acid (*4-AB*), 6-aminocaproic acid (*6-AC*). The experimental points are calculated from the NMR results shown in Fig. 5. The solid curve corresponds to the prediction of counterion condensation theory (Eq. 5).

obtained when the NaDNA solutions were titrated by hexamethonium$^{2+}$ ($Hex^{2+}$). Using the method described above (in this case $R_{2b}$ was found equal to 198 $s^{-1}$, a value very similar to that reported above), we calculated the fraction of DNA phosphate charges neutralized by sodium ions and show them in Table 2. Values of $\theta$ calculated in the absence of $Hex^{2+}$ are, within experimental accuracy, identical to the values reported above in the presence of 1 M aminocarboxylic acids (Fig. 6). The fraction of phosphate charges neutralized by sodium ions at the end of the titration, when $[Hex^{2+}]/[Na^+] = 1.1$, does not seem to be correlated with the length of the zwitterionic compounds used, unlike in the case of $\theta$ values obtained in the absence of $Hex^{2+}$. However, the NMR technique is not adequate to yield with a good degree of accuracy the values of $\theta$ when the majority of the sodium nuclei are free in solution (see Eq. 3), and no definitive conclusion can be formulated about the effect of aminocarboxylic acids on the fraction of phosphate charges remaining neutralized by sodium ions at the end of the titration experiments. The major information to get from these experiments is that zwitterionic compounds are unable to prevent competition between sodium ions and hexamethonium.

## DISCUSSION

Although it was not possible to determine a slow and a fast transverse relaxation rate from experiments on DNA solution at 5°C, the value of the longitudinal relaxation rate of sodium ions bound to DNA ($R_{1b}$ = 155 $s^{-1}$) agrees well with the previously reported value (van Dijk et al., 1987). Indeed, we have calculated from Eq. 4 a similar value of $R_{1b}$ (160 $s^{-1}$) from their longitudinal relaxation-rate value ($R_1$ = 123 $s^{-1}$) obtained at the same temperature, a similar ratio $C_p/C_1$, and a slightly lower frequency (71.569 MHz). Other $^{23}$Na NMR measurements of DNA solutions have been reported in the literature (Padmanabhan et al., 1988; Braunlin et al., 1986; Delville et al., 1986; Nordenskiöld et al., 1984; Bleam et al., 1983; Anderson et al., 1978; Reuben et al., 1975). Unfortunately, because of differences in $C_p/C_1$, temperature, and magnetic field, valuable comparisons with our results are impossible.

At 5°C the departure of the $^{23}$Na NMR band from the Lorentzian form is slight. This explains why we failed in the determination of slow and fast transverse relaxation rates by the fitting procedure, a technique less time consuming but also less efficient for determining these two relaxation rates than the spin-echo method used by van Dijk et al. (1987). Other studies (Padmanabhan et al., 1988; van Dijk et al., 1987; Delville et al., 1986; Nordenskiöld et al., 1984) have also revealed a bi-Lorentzian shape for the $^{23}$Na NMR spectra of DNA solutions, at 20°C. However, in our research, no significant deviation from the single Lorentzian form was found above 10°C. Therefore, in the experiments conducted at 20°C, the line widths of the $^{23}$Na NMR bands were used directly to determine the transverse relaxation rate of the sodium ions.

The modification of the $^{23}$Na NMR spectra when organic osmolytes are added to NaDNA solution results in distinct behaviors for zwitterionic compounds and for polyols (Fig. 3). We will thus discuss separately the effects of these two

**TABLE 2 Fraction of DNA phosphate charges neutralized by sodium ions ($\theta$) during titration of DNA solutions (8.9 mM with $[P^-]/[Na^+]$ = 0.99) in the absence and presence of 1 M glycine (Gly), β-alanine (β-ala), 4-aminobutyric acid (4-AB) and 6-aminocaproic acid (6-AC) by hexamethonium$^{2+}$ ($Hex^{2+}$)**

| $[Hex^{2+}]/[Na^+]$: | DNA | DNA + Gly | DNA + β-ala | DNA + 4-AB | DNA + 6-AC |
|---|---|---|---|---|---|
| 0 | 0.716 | 0.586 | 0.553 | 0.527 | 0.461 |
| 0.10 | 0.506 | 0.394 | 0.398 | 0.362 | 0.353 |
| 0.21 | 0.339 | 0.296 | 0.266 | 0.256 | 0.262 |
| 0.31 | 0.267 | 0.214 | 0.197 | 0.197 | 0.183 |
| 0.42 | 0.206 | 0.162 | – | – | – |
| 0.52 | – | 0.148 | 0.146 | 0.144 | 0.146 |
| 0.73 | 0.138 | 0.119 | 0.123 | 0.108 | 0.118 |
| 1.1 | 0.103 | 0.092 | 0.097 | 0.084 | 0.100 |

types of compound. We must also address the question why the relaxation rates of sodium ions in NaCl solution increase with the addition of progressively larger amounts of organic osmolytes. This enhancement is small, ±11%, in the presence of glycine 0.5 M, but it is dependent on the increase of amino acid concentration (Fig. 3) and reaches 27% when the concentration is 1.5 M. The relaxation-rate increase is more important in the presence of sorbitol, as the relaxation rate doubles at 1 M concentration in this polyol. We can understand these effects by considering the results of a study made by Eisenstedt and Friedman (1967) of $^{23}$Na NMR in solutions containing simple electrolytes. By examining a wide variety of systems, they established a correlation between increases in the sodium relaxation rate and increases in the bulk viscosity and partial molar volume of the solvent brought about by varying the solute concentration. We show in Fig. 7 the free sodium relaxation rates ($R_f$) measured in aminocarboxylic solutions at 20°C as a function of the relative viscosity $\eta_r$ of the solution (obtained from Devine and Lowe, 1971). The solution viscosity appears to have a nonnegligible effect on the sodium relaxation rate, and that could make difficult the analysis of the relaxation rates obtained in the presence of DNA owing to the additional contribution of this macromolecule to the viscosity increase. However, other studies (Bleam et al., 1983; Padmanabhan et al., 1988) have shown by extrapolation to zero DNA concentration that the relaxation rates of sodium ions free in DNA solution only slightly exceed the relaxation rates measured at the same temperature in NaCl solutions containing no DNA. Thus, the assumption that the relaxation rates of sodium ions not condensed to DNA are given by the relaxation rates measured in equivalent NaCl solutions seems justified.

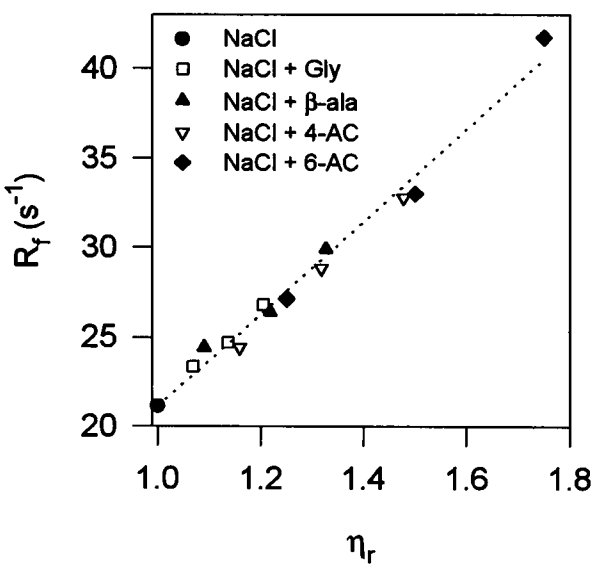


FIGURE 7 Modification of the free sodium relaxation rates $R_f$ measured in NaCl (8 mM) solutions at 20°C as a function of the relative viscosity $\eta_r$ of the aminocarboxylic solutions (calculated according to data of Devine and Lowe, 1971).

## Effect of zwitterionic compounds on DNA

The effects of glycine, β-alanine, 4-aminobutyric acid, and 6-aminocaproic acid on the fraction of DNA phosphate charges neutralized are clearly correlated to the ability of these zwitterionic compounds to increase the medium dielectric constant (Fig. 6). In an earlier study (Flock et al., 1996) we correlated this increase in dielectric constant with the protective effect of these compounds on the DNA precipitation induced by spermine$^{4+}$. The conclusion is thus evident: By increasing the medium's dielectric constant in DNA solutions, these zwitterionic compounds increase the macromolecule charge, which thus becomes more difficult to aggregate. In addition, the experimental dependence of $\theta$ on $\epsilon_r$ is in relatively good agreement with the theoretical relationship of condensation theory (Fig. 6 and Eq. 5).

However, according to Fig. 6, we could think that the disagreement between experiments and theory is the result of a systematic deviation. A similar experiment conducted at 27°C shows an identical departure from theory and leads to the same conclusion (data not shown). A slight modification of the distance between base pairs of DNA (this parameter appears in the charge density parameter $\xi$ and thus in the fraction of phosphate charge that is neutralized), of the relaxation rate of sodium ions bound to DNA with the dielectric constant of solutions, or of both, could explain this systematic deviation. The slight modifications of the intensity of the CD band at 280 nm (Fig. 8) indicate a small variation of the DNA structure in the presence of aminocarboxylic acids and thus partially validate this hypothesis.

The divergence between theory and experiment thus raises some interesting questions. However, to answer them, some complementary experiments would be necessary. These are, for example, the precise determination of $R_b$ and $p_b$ values by $^{23}$Na NMR experiments and the experimental detection of an eventual slight modification of distance between DNA base pairs when the dielectric constant is modified. On the other hand, although counterion condensation theory seems to give a good qualitative prediction of the DNA behavior in solution, it would be illusory to attempt to describe quantitatively the behavior of this rather complex macromolecule by this rather "simple" model. In the same way, identifying the bound state in the NMR two-state model with the condensed state of counterion condensation theory can be seen as a rough approximation. Work is also in progress to permit a comparison between experimental data and predictions from more-elaborate theories such as Poisson–Boltzmann and Monte Carlo calculations.

## Titration by $Hex^{2+}$

We have compared (Fig. 9) the values of $\theta$ listed in Table 2 for titration of a Na–DNA solution by $Hex^{2+}$ with the

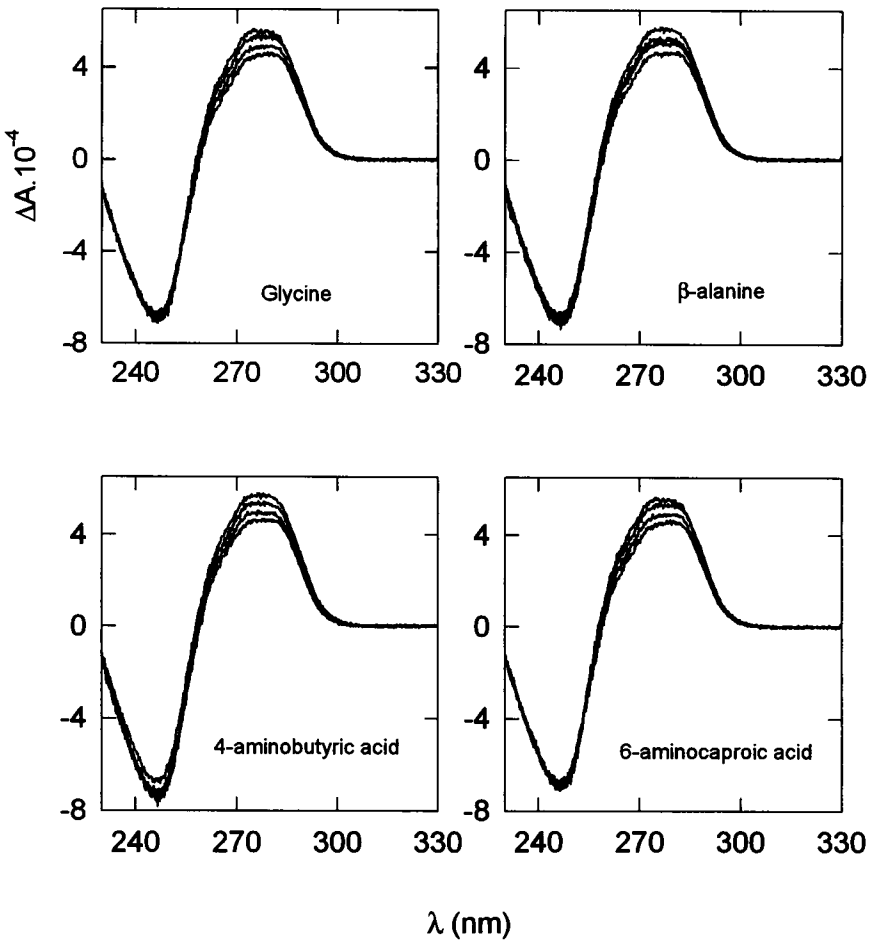


FIGURE 8 Circular dichroism signals of DNA solutions (1 mM, with a cell path length of 2 mm) in the absence and presence of aminocarboxylic acids at concentrations of 0.5, 1, and 1.5 M (*top to bottom*), at 280 nm.

prediction of counterion condensation theory calculated according to the following equations (Manning, 1978):

$$\ln\left(\frac{\theta}{V(C_1 - \theta C_p)}\right) + 1 = -2(1 - \theta - N\theta_N)\xi \ln(1 - e^{-\kappa b}), \tag{7}$$

$$\ln\left(\frac{\theta_N}{V(C_N - \theta_N C_p)}\right) + 1 = -2N(1 - \theta - N\theta_N)\xi \ln(1 - e^{-\kappa b}), \tag{8}$$

where $\theta_N$ is the number $M^{N+}$ of ions bound per DNA phosphate charge and $C_N$ is their total concentration.

Once again, theory fails in the quantitative prediction of $\theta$ values. Indeed, the amount of sodium ions remaining in the DNA condensation layer at the end of the titration is much more important than predicted by theory for $N = 2$.

It must be kept in mind that the theoretical formalism developed by Manning describes the binding of counterions on a polyelectrolyte chain as delocalized nonspecific binding of punctual charges. $Hex^{2+}$ is a rather long chain with functional groups that are spatially dispersed and cannot be considered punctual. In a previous study with spermidine$^{3+}$ and spermine$^{4+}$ we proposed replacing the punctual charge by a lower effective charge resulting from the spatial dispersion of the charge on these molecules (Flock et al., 1995). We thus show in Fig. 9 the theoretical curve obtained by taking a lower charge ($N = 1.47$) for this cation. In this case, the fraction of phosphate charges neutralized by sodium ions at the end of the titration ($[Hex^{2+}]/[Na^+] > 0.7$) is correctly predicted, but the remainder of the curves where the ability of $Hex^{2+}$ to replace $Na^+$ is underestimated is not. This would seem to indicate that titration of NaDNA solution by $Hex^{2+}$ cannot be quantitatively described by counterion condensation theory, except if the $Hex^{2+}$ charge is decreased during the titration. This is clearly apparent in Fig. 9, where experimental data are illustrated by the two theoretical curves obtained with $N = 2$ and $N = 1.47$.

In conclusion, counterion condensation theory is in qualitative agreement with the experimental results obtained. The decrease in the amount of sodium ions in the vicinity of DNA when the dielectric constant increases and when DNA is titrated by a divalent cationic compound (analog of a polyamine) is correctly described by theory. However, some departures are apparent when quantitative comparisons are attempted. Padmanabhan et al. (1988, 1991) have proposed

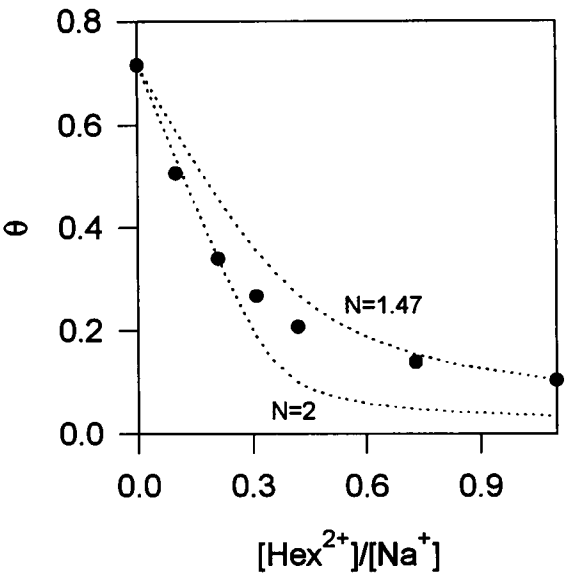


FIGURE 9 Fraction $\theta$ of phosphate charges neutralized by sodium counterions in DNA (8.5 mM) at 20°C during titration by $Hex^{2+}$. The experimental points are calculated from the NMR results. Curves are predictions of counterion condensation theory with $N = 1.47$ and $N = 2$ (Eqs. 8 and 9).

another model to analyze such experimental titrations. Ion exchange in the vicinity of DNA is described by two parameters $D$ and $n$, given by

$$D = (p_b^{Hex})(p_f^{Na})^n/(p_f^{Hex})(p_b^{Na})^n, \quad (9)$$

$$n = (\theta_0 C_p - p_b^{Na}[Na^+])/p_b^{Hex}[Hex^{2+}], \quad (10)$$

where $p_b^{Hex}$ is the fraction of $Hex^{2+}$ ions that are associated with DNA ($p_b^{Hex} = 1 - p_f^{Hex}$) and $\theta_0$ is the number of $Na^+$ ions bound per DNA phosphate in the absence of the competing $Hex^{2+}$ ion. According to these definitions, $D$ characterizes the relative distribution of $Hex^{2+}$ and $Na^+$ between the bound and the free states and $n$ is the number of $Na^+$ ions displaced by $Hex^{2+}$ at any point in the titration. These two parameters are assumed to be invariant, and a value of $n < 2$ indicates that the total number of cationic charges near DNA increases during the titration of NaDNA with $Hex^{2+}$. By fitting the NMR experimental results, it is thus possible to determine $D$ and $n$. We show in Table 3 the $D$ and $n$ values obtained for different value of $\theta_0$. The parameter $D$ obtained from DNA titration by $Hex^{2+}$ is approximately three times less important than values reported by Padmanabhan et al. (1991). This could probably be explained by the differences in DNA and sodium concentrations between the two experiments. Values of $n$ obtained in these conditions are also largely different from those reported by Padmanabhan et al. (1991). In our case $n$ was most often inferior to 2, the "Coulombic" limit.

For a given value of $\theta_0$, addition of aminocarboxylic acids seems to have no systematic effect on the $n$ parameter. On the contrary, the ion-exchange parameter $D$ appears to decrease in the presence of these compounds. Except in the presence of 6-aminocaproic acid, this effect is small. These values of $D$ and $n$ are clearly dependent on the choice of $\theta_0$, and an increase in this parameter generally induces an enhancement of $D$ and $n$. As the fraction of phosphate charges neutralized by sodium nuclei before titration has been shown to decrease in the presence of aminocarboxylic acids, we also show in Table 3 the $D$ and $n$ values obtained with $\theta_0$ given by experiments. The correlation between $D$ and the length of the zwitterionic compound added is now more evident (and outside the uncertainties in the fit). This would indicate a decrease in the $Hex^{2+}$ affinity for DNA as the medium's dielectric constant increases. Parameter $n$ is less influenced by these compounds. As it is always inferior to 2, the total amount of cationic charge near DNA increases

**TABLE 3 Results of fitting of NMR experimental data obtained by titration of different DNA solutions with $Hex^{2+}$ according to the model of Padmanabhan et al. (1991) (Eqs. 3, 9, and 10)**

| $\theta_0$ | DNA | DNA + Gly | DNA + β-ala | DNA + 4-AB | DNA + 6-AC |
|---|---|---|---|---|---|
| Best fitted values of $D$ for various fixed values of $\theta_0$ | | | | | |
| 0.4 | 17 ± 1 | 18 ± 2 | 18.5 ± 2.5 | 15.1 ± 1.5 | 10.7 ± 2.4 |
| 0.6 | 21 ± 1.5 | 21 ± 2 | 20 ± 2 | 18 ± 2 | 11.0 ± 1.8 |
| 0.8 | 24 ± 2 | 23 ± 2 | 19.3 ± 1.5 | 19 ± 2 | 10.3 ± 1 |
| Best fitted values of $n$ for various fixed values of $\theta_0$ | | | | | |
| 0.4 | 1.48 ± 0.04 | 1.6 ± 0.1 | 1.71 ± 0.09 | 1.52 ± 0.06 | 1.6 ± 0.1 |
| 0.6 | 1.70 ± 0.05 | 1.84 ± 0.08 | 1.95 ± 0.08 | 1.7 ± 0.1 | 1.9 ± 0.1 |
| 0.8 | 1.93 ± 0.08 | 2.06 ± 0.09 | 2.17 ± 0.09 | 1.9 ± 0.1 | 2.1 ± 0.1 |

Best values of $D$ and $n$ for values of $\theta_0$ calculated from experimental data in the absence of $Hex^{2+}$

| | $\theta_0$ | $D$ | $n$ |
|---|---|---|---|
| DNA | 0.716 | 22.9 ± 1.7 | 1.84 ± 0.07 |
| DNA + Gly | 0.586 | 21 ± 2 | 1.82 ± 0.08 |
| DNA + β-ala | 0.553 | 19.6 ± 1.6 | 1.90 ± 0.06 |
| DNA + 4-AB | 0.527 | 16.8 ± 1.8 | 1.66 ± 0.09 |
| DNA + 6-AC | 0.461 | 11 ± 3 | 1.7 ± 0.2 |

during the titration by $Hex^{2+}$, in agreement with counterion condensation theory.

However, in this model $D$ and $n$ are assumed to remain constant as the binding densities of $Hex^{2+}$ and $Na^{+}$ vary. At present there is no independent experimental or theoretical evidence to support this hypothesis, and the values of $D$ and $n$ given in Table 3 must be considered with caution. Nevertheless, these results are consistent with the inferences that the electrostatic strength is responsible for DNA–polyamine interactions and that the protection effect of aminocarboxylic acids is due to the ability of the latter compounds to increase the medium dielectric constant. Indeed, an increase in the dielectric constant results in a decrease in the electrostatic interactions and thus in a decrease in the affinity of $Hex^{2+}$ for DNA.

### Effect of polyols on DNA

The increase of the $^{23}Na$ line width in the presence of sorbitol and mannitol (Fig. 3) cannot be attributed simply to a modification in the amount of sodium ions bound to DNA. Calculations based on the relaxation rates measured at 5°C (Fig. 4) show clearly that one cannot formulate an explanation for this behavior without changing the relaxation rate of sodium ions bound to DNA. No rigorous analysis appears possible in the conditions of this experimental work without a precise determination of $p_b$ by an independent technique.

According to some experiments on DNA precipitation induced by $spermine^{4+}$, sorbitol has very little effect on this transition (data not shown). This could mean that the protection effect of these polyols evidenced by Buche et al. (1990) in chromatin precipitation experiments cannot be explained on the same basis as DNA precipitation experiments and that the origin of this protection effect probably must be searched at the level of histones.

## CONCLUSION

We have clearly shown that glycine, taurine, sorbitol, and mannitol do not have the same effects on DNA, although all have been shown to prevent chromatin precipitation at high salt concentrations.

Polyols increase the relaxation rate of sodium ions in the vicinity of DNA. As the dynamic of water molecules is one of the most likely candidates to produce the fluctuating electric field gradients that are responsible for this relaxation, such modification of the relaxation rate of sodium ions bound to DNA could result from perturbation of this water dynamic by polyols. It could also result from a modification of the primary or the secondary hydration shell of these sodium ions.

However, amino acids seem to have very little effect on this relaxation rate. Indeed, NMR data obtained in the presence of these compounds can easily be explained by a decrease in the amount of sodium ions in the vicinity of the macromolecule. According to experiments conducted with glycine, β-alanine, 4-aminobutyric acid, and 6-aminocaproic acid, this sodium ejection from the DNA condensation layer is the result of an increase in a medium's dielectric constant following the addition of these zwitterionic compounds to the solution. This is in accord with previous experiments that showed that the protection effect of these compounds, evidenced in DNA precipitation experiments induced by $spermine^{4+}$, is correlated with the ability of these zwitterionic compounds to increase the medium dielectric constant (Flock et al., 1996). As interactions between DNA and cationic compounds are due mainly to electrostatic forces, this increase in dielectric constant results in a decrease of these interactions. Reaching a sufficient level of phosphate charges neutralization to produce DNA precipitation thus requires a greater amount of cationic compound. This also explains the effects of glycine and taurine in the chromatin precipitation experiments of Buche et al. (1989, 1990, 1993) and probably their presence in the euryhaline cells that are able to support osmotic stress.

Counterion condensation theory gives a good qualitative prediction of this DNA behavior in the presence of these different compounds but fails when quantitative comparisons with experimental data are attempted. Better agreement could be achieved by a slight modification of the relaxation rate of bound sodium or of the distance between DNA base pairs, with the dielectric constant. On the other hand, we cannot exclude the possibility that this theory may be too simple to give accurate prediction of the complex behavior of this macromolecule in solution. Some experiments and comparisons with other theories are in progress in this regard.

The exact role played by polyols and some sugarlike trehalose in resistance of euryhaline cells to osmotic stress must still be found, and experiments to solve this problem are also planned.

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