# NUCLEAR MAGNETIC RESONANCE OF $^{23}$NA IONS INTERACTING WITH THE GRAMICIDIN CHANNEL

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ABSTRACT Basic nuclear magnetic resonance (NMR) features of $^{23}$Na ions bound to the gramicidin channel (packaged into lecithin liposomes) were studied. The first binding constant $K_1$ of $Na^+$ was not significantly dependent on channel models employed. With the two-identical-site model (Model I), $K_1$ was 13.7 (±1.4) molal$^{-1}$ (in the activity basis) at 25°C; when the binding of a third ion was included (Model II), it was 13.0 (±2.0) molal$^{-1}$. The second binding constant $K_2$ was model dependent; it was 1.6 (±0.2) and 3–4 molal$^{-1}$ for Models I and II, respectively. The rate constants, $k_{-1}$ and $k_{-2}$, of $Na^+$ for exit from singly and doubly loaded channels, respectively, were $8 \times 10^5\ s^{-1} \lesssim k_{-1} \lesssim 3 \times 10^6\ s^{-1}$ and $8 \times 10^5\ s^{-1} \lesssim k_{-2} \lesssim 1.0 \times 10^7\ s^{-1}$ at 25°C; the lower bound represents a rough approximation of $k_{-1}$. The ratio $k_{-2}/k_{-1}$ was greater than one and did not greatly exceed 20. From the competition experiment, $K_1$ of $Tl^+$ was 5.7 (±0.6) × $10^2$ molal$^{-1}$. The longitudinal relaxation time $T_1$ of bound $^{23}$Na in the state of single occupancy ($T_{1B}^{sing}$) was virtually independent of models, 0.56 (±0.03) and 0.55 (±0.04) ms at 25°C for Models I and II, respectively. For the state of double occupancy, $T_1$ of bound $^{23}$Na ($T_{1B}^{doub}$) was model dependent: 0.27 (±0.01) and 0.4–0.6 ms for Models I and II. The correlation time $\tau_c$ of bound $^{23}$Na was 2.2 (±0.2) ns at 25°C for single occupancy; $\tau_c$ for double occupancy was not significantly different from this value. The estimated $\tau_c$ was found to involve no appreciable contribution of the exchange of $^{23}$Na between the channel and the bulk solution. The quadrupole coupling constant $\chi$ was 1.0 (±0.1) MHz for $^{23}$Na in single occupancy; $\chi$ for double occupancy was 0.9–1.4 MHz, depending on models. A lower bound of the average quadrupole coupling constant $\chi_\alpha$ was 0.13–0.26 MHz at 25°C for $^{23}$Na in single occupancy; this value represents a rough approximation of $\chi_\alpha$ at this temperature. An argument based on the estimated $\chi_\alpha$ and the known conformation of the gramicidin channel suggests that the binding site is a small domain near the channel end. Within the framework of Model I, $T_{1B}^{doub}$ was faster than $T_{1B}^{sing}$; this inequality was attributed to an increased $\chi$ in the presence of a second cation, which was not explained in terms of electrostatic interactions between bound cations, implying a conformation change upon binding of cations.

## INTRODUCTION

The nonionic pentadecapeptide gramicidin (Dubos, 1939*a*,*b*; Hotchkiss and Dubos, 1941) forms transmembrane channels permeable to monovalent cations (Hladky and Haydon, 1970; Krasne et al., 1971) and water molecules (Finkelstein, 1974; Levitt et al., 1978; Rosenberg and Finkelstein, 1978*a*,*b*) when incorporated into lipid bilayer membranes. The molecular structure responsible for the permeation of cations and waters is known to be the head-to-head dimer of two $\pi^6$(L,D)-helical monomers (e.g., Urry et al., 1971; Bamberg et al., 1977; Veatch and Stryer, 1977; Weinstein et al., 1979, 1980). The gramicidin channel exhibits several interesting transport characteristics that are shared by ion channels of biological membranes (e.g., Hladky and Haydon, 1972; Myers and Haydon, 1972; Neher, 1975; Schagina et al., 1978; Eisenman et al., 1978*a*,*b*; Neher et al., 1978).

The binding constant of a cation for the gramicidin channel reported by different authors shows a considerable variation. Reported values of the first binding constant $K_1$ of $Na^+$, for example, vary over three orders of magnitude (Table I). Estimates for the ratio of the first to the second binding constant ($K_1/K_2$) of $Na^+$ range from 8 (Levitt, 1978*b*) to 600 (Urban et al., 1980). According to Finkelstein and Andersen (1981), the gramicidin channel never contains more than one $Na^+$ ion at all attainable concentrations of NaCl. The rate constant of a cation evaluated by different authors also shows a remarkable variation (see Discussion). Such variability of experimental binding and rate constants is chiefly due to different theoretical models that are used to analyze experimental results and different weights that are given to different types of experiments. The variability may partly reflect the use of different lipids to form bilayer membranes (Bamberg et al., 1976; Kolb and Bamberg, 1977; Fröhlich, 1979; Rudnev et al., 1981).

The shape of the conductance-voltage ($G$-$V$) characteristic of the single channel of gramicidin is known to be sublinear at low ionic concentrations and supralinear at high ionic concentrations (Hladky and Haydon, 1972; Hägglund et al., 1979; Eisenman et al., 1980; Andersen and Procopio, 1980). The sublinear $G$-$V$ relation is interpreted to indicate that, at low ionic concentrations, the

TABLE I
ESTIMATED BINDING CONSTANTS OF $Na^+$ FOR THE GRAMICIDIN CHANNEL

| $K_1$ | $K_2$ | $K_1/K_2$ | Model: Site | Model: Maximum occupancy | Membrane | Experiment | Reference |
|---|---|---|---|---|---|---|---|
| 3.3* | — | — | One site | 1 | GMO** | Electrical | Läuger (1973) |
| 3.1§ | — | — | One site | 1 | GMO | Electrical | Neher (1975) |
| 10* | 10 (1.0)*,‖ | 1 (10¶) | Four sites (First two sites are identical and noninteracting.) | 4 | GMO | Electrical | Eisenman et al. (1978) |
| 1.4* | 0.18* | 8 | Two identical sites | 2 | GMO | Electrical | Levitt (1978*b*) |
| 120‡ | 0.20‡ | 600 | Two identical sites | 2 | GMO | Electrical | Urban et al. (1980) |
| 3.2* | — | — | One site | 1 | PE‡‡ | Electrical | Finkelstein and Andersen (1981) |
| ≤30§ | — | — | Two identical sites | — | PC§§ | Equilibrium dialysis | Veatch and Durkin (1980) |
| 100§ | 3§ | 33 | Two identical sites | 2 | LysoPC‖ | $^{23}Na$ NMR | Urry et al. (1980) |
| 300§ | 1§ | 300 | Three sites (Last two sites are identical and noninteracting.) | 3 | LysoPC‖ | $^{23}Na$ NMR | Urry et al. (1980) |
| 14‡ | 1.6‡ | 8 | Two identical sites | 2 | PC | $^{23}Na$ NMR | This study |
| 13‡ | 3–4‡ | 3–5 | Two identical sites, including the binding of an additional ion | 3¶¶ | PC | $^{23}Na$ NMR | This study |

*In $M^{-1}$ (activity basis).
‡In $molal^{-1}$ (activity basis.)
§In $M^{-1}$ (concentration basis).
‖$K_1 = K_2$ is postulated; the numeral in the parentheses refers to $K_3$.
¶$K_1/K_3$.
**Glyceryl monooleate black membrane.
‡‡Phosphatidylethanolamine black membrane.
§§Phosphatidylcholine liposomes.
‖Lysophosphatidylcholine micelles.
¶¶Three cations or (two cations + one anion).

major barrier to ion movement through the channel is the entry of ions into, and/or exit from, the channel (Läuger, 1973; Urban and Hladky, 1979; Hladky et al., 1979; Finkelstein and Andersen, 1981). Recent models for ion permeation through the gramicidin channel thus assume that cations are not necessarily in equilibrium distribution between the bulk phase and each end of the channel (when there is a net flux of ions through the channel). The substantial fraction of the variance of reported values of binding and rate constants arises in connection with this assumption, under which binding and rate constants have no longer a simple relationship with the rate of ionic passage through the channel. To estimate these parameters, it would thus be preferable to use samples in which (*a*) an equilibrium distribution of ions is attained even at low ionic activities, and (*b*) the transmembrane potential is negligibly low.

One of the purposes of the present study is to estimate the binding and rate constants of $Na^+$ for the gramicidin channel. Interactions between $Na^+$ and the channel were examined by $^{23}Na$ NMR spectroscopy. Samples used are suspensions of lecithin liposomes that are doped with gramicidin. In preparing samples, care was taken that conditions (*a*) and (*b*) mentioned above were satisfied. Some fraction of the variance of experimental values of binding and rate constants may have appeared in association with procedures for fitting curves to data involving so many parameters that they cannot necessarily be determined uniquely. The process of the estimation of parameters is thus described in some details (see the Results section entitled Binding Constant and Longitudinal Relaxation Time of Bound $^{23}Na$).

Cornélis and Laszlo (1979) and Monoi and Uedaira (1979) working with gramicidin in ethanol-water mixtures and aqueous emulsions, respectively, found that gramicidin markedly accelerates the nuclear spin relaxation of $^{23}Na$ ions. In spite of a more detailed study of Urry et al. (1980) and Venkatachalam and Urry (1980), basic NMR characteristics of $^{23}Na$ ions bound to the gramicidin channel remain to be investigated. In this work, emphasis is also placed on estimating fundamental NMR parameters for bound $^{23}Na$ and on elucidating implications of these parameters for the transport properties of the channel. In suspensions of gramicidin-doped liposomes, $^{23}Na$ nuclei bound to gramicidin experience nonzero-averaging fluctuations of electric field gradients, as will be shown later. Basic principles of quadrupole relaxation involving nonzero-averaging interactions, upon which the present investigation is founded, are described elsewhere (Monoi, 1985).

## MATERIALS AND METHODS

### Gramicidin-doped Liposomes

Weighed amounts of gramicidin (a mixture of gramicidins A, B, and C; ICN Nutritional Biochemicals, Cleveland, OH) and egg-yolk L-$\alpha$-lecithin (Sigma Chemical Co., St. Louis, MO; type V-E or V-EA) were dissolved in 2,2,2-trifluoroethanol. After the solution was allowed to stand at room temperature for ~20 h under $N_2$ gas, the solvent was evaporated. An aqueous NaCl solution containing 10 mM HEPES-NaOH buffer was added to the residue unless otherwise stated. The sample was sonicated and maintained at 65°C for ~20 h (Masotti et al., 1980). The sample was again sonicated and was served for NMR experiment without further alteration in composition. A 20-h incubation at 65°C was sufficient to cause maximal shortening of the relaxation time of $^{23}Na$ in the sample. The concentrations of gramicidin and egg-yolk lecithin were normally 0.25–1.0 g and 2.5–10 g per 100 g $H_2O$. Unless otherwise noted, the gramicidin/lecithin ratio was 1:10 by weight and the pH was 6.5–7.2.

### Pulse NMR Spectroscopy

NMR spectrometers JEOL FSE60A (15.80 MHz), Varian XL-200 (52.92 MHz), and Nicolet NT-300 and Bruker CXP-300 (79.37 MHz) were used. Longitudinal and transverse relaxation times $T_1$ and $T_2$ were determined from conventional 180°-$\tau$-90° and 90°-$\tau$-180° pulse sequences, respectively. The 90° pulses were 12–24 $\mu$s in duration.

For an assembly of like spins or of spins that are rapidly exchanging between different states, the longitudinal relaxation following a $\theta$-degree pulse is expressed as a sum of two exponentials when the spin quantum number is 3/2 (Hubbard, 1970; Rubinstein et al., 1971; Bull, 1972):

$$\langle I_z\rangle - \langle I_z\rangle_0 = \langle I_z\rangle_0(\cos\theta - 1) [0.8\exp(-t/T_1') + 0.2\exp(-t/T_1'')], \quad (1)$$

where $I_z$ is the component of spin angular momentum $I$ along the direction of the applied static magnetic field $H_0$, and $\langle I_z\rangle_0$ is the thermal equilibrium value of $\langle I_z\rangle$, and $T_1'$ and $T_1''$ are the slow and the fast $T_1$, respectively.

On the basis of Eq. 1, we may define $T_1^p$, or pooled $T_1$, as

$$1/T_1^p \equiv 0.8/T_1' + 0.2/T_1''; \quad (2)$$

when $T_1' \approx T_1''$, the decay curve can be approximated by a single exponential whose time constant is equal to $T_1^p$. Similarly, the pooled $T_1$ of bound $^{23}Na$, $T_{1B}^p$, may be defined by

$$1/T_{1B}^p \equiv 0.8/T_{1B}' + 0.2/T_{1B}'', \quad (3)$$

where $T_{1B}'$ and $T_{1B}''$ are the slow and the fast $T_1$ of bound $^{23}Na$.

The longitudinal relaxation of $^{23}Na$ in liposomal samples doped with gramicidin was biexponential at high radio frequencies (see Fig. 2). However, the ratio of $T_1'$ to $T_1''$ was not large; it was 1.9, at most, at 79.4 MHz. If appropriate sets of $\tau$ values were adopted in inversion-recovery experiments, the decay was approximated by an exponential regression line whose time constant agreed with $T_1^p$ within an error of 1%. In what follows, $T_1^p$ and $T_{1B}^p$ are simply denoted by $T_1$ and $T_{1B}$, respectively, when no confusion will arise.

### Continuous-Wave Wide-Line NMR Spectroscopy

The peak-to-peak (pp) height of the wide-line signal (derivative-of-absorption mode) of $^{23}Na$ was determined at 24–25°C with a Varian V-4200B wide-line NMR spectrometer (with a Varian V-3606 electromagnet; Varian Associates, Palo Alto, CA) operating at a radio frequency of 11.262 MHz. The instrumental conditions employed were the same as reported before (Monoi and Uedaira, 1980). The pp height is expressed in percentage of that for a NaCl solution containing the same amount of $Na^+$ ions per volume. Signal heights from 10 or more succesive upward and downward sweeps were averaged.

Within the framework of the instrumental conditions adopted and the nature of the $^{23}Na$ lines of the samples (see the Results section entitled Basic Features of the Resonance Line and Spin Relaxation), a single Lorentzian line is expected to show a pp height of ~100%, and loss in the signal height implies that the signal consists of narrow and broad components; when the broad component is sufficiently broader, the pp height is approximately equal to, or slightly less than, 40% (see Appendix).

### Curve Fitting

Curves were fitted to the data given in Fig. 5 by the least squares according to the procedure described by Deming (1943). The weight of a single observation for the reciprocal of the excess longitudinal relaxation rate $\Delta R_1$ ($\equiv 1/T_1 - 1/T_{1F}$; $T_{1F}$ is $T_1$ in the absence of gramicidin) was assumed to be proportional to the square of $\Delta R_1$. Unless otherwise noted, the numeral after a ± sign represents the estimated standard error (by external consistency) of an estimated adjustable parameter.

### Electrostatic Calculation

Electrostatic field gradients due to image forces were calculated by use of essentially the same procedure as reported previously (Monoi, 1983). The gramicidin channel was supposed to be a cylindrical pore with a length of 26 Å (Urry et al., 1971; Koeppe et al., 1979). Local interactions between bound cations and the channel wall were not included. The antishielding effect of the electronic shell of a bound cation was taken to be the same irrespective of the state of ion loading of the channel. Calculation was performed for an effective pore radius of 2–4 Å, an effective membrane thickness of 26–36 Å, and binding sites 1–3.5 Å from the pore ends with dimples of 0–5 Å in depth.

## RESULTS

### Basic Features of the Resonance Line and Spin Relaxation

The samples examined in this study are suspensions of gramicidin-doped lecithin liposomes. As illustrated in Fig. 1, the absorption line of $^{23}Na$ ions in this system consisted of a narrow and a broad component, which comprised ~40 and 60%, respectively, of the total resonance intensity. The dispersion-vs.-absorption plot (Marshall et al., 1982) indicated that the frequency shift between the two components was small if any. (In the absence of gramicidin, suspensions of lecithin liposomes showed single Lorentzian lines and exponential relaxations, and relaxation times were virtually frequency independent and slightly shorter than those of NaCl solutions. $T_2 \simeq T_1 = 51.6$ [±0.4 SE] ms at 25°C [79.4 MHz] for 100 g lecithin and 100 mmol $Na^+$/kg $H_2O$.) The transverse relaxation was approximated by a sum of two exponentials. The reciprocal half-width of the narrow component of the resonance line agreed, within experimental errors, with the slow $T_2$ value. Excess longitudinal relaxation rate was proportional to the concentration of gramicidin when the concentration of $Na^+$ was sufficiently high as compared with that of gramicidin. These characteristics of the $^{23}Na$ resonance are similar to those reported for gramicidin-doped emulsions of nonionic surfactant and higher alcohol in saline (Monoi and Uedaira, 1979).

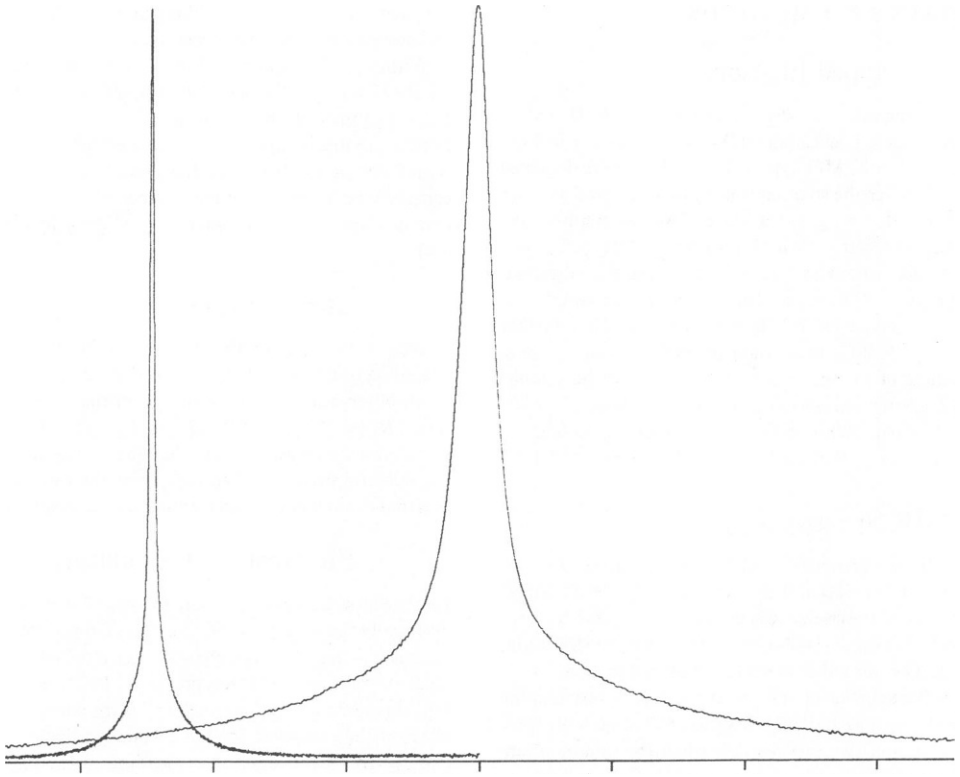

FIGURE 1 Absorption line of $^{23}Na$ ions in a suspension of gramicidin-doped liposomes. The sample contains 1 mol $Na^+$, 1 g gramicidin, and 10 g egg lecithin/kg $H_2O$. 400 transients were collected at a radio frequency of 79.4 MHz (25°C). Curve *a* shows the whole spectrum; its central portion is enlarged in *b*. Scale markers on the abscissa are at intervals of 350 (*a*) and 50 (*b*) Hz. This absorption line is approximated by a narrow and a broad Lorentzian components with relative areas of 0.38:0.62. The reciprocal of the half-width at half-height of the broad component is 2.5 ms, which is not significantly different from the fast $T_2$ value (2.7 ms) determined by the spin-echo method, implying a super-Lorentzian nature of this line.

For high $Na^+$ levels (e.g., 1 mol/kg $H_2O$ or higher), the reciprocal half-width of the broad component was equal to the fast $T_2$ value (see Fig. 1). For low $Na^+$ levels (e.g., 100 mmol/kg $H_2O$ or lower), the case was true only when the temperature was well above room temperature; at lower temperatures (e.g., 5–10°C), the reciprocal half-width of the broad component was shorter than the fast $T_2$, thus the broad component possessing a distribution of Larmor precessional frequencies (Monoi, 1985).

As evident from the above results, the narrow and broad components of the absorption line correspond to different transverse components of the magnetization of the spins, and, for high $Na^+$ levels (as well as for low $Na^+$ levels at high temperatures), the line is a sum of two Lorentzian, each characterized by a single Larmor frequency, implying that the rate of exchange of $^{23}Na$ between free and bound states is sufficiently rapid. (Such a line will be referred to as a super-Lorentzian.) For low $Na^+$ levels at temperatures lower than room temperature, the broad component resembles a Lorentzian but shows a heterogeneous broadening (i.e., distribution of Larmor precessional frequencies), whereas the narrow component is still a true Lorentzian, implying that the exchange rate is sufficiently rapid with respect to the narrow component but not so with respect to the broad component. (Such a line will be called a pseudo-super-Lorentzian.) For low $Na^+$ levels at room temperature, the line was only approximately represented in terms of a super-Lorentzian.

The longitudinal relaxation of $^{23}Na$ in the present samples was exponential at a low level of radio frequency (15.8 MHz). At a higher radio frequency (79.4 MHz), the decay was nonexponential (Fig. 2). When it was approximated by a sum of two exponentials, the longer $T_1$ was found to comprise 77–81% of the total resonance intensity, in agreement with the theoretical expectation (80%) for the super- and pseudosuper-Lorentzians of spin-3/2 nuclei (see Eq. 1). Note that the rapid-exchange condition with respect to the longitudinal magnetization is fulfilled because the fast $T_1$ is expected to be of the order of the slow $T_2$.

## Effect of $Tl^+$

Fig. 3 shows the effect of $Tl^+$ ions on the pp height of the continuous-wave wide-line signal of $^{23}Na$ in the presence of liposome-packaged gramicidin. The pp height increased to 100% with the increase of the $Tl^+$ concentration.

Within the framework of the instrumental conditions employed and the nature of the $^{23}Na$ line of the present samples (super- and pseudosuper-Lorentzians), an

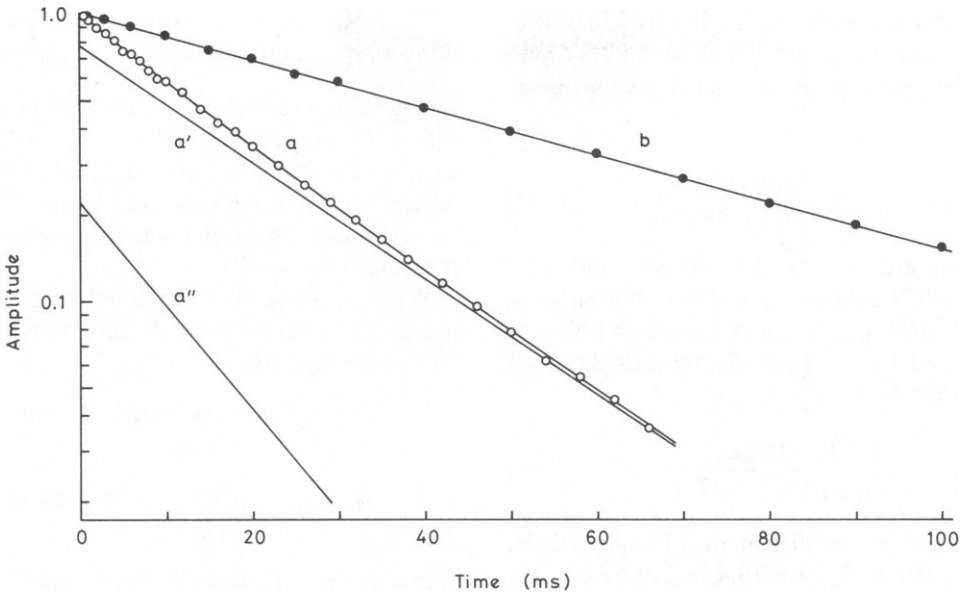


FIGURE 2 Nonexponential longitudinal relaxation of $^{23}Na$ in a suspension of gramicidin-doped liposomes. The sample contains 25 mmol $Na^+$ and 50 g egg lecithin/kg $H_2O$ in the presence (*a*) and the absence (*b*) of gramicidin (5 g/kg $H_2O$); 79.4 MHz, 25°C. Curve *a* is a biexponential regression line of the type: $A' \exp(-t/T_1') + A'' \exp(-t/T_1'')$; $T_1' = 21.5$ ms, $T_1'' = 12.0$ ms, and $A'/A'' = 0.78/0.22$. The lines *a'* and *a''* represent the slow and the fast component, respectively, of *a*. When the decay *a* was approximated by a simple exponential, the regression coefficient $T_1$ was 18.7 ms, which is not significantly different from $T_1^0$ ($1/T_1^0 = 0.8/T_1' + 0.2/T_1''$) = 18.6 ms.

increase in the fractional pp height signifies increased relaxation times and a decreased ratio $\rho^*$ of the linewidth of the broad component of the absorption line to that of the narrow (see Appendix). A pp height of 100% infers that the absorption line corresponds (approximately) to a Lorentzian ($\rho^* \simeq 1$). As shown in Fig. 4, the loss in the pp height had an inverse-sigmoidal relationship to the concentration of gramicidin and hence to the amount of bound $Na^+$ ions. When the plot given in Fig. 3 is corrected for this standard curve, the $Tl^+$ concentration, $(c_{Tl})_{1/2}$ that causes a half-maximum inhibition is found to be 3.1 (±0.2) mmol/kg $H_2O$.

Here we assume for the gramicidin channel two identi-

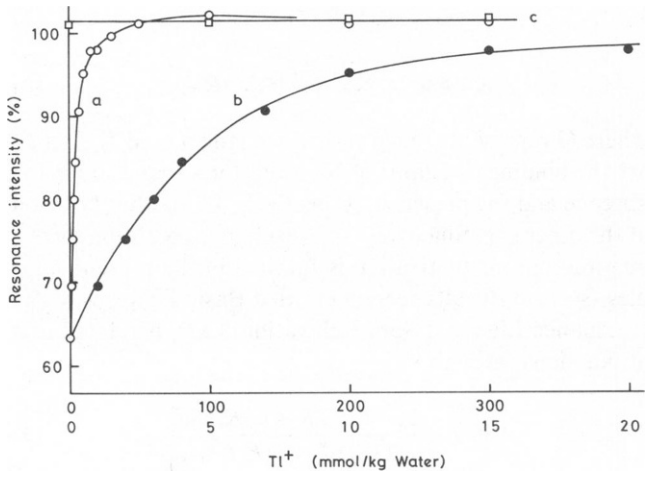


FIGURE 3 Effect of the $Tl^+$ concentration on the pp height of the wide-line signal of $^{23}Na$ in suspensions of gramicidin-doped liposomes. The samples are $NaNO_3$ (100 mmol/kg $H_2O$)—$TlNO_3$ (0–300 mmol/kg $H_2O$)—gramicidin (2 g/kg $H_2O$)—egg lecithin (20 g/kg $H_2O$). The ordinate is the pp height expressed in percentage of the height for a NaCl solution containing the same number of $Na^+$ ions per volume. Each point is the average of three samples. Curve *a* represents data for $Tl^+$ concentrations of 0–300 mmol/kg $H_2O$; its portion (0–20 mmol $Tl^+$/kg $H_2O$) is enlarged in *b*; *c*, no gramicidin (0–300 mmol $Tl^+$/kg $H_2O$).

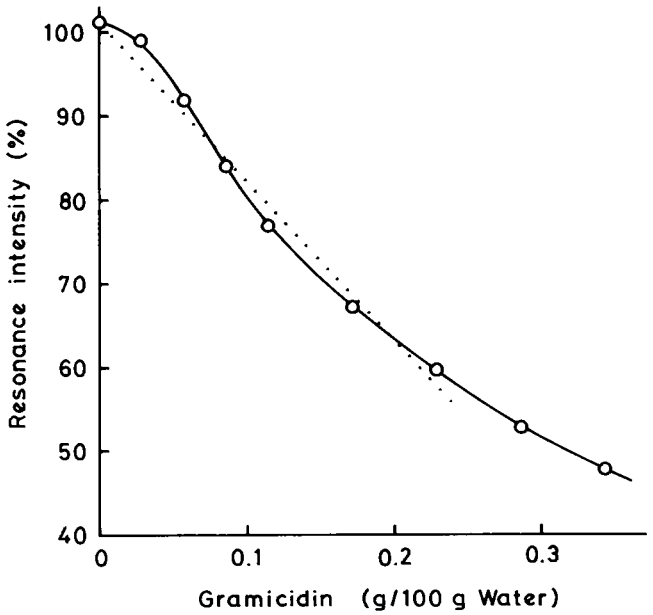


FIGURE 4 Effect of the concentration of gramicidin on the pp height of the wide-line signal of $^{23}Na$. The samples are $NaNO_3$ (100 mmol/kg $H_2O$)—gramicidin—egg lecithin; the gramicidin/lecithin ratio is 1:10 by weight. The ordinate is the same as in Fig. 3. Each point is the average of three samples.

cal binding sites interacting with each other (two-identical-site model).[1] Then, the concentration of bound $Na^+$ ions, $(c_{Na})_{bound}$, is (the binding of a second cation being neglected)

$$(c_{Na})_{bound} = \frac{2K_{Na}a_{Na}c_G}{1 + 2K_{Na}a_{Na} + 2K_{Tl}a_{Tl}}, \quad (4)$$

where $a_{Na}$ and $a_{Tl}$ are the activities of $Na^+$ and $Tl^+$, respectively, in the bulk solution, $c_G$ is the concentration of the gramicidin channel, and $K_{Na}$ and $K_{Tl}$ are the binding constants of $Na^+$ and $Tl^+$, respectively, for a single site of the channel. We then have

$$K_{Tl} = \frac{1}{2\gamma_{Tl}} \frac{1 + 2K_{Na}a_{Na}}{(c_{Tl})_{1/2} - c_G/2}, \quad (5)$$

where $\gamma_{Tl}$ is the activity coefficient of $Tl^+$. As will be shown in the next section, $K_{Na}$ was 13.4 (±2.0) $molal^{-1}$ (in the activity basis) for the binding of a first $Na^+$ ion at 25°C. Substituting $a_{Na}$ = 78 mmolal, $c_G$ = 0.53 mmol/kg $H_2O$, $(c_{Tl})_{1/2}$ = 3.1 (±0.2) mmol/kg $H_2O$, and $\gamma_{Tl}$ = 0.94, we get $K_{Tl}$ = 5.7 (±0.6) × $10^2$ $molal^{-1}$ for the binding of a first $Tl^+$ ion.

This value is consistent with the values of 500–1,000 $M^{-1}$ reported by Veatch and Durkin (1980) and 900 $M^{-1}$ by Hinton et al. (1982). The former value was estimated from an equilibrium dialysis experiment with gramicidin-doped lecithin vesicles. The latter was obtained from the NMR frequency shift of $^{205}Tl$ ions interacting with gramicidin-doped lysolecithin micelles by using a single-site model; this value decreases to 450 $M^{-1}$ when two identical sites are postulated.

The value of $K_{Tl}$ estimated from transport or kinetic properties shows a considerable variation (see Veatch and Durkin, 1980, and Dani and Levitt, 1981, which list recent estimates of $K_{Tl}$ for the gramicidin channel). Reported values of $K_{Tl}$ for the binding of a first $Tl^+$ ion range from 300 to 9,000 $M^{-1}$. Most are in the range 400–1,100 $M^{-1}$. Our value and others obtained from equilibrium experiments given above are within this range.

In lecithin liposomes, gramicidin is expected to be in nearly all the forms of head-to-head helical dimers, which is relevant to the functional channel conformation (Veatch et al., 1975; Veatch and Stryer, 1977; Weinstein et al., 1979, 1980; Masotti et al., 1980). The observation that the affinity of $Tl^+$ ions in the present samples is comparable to that obtained from kinetic properties in black lipid membranes is also consistent with the functional channel state of gramicidin.

[1]The term identical is used here to refer to a situation in which sites within a channel possess the same transport and NMR parameters except for the orientation of the principal axes of electric field (and average electric field) gradients, which is not necessarily the same.

## Binding Constant and Longitudinal Relaxation Time of Bound $^{23}Na$

In Fig. 5, the reciprocal of excess longitudinal relaxation rate $\Delta R_1$, weighted by the activity coefficient $\gamma$ of $Na^+$ ions, is plotted against the activity $a$ of $Na^+$ ions for two different levels of temperature (25 and 10°C). The curves are sublinear. This trend was less marked at higher temperatures (e.g., 45°C).

When straight lines are fitted to data for low (≤0.1 molal) and high (≥1 molal) activity ranges, their slopes and $x$-intercepts are

$$(\text{slope})_{\text{low } a} = 0.138\ (25°C) \text{ and } 0.140\ (10°C)\ \text{s/molal}, \quad (6a)$$

$$(\text{slope})_{\text{high } a} = 0.057\ (25°C) \text{ and } 0.041\ (10°C)\ \text{s/molal}, \quad (6b)$$

$$|(x\text{-intercept})_{\text{low } a}| = 0.062\ (25°C) \text{ and } 0.053\ (10°C)\ \text{molal}, \quad (6c)$$

$$|(x\text{-intercept})_{\text{high } a}| = 0.58\ (25°C) \text{ and } 1.07\ (10°C)\ \text{molal}. \quad (6d)$$

The above data will be analyzed in terms of two species of channel models.

*Analysis in Terms of the Two-Identical-Site Model (Model I).* Suppose two binding sites for cations in the gramicidin channel. On the basis of the symmetrical nature of the channel structure, the binding sites are assumed to be identical. We consider the binding of maximally two $Na^+$ ions:

$$G + Na^+ \overset{K_1}{\rightleftharpoons} G \cdot Na^+, \quad (7)$$

$$G \cdot Na^+ + Na^+ \overset{K_2}{\rightleftharpoons} G \cdot 2Na^+, \quad (8)$$

where G represents the gramicidin channel, and $K_1$ and $K_2$ are the binding constants of $Na^+$ ions for a single site in the absence and the presence, respectively, of another $Na^+$ ion at the other site. Since the rapid-exchange condition for the longitudinal magnetization is fulfilled in the present samples (see the Results section entitled Basic Features of the Resonance Line and Spin Relaxation), $\Delta R_1$ is related to $a$ of $Na^+$ ions through

$$\gamma \Delta R_1^{-1} = \frac{1}{c_G} \frac{1 + 2K_1a + K_1K_2a^2}{2K_1/t_{1B}^{sing} + 2K_1K_2a/t_{1B}^{doub}}, \quad (9)$$

$$1/t_{1B}^{sing} = 1/T_{1B}^{sing} - 1/T_{1F}, \quad 1/t_{1B}^{doub} = 1/T_{1B}^{doub} - 1/T_{1F}, \quad (10)$$

where $\gamma$ is the activity coefficient of $Na^+$ ions, $c_G$ is the concentration of the gramicidin channel ($c_G \ll a/\gamma$), $T_{1B}^{sing}$ and $T_{1B}^{doub}$ are the respective $T_1$ of bound $^{23}Na$ in the states of single and double occupancies. As shown later, $T_{1B}^{sing}$ and $T_{1B}^{doub}$ are far shorter than $T_{1F}$. We then put $t_{1B}^{sing} \simeq T_{1B}^{sing}$ and

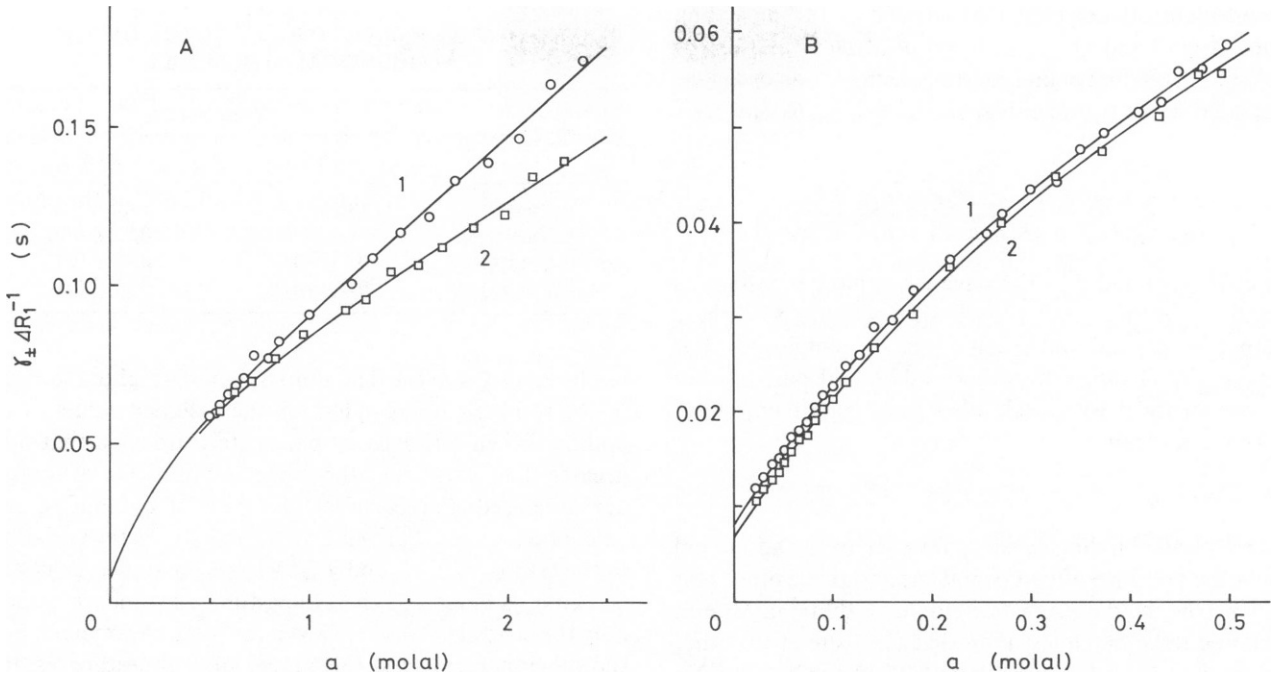


FIGURE 5 Dependence of the reciprocal of excess longitudinal relaxation rate $\Delta R_1$ on ionic activity $a$. The temperature is 25° (○) or 10°C (□), and the radio frequency, 79.4 MHz. Data for $a \geq 0.5$ and $> 0.5$ molal are plotted in *A* and *B*, respectively. Each point normally represents the average of two samples. Curves are regression lines according to Eq. 14. The regression lines according to Eq. 9 deviate slightly downwards at high levels of $a$. The value for $\Delta R_1^{-1}$ is expressed with reference to a gramicidin concentration of 1 g/100 g $H_2O$.

$t_{1B}^{doub} \simeq T_{1B}^{doub}$ beforehand. The mean activity coefficient $\gamma_\pm$ of NaCl will be used for $\gamma$ of $Na^+$ ions.

When $\gamma \Delta R_1^{-1}$ is plotted against $a$, the slopes and $x$-intercepts of the tangents in the limits $a \to 0$ and $a \to \infty$ are

$$(\text{slope})_{a\to 0} = \frac{T_{1B}^{sing}}{c_G}\left(1 - \frac{K_2 T_{1B}^{sing}}{2K_1 T_{1B}^{doub}}\right), \tag{11a}$$

$$(\text{slope})_{a\to \infty} = \frac{T_{1B}^{doub}}{2c_G}, \tag{11b}$$

$$(x\text{-intercept})_{a\to 0} = -\frac{1}{2K_1}\left(1 - \frac{K_2 T_{1B}^{sing}}{2K_1 T_{1B}^{doub}}\right)^{-1}, \tag{11c}$$

$$(x\text{-intercept})_{a\to \infty} = -\frac{2}{K_2}\left(1 - \frac{T_{1B}^{doub}}{2T_{1B}^{sing}}\right). \tag{11d}$$

From these relations, an upper bound of $1/K_1$ and a lower bound of $1/K_2$ are $-2(x\text{-intercept})_{a\to 0}$ and $-1/2(x\text{-intercept})_{a\to\infty}$, respectively; a lower bound of $T_{1B}^{sing}$ is $c_G(\text{slope})_{a\to 0}$.

Eq. 9 is supralinear or sublinear with respect to $a$ when

$$\frac{T_{1B}^{doub}}{T_{1B}^{sing}} + \frac{K_2 T_{1B}^{sing}}{K_1 T_{1B}^{doub}} > \text{or} < 2, \tag{12}$$

respectively. Sublinear curves demand that $K_1 > K_2$ and $2T_{1B}^{sing} > T_{1B}^{doub}$.

From Eqs. 6 and 11, these parameters will possess the values

$$K_1 \gtrsim 8.0\ (25°C) \text{ and } 9.5\ (10°C)\ \text{molal}^{-1}, \tag{13a}$$

$$K_2 \lesssim 3.4\ (25°C) \text{ and } 1.86\ (10°C)\ \text{molal}^{-1}, \tag{13b}$$

$$T_{1B}^{sing} \gtrsim 0.37\ (25°C) \text{ and } 0.37\ (10°C)\ \text{ms}, \tag{13c}$$

$$T_{1B}^{doub} \lesssim 0.30\ (25°C) \text{ and } 0.22\ (10°C)\ \text{ms}, \tag{13d}$$

where $T_{1B}^{doub}$ is expected to be only slightly less than the value given in the right-hand side of Eq. 13d.

Starting from initial guesses that satisfy Eq. 13, we get unique least-square estimates of the adjustable parameters, which are listed in Table II. The ratio $K_1/K_2$ was 8.6 (25°C) and 19 (10°C). Relaxation time $T_1$ of bound $^{23}Na$ was ~100 times or more shorter than $T_1$ of $^{23}Na$ ions in NaCl solutions, and $T_1$ for double occupancy was faster than $T_1$ for single occupancy by a factor of two or greater.

*Analysis in Terms of More Involved Models (Model II).* According to Sandblom et al. (1977) and Eisenman et al. (1980), the gramicidin channel may be occupiable by more than two cations. As suggested in previous works (Monoi, 1982, 1983), anions may form ion pairs with cations that have already been bound near the ends of gramicidin-like channels. It is hence interesting to see what estimates of parameters are obtained when the binding of additional ions (cations or anions) is included in

the two-identical-site model considered in the preceding section (Eqs. 7 and 8).

When the binding of one additional ion (cation or anion) is included in the two-identical-site model, $\gamma\Delta R_1^{-1}$ appears as

$$\gamma\Delta R_1^{-1} = \frac{1}{c_G}\frac{1 + 2K_1a + K_1K^{\dagger}a^2 + K_1K^{\dagger}K^{\ddagger}a^3}{2K_1/T_{1B}^{sing} + 2K_1K^{\dagger}a/T_1^{\dagger} + K_1K^{\dagger}K^{\ddagger}a^2/T_1^{\ddagger}}, \quad (14)$$

where $K^{\dagger}$ ($K^{\ddagger}$) and $T_1^{\dagger}$ ($T_1^{\ddagger}$) have the same dimensions as $K_1$ and $T_1$, respectively; the physical meanings of these parameters depend on specific models employed. For example, for channels having an additional pair of sites that are identical with each other (e.g., Sandblom et al., 1977), we may put

$$K^{\dagger} = K_2, \quad K^{\ddagger} = 2K_3, \quad T_1^{\dagger} = T_{1B}^{doub}, \quad T_1^{\ddagger} = T_{1B}^{tri}/3, \quad (15)$$

where $K_3$ is the binding constant for each of the additional sites in the presence of two bound cations at the other pair of sites (the binding constant for an additional site is postulated to be much less than that for a site of the other pair), and $1/T_{1B}^{tri}$ is the average of $1/T_1$ of the bound $^{23}$Na ions in the state of triple occupancy.

For two-identical-site channels involving the formation of an ion pair, we may write

$$K^{\dagger} = K_2, \quad K^{\ddagger} = 2K_a^{doub}, \quad T_1^{\dagger} = T_{1B}^{doub}, \quad T_1^{\ddagger} = T_{1B}^{an}/2, \quad (16)$$

where $K_a^{doub}$ is the binding constant of anions for the formation of an ion pair in a channel occupied by two $^{23}$Na ions alone (it is simply postulated that the anion binding constant, $K_a^{sing}$, in a channel occupied by one $^{23}$Na ion is much less than $K_2$; $K_a^{sing}$ is expected to be less than $K_a^{doub}$, and $K_a^{sing}$ and $K_a^{doub}$ are expected to be less and greater, respectively, than the anion binding constant in the presence of an ion pair at the other site [Monoi, 1983]), and $T_{1B}^{an}$ is such that $1/T_{1B}^{an}$ is the average of $1/T_1$ of the $^{23}$Na ions in a channel occupied by a $^{23}$Na ion paired with an anion at one site and an unpaired $^{23}$Na ion at the other site.

Eq. 14 has six adjustable parameters, so many parameters that they cannot uniquely be determined. Here we introduce noncritical assumptions

$$q_1 \equiv (\text{slope})_{a\to\infty}/(\text{slope})_{\text{high } a} = 0.94\text{–}1.0\ (25^{\circ}\text{C}) \text{ and } 0.88\text{–}1.0\ (10^{\circ}\text{C}), \quad (17)$$

$$q_2 \equiv (x\text{-intercept})_{a\to\infty}/(x\text{-intercept})_{\text{high } a} \geq 1. \quad (18)$$

On the other hand, we have, from Eq. 14,

$$T_1^{\ddagger} = c_G(\text{slope})_{a\to\infty}, \quad (19)$$

$$K^{\ddagger} = -(1 - 2T_1^{\ddagger}/T_1^{\dagger})/(x\text{-intercept})_{a\to\infty}. \quad (20)$$

For each set of values of $(\text{slope})_{a\to\infty}$ and $(x\text{-intercept})_{a\to\infty}$ that satisfy Eqs. 6, 17, and 18, one can get the least-square estimates of four adjustable parameters ($K_1$, $K^{\dagger}$, $T_{1B}^{sing}$, and $T_1^{\dagger}$) by means of Eq. 14 together with Eqs. 19 and 20. They are listed in Table III. The sum, $S$, of the weighted squares of the residuals is dependent on the assigned values of $q_1$ and $q_2$. When estimates of parameters are such that $S$ is greater than 1.05 times the minimum of $S$, the estimates are discarded. For each set of values of $q_1$ and $q_2$, are calculated $K^{\ddagger}$ and $T_1^{\ddagger}$ from Eqs. 19 and 20. As the variation in the estimates of $K_1$ and $T_{1B}^{sing}$ with the assigned values of $q_1$ and $q_2$ is much less than the estimated standard errors, only the central values are shown for these parameters. For the other pararmeters, the ranges of their estimates are given.

TABLE II
ESTIMATED PARAMETERS IN TERMS OF THE TWO-IDENTICAL-SITE MODEL

| Parameter | Temperature | |
|---|---|---|
| | 25°C | 10°C |
| $K_1$ (molal$^{-1}$) | 13.7 ± 1.4 | 13.7 ± 1.5 |
| $K_2$ (molal$^{-1}$) | 1.6 ± 0.2 | 0.72 ± 0.12 |
| $T_{1B}^{sing}$ (ms) | 0.56 ± 0.03 | 0.49 ± 0.03 |
| $T_{1B}^{doub}$ (ms) | 0.27 ± 0.01 | 0.17 ± 0.01 |

*Estimated Parameters.* As seen from Tables II and III, the estimates of $K_1$ and $T_{1B}^{sing}$ were not significantly dependent on channel models employed. For the two-identical-site model (Model I), $K_1$ and $T_{1B}^{sing}$ were 13.7 (±1.4) molal$^{-1}$ and 0.56 (±0.03) ms, respectively, at 25°C; when the binding of an additional ion was included in the two-identical-site model (Model II), they were 13.0 (±2.0) molal$^{-1}$ and 0.55 (±0.04) ms. The temperature dependence of these parameters was small. Note that $T_{1B}^{sing}$ is ~100 times shorter than $T_1$ of $^{23}$Na ions in NaCl solutions. (As shown later, the accerelated $T_1$ of bound $^{23}$Na ions is due to their correlation time $\tau_c$ that is longer, by three orders of magnitude, than $\tau_c$ of $^{23}$Na ions free in aqueous solutions.) In following sections, an average value of 0.55 ms is used for $T_{1B}^{sing}$ (at 25°C) in order to calculate the quadrupole coupling constant and some other parameters for bound nuclei.

Within the framework of Model I, $T_{1B}^{doub}$ at 25 and 10°C was 0.27 (±0.01) and 0.17 (±0.01) ms, which are faster than $T_{1B}^{sing}$ by a factor of ~2 and ~3, respectively. Within

TABLE III
ESTIMATED PARAMETERS IN TERMS OF MORE INVOLVED MODELS

| Parameter | Temperature | |
|---|---|---|
| | 25°C | 10°C |
| $K_1$ (molal$^{-1}$) | 13.0 ± 2.0 | 13.7 ± 2.3 |
| $K^{\dagger}$ (molal$^{-1}$) | 3–4 | 2–3 |
| $T_{1B}^{sing}$ (ms) | 0.55 ± 0.04 | 0.49 ± 0.05 |
| $T_1^{\dagger}$ (ms) | 0.4–0.6 | 0.3–0.5 |
| $K^{\ddagger}$ (molal$^{-1}$) | 0.5–0.8 | 0.3–0.5 |
| $T_1^{\ddagger}$ (ms) | 0.14–0.15 | 0.10–0.11 |

the framework of Model II, $T_{1B}^{doub}$ was comparable to (or not greatly different from) $T_{1B}^{sing}$. The estimate of $K_2$ was 1.6 and 0.7 molal$^{-1}$ or greater at 25 and 10°C, respectively, depending on models employed. The ratio $K_1/K_2$ was 9 and 19 or less at 25 and 10°C, depending on models used.

(If some fraction of the gramicidin molecules is not in the functional channel state, the estimate for $T_1$ of bound $^{23}$Na decreases correspondingly, whereas the estimates for binding constants as well as the relative magnitudes of $T_{1B}^{sing}$ and $T_{1B}^{doub}$ remain the same.)

## Correlation Time

The correlation time $\tau_c$ of $^{23}$Na bound to the gramicidin channel can be related to the ratio $\kappa_1^p$ of the excess pooled longitudinal relaxation rates ($\equiv 1/T_1^p - 1/T_{1F}$) at different radio frequencies $\omega_0 = \omega'$ and $\omega''$ through the equation

$$\kappa_1^p \equiv \frac{(1/T_1^p - 1/T_{1F})_{\omega'}}{(1/T_1^p - 1/T_{1F})_{\omega''}} = \frac{0.8/(1 + 4\omega'^2\tau_c^2) + 0.2/(1 + \omega'^2\tau_c^2)}{0.8/(1 + 4\omega''^2\tau_c^2) + 0.2/(1 + \omega''^2\tau_c^2)}. \quad (21)$$

For $\omega' = 9.93 \times 10^7$ rad/s and $\omega'' = 4.99 \times 10^8$ rad/s, the found value of $\kappa_1^p$ was 3.8 (±0.3) at 25°C for a low $Na^+$ level (100 mmol $Na^+$/kg $H_2O$; average of three samples for each $\omega_0$ level). This leads to a $\tau_c$ value of 2.2 (±0.2) ns. At higher $Na^+$ levels (1.0–1.5 mol/kg $H_2O$), $\tau_c$ of bound $^{23}$Na was 1.7 (±0.3) ns at 25°C, not significantly different from that for the lower $Na^+$ level.

As shown in the preceding section, the binding constant for a first $Na^+$ ion was 13–14 molal$^{-1}$; the second binding constant was 1.6 molal$^{-1}$ or greater at 25°C. Therefore, the value 2.2 ns may be considered to be $\tau_c$ of bound $^{23}$Na in the state of single occupancy, and $\tau_c$ for double occupancy is not significantly different from that for single occupancy.

The rotational correlation time $\tau_r$ for the diffusional rotation of lecithin liposomes is evidently far longer than both of the Larmor period $\omega_0^{-1}$ (2–10 ns in the present case) and the found $\tau_c$ value (2 ns). Therefore, the spectral density $J(\omega)$ at $\omega = k\omega_0$ ($k = 1, 2$) and the estimated $\tau_c$ for bound $^{23}$Na involve no contribution of $\tau_r$. On the other hand, the ratio, $\rho_2$, of the fast and the slow excess transverse relaxation rates (fast/slow) was 15–21 (average 18) at $\omega_0 = 4.99 \times 10^8$ rad/s (50 mmol $Na^+$/kg $H_2O$, 25°C). We may write

$$\rho_2 \equiv \frac{1/T_2'' - 1/T_{2F}}{1/T_2' - 1/T_{2F}} = \frac{J(0) + J(\omega_0)}{J(\omega_0) + J(2\omega_0)}, \quad (22)$$

where $T_2'$ and $T_2''$ are the slow and the fast $T_2$, respectively, and $T_{2F}$ is $T_2$ in the absence of gramicidin. If the residence time $\tau_B$ is equal, or comparable, to $\tau_c$ of bound $^{23}$Na (hence $\tau_B \approx 2$ ns), then we may put

$$\rho_2 = \frac{1 + 1/(1 + \omega_0^2\tau_c^2)}{1/(1 + \omega_0^2\tau_c^2) + 1/(1 + 4\omega_0^2\tau_c^2)}. \quad (23)$$

This equation indicates that for $\omega_0 = 4.99 \times 10^8$ rad/s (with $\tau_c \approx 2$ ns), $\rho_2$ would be of the order of 2, which is inconsistent with the found value of $\rho_2$. We, therefore, conclude that the observed $\tau_c$ of bound $^{23}$Na involves no $\tau_B$-contribution either, thus representing the intrinsic correlation time $\tau_c'$ of bound $^{23}$Na (i.e., $\tau_c$ of bound $^{23}$Na in the absence of both of the rotation of liposomes and the nuclear exchange between free and bound states). Note that the estimated $\tau_c$ of bound $^{23}$Na remains unchanged even if some fraction of gramicidin is in forms noninteracting with $Na^+$ ions.

So far, two different values have been reported for $\tau_c$ of $^{23}$Na bound to gramicidin, 2 ns (Cornélis and Laszlo, 1979) and 25 ns (Urry et al., 1980). Our estimate agrees with the former, but is different from the latter by a factor of ~10. The former value refers to gramicidin in an ethanol-water (9:1) mixture. The conformation of gramicidin in this solvent is known to be considerably different from that in lecithin liposomes (Veatch et al., 1974; Urry et al., 1975; Masotti et al., 1980), which are used in the present study. It is interesting that $\tau_c$ of bound $^{23}$Na is approximately the same for different conformations of gramicidin.

The latter value, reported by Urry et al. (1980), is for gramicidin packaged into lysolecithin micelles in saline. The conformation of gramicidin in this system is essentially the same as in lecithin liposomes (Masotti et al., 1980). How can the disagreement with our estimate then be accounted for? The value from Urry's laboratory was computed from $\rho_2$ (measured at $1.66 \times 10^8$ rad/s) by using Eq. 23. This equation, however, is applicable only when the average quadrupole interaction $\chi_\alpha$ for bound nuclei is negligibly small and/or residence time $\tau_B$ is sufficiently short (see Eq. 25). This prerequisite (i.e., negligible $\chi_\alpha$ and/or sufficiently short $\tau_B$) fails, at least, for liposomal samples, as shown later. Note that if Eq. 23 were to be applied to our samples, the resulting estimate of $\tau_c$ would be 8–10 ns ($\rho_2 = 15$–23 at $\omega_0 = 4.99 \times 10^8$ rad/s for 0.05 and 1.0–1.5 mol $Na^+$/kg $H_2O$, 25°C), which is substantially greater than $\tau_c$ obtained above from $\kappa_1^p$ (Eq. 21), but is still shorter than Urry's value. The difference between the two values calculated from $\rho_2$ reflects, at least in part, different $\omega_0$ levels employed, because the estimate of $\tau_c$ would be frequency dependent(!) if Eq. 23 were to be applied to cases for which this equation does not hold.

## Quadrupole Coupling Constant

The pooled longitudinal relaxation time, $T_{1B}^p$, of bound $^{23}$Na is expressed (axially symmetrical field gradients being assumed) as

$$1/T_{1B}^p \equiv 0.8/T_{1B}' + 0.2/T_{1B}'' = \frac{1}{10}\chi^2\tau_c\left(\frac{0.8}{1 + 4\omega_0^2\tau_c^2} + \frac{0.2}{1 + \omega_0^2\tau_c^2}\right), \quad (24)$$

where $\chi$ is the quadrupole coupling constant for bound

$^{23}$Na. As shown already, $\tau_c$ of bound $^{23}$Na was 2.2 (±0.2) ns at 25°C for the state of single occupancy, and $T_{1B}^{p}$ for this state of occupancy was 0.55 (±0.04) ms. From Eq. 24, $\chi$ is estimated as 1.0 (±0.1) MHz at 25°C for singly loaded channels. For the state of double occupancy, the estimates of $T_{1B}^{p}$ and hence of $\chi$ depend on theoretical channel models adopted for data analysis (see the Results section entitled Binding Constant and Longitudinal Relaxation Time of Bound $^{23}$Na); $\chi$ for this state was 1.4 and 0.9–1.1 MHz for Models I and II, respectively.

These values are comparable to, or slightly greater than, $\chi$ for $^{23}$Na ions in aqueous solutions (~1 MHz) and less than the value for $^{23}$Na bound to gramicidin in an ethanol-water mixture (1.7 MHz [Cornélis and Laszlo, 1979]). If some fraction of gramicidin is in inactive forms, then $T_{1B}^{p}$ decreases correspondingly, and hence $\chi$ tends to increase.)

## Average Quadrupole Coupling Constant

As shown in the Results section entitled Correlation Time, the residence time $\tau_B$ of bound $^{23}$Na (as well as the rotational correlation time $\tau_r$ of liposomal particles) is much longer than $\tau_c'$ (2 ns) and hence $\omega_0^{-1}$ (2 ns for a radio frequency of 79.4 MHz). In this situation, $\rho_2$ for super-Lorentzians is expressed as (Monoi, 1985)

$$\rho_2 = \frac{1 + 1/(1 + \omega_0^2\tau_c^2) + (\tau_B\chi_\alpha^2)/(\tau_c\chi^2)}{1/(1 + \omega_0^2\tau_c^2) + 1/(1 + 4\omega_0^2\tau_c^2)}, \qquad (25)$$

where $\chi_\alpha$ is the average quadrupole coupling constant of bound $^{23}$Na. One of the rapid-exchange conditions can be written as

$$\tau_B\chi_\alpha \lesssim \xi < 2, \qquad (26)$$

where $\xi$ is the maximum value of $\tau_B\chi_\alpha$ that causes complete merging of the transverse magnetizations of the free and the bound nuclei. The factor 2 appearing in the above relation is derived from the fact that half the quadrupole distribution of the resonance line of bound $^{23}$Na in the absence of chemical exchange is equal to $\chi_\alpha/2$. An upper bound of $\xi$ is 1. With $\rho_2 = 18$, $\tau_c = 2.2$ ns, and $\chi = 1.0$ MHz or $6.3 \times 10^6$ rad/s (at $\omega_0 = 4.99 \times 10^8$ rad/s; 25°C), we estimate that a lower bound of $\chi_\alpha$ is 0.13 MHz for singly loaded channels.

The found value of $\rho_2$ for high $Na^+$ levels (1.0–1.5 mol/kg $H_2O$) was 16–23 (average 20) at $\omega_0 = 4.99 \times 10^8$ rad/s (25°C), not significantly different from that for low $Na^+$ levels. Together with the values $\tau_c = 1.7$ ns and $\chi \geq 0.9$ MHz, the same line of calculation as employed above leads to a value of 0.13 MHz as a lower bound of $\chi_\alpha$ for the state of double occupancy. A lower bound of ratio $\chi_\alpha/\chi$ in this state was 0.15–0.23, depending on models. (The estimate for $\chi_\alpha$ in both states of occupancy increases if some fraction of gramicidin is in inactive states, and ratio $\chi_\alpha/\chi$ increases as well.)

In the above calculation, the whole cavity of the channel is regarded as a single site. For multiple-site models for the channel, this treatment is equivalent to the assumption that the translocation of $Na^+$ ions between the sites within a channel makes no appreciable contribution to the relaxation rates. When the contribution of this translocation step is taken into account, the last term of the numerator of Eq. 25 is multiplied by a factor $f^2$ such that $1/2 \leq f \leq 1$ (Monoi, 1985; $f = 1$ corresponds to a negligible contribution of the translocation step), and, therefore, the above estimate for a lower bound of $\chi_\alpha$ increases by a factor of two at the most. A lower bound of $\chi_\alpha$ for single occupancy is thus 0.13–0.26 MHz at 25°C. Because, at this temperature, the absorption line for low $Na^+$ levels is only approximately represented in terms of a super-Lorentzian line (i.e., the rate of exit of $^{23}$Na from the channel into the bulk phase is only marginally rapid), the value 0.13–0.26 MHz corresponds to a rough approximation of $\chi_\alpha$ for singly loaded channels at 25°C.

On the other hand, there is evidently no (appreciable) contribution of the translocation step in the state of double occupancy, so that the lower bound (0.13 MHz) for $\chi_\alpha$ in this state remains unchanged. At high $Na^+$ levels, the absorption line is a super-Lorentzian (e.g., the rate of exit from the channel is more rapid). Therefore, $\chi_\alpha$ for this state of occupancy is >0.13 MHz at 25°C.

Clearly, the intrinsic average quadrupole coupling constant $\chi_\alpha'$ of bound $^{23}$Na (i.e., $\chi_\alpha$ in the absence of rotation of liposomes) is not $<\chi_\alpha$. From the estimate of $\chi_\alpha$ (0.13 MHz or more), the critical diameter $d_{crit}$ (Monoi and Uedaira, 1980; Monoi, 1985) is calculated to be 21 nm at most. Since the diameter of lecithin liposomes is evidently much greater than $d_{crit}$, parameter $\chi_\alpha$ involves no appreciable contribution of $\tau_r$: $\chi_\alpha \simeq \chi_\alpha'$. Thus, the estimated lower bound of $\chi_\alpha$ represents an intrinsic property of the (first two) cation sites of the gramicidin channel.

## Rate Constants for Entry into, and Exit from, the Channel

Cornélis and Laszlo (1979) and Urry et al. (1980) postulated that the mean residence time $\tau_B$ of $^{23}$Na ions bound to the gramicidin channel is approximately equal to their correlation time $\tau_c$. If this is the case, our data imply that the rate constant, $k_-$, for exit from the channel is $5 \times 10^8$ $s^{-1}$ ($k_- \simeq \tau_c^{-1}$). This value seems too large when we consider that the maximum unidirectional flux of $Na^+$ ions through the channel at zero transmembrane potential is $<10^7$ ions/s (e.g., $2.3 \times 10^6$ ions/s for phosphatidylethanolamine [PE] membranes [Finkelstein and Andersen, 1981]; the single-channel conductance in PE membranes is approximately equal to that in lecithin membranes [Bamberg et al., 1976]), and that $k_-$ will not be sufficiently faster than the rate constant, $k_{loc}$, for translocation between sites within the channel (Läuger, 1973; Hladky et al., 1979; Urban and Hladky, 1979; Finkelstein and Andersen, 1981).

As shown in a foregoing section, $\tau_B$ is sufficiently longer

than $\tau_c'$ and $\omega_0^{-1}$. Thus, $k_-$ is much less than the reciprocal of $\tau_c'$: $k_- \ll 5 \times 10^8\ s^{-1}$. Now let's estimate a lower and an upper bound of $k_-$. When the relaxation rates involve no appreciable contribution of $k_{loc}$, then we may write, from Eq. 25,

$$\tau_B = \tau_c \left(\frac{\chi}{\chi_\alpha}\right)^2 \left[\rho_2 \left(\frac{1}{1+\omega_0^2\tau_c^2} + \frac{1}{1+4\omega_0^2\tau_c^2}\right) - \left(1 + \frac{1}{1+\omega_0^2\tau_c^2}\right)\right]; \quad (27)$$

when a $k_{loc}$-contribution is taken into account, $\chi_\alpha$ in this equation is replaced by $f\chi_\alpha$ (where $1/2 \leq f \leq 1$), and the value 0.13 MHz is a lower bound, and a rough approximation, of $f\chi_\alpha$ for the state of single occupancy at 25°C (see the Results section entitled Average Quadrupole Coupling Constant). It may be safely assumed that $f\chi_\alpha$ is not greater than about twice this value; hence $0.13 \lesssim f\chi_\alpha/\chi \lesssim 0.26$. Together with the found values of $\tau_c$ and $\rho_2$ (2.2 ns and 18, respectively, at $\omega_0 = 4.99 \times 10^8$ rad/s; 25°C), $\tau_B$ for a first $Na^+$ ion is calculated from Eq. 27 to be $3 \times 10^{-7}\ s \lesssim \tau_B \lesssim 1.3 \times 10^{-6}\ s^{-1}$. Consequently, the rate constant, $k_{-1}$, for exit from a singly loaded channel is

$$8 \times 10^5\ s^{-1} \lesssim k_{-1} \lesssim 3 \times 10^6\ s^{-1} \quad (28)$$

at 25°C; the lower bound represents a rough approximation of $k_{-1}$.

For the state of double occupancy, a lower bound of $\chi_\alpha/\chi$ was 0.15–0.23 depending on models (see the Results section entitled Average Quadrupole Coupling Constant). Together with the values $\tau_c = 1.7$ ns and $\rho_2 = 20$, an upper bound of $\tau_B$ for this state is estimated to be $0.5\text{–}1.2 \times 10^{-6}\ s^{-1}$ at 25°C. On the basis of the known conformation of the gramicidin channel, the first two binding sites are expected to be identical, and the local interaction between a bound cation and the channel wall in the state of double occupancy is expected not to be very different from that in the state of single occupancy. In fact, $\tau_c'$ and $\chi$ of bound $^{23}Na$ in the former state are not significantly, or are not very, different from those in the latter (1.7 vs. 2.2 ns and 0.9–1.4 vs. 1.0 MHz, respectively, for former vs. latter). It may thus be supposed that ratio $\chi_\alpha/\chi$ in the former is not greater than about twice that (= 0.13–0.26) in the latter. Putting $\chi_\alpha/\chi \lesssim 0.5$, we estimate that a lower bound of $\tau_B$ in doubly loaded channels is $1.0 \times 10^{-7}$ s. Consequently, the rate constant, $k_{-2}$, for exit from a doubly loaded channel is

$$8 \times 10^5\ s^{-1} \lesssim k_{-2} \lesssim 1.0 \times 10^7\ s^{-1} \quad (29)$$

at 25°C. The lower bound depends on models used; it increases to $2 \times 10^6\ s^{-1}$ if the two-identical-site model (Model I) is employed. Because, at high $Na^+$ levels, the absorption line is a super-Lorentzian (i.e., $\tau_B\chi_\alpha$ is less than y; see the Results section entitled Average Quadrupole Coupling Constant), $k_{-2}$ is faster than this lower bound. Therefore, $k_{-2}$ is not slower than $k_{-1}$. As also inferred by Eqs. 28 and 29, ratio $k_{-2}/k_{-1}$ does not greatly exceed 20.

With a value of 13.4 $molal^{-1}$ for the binding constant, $K_1$, of a first $Na^+$ ion (see the Results section entitled Binding Constant and Longitudinal Relaxation Time of Bound $^{23}Na$), the single-site rate constant, $k_{+1}$, for entry into a site of an empty channel is, from Eq. 28,

$$1.1 \times 10^7\ molal^{-1} \cdot s^{-1} \lesssim k_{+1} \lesssim 4 \times 10^7\ molal^{-1} \cdot s^{-1}. \quad (30)$$

The lower bound represents a rough approximation of $k_{+1}$. The second binding constant $K_2$ was 1.6–4 $molal^{-1}$. Accordingly, the single-site rate constant, $k_{+2}$, for entry into a singly loaded channel is, from Eq. 29,

$$1.3 \times 10^6\ molal^{-1} \cdot s^{-1} \lesssim k_{+2} \lesssim 4 \times 10^7\ molal^{-1} \cdot s^{-1}. \quad (31)$$

This range for $k_{+2}$ is more restricted when specific channel models are postulated; when Model I is applied, $K_2$ was

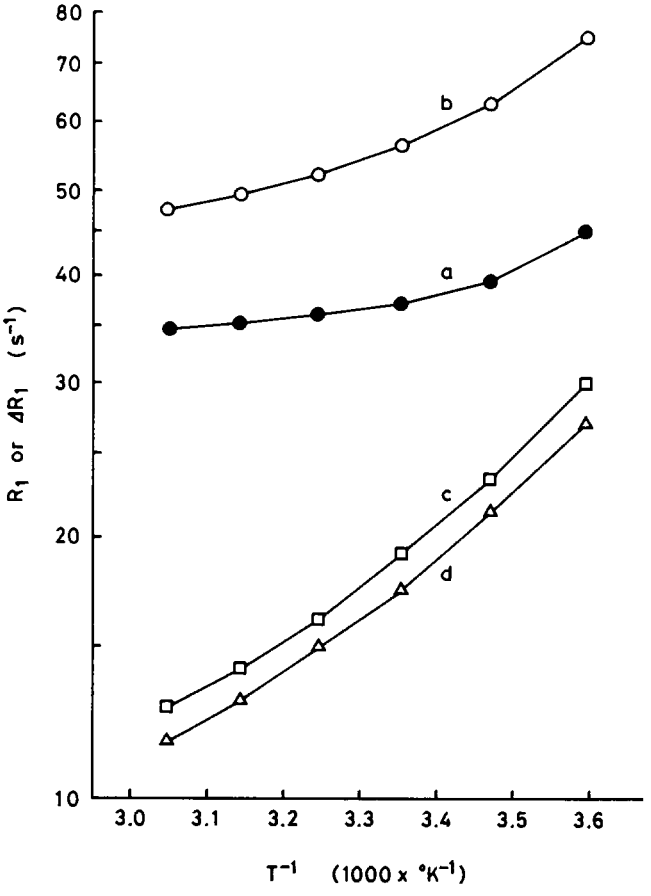


FIGURE 6 Temperature dependence of the longitudinal relaxation rate of $^{23}Na$ in suspensions of gramicidin-doped liposomes at a high level of radio frequency (79.4 MHz). The samples contain (*a*, *b*) 100 mmol $Na^+$, 10 g gramicidin, and 100 g lecithin/kg $H_2O$, (*c*) the same as in *a* and *b* except that no gramicidin is added, and (*d*) NaCl (100 mmol/kg $H_2O$) alone. The ordinate represents (*a*) excess longitudinal relaxation rate $1/T_1 - 1/T_{1F}$ or (*b*, *c*, *d*) longitudinal relaxation rate $1/T_1$.

1.6 molal$^{-1}$, and hence the upper bound for $k_{+2}$ decreases to $1.6 \times 10^7$ molal$^{-1} \cdot s^{-1}$.

### A Comment on the Temperature Dependence of Relaxation Rates

Urry et al. (1980) studied the temperature dependence of $T_1$ of $^{23}$Na ions in samples composed of gramicidin-doped lesolecithin micelles in saline. On the basis of the observed temperature coefficient of $T_1$, they concluded that the activation energy $E_a$ for the exchange of $Na^+$ ions between the gramicidin channel and the bulk solution is 7.2 kcal/mol. This value is in the same range as $E_a$ reported for the transport of $Na^+$ ions through the gramicidin channel in black lipid membranes (4.9 kcal/mol for glyceryl monooleate–decane membranes [Hladky and Haydon, 1972] and 7.3 kcal/mol for dioleyllecithin–decane membranes [Bamberg and Läuger, 1974]). This result was interpreted by them to require that no barriers in translocation within the channel are higher than the barrier encountered on the exchange step between the solution and the cation-binding site.

We reexamined this point. As shown in Fig. 6, the temperature coefficient of $T_1$ was substantially low at a radio frequency of 79.4 MHz: $d(\ln T_1^p)/dT = 0.004$ for 45–55°C. This value corresponds to an $E_a$ of 0.8 kcal/mol, which is far lower than the $E_a$ value for $T_1$ reported by Urry and his collaborators: 7.2 kcal/mol for ~46–~66°C at 26.3 MHz. Further investigation showed that the temperature coefficient of $T_1$ has a marked dependence on the radio frequency; at 15.8 MHz, $E_a$ was even greater than Urry's value. This finding demonstrates that the temperature coefficient (and hence $E_a$) of $T_1$ cannot be related directly to the energy barriers that permeant ions encounter on diffusion through the gramicidin channel.

The temperature coefficient of the excess longitudinal relaxation rate $\Delta R_1$ involves three contributions:

$$\frac{d(\ln \Delta R_1)}{dT} = \frac{d(\ln p_B)}{dT} + 2\frac{d(\ln \chi)}{dT} + c(\omega_0)\frac{d(\ln \tau_c)}{dT}, \quad (32)$$

where $p_B$ is the fractional population of bound $^{23}$Na, and $c(\omega_0)$ is a decreasing function of $\omega_0\tau_c$. In the right-hand side of this equation, $c(\omega_0)$ alone is frequency dependent. Parameter $c(\omega_0)$ varies from 1 to $-1$ as $\omega_0\tau_c$ increases from null to a value sufficiently greater than unity. When the temperature coefficient of $\tau_c$ is not negligibly small relative to that of $p_B\chi^2$, the temperature coefficient of $\Delta R_1$ thus shows a frequency dependence unless $\tau_c$ is sufficiently shorter, or longer, than the Larmor period.

As shown in a previous section, $\tau_c$ of bound $^{23}$Na involves no contributions of $k_-$ (rate constant for exit from the channel) and $\tau_r$ (rotational correlation time for the diffusional rotation of liposomal particles). It involves no $k_{loc}$-contribution either.[2] Therefore, $E_a$ of $\tau_c$ for the bound state represents a parameter characterizing the cation sites of the gramicidin channel. Note that $E_a$ of $\tau_c$ (as well as that of $T_1$) bears no relationship with $E_a$ for the exchange between the channel and the bulk solution.

The pronounced frequency dependence of the excess relaxation rate indicates that the activation energy of $\tau_c$ for bound $^{23}$Na ions is considerable. The precise magnitude of this activation energy and its implications for the transport properties of the gramicidin channel are to be investigated.

## DISCUSSION

### The Number of Maximum Occupancy

So far, one- to four-cation-occupiable models have been proposed for the gramicidin channel (e.g., Sandblom et al., 1977; Urban et al., 1980; Finkelstein and Andersen, 1981). Data given in Fig. 5 are incompatible with occupancy by maximally one $Na^+$ ion.

According to Finkelstein and Andersen (1981), the gramicidin channel is not occupied by more than one $Na^+$ ion at any instant. Their argument is based on two principal reasons. The first is that the dependence of the single-channel conductance upon the $Na^+$ ion activity is in accord with the kinetics of one-ion occupancy. The second reason is that the flux-ratio exponent (Ussing, 1949) for $Na^+$ ions is equal to unity at all attainable concentrations of NaCl. Some comments will be added below on these points.

When a single salt is present symmetrically on both sides of a membrane across which a small voltage is applied, the single-channel conductance $G$ for a two-cation-occupiable two-identical-site channel is (Levitt, 1978*b*; Urban and Hladky, 1979)

$$G = \frac{eF}{RT} \cdot \frac{k_{loc}K_1a}{(1 + 2K_1a + K_1K_2a^2)[1 + 2k_{loc}/(k_{-1} + k_{+2}a)]}, \quad (33)$$

where $k_{loc}$ is the rate constant for translocation between the two sites, $e$ is the elementary charge, $F$ is the Faraday

[2]This is shown as follows. According to Finkelstein and Andersen (1981), the unidirectional flux of water through an ion-free gramicidin channel is $6.1 \times 10^7$ waters/s at room temperature. We may assume that a cation site is near each channel end. The average number of waters coupled to the flux of $Na^+$ ions is 6–12 waters/ion at low $Na^+$ concentrations (Rosenberg and Finkelstein, 1978*b*; Levitt et al., 1978). Consequently, an approximate upper bound of $k_{loc}$ for $Na^+$ ions at zero transmembrane potential is then only slightly greater than (and less than twice) $1–0.5 \times 10^7$ ions/s ($6.1 \times 10^7$ waters/s divided by 6–12 waters/ion), which is considerably smaller than the reciprocal $\tau_c$ ($\approx 5 \times 10^8$ s$^{-1}$) of bound $^{23}$Na. Therefore, $\tau_c$ of the bound state involves no $k_{loc}$-contribution.

constant, $R$ is the gas constant, and $T$ is the absolute temperature. This equation indicates that when $G$ is plotted against $G/a$ (an Eadie-Hofstee plot), the curve has a foot in the range of low ionic activities unless $k_{loc} \ll k_{-1}$ (this inequality does not hold for gramicidin [Läuger, 1973; Urban and Hladky, 1979; Hladky et al., 1979; Finkelstein and Andersen, 1981]). At very high ionic activities ($K_2 a \gg 1$), the curve deviates towards lower conductance values.

From Eq. 33, when $G$ is plotted against $G/a$, the reciprocal, $-K^{app}$, of the slope in the limit $a \to 0$ is given by

$$K^{app} = -\lim_{a \to 0} \frac{d(G/a)}{dG} = 2K_1 \left(1 - \frac{k_{+2}}{k_{+1}} \frac{k_{loc}}{k_{-1} + 2k_{loc}}\right). \quad (34)$$

If $k_{loc} \ll k_{-1}$ or $k_{loc} \gg k_{-1}$, then Eq. 34 is reduced to

$$K^{app} = 2K_1 \qquad (k_{loc} \ll k_{-1}), \quad (35)$$

$$K^{app} = 2K_1 \left(1 - \frac{k_{+2}}{2k_{+1}}\right) \qquad (k_{loc} \gg k_{-1}). \quad (36)$$

It may be assumed that $k_{+2}$ is $< k_{+1}$ due to electrostatic repulsion between permeant cations, so that $K^{app} \geq K_1$. From Eqs. 35 and 36, we then have, for any value of $k_{loc}$,

$$K_1 \leq K^{app} \leq 2K_1 \quad \text{or} \quad K^{app}/2 \leq K_1 \leq K^{app}. \quad (37)$$

This relation infers that with nonequilibrium channels, $K_1$ cannot directly be calculated from the slope of the low-concentration asymptote in the $G$ vs. $G/a$ plot.

In Fig. 7, experimental values of $G$ are plotted against $G/a$. Data are taken from Urban et al. (1980). The plot

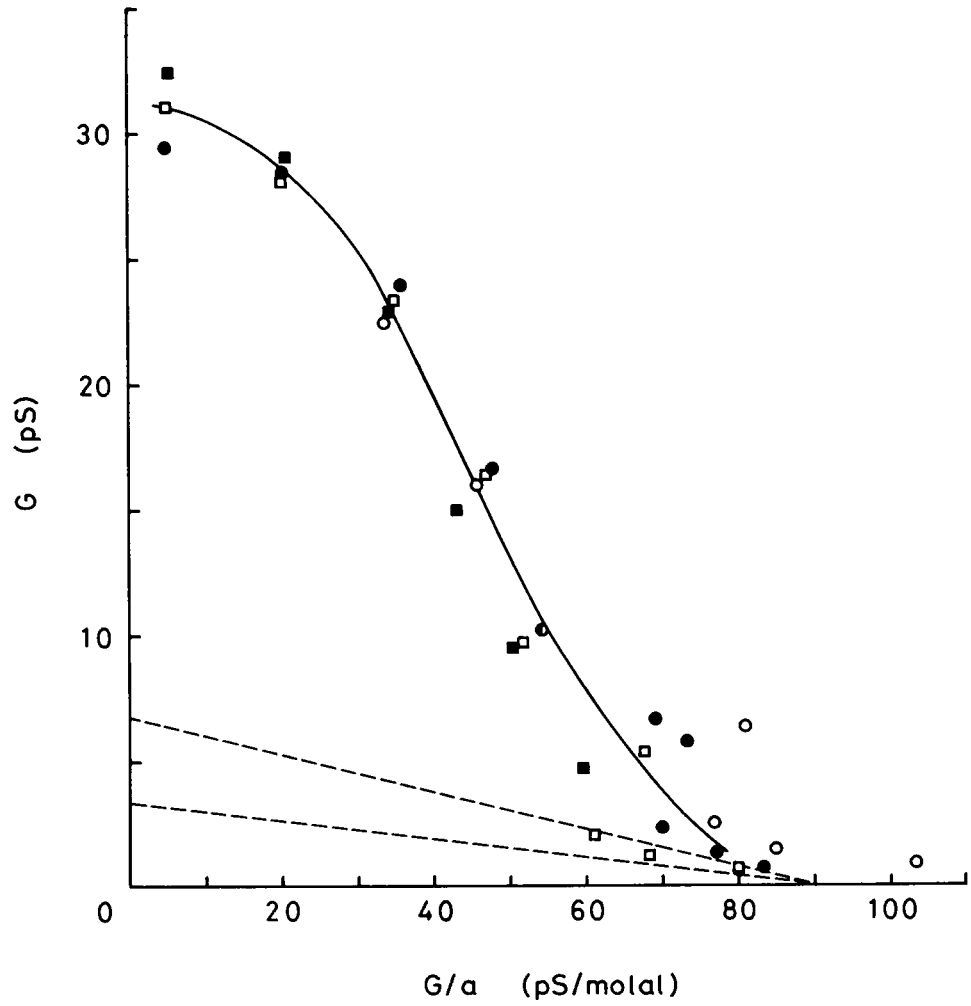


FIGURE 7 $G$ vs. $G/a$ plot for NaCl. The applied potential is: ○, 50 mV; ●, 100 mV; □, 150 mV; ■, 200 mV (glyceryl monooleate–hexadecane black membrane; 20–22°C). The curve drawn has no statistical meaning. The two broken lines represent a possible range for the low-concentration asymptote. Their slopes are equal to $-K_1^{-1}$ and $-(2K_1)^{-1}$ estimated from the present NMR experiment. The magnitudes of their $y$-intercepts are mere guesses.

shows a tendency to have a foot at low ionic activities. Although this foot is observed for some other cations such as $K^+$, $Rb^+$, $Cs^+$, and $Tl^+$ (Neher et al., 1978), whether or not this tendency is also real for $Na^+$ is uncertain because of relatively large errors (scatter along the horizontal direction) of the observed points in the low activity range. Conductance data reported by Finkelstein and Andersen (1981; Fig. 5) cannot discriminate the two alternatives (i.e., presence or absence of the foot) either. Thus, there still leave two possibilities: single and multiple occupiabilities of $Na^+$.

The second reason that Finkelstein and Andersen raised is the observed value ($\simeq 1$) of the flux-ratio exponent $n^*$. For a two-cation-occupiable two-identical-site channel, $n^*$ is related to rate constants through (Urban and Hladky, 1979)

$$n^* = \frac{(k_{-1} + k_{+2}a)(4k_{loc} + 2k_{-1} + k_{+2}a)}{(2k_{-1} + k_{+2}a)(2k_{loc} + k_{-1} + k_{+2}a)}. \quad (38)$$

Hence, $n^*$ takes a value between 1 and 2, depending on $k_{loc}/k_{-1}$ and $k_{+2}a/k_{-1}$; $n^*$ is equal to 1 if $k_{loc} \ll k_{-1}$. As immediately seen, $n^*$ is not greatly different from one even when $k_{loc}$ is comparable to $k_{-1}$. Reported values of $k_{loc}/k_{-1}$ show a considerable variation. They range from 0.5 (Levitt, 1978*b*) to 29 (Urban et al., 1980). The estimate by Andersen and Procopio (1980) is 1.7. Within the framework of the diverse reported values of $k_{loc}/k_{-1}$, we cannot exclude, again, the possibility of multiple occupancy by $Na^+$ ions, in spite of the argument of Finkelstein and Andersen (1981) for single occupancy.

Now consider $^{23}Na$ NMR data. For low ion concentrations, the reciprocal of excess relaxation rate $\Delta R_1$ can usually be measured far more accurately than single-channel conductance $G$, as will be seen by comparing Figs. 5 and 7. A plot of $\Delta R_1^{-1}$ (weighted by $\gamma$) against $a$ demonstrated that more than one $Na^+$ ion can be present at the same time within a channel (Fig. 5). The estimate of $K_1$ was not significantly different ($\sim 13$ molal$^{-1}$) within the framework of the channel models employed. On the basis of this NMR value of $K_1$, is drawn in Fig. 7 an expected range for the low-concentration asymptote of the $G$ vs. $G/a$ plot according to Eq. 37. This reveals the existence of a foot of this plot in the range of low ionic activities. (Data plotted in Fig. 7 are for glyceryl monooleate [GMO] membranes, whereas phosphatidylcholine [PC] is used in the present study. The single channel conductance in GMO is roughly twice as large as in PC [Bamberg et al., 1976]. According to Jordan [1983], this difference will reflect a membrane dipole potential [Haydon, 1975], which arises from oriented dipoles at the surface of membranes. Thus, $K_1$ for GMO membranes may be expected to be more or less greater [hence the slope of the low-concentration asymptote tends to be smaller] than that for PC membranes.)

As has recently been suggested by Andersen (1983), the foot of the $G$ vs. $G/a$ plot may involve a substantial contribution of the interfacial polarization associated with applied potentials, and it may thus be possible to misinterpret the physical basis of the foot of this plot and erroneously assign the foot to be an indication of an additional binding site. The contribution of the interfacial polarization, however, will not be involved in equilibrium binding experiments. Therefore, the existence of the foot as shown above implies multiple occupancy. From analogy, it may be possible that the foot observed in the $G$ vs. $G/a$ plot of other permeant cations ($K^+$, $Rb^+$, $Cs^+$, $Tl^+$, and $H^+$ [Neher et al., 1978; Eisenman et al., 1978*a*]) involves a nondipolar contribution and reflects multiple occupancy.

On the other hand, loading of more than two cations in gramicidin-like channels is very unlikely from electrostatic reasons (Levitt, 1978*a*; Monoi, 1983). Therefore, the most reasonable conclusion at present is that the gramicidin channel accomodates maximally two $Na^+$ ions. To explain the observed value of the flux-ratio exponent $n^*$ ($\simeq 1$ [Finkelstein and Andersen, 1981]) in terms of two-cation-occupiable channels, it is required that $k_{loc}$ is of the order of $k_{-1}$. This relation may also explain why the foot of the $G$ vs. $G/a$ plot is not prominent for $Na^+$ ions.

## Localization of Bound $Na^+$ Ions

The found value (0.13–0.26 MHz or more) for a lower bound of $\chi_\alpha$ is much greater than the quadrupolar frequency distribution observed for $^{23}Na$ ions in aqueous unoriented liquid crystals of anionic amphiphiles (20–30 kHz at most [e.g., Lindblom, 1971; Shporer and Civan, 1972; Tiddy et al., 1978; also see Chen and Reeves, 1972]). In oriented Na-DNA fibers, two times the distance of quadrupole splitting is 26 kHz (Berendsen and Edzes, 1973). The high level of $\chi_\alpha$ implies that bound $^{23}Na$ ions are partially dehydrated. It follows that the bound $^{23}Na$ ions that are being considered in this study do not correspond to the $Na^+$ ions attached to channel entrances with full primary hydration spheres, thus representing $Na^+$ ions within the channels.

The wall of the gramicidin channel is known to consist of the peptide backbone of the head-to-head dimer of two $\pi^6$(L,D)-helical monomers (e.g., Urry et al., 1971); one pitch of the helix is $\sim 5$ Å, and one helical turn involves 6.3 hydrogen-bonded carbonyl oxygens except for the extremities of the pore, where three unhydrogen-bonded carbonyl oxygens push out into the bulk solution. The ligands for cation binding are most likely carbonyl oxygens, which are aligned spirally on the pore wall. When a cation passes through the channel, it will move spirally along the alignment of the ligand oxygens. Aligned ligands constitute a series of energy wells for the passage of cations through the channel.

Two types of energy wells can be distinguished. In inner wells, which are in the inner compartment of the channel cavity, ligands for cation binding are hydrogen-bonded carbonyl oxygens; from conformational restrictions, each well will involve only few (presumably two or three) adjacent carbonyl oxygens. Inner wells are likely separated

from their neighboring inner wells by relatively low energy barriers. Outer, or superficial, wells are located in the superficial compartment near the channel end and are constituted by unhydrogen-bonded carbonyl oxygens. There might be a third type of energy wells, intermediate wells, which involve both kinds of carbonyl oxygens and are situated at the boundary between the two compartments. The oxygen of the ethanolamine group may participate in cation binding in superficial and intermediate wells.

For a cation in an inner well, main sources of the field gradient will be hydrogen-bonded carbonyl oxygens coordinated to the cation and primary waters of hydration that precede and follow the cation (as well as the distorted electron shell of the cation caused by these polar species). The principal axis $Z_\alpha$ of the average field gradient at each inner well lies, most likely, near the plane perpendicular to the channel axis. When a cation passes through from the outermost inner well at one channel end to the corresponding well at the opposite end, the $Z_\alpha$ axis rotates around the channel axis by $\sim 2\pi$ rad per each helical pitch ($\approx$5Å) of the $\pi^6$(L,D)-conformation. In addition, adjoining hydrogen-bonded carbonyl groups are oriented toward opposite channel ends. As a consequence, if a permeant cation does occur with an equal probability over the full length of the inner compartment or the whole channel cavity, then the quadrupole interaction is averaged to a low level. This is not the case, as is indicated by the large found value of $\chi_\alpha/\chi$ (0.13–0.26 or greater).

A conclusion is that $Na^+$ ions spend a large fraction of their residence time (for a stay in a whole channel) in more or less restricted domains, or binding sites, within the channel. If a binding site belongs to the inner compartment, its dimensions along the channel axis must be much less than one helical pitch ($\approx$5Å) of the $\pi^6$(L,D) conformation. Consequently, whichever compartment a binding site may be assigned to, it involves few carbonyl oxygens as ligands for cation binding. Because local interactions between a permeant cation and the channel wall are expected to be virtually the same within the inner compartment, we must consider that the binding site is located in the superficial compartment and/or at the boundary between the two compartments. (This remains valid when we take into account the profile of the electrostatic potential due to image forces [Levitt, 1978*a*; Monoi, 1983].) We conclude that the binding site (for first two cations) is located near the channel end, and that the superficial compartment and hence unhydrogen-bonded carbonyl oxygens are included in cation binding.

It has usually been assumed, in analyzing transport data of the gramicidin channel, that the binding site for permeant cations is located close to either end of the channel (e.g., Sandblom et al., 1977; Eisenman et al., 1978*b*; Levitt, 1978*b*; Andersen and Procopio, 1980; Läuger, 1980). This assumption is chiefly based on the profile of the image-force energy along the channel axis; this energy is lowest at the channel entrances.

The first experimental evidence for the localization of cation sites in gramicidin was presented by Koeppe et al. (1979) on the basis of an x-ray diffraction study of crystals of cation-complexed gramicidin. They found two locations of cations per channel length of 26 Å; the sites are separated by 21 and 5 Å along the mouth-to-mouth aligned channels. This means that the two locations are either 2.5 Å from the channel ends or, alternatively, 2.5 Å from the center of the channel. The two alternatives could not be resolved.

With crystals of gramicidin, we can expect no appreciable variation in image-force energy along the direction of the channel axis. This implies that the binding site can be localized without the effect of image forces. We are thus again tempted to consider that the binding site is located near the channel end, and that unhydrogen-bonded carbonyl oxygens are included in cation binding since local interactions between a cation and the channel wall will be virtually the same within the inner compartment of the channel cavity. Presumably, unhydrogen-bonded carbonyl oxygens near the channel end are more favorable for cation binding than hydrogen-bonded carbonyl oxygens which form the helical structure of the channel wall.

From a $^{13}C$ NMR study, Urry et al. (1982) have recently reported that the gramicidin channel possesses two cation sites near the channel ends. This is compatible with the present results. According to them, the distance of separation between the sites was estimated to be slightly more than 20 Å, which is very close to the value for gramicidin crystals observed by Koeppe et al. (1979) if the site in crystals is supposed to be near the channel end.

Thus, experimental evidences and considerations now available point to the notion of the localization of the binding site (for first two cations) near the channel entrance. The localization of the binding site will be a result of the combined effects of the image force and the short-range interaction between cations and the channel wall. It seems doubtful that the image force alone is able to confine a cation to such a small domain so as to explain the found value of $\chi_\alpha$.

## Binding Constants

The estimate for $K_1$ of $Na^+$ ions was not significantly dependent on channel models employed. For the two-identical-site model, $K_1$ was 13.7 ($\pm$1.4) molal$^{-1}$ at 25°C; when the binding of an additional ion (cation or anion) was included in the two-identical-site model, $K_1$ was 13.0 ($\pm$2.0) molal$^{-1}$ at 25°C. In estimating some other parameters, an average value 13.4 molal$^{-1}$ was tentatively used for $K_1$. The temperature coefficient of $K_1$ was small (Tables II and III).

The estimate for $K_2$ was dependent on channel models employed. For the two-identical-site model, $K_2$ was 1.6 ($\pm$0.2) molal$^{-1}$ at 25°C; the standard entropy change $\Delta S^0$ for the binding of a second $Na^+$ ion was $\sim$30 e.u. (10–

25°C), which was much greater than that for the binding of a first ion (5 e.u.). When the binding of an additional ion was included in the two-identical-site model, $K_2$ was 3–4 molal$^{-1}$, and the temperature dependence was less marked.

Table I tabulates reported values of binding constants of $Na^+$ ions for the gramicidin channel. As shown in this table, estimates of $K_1$ by different authors vary over three orders of magnitude: 1.4 molal$^{-1}$ (Levitt, 1978*b*) to 120 molal$^{-1}$ (Urban et al., 1980). The estimate by Urry et al. (1980) in terms of a three-cation-occupiable three-site model is as high as 300 M$^{-1}$. The origin of this divergence of estimated binding constants is not necessarily clear.

When the single-site model is used, the estimate for $K_1$ is rather reproducible: 3.1 M$^{-1}$ (Neher, 1975) to 3.3 M$^{-1}$ (Läuger, 1973). This is because in this case, $K_1$ has a simple relationship to conductance $G$. When a more-than-one-cation-occupiable channel is assumed, the $G$-$a$ relationship involves at least five adjustable parameters (see Eq. 33) so that they are not easy to estimate properly. Urban et al. (1980) reported a considerably large estimate for $K_1$ (120 molal$^{-1}$). This value was not necessarily demanded by the observed $G$-$a$ relationship; it developed chiefly to explain the observed inequality of the permeability and conductance ratios for $Na^+$ and $K^+$ ions at low ionic activities (e.g., 10 mmolal). The estimate of $K_1$ by Eisenman et al. (1978*b*) seems somewhat arbitrary since the presence of the foot in the $G$ vs. $G/a$ plot was assumed from analogy with other alkali cations. The values (100 and 300 M$^{-1}$, depending on models) calculated by Urry et al. (1980) from NMR data are at least eight times greater than that obtained in the present NMR study in spite of the fact that like models are used for data analysis. On the basis of the equilibrium dialysis study of Veatch and Durkin (1980), values of $K_1$ greater than a few tens in molal$^{-1}$ seem overestimated.

The estimate for the ratio $K_1/K_2$ also shows a considerable variation, ranging from 8 (Levitt, 1978*b*) to 600 (Urban et al., 1980). The present estimate is 8 or ~4, depending on models used. If the inequality of $K_1$ and $K_2$ is solely attributable to the electrostatic interaction between the bound cations within a channel, then $K_1/K_2$ values such as 300 (Urry et al., 1980) and 600 (Urban et al., 1980) seem too large (see Table 3 of Monoi, 1983). In other words, if these values are correct, it is required that a considerable change in channel conformation occurs upon binding of cations. According to Wallace et al. (1981), however, the circular dichroism spectra of gramicidin-packaged lecithin vesicles exhibit no change over a wide range of NaCl or CsCl concentrations (0–2 M), indicating that no major change in channel conformation is produced by the binding of ions.

## Rate Constants

Experimental values of $k_{-1}$ of $Na^+$ ions for the gramicidin channel reported by different authors show a considerable variation. Estimates from electrical measurements range from $4.5 \times 10^5$ s$^{-1}$ (Urban et al., 1980) to $8 \times 10^7$ s$^{-1}$ (Läuger, 1973); according to Levitt (1978*b*), $k_{-1}$ is $4 \times 10^7$ s$^{-1}$. Values of $k_{-1}$ obtained from $^{23}$Na-NMR study were $5.5 \times 10^8$ s$^{-1}$ (Cornélis and Laszlo, 1979; in an ethanol-water mixture) and $3 \times 10^5$ s$^{-1}$ (Urry et al., 1980; in a micellar system). The latter is measured from the frequency shift of $^{23}$Na lines. The present study shows that the value $8 \times 10^5$ s$^{-1}$ represents a rough approximation of $k_{-1}$ at 25°C. This estimate is consistent with the values reported by Urban et al. (1980) and Urry et al. (1980).

On the other hand, the estimate of $k_{-2}$ ($2.6 \times 10^8$ s$^{-1}$) by Urban et al. (1980) is considerably different from the present estimation: $k_{-2} \lesssim 1 \times 10^7$ s$^{-1}$. This is rather similar to the magnitude ($4 \times 10^7$ s$^{-1}$) reported by Urry et al. (1980). This agreement seems to be a fortuitous one, since the value from Urry's laboratory was calculated under the two assumptions: (*a*) $k_{-2}$ is equal to the reciprocal of $\tau_c$ of bound $^{23}$Na at high $Na^+$ levels, and (*b*) $\tau_c$ for bound $^{23}$Na can be obtained from $\rho_2$ by using Eq. 23. These assumptions do not hold, as argued in the Results section entitled Correlation Time.

According to Urban et al. (1980), the ratio $k_{-2}/k_{-1}$ is 600, which is inconsistent with our estimation that this ratio does not greatly exceed 20. It is usually assumed that $k_{-2}$ is $> k_{-1}$ owing to electrostatic repulsion between bound cations. If the distance of separation between the bound cations is 20–21 Å (Koeppe et al., 1979; Urry et al., 1982), this repulsive force is calculated to increase the second binding constant (and hence $k_{-2}$) by a factor of several tens at most (Monoi, 1983). The estimate for $k_{-2}/k_{-1}$ by Urban et al. (1980) seems thus difficult to explain in terms of simple electrostatic interactions between bound cations.

## Conformation Change upon Binding of Cations?

Within the framework of the two-identical-site model, $T_{1B}^{doub}$ was faster than $T_{1B}^{sing}$ by a factor of 2.1 (25°C) or 2.9 (10°C). The correlation time $\tau_c$ for bound $^{23}$Na in both states of occupancy was 2 ns, which is near the $T_1$-minimum at the radio frequency employed (79.4 MHz). Therefore, the observed difference between $T_{1B}^{sing}$ and $T_{1B}^{doub}$ is chiefly due to different magnitudes of the quadrupole coupling constants $\chi$ for single and double occupancies (denoted by $\chi^{sing}$ and $\chi^{doub}$, respectively). The ratio $\chi^{doub}/\chi^{sing}$ was 1.4 (approximately the square root of 2.1) at 25°C; at 10°C, it will be greater than this value.

The same trend (i.e., $\chi^{doub}/\chi^{sing} > 1$) was also reported by Urry et al. (1980). They attributed this finding to the electrostatic interaction between bound cations within a channel. This is not the case, however, as shown in Table IV, which gives calculated values for the electrostatic field gradient at the position of a bound cation in the presence and absence of a second cation near the opposite end. As is immediately clear, the electrostatic field gradient remains virtually unchanged (even tends to decrease) upon binding

TABLE IV
ELECTROSTATIC-FIELD GRADIENT AT THE POSITION OF A BOUND CATION IN TWO-CATION-LOADED CHANNELS

| Dimple | Effective pore radius | Position | | Field gradient* |
|---|---|---|---|---|
| | | Cation 1 | Cation 2 | |
| | Å | Å | Å | |
| None | 3 | 12.0 | −12.0 | 0.73 |
| | 3 | 10.5 | −10.5 | 0.96 |
| | 3 | 9.5 | −9.5 | 0.98 |
| | 4 | 10.5 | −10.5 | 0.91 |
| | 2 | 10.5 | −10.5 | 0.99 |
| 5-Å dimple | 3 | 12.0 | −12.0 | 0.97 |
| | 3 | 10.5 | −10.5 | 0.99 |
| | 3 | 9.5 | −9.5 | 0.99 |
| | 4 | 10.5 | −10.5 | 0.98 |
| | 2 | 10.5 | −10.5 | 0.99 |

*Numerals represent the components of the field gradients along the channel axis, expressed relative to the values in the absence of a second cation. The electric charge of the second cation is supposed to be the same as of the first. For details, see text.

of a second cation if the local interaction between a bound cation and the channel wall is the same in the presence and the absence of a second cation. Note that even if the gradient is greater in the presence of a second cation, $T_1$ for a first cation is not affected because the mean residence time of bound $^{23}Na$ is much longer than its intrinsic $\tau_c$ and the Larmor period (see the Results section entitled Correlation Time), whereas $T_1$ involves no contribution of the spectral density at low frequencies.

Therefore, the two-identical-site model demands that local interactions between the bound cation and the wall of the gramicidin channel are dependent on the state of ion loading of the channel. This would be accounted for only if a considerable change in channel conformation occurs upon binding of cations. With more involved models, such a conformation change is not necessarily required since $T_{1B}^{sing}$ is not necessarily different from $T_{1B}^{doub}$ (see Tables II and III and Eqs. 15 and 16).

Again within the framework of the two-identical-site model, the standard entropy change for the binding of a second $Na^+$ ion was much greater than that for a first $Na^+$ ion (see the Discussion section entitled Binding Constants), also consistent with a change in channel conformation. Thus, the simple two-identical-site model leads to the notion that the gramicidin channel undergoes a conformation change upon binding of cations. Such a conformation change, however, has not been detected (Wallace et al., 1981).

On the other hand, if we apply more involved channel models, such as two-identical-site channels involving the binding of an additional ion (cation or anion), then a conformation change upon binding of cations is not necessarily required to explain observed data. Nevertheless, the binding of a third cation seems very unlikely for electrostatic reasons (Levitt, 1978*a*; Monoi, 1983), and the binding of anions has recently been suggested only theoretically (Monoi, 1982, 1983).

## APPENDIX

## Effects of Instrumental Parameters in Continuous-Wave Wide-Line NMR Spectroscopy

The Appendix describes combined effects of field inhomogeneity, radio-frequency power, modulation amplitude, and response time in continuous-wave wide-line spectroscopy (in which the method of phase-sensitive detection is used) on the recorded signal of a super-Lorentzian line of spin-3/2 nuclei.

We suppose that an assembly of nuclear spins is in a uniform static magnetic field $H_0$ oriented along the $z$-axis of the laboratory frame $O$-$xyz$ and is perturbed by a linearly polarized radio-frequency field $H_x = 2H_1 \cos \omega t$, $H_y = H_z = 0$. We suppose further that quadrupole relaxation is the only relaxation mechanism, and that the transverse magnetization is represented in terms of a super-Lorentzian.

*Super-Lorentzian in the Presence of Saturation.* In the absence of saturation, the normalized shape function for the $1/2 \leftrightarrow -1/2$ and $\pm 3/2 \leftrightarrow \pm 1/2$ components of a super-Lorentzian line are, under the condition of slow passage,

$$g'(\omega) = \frac{1}{\pi} \frac{T_2'}{1 + T_2'^2(\omega_0' - \omega)^2}, \tag{A1}$$

$$g''(\omega) = \frac{1}{\pi} \frac{T_2''}{1 + T_2''^2(\omega_0'' - \omega)^2}, \tag{A2}$$

respectively, where $T_2'$ and $T_2''$ are the slow and the fast $T_2$, and $\omega_0'$ and $\omega_0''$ are the Larmor frequencies for the narrow and the broad component. In what follows, we consider a limiting case in which $\omega_0' = \omega_0'' (\equiv \omega_0)$.

In the presence of saturation, the absorption line is proportional to (Monoi, H., unpublished results)

$$v = 0.4\, v' + 0.6\, v'', \tag{A3}$$

$$v' = -\frac{\gamma H_1 T_2' M_0}{1 + T_2'^2(\omega_0 - \omega)^2 + \gamma^2 H_1^2 T_1' T_2'} F', \tag{A4}$$

$$v'' = -\frac{\gamma H_1 T_2'' M_0}{1 + T_2''^2(\omega_0 - \omega)^2 + \gamma^2 H_1^2 T_1'' T_2''} F'', \tag{A5}$$

$$F' = \frac{\begin{array}{l} 1 + \pi\gamma^2 H_1^2 T_1' g'(\omega) + 6\pi\gamma^2 H_1^2 T_1'' g''(\omega) \\ \quad + 6\pi^2\gamma^4 H_1^4 T_1' T_1'' g'(\omega) g''(\omega) \end{array}}{\begin{array}{l} 1 + 2\pi\gamma^2 H_1^2 (T_1' + T_1'') g'(\omega) \\ \quad + 3\pi\gamma^2 H_1^2 T_1'' g''(\omega) \\ \quad + 6\pi^2\gamma^4 H_1^4 T_1' T_1'' g'(\omega) g''(\omega) \end{array}}, \tag{A6}$$

$$F'' = \frac{\begin{array}{l} 1 + 2\pi\gamma^2 H_1^2 (T_1' + 2T_1'') g'(\omega) \\ \quad + \pi\gamma^2 H_1^2 T_1'' g''(\omega) \\ \quad + 2\pi^2\gamma^4 H_1^4 (T_1' + 2T_1'') T_1'' g'(\omega) g''(\omega) \end{array}}{\begin{array}{l} 1 + 2\pi\gamma^2 H_1^2 (T_1' + T_1'') g'(\omega) \\ \quad + 3\pi\gamma^2 H_1^2 T_1'' g''(\omega) \\ \quad + 6\pi^2\gamma^4 H_1^4 T_1' T_1'' g'(\omega) g''(\omega) \end{array}}, \tag{A7}$$

where $\gamma$ is the gyromagnetic ratio, $M_0$ is the thermal equilibrium value of the macroscopic magnetization $M$, and $T_1'$ and $T_1''$ are the slow and the fast $T_1$, respectively. The narrow and the broad component are proportional to $0.4\,|v'|$ and $0.6\,|v''|$, respectively. Coefficients $F'$ and $F''$ are equal to one when $T_1' = T_1''$ and $T_2' = T_2''$. The components of a

super-Lorentzian generally retain no Lorentz form in the presence of saturation (see Fig. 6 of Monoi, 1985).

*Field Inhomogeneity.* Suppose that $h(x)$ (normalized to $\int h[x]\mathrm{d}x = 1$) is the distribution function of $\omega_0$ due to the field inhomogeneity and that $h(x)\mathrm{d}x$ represents the relative weight of spins with $\omega_0$ between $\omega_0^0 + x$ and $\omega_0^0 + x + \mathrm{d}x$ ($\omega_0^0$ is the value of the Larmor frequency with the maximum weight). Then, the out-of-phase component, $v^\dagger(\Delta\omega)$ (where $\Delta\omega = \omega - \omega_0$), of $M$ in an inhomogeneous field is related to $v(\Delta\omega)$ through (Abragam, 1961)

$$v^\dagger(\Delta\omega) = \int_{-\infty}^{+\infty} v(\omega - \omega_0^0 - x)h(x)\mathrm{d}x. \qquad \text{(A8)}$$

We simply put $\omega_0^0 = \omega_0$ for the sake of simplicity. Assume a Lorentz form for $h(x)$:

$$h(x) = \frac{1}{\pi}\frac{T_2^\dagger}{1 + T_2^{\dagger 2}x^2}, \qquad \text{(A9)}$$

where $1/T_2^\dagger$ is the half-width at half-height, due to the field inhomogeneity. The half-width in the presence of field inhomogeneity and of negligible saturation is $1/T_2^* = 1/T_2 + 1/T_2^\dagger$.

*Modulation Amplitude.* Let $\omega_s(t)$ be the angular frequency of the radio-frequency field whose time dependence involves only a sufficiently slow sweep across the absorption line and $\omega_m$ be the amplitude of the sinusoidal frequency modulation with frequency $\omega_\mu$, which is sufficiently small. (Field modulation is usually employed in the wide-line method. The use of a fixed magnetic field and modulated radio frequency is known to be equivalent to the use of a fixed radio frequency and modulated magnetic field when the modulation frequency is sufficiently low.)

When phase-sensitive detection at the modulation frequency is used, the output signal is proportional to (Wahlquist, 1961)

$$a_1(\omega_\delta) = \frac{2}{\pi}\int_0^\pi v^\dagger(\omega_\delta + \omega_m \cos\omega_\mu t)\cos\omega_\mu t\,\mathrm{d}(\omega_\mu t), \qquad \text{(A10)}$$

where $\omega_\delta = \omega_s - \omega_0$.

*Response Time.* We may write the linear scan as

$$\omega_s - \omega_0 = kt, \qquad \text{(A11)}$$

where $k$ is the constant sweep rate. The output of the phase-sensitive detector, proportional to $a_1(kt)$, frequently traverses a time-constant ($\tau$) network, which converts it to a new shape function proportional to (Štirand, 1962)

$$a_1^p(kt) = \frac{1}{\tau}\exp(-t/\tau)\int_{-\infty}^{t}\exp(t'/\tau)\,a_1(kt')\mathrm{d}t'. \qquad \text{(A12)}$$

*Calculated Peak-to-Peak Height.* The line shape of the output derivative-of-absorption-mode signal, $a_1^p$, of a super-Lorentzian line can be calculated by using Eqs. A1–A12. We simply postulate $T_1' = T_1'' = T_2'$ because this relation is often approximately valid for ions in aqueous heterogeneous samples. The absorption line used as the standard is supposed to be a simple Lorentzian and characterized by single values of $T_1$ and $T_2$ ($T_1 = T_2$). The superscript s will be used to designate quantities for the standard. The value of $^{23}$Na ions in various aqueous materials including NaCl solutions falls into a sixfold range (57 to 9.5 ms at 25°C). In the present wide-line experiments, it falls within a fourfold range; i.e., the ratio $T_2^s/T_2'$ is from 1 to 4 when a dilute NaCl solution is used as the standard. The ratio $T_2'/T_2''$ will be denoted by $r$.

In Fig. 8, the pp height of $a_1^p$ is plotted against $1/T_2'$ at various levels of $r$. The values of instrumental parameters assumed are: $T_2^s/T_2^\dagger = 15$, $\omega_m T_2^{*s} = 3$, $\gamma H_1(T_1^s T_2^s)^{1/2} = 0.47$, and $\tau/\tau_{pp} = 0.15$. $T_1' = T_1'' = T_2'$ is assumed. Numerals in the figure represent $r = T_2'/T_2''$. The pp height for the standard sample is normalized to one. The abscissa is the intrinsic width $1/T_2'$ of the narrow component normalized with respect to $1/T_2^s$.

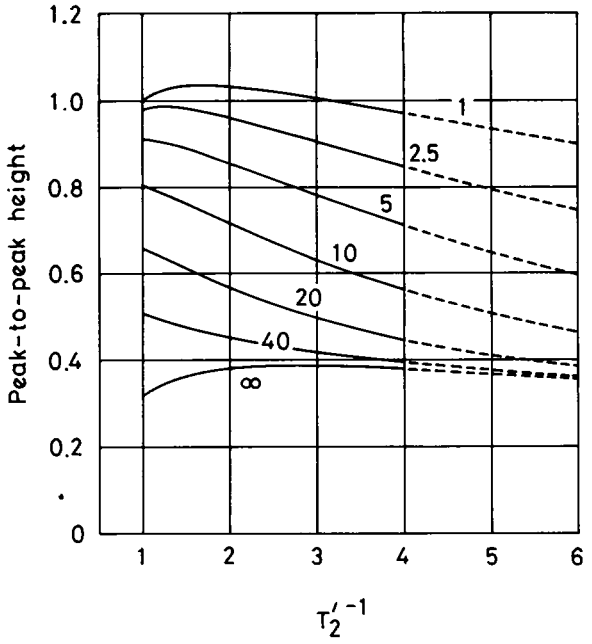


FIGURE 8 Effect of the ratio $T_2'/T_2''$ on the pp height of $a_1^p$ for a super-Lorentzian line of spin-3/2 nuclei. $T_2^s/T_2^\dagger = 15$, $\omega_m T_2^{*s} = 3$, $\gamma H_1(T_1^s T_2^s)^{1/2} = 0.47$, and $\tau/\tau_{pp} = 0.15$. $T_1' = T_1'' = T_2'$ is assumed. Numerals in the figure represent $r = T_2'/T_2''$. The pp height for the standard sample is normalized to one. The abscissa is the intrinsic width $1/T_2'$ of the narrow component normalized with respect to $1/T_2^s$.

As shown in this figure, when $r = 1$ (i.e., the absorption line is a Lorentzian), the pp height of the recorded signal is ~100%; it is within 100 ± 3% for the range of $T_2^s/T_2'$ between 1 and 4. When $r$ is sufficiently large, the pp height is roughly equal to, or slightly less than, 40%. For intermediate values of $r$, the pp height ranges from 40 to 100%, depending on both $r$ and $T_2'$. (The pp width is practically independent of $r$ and $T_2'$.) These results also hold for pseudosuper-Lorentzians if the reciprocal of the apparent half-width of the broad component is taken as $T_2''$. The values for the instrumental parameters used in the present wide-line experiments correspond approximately to instrumental condition I given above.

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