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Thallium-205 NMR Studies of the Tl(I) Ion Complexation by Gramicidin in Non-Aqueous and Micelle Solutions

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Abstract - The potential use of thallium-205 nmr spectroscopy for the study of ion-transport across biological membranes mediated by antibiotic molecules is discussed. The complexation of the Tl(I) ion by the antibiotic, gramicidin, in non-aqueous solutions of dioxane, DMSO, trifluoroethanol and micelle solutions is used as a model system.

INTRODUCTION

The advent of pulsed-Fourier transform nuclear magnetic resonance (nmr) spectroscopy together with wide-bore, very high-field spectrometers have opened the periodic table of elements to investigation in an extraordinary manner. With a few exceptions, any nuclide with spin can now be observed directly in solution and in many cases in the solid state with the use of multipulse techniques and magic angle spinning. One element that is particularly well suited for the nmr experiment due to it's intrinsic nuclear properties is thallium. Thallium has two isotopes Tl-203 (29.5% natural abundance) and Tl-205 (70.5% natural abundance) which have a nuclear spin of ½. The relative receptivity of the more abundant isotope, Tl-205, is 0.1355 compared to the proton which has a value of 1. This makes the Tl-205 nucleus the third most receptive spin ½ nuclide in the periodic table and, therefore, one that is easy to directly observe in the nmr experiment. The ease with which thallium nmr signals can be observed is illustrated in Fig. 1. The spectrum shows

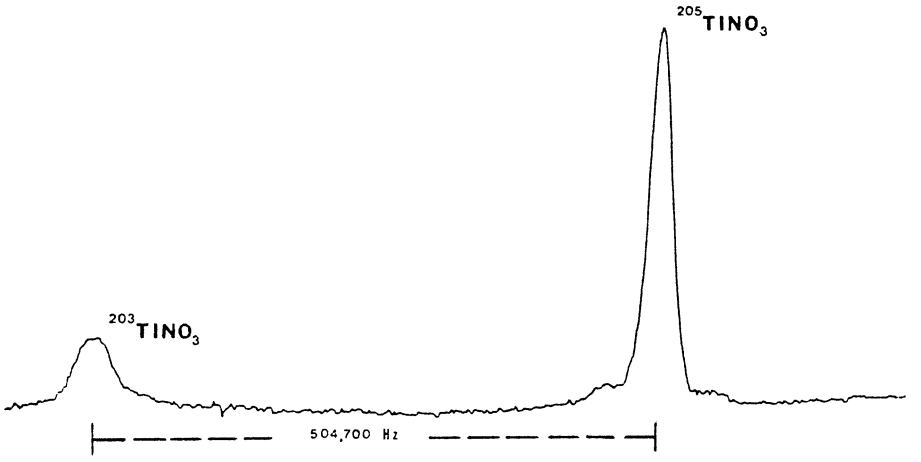

Fig. 1. Solid state Tl-203 and Tl-205 nmr spectrum of $TlNO_3$.

the simultaneous observation of both isotopes of thallium in a solid state $TlNO_3$ sample whose resonance frequencies are over 500,000 Hz apart. This spectrum was obtained without using any of the more sophisticated techniques such as magic angle spinning, but just the normal pulsed-Fourier transform technique with the solid contained in a 5 mm tube. The very high sensitivity to detection also permits one to obtain spectra from solutions whose thallium concentration is about 5×10^{-4} M.

Not only do the nuclear properties of the thallium isotopes make them easy to observe in the nmr experiment, but the spectral parameters of chemical shift, coupling constant and spin-lattice relaxation time are exceptionally sensitive to the chemical environment in which the thallium nucleus is placed. The chemical shift range for the Tl-205 nucleus extends over 7000 ppm; a one bond thallium-carbon coupling constant can be over 10,000 Hz; the presence of dissolved oxygen can change the spin-lattice relaxation time of the Tl(I) ion in water by over an order of magnitude.

Thallium exists in two oxidation states (+1 and +3) which provides a significant variety in the chemistry of this element. Organothallium compounds contain the thallium in the +3 state most frequently while simple inorganic salts are normally of the +1 state. Furthermore, the Tl(I) ion is a good replacement probe for the Na(I) and K(I) ions in many biological systems, thus providing a convenient spectroscopic alternative for the study of such phenomena as ion transport across membranes, activation and regulation of enzymes.

The very high receptivity to detection, the exceptional sensitivity of the resonance frequency of the Tl(I) ion to its chemical environment, and the similarity in behavior to the Na(I) and K(I) ions have led us to explore the use of Tl-205 nmr spectroscopy as a probe for studying ion-transport across biological membranes mediated by antibiotics. We have shown recently that the Tl-205 resonance frequency is extremely sensitive to the number and types of functional groups in the antibiotic that bind the Tl(I) ion (1). This resonance frequency dependence appears to have the potential for acting as a "fingerprint" in identifying unknown transport antibiotics in naturally occurring membrane systems. Not only can one use the resonance frequency to identify functional groups within an antibiotic responsible for binding cations but it can also be used to determine binding constants, thermodynamic parameters, and binding stoichiometries. In order to test the Tl(I)-205 nmr probe for obtaining this information we have studied the binding of the Tl(I) by the antibiotic, gramicidin, in non-aqueous solutions and in model membrane systems (i.e. micelles).

Gramicidin-A (Fig. 2), a pentadecapeptide isolated from Bacillus brevis, is known to form channels which assist the passive transport of cations across membranes (2-6). In membranes the channel is formed by helical gramicidin dimers (7-10) connected by N-terminal

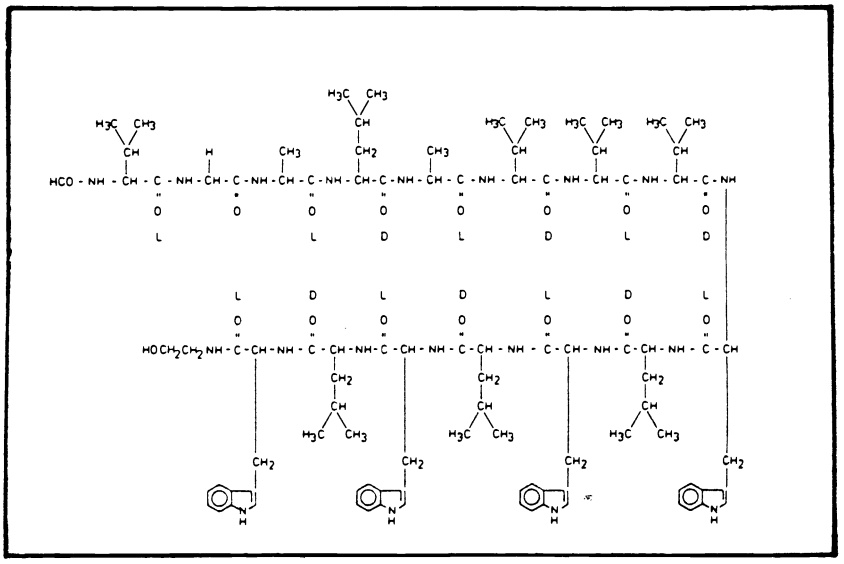

Fig. 2. Gramicidin-A molecular structure.

to N-terminal interaction (9,11-14). In solvents such as trifluoroethanol and dioxane gramicidin also forms helical dimers (15-18). Complexes formed with gramicidin and monovalent cations have also been studied (19-22). Specifically, it was of interest to compare the Tl-205 nmr results with those obtained by equilibrium dialysis (23) and electrical measurements (24-26) where gramicidin was incorporated in a membrane, to obtain the number of strong binding sites per dimer in solution and in micelles and for the monomer in solution, to determine the binding constant and to determine the extent of solvation of the ion in the channel.

EXPERIMENTAL PROCEDURES

The thallium(I) salts (acetate and nitrate) used were multiply recrystallized from water and dried at 90°C and 40 mm Hg for at least 24 hours in a vacuum oven. All solvents were purified by standard procedures. A commercial preparation of gramicidin was obtained from ICN Pharmaceuticals. All of the samples were prepared in a glove box under dry nitrogen and sealed in 5 mm nmr tubes.

Gramicidin was incorporated into lysolecithin micelles using the procedures of Urry (27-28). Samples for Tl-205 NMR spectroscopy were prepared by diluting aliquots of the lysolecithin-gramicidin suspension with 0.2 M $TlNO_3$ degassed aqueous solution to give the desired $TlNO_3$ and gramicidin concentrations. Samples were stored at 68°C until placed in the spectrometer.

For each gramicidin-containing sample an aqueous $TlNO_3$ solution at the same Tl^+ concentration was prepared. The resonance frequency of this sample was used as the "free" ion resonance frequency for that $TlNO_3$ concentration in calculating the gramicidin induced shift.

For correction of the effect of just the lysolecithin micelles on the $TlNO_3$ resonance frequency, a series of samples containing the same lysolecithin concentration as the gramicidin samples and with $TlNO_3$ concentration varying over the same range were prepared. The lysolecithin-induced shift in the Tl-205 resonance frequency at a given $TlNO_3$ concentration was subtracted from the gramicidin-induced shift to remove the lysolecithin effect. The assumption is made that the incorporation of gramicidin into the micelle does not significantly alter the properties of the micelle toward the binding of the thallous ion. The correction for micelle binding was most significant in the high thallous ion concentration range. It was found that the lysolecithin micelles induced a shift to high frequency in the Tl-205 resonance frequency while the gramicidin-induced shift was to low frequency referenced to an aqueous solution of $TlNO_3$ at the same salt concentration.

The pertinent data obtained were, therefore, the gramicidin-induced shift of the thallous ion resonance frequency for a fixed gramicidin concentration as a function of varying thallous ion concentration.

In the study of the complexation of the Tl(I) ion by gramicidin in trifluoroethanol and DMSO a constant concentration of the ion (0.012 M) was "titrated" with gramicidin. Again, the induced shift in the Tl-205 resonance frequency was used to obtain the desired information about the binding process. In all solutions, both the non-aqueous and micelle, only one resonance signal was observed indicating fast exchange between all of the chemical environments associated with the ion.

The intact (non-exchanging) Tl(I)-gramicidin complex was studied in p-dioxane. p-Dioxane was used as the solvent because thallium salts are insoluble in it, the thallium-antibiotic complex does not dissociate and because gramicidin is known to exist as the helical dimer in this solvent (16). The complex was prepared by standard methods (30).

The spectrometer used for all measurements was a modified Bruker HFX-90 that has been described in the literature (29).

RESULTS AND DISCUSSION

The Tl-205 nmr signal of the Tl(I)-gramicidin complex in p-dioxane was found to occur at the lowest resonance frequency (highest field) observed for any of the antibiotic complexes studied thus far. A comparison of the resonance frequency of the Tl(I)-gramicidin complex with those of other Tl(I)-antibiotic complexes previously studied is shown in Table 1. The position of the chemical shift of the Tl(I) ion in the gramicidin complex is consistent with the ordering resulting from the basicity of the binding functional group(s) previously established (31) and shown in Table 1. The chemical shift range of 1000 ppm for the Tl(I) ion complexed by the various antibiotics strongly suggests that the chemical shift might be used as a "fingerprint" for binding functionally (1).

TABLE 1. Relationship between the number and type of ligand atoms and the Tl-205 chemical shift of some Tl^+-antibiotic complexes.

Antibiotic[a]	(Tl-205)[b,c] (ppm)	Number and type of ligands[d]			
		C=O	C-O-C	C-OH	COO^-
Lasalocid	297.6	1(or 2)	1	2	1(or 2)
Monensin-H	134.1		4	2	
Nigericin$^-$	126.6	4(or 5)			1(or 0)
Monensin$^-$	106.7		4	2	
18-Crown-6	-162.0		6		
Nonactin	-261.5	4	4		
Monactin	-261.7	4	4		
Dinactin	-262.0	4	4		
Valinomycin	-540.0	6			
Gramicidin[e]	-675.3	4 to 8 (plus 4 tryptophan rings)			
	-685.6[f]				
	-700.0[g]				

[a] 0.1 M solutions at 24°C in $CHCl_3$.
[b] B_o = 2.114 T.
[c] Reference is Tl^+ in H_2O at infinite dilution.
[d] Basicity increases in the order C=O < COC < COH < COO^-.
[e] Complex concentration was approximately 0.003 M in p-dioxane at 50°C.
[f] Solution contained 0.020 M trifluoroethanol at 50°C.
[g] Solution contained 0.220 M H_2O at 50°C.

Having established the chemical shift of the Tl(I) ion in the gramicidin complex as being in the carbonyl functionality range, it was important to try to locate the tight binding site in the gramicidin channel. The X-ray crystallographic results for the K(I)-gramicidin complex suggested that the tight binding site was located either 2.5 A from the channel entrance or in the middle of the channel dimer (32). The helical dimer of the type presented by Ovchinnikov (33) incorporating the cation at the channel entrance is shown in Fig. 3. At the channel entrance one could assume four to eight carbonyl oxygens

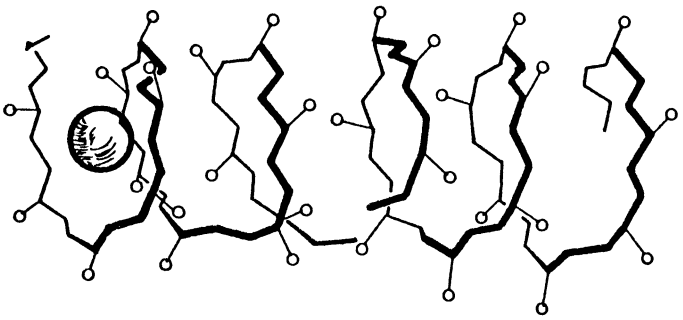

Fig. 3. Helical gramicidin dimer with bound cation at channel entrance.

available for binding the cation. However, the interior of the channel is also lined with carbonyl groups so that one must try to resolve the question of the location of the tight binding site by other experimental means. We have attempted to answer this question by observing the effect on the Tl(I) chemical shift of the addition of very small amounts of water or trifluoroethanol to the p-dioxane solution containing the Tl(I)-gramicidin complex. As shown in Table 1, the Tl-205 resonance signal moved to lower frequency (higher field) upon the addition of water or trifluoroethanol. Several important conclusions may be drawn from these results. The direction of the shift in resonance frequency implies that no water entered the channel to interact with the Tl^+ ion since a shift to higher resonance frequency would have been observed. It also implies that the Tl^+ ion is close to the CH_3COO^- ion to sense the effect of partial solvation of the anion and hence the reduction of the ion-pair interaction. It is known that decreasing ion-pair formation of $Tl^+CH_3COO^-$ in H_2O produces a shift to low frequency in the ^{205}Tl resonance frequency. Since the anion cannot enter the channel because of electrostatic repulsion from the carbonyl groups that line the interior of the channel, this strongly suggests that the cation is located at the channel entrance rather than in the center of the channel, where ion-pair interaction would be minimal. The same experiment was performed by adding trifluoroethanol to a p-dioxane solution of the Tl^+-gramicidin complex instead of water. Trifluoroethanol is known to solvate anions very effectively and to interact relatively weakly with cations (33), and because of its size is much less likely to enter the channel than water. Again, a shift to low resonance frequency was observed (see Table 1). The reduction of ion-pair formation of $Tl^+CH_3COO^-$ in trifluoroethanol produces a low-frequency shift. This same type of effect was observed for Cs^+-cryptate complexes (34).

To further define the ion binding properties of the gramicidin dimer channel, the complexation of the Tl(I) ion by gramicidin has been studied in trifluoroethanol, a solvent in which gramicidin is in the helical dimer form, and in DMSO, a solvent in which the monomer form is dominant (35). It was of interest to determine the number of strong binding sites in the gramicidin dimer and monomer and, if possible, the thermodynamic parameters for complexation.

Figure 4 shows the relationship between Tl(I)-205 chemical shift and gramicidin concentration in trifluoroethanol. A cursory examination of the data obtained for the trifluoroethanol system shows a very large change in the chemical shift of Tl(I), about 360 ppm, with the addition of gramicidin which indicates a relatively strong interaction between the ion and antibiotic. The experimental data used for the model fitting procedure were for ratios of less than 1 to ensure that an excess of gramicidin was present. Since the gramicidin dimer presents identical ends to the solution for complexing ions, we

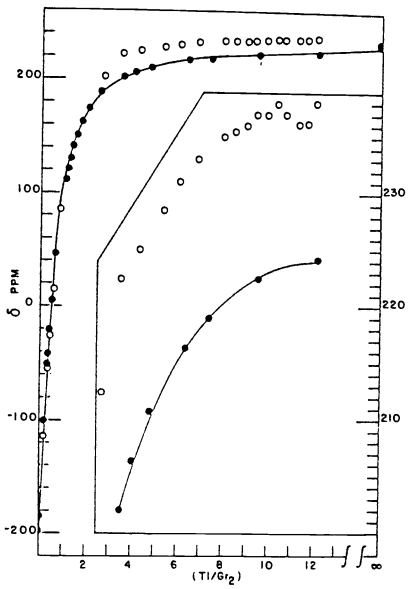

Fig. 4. Plot of the thallium chemical shift as a function of the mole ratio of total available thallium concentration (i.e., total thallium concentration minus the Tl^+OAc^- ion pair concentration) to the gramicidin dimer concentration. The inset is the same plot but with an expanded parts per million scale. The same mole ratio scale is used for both plots. The temperature is 298 K. The open circles are experimental data, and the solid line with filled circles represents the theoretical curve of the strong binding sites only.

chose a two-site binding model for each dimer compatible with models suggested by Sandblom (36) and Laszlo (19). The pertinent equilibrium expressions are as follows:

$$Tl^+ + OAc^- \rightleftharpoons Tl^+OAc^-$$

$$K_1 = \frac{[Tl^+OAc]\gamma_{Tl^+OAc^-}}{[Tl^+]\gamma_{Tl^+}[OAc^-]\gamma_{OAc^-}} \quad (1)$$

$$Tl^+ + Gr_2 \rightleftharpoons (Tl^+Gr_2)$$

$$K_2 = \frac{[Tl^+Gr_2]\gamma_{(Tl^+Gr_2)}}{[Tl^+]\gamma_{Tl^+}[Gr_2]\gamma_{Gr_2}} \quad (2)$$

$$Tl^+ + (Tl^+Gr_2) \rightleftharpoons (Tl_2^{2+}Gr_2)$$

$$K_3 = \frac{[Tl_2^{2+}Gr_2]\gamma_{(Tl_2^{2+}Gr_2)}}{[Tl^+]\gamma_{Tl^+}[Tl^+Gr_2]\gamma_{(Tl^+Gr_2)}} \quad (3)$$

where the γ's represent the activity coefficients of the species involved in the equilibria.

Taking into account the ion-pairing equilibrium process between Tl(I) and the acetate counterion and assuming that the binding constant at both ends of the molecule was the same, the chemical shift of the Tl(I)-205 ion as a function of gramicidin dimer concentration below a mole ratio of 1 was calculated and found to agree quite well with the experimental data as shown in Fig. 4. (See reference 36 for the details of the calculation.) Equilibrium or binding constants were obtained from the model calculations. The binding constants, determined as a function of temperature, were used to calculate the thermodynamic parameters of ΔH = -2.13 kcal/mole and ΔS = +5.45 e.u. Other models, such as one tight binding site per dimer, were explored but found not to fit the experimental data.

Further verification that the tight binding sites of the gramicidin dimer are at the entrance to the channel and not in the middle was obtained with C-13 nmr spectroscopy. Figure 5 shows the carbonyl resonance signals for gramicidin (top) and gramicidin-Tl(I) (bottom and inverted) in trifluoroethanol. The two peaks at 165.4 ppm and 172.2 ppm are assigned to the formyl and glycyl carbonyl carbons which are located at the end of the molecule opposite to the channel entrance and in the middle of the dimer. While it is

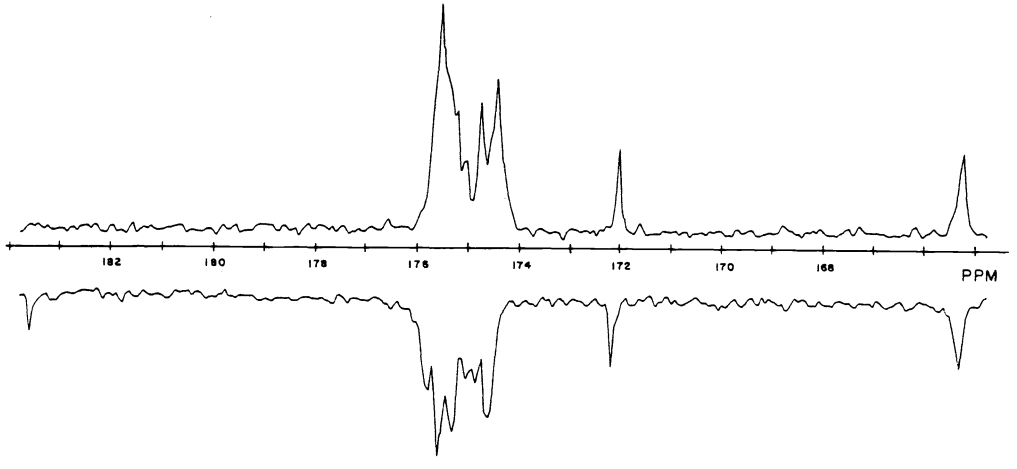

Fig. 5. C-13 nmr spectrum of carbonyl carbon region of gramicidin. Bottom inverted spectrum is that of the Tl(I)-gramicidin complex and the top spectrum is that of just gramicidin.

obvious that some carbonyl carbon resonances are shifted upon ion complexation, the two in the middle of the dimer are unchanged.

The complexation of the Tl(I) ion by the gramicidin monomer in DMSO solution has been studied using the Tl-205 nmr technique developed. Figure 6 shows the experimental chemical shift data obtained for this system as a function of Tl(I)-gramicidin mole fraction. A model consisting of only one tight binding site per gramicidin monomer was the only one that would adequately fit the experimental data. The agreement between the theoretically predicted chemical shift for the Tl(I) ion and the experimental values is shown in Fig. 6. The intersection of the extrapolated linear portions of the experimental data also occurs at a mole ratio of 1.

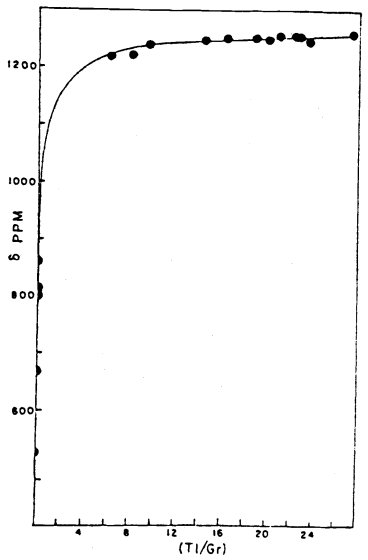

Fig. 6. Plot of thallium chemical shift as a function of the mole ratio of total thallium concentration to the gramicidin monomer concentration at 303 K in DMSO. Solid line represents the theoretical curve and circles are experimental data.

Carbon-13 nmr studies of the Tl(I)-gramicidin monomer complex also indicate that only one end of the monomer (i.e. the tryptophan end) is involved in binding the cation and that the formyl end of the molecule is not involved to any detectable extent. Figure 7 shows the carbonyl carbon resonance signals for the gramicidin monomer in DMSO and in solutions containing the Tl(I) ion. Again, the formyl and glycyl carbonyl carbon signals remain unchanged.

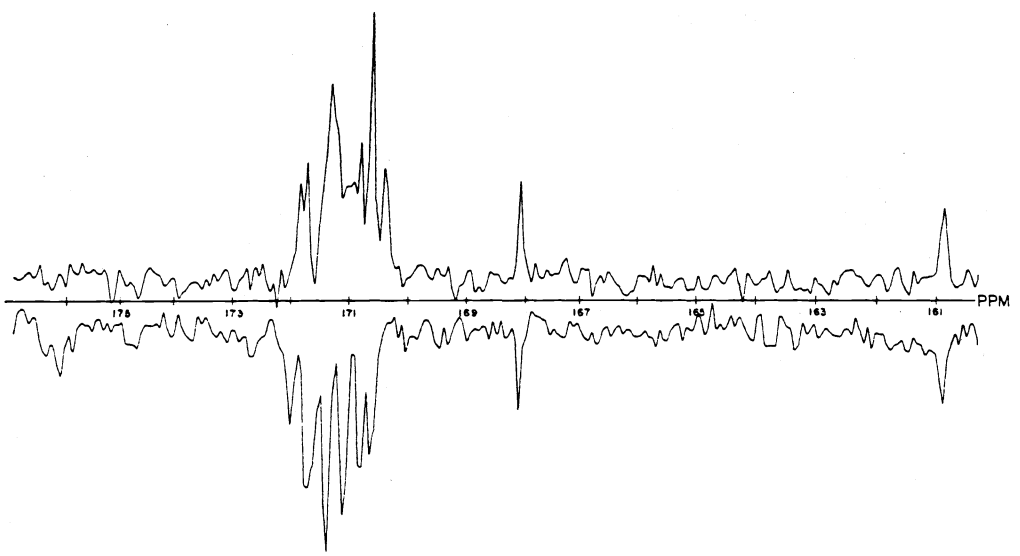

Fig. 7. C-13 nmr spectrum of gramicidin carbonyl carbon region (top) and carbonyl region of Tl(I)-gramicidin complex (bottom, inverted).

Finally, the complexation of the Tl(I) ion by gramicidin incorporated into a model membrane (i.e. micelles) has been studied by the Tl-205 nmr technique. In these experiments the gramicidin dimer concentration in the micelles was fixed at 0.0052 M while the Tl(I) ion concentration was varied from 0.065 M to 0.0025 M.

For Tl^+ ions rapidly exchanging between a free state (f) in solution and a gramicidin bound state (b), the observed chemical shift, δ_o, is given by

$$\delta_o = x_f\delta_f + x_b\delta_b \tag{4}$$

where x_f, x_b, δ_f, and δ_b are the mole fractions and chemcal shifts of the free and bound state, respectively. If we let $\delta_f = 0$ (i.e., the free state chemical shift is used as the reference), then

$$\delta_o = x_b\delta_b \tag{5}$$

Model I. If in considering the Tl^+-gramicidin interaction, we assume a single tight binding site per dimer, one may derive the following equation for the observed chemical shift.

$$Tl^+ + Gr_2 \rightleftharpoons Tl^+Gr_2 \tag{6}$$

Let $A = Tl^+$, $E = Gr_2$, and $X = Tl^+Gr_2$ so that

$$A + E \rightleftharpoons X \tag{7}$$

and the equilibrium constant is then

$$K = \frac{X}{AE} \tag{8}$$

Therefore

$$x_b = \frac{X}{A_o} \tag{9}$$

Where A_o = total $[Tl^+]$. Therefore

$$\delta_o = \left(\frac{X}{A_o}\right)_b \tag{10}$$

or

$$X = A_o(\delta_o/\delta_b) \tag{11}$$

Now since

$$A = A_o - X = A_o - \left(\frac{\delta_o}{\delta_b}\right)A_o \tag{12}$$

$$E = E_o - X = E_o - (\delta_o/\delta_b)A_o \tag{13}$$

the equilibrium or binding constant is then

$$K = \frac{(\delta_o/\delta_b)A_o}{[A_o-(\delta_o/\delta_b)A_o][E_o-(\delta_o/\delta_b)A_o]} \tag{14}$$

or solving for the observed chemical shift

$$\delta_o = \frac{(KA_o+KE_o+1) - \sqrt{(KA_o+KE_o+1)^2 - 4(KA_o)(KE_o)}}{2(KA_o/\delta_b)} \tag{15}$$

For the case in which there are two available tight binding sites per dimer, but the occupation of one forbids the occupation of the other, the mathematical treatment will be identical with the above except that K is replaced by $2K_s$, where K_s is the site binding constant.

Model II. Finally, if we consider the case of simultaneously occupiable, and noninteracting identical sites per dimer, we have

$$Tl^+ + Gr \rightleftharpoons Tl^+Gr \qquad (16)$$

where Gr represents the total gramicidin concentration (i.e., the monomer concentration). The observed chemical shift is then

$$\delta_o = \frac{(KA_o+KE_o+1) - \sqrt{(KA_o+KE_o+1)^2-4(KA_o)(KE_o)}}{2(KA_o/\delta_b)}$$

As can be seen in Fig. 8, model I is consistent with the experimental data. This result is consistent with that obtained by equilibrium dialysis (23) which indicated a maximum of only one occupied highest affinity binding site per channel. The binding or equilibrium

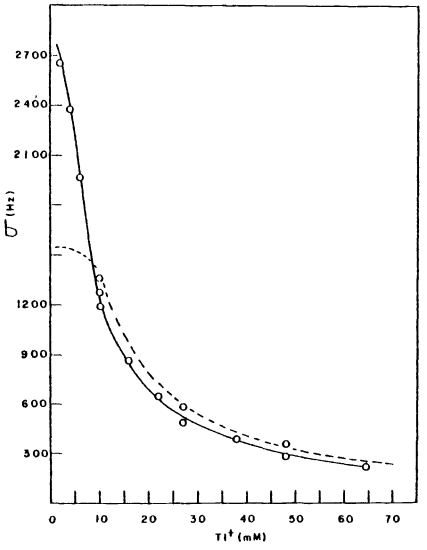

Fig. 8. Corrected ^{205}Tl chemical shift plotted as a function of the thallous ion concentration (open circles). The solid line is a theoretical representation of the shift-concentration relationship for model I. The dashed line is a theoretical representation of the shift-concentration relationship for model II. The temperature is 30°C.

constant obtained from the mathematical analysis is K_s = 900 M^{-1} at 30°C. This value is consistent with the value of 500-1000 M^{-1} obtained by using other techniques (2,23,24,37). The analysis of the data also gave a value for the complex shift which strongly suggests that the Tl(I) ion is appreciably hydrated in the channel.

The variation of the binding constant over the temperature range of 303 to 323 K was used to obtain a value for the enthalpy change for the binding process of -11.3 ± 4 kcal/mole.

SUMMARY

Thallium-205 nmr spectroscopy appears to be an extremely useful replacement probe for sodium and potassium ions in the study of antibiotic complexation that mediates ion transport through biological membranes. The potential for identifying the types of functional groups involved in ion binding in real biological membrane systems and for determining thermodynamic parameters associated with the binding process seems quite good.

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