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Hydrogen-1 and Gallium-71 Nuclear Magnetic Resonance Study of Gallium Citrate in Aqueous Solution[1a]

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Abstract: Gallium citrate complexes which occur in aqueous solution were studied by ^1H and ^{71}Ga NMR and by equilibrium dialysis. In strongly acidic solution low molecular weight complexes having a Ga:citrate ratio of 1:1 form. At pH 2–6 gallium citrate polymers were detected by broadening of the citrate ^1H NMR resonance, decrease in the citrate ^1H spin–lattice relaxation time, and retardation of the rate of dialysis of the metal. Near neutral pH smaller gallium citrate complexes are observed. Chemical exchange between free and metal-bound citrate is slow on the NMR time scale. In highly basic solution (pH $\gtrsim$12) $Ga(OH)_4^-$ is the predominant species, even in the presence of citrate.

Localization of the radioisotope ^{67}Ga in malignant tissue has been employed in the clinical detection of a broad range of tumors.[2,3] The clinical procedure involves intravenous injection of gallium-67 citrate (typical dose: 2 mg of sodium citrate, 25 pmol (2 mCi) of carrier-free ^{67}Ga), clearance of the nuclide from normal tissue during a 2 day waiting period, and scintigraphic detection of γ radiation from regions of isotope accumulation. Our studies of the molecular mechanism of incorporation of ^{67}Ga in normal and malignant cells[4,5] have been directed toward improving methods of tumor detection, obtaining information about the nature of malignant cells, and gaining a better understanding of how cells bind metals which do not normally occur in their environment.

The impetus for the present study of the chemistry of gallium citrate in aqueous solution is the observation in this laboratory[4,5] that citrate inhibits in vitro uptake of ^{67}Ga by L1210 leukemic cells. It was suggested that formation of gallium citrate complexes, which had been detected by ion exchange chromatography[6] and by differential thermal analysis,[7] may cause this inhibition.[5] However, alternate mechanisms involving gallium citrate polymers may also explain this process.[8] Such polymers may be similar to gallium perchlorate polymers detected by Tyree and coworkers[9-11] and iron nitrate[12] and citrate[13,14] polymers characterized by Spiro et al. If tumor cells bind Ga polymers preferentially, then excess citrate may inhibit incorporation of the metal by favoring the formation of smaller citrate complexes. Additional data on the aqueous chemistry of Ga and on the structure of gallium citrate complexes is required for a better understanding of the molecular mechanism of cellular binding of Ga and the inhibition of this process by citrate and other buffers.[5] Characterization of gallium citrate complexes which occur in aqueous solution is the purpose of this investigation.

NMR spectroscopy is the principal method employed in this study. Other NMR investigations of Ga in solution include the demonstration by ^{17}O NMR that $Ga(NO_3)_3$ and $Ga(ClO_4)_3$ dissociate in aqueous solution to yield $Ga(H_2O)_6^{3+}$.[15-17] Fiat and Connick[16] also studied the exchange of water molecules between the solvation sphere of the metal and the bulk solvent. Employing broadline techniques, Akitt et al.[18] observed that ^{71}Ga resonances of a series of symmetrical Ga complexes had chemical shifts ranging over 1367 ppm. Lincoln[19] used ^1H and ^{71}Ga NMR to describe the solvation of $GaCl_3$ in acetonitrile.

In the present study we employed ^{71}Ga and ^1H NMR to monitor the chemical environment of both the metal and the ligands in gallium citrate complexes. ^{69}Ga and ^{71}Ga occur at natural abundances of 60.2 and 39.8%, respectively, and are both spin $\frac{3}{2}$ nuclei with large quadrupole moments. ^{71}Ga is generally the isotope studied by NMR because its lower natural abundance is offset by a greater sensitivity and lower quadrupole moment ($0.11–0.15e \times 10^{24}$ cm^2). Only resonances of Ga in a highly symmetrical local environment are sharp enough to be detected. The ^{71}Ga res-

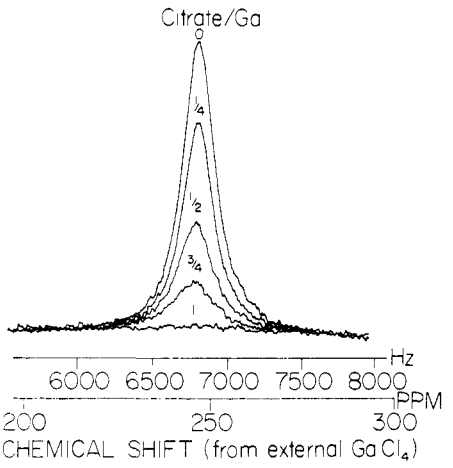

Figure 1. ^{71}Ga NMR spectra of 1.00 *M* $Ga(NO_3)_3$ in D_2O after addition of 0, 0.25, 0.50, 0.75, and 1 equiv of trisodium citrate monohydrate.

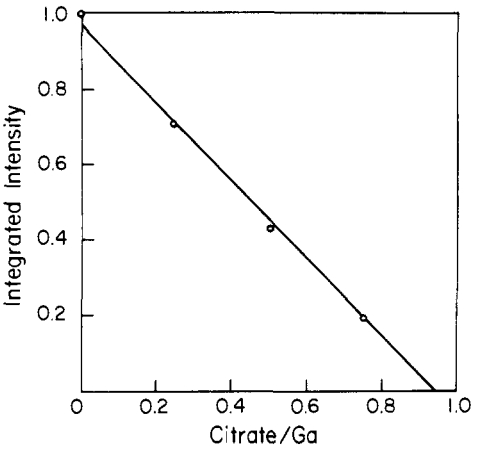

Figure 2. The relative intensities of spectra in Figure 1 corrected for volume changes of the sample resulting from solution of the citrate.

onance of gallium citrate is broadened beyond detection because quadrupolar relaxation is enhanced by the lower symmetry of this complex. Consequently, our ^{71}Ga NMR experiments were limited to detection of resonances of octahedral $Ga(D_2O)_6^{3+}$ in acidic solution, and of tetrahedral $Ga(OD)_4^-$ in alkaline solution. ^{1}H NMR made it possible to extend our nmr measurements over the entire pH range. The approach illustrated in this study, whereby metal complexes can be characterized by means of resonances of both the metal and its ligands, may prove useful in the investigation of other Ga complexes as well as complexes of other metals whose resonances can now be routinely detected by improved multinuclear nmr techniques.[20-22]

Experimental Section

Materials. $Ga(NO_3)_3 \cdot 9H_2O$ (Alfa Inorganics, Beverly, Mass.), trisodium citrate monohydrate (Fisher Scientific Company, Fair Lawn, N.J., certified reagent), citric acid monohydrate (Matheson Coleman and Bell, East Rutherford, N.J., A.C.S. reagent), and D_2O (Merck, Sharp, and Dohme, Montreal, Canada) were used in these studies. A representative solution of $Ga(NO_3)_3$ was analyzed by EDTA titration with Cu-PAN indicator[23] (Calcd: 1.00 mg Ga/ml. Found: 1.00 mg Ga/ml). Titration of a representative sodium citrate solution with standard NaOH indicated a citrate concentration within 2% of the calculated value. In D_2O solution the pD_c (pH meter reading + 0.40) was adjusted with DCl (Thompson Packard Inc., Little Falls, N.J.) and NaOD (prepared by dissolving NaOH in D_2O).

NMR Spectra. Single scan continuous wave ^{1}H (90.0 MHz) and ^{71}Ga (27.45 MHz) NMR spectra (frequency sweep mode) were recorded on a Bruker HX-90 (18-in. magnet) spectrometer. All spectra were measured at ambient probe temperature (28°). Proton spin-lattice relaxation times were determined by the inversion recovery technique[24] employing a NIC-293 pulse programmer (Nicolet Instrument Corp., Madison, Wis.). Least-squares analysis of citrate ^{1}H spectra were performed on a Nicolet-1085 computer employing ITERCAL, an iterative program for implementing the LAOCN3 algorithm of Castellano and Bothner-By.[25] Sample tubes were 5 and 10 mm in diameter for ^{1}H and ^{71}Ga measurements, respectively. Chemical shifts were referred to the ^{71}Ga resonance of external $GaCl_4^-$, and the methyl ^{1}H resonance of internal *tert*-butyl alcohol. Integrated spectral intensities were measured with a planimeter.

Dialysis. Dialysis experiments were performed with an EMD 101 equilibrium microdialyzer (Hoefer Scientific Instruments, San Francisco, Calif.). At the beginning of the experiment the dialysate chamber contained a solution prepared by mixing a 2.00×10^{-3} *M* $Ga(NO_3)_3$ solution with an equal volume of a 2.00×10^{-3} *M* citric acid solution and then adjusting the pH with HCl and NaOH. The starting solution in the retentate chamber was prepared in an identical manner except for addition of a trace quantity (10^{-12}–10^{-11} *M*) of gallium-67 citrate *in* the $Ga(NO_3)_3$ solution. In order to ensure uniform incorporation of the radioactive label, ^{67}Ga was added before addition of citrate or adjustment of pH. Radioactivity was determined with a Model 1185 automatic well scintillation counter (Nuclear Chicago, Des Plaines, Ill.).

Results

^{71}Ga NMR. Formation of gallium citrate complexes was studied by ^{71}Ga NMR spectroscopy. Figure 1 displays spectra of 1.00 *M* $Ga(NO_3)_3$ with various proportions of trisodium citrate. A single resonance is observed, which originates from $Ga(D_2O)_6^{3+}$.[15-18] The intensity of the $Ga(D_2O)_6^{3+}$ peak progressively diminishes as gallium citrate is formed; because of quadrupolar relaxation, the gallium citrate resonance is broadened beyond detection. The width at half-height of the $Ga(D_2O)_6^{3+}$ peak is: 252 Hz (0.0), 242 Hz (0.25), 269 Hz (0.50), and 320 Hz (0.75) (the citrate/Ga ratio is shown in parentheses). These spectral characteristics indicate that chemical exchange between free and bound citrate is slow on the NMR time scale. A plot of the integrated intensity of the $Ga(D_2O)_6^{3+}$ peak against the citrate/Ga ratio is characteristic of a complex in which the ratio of metal to ligand is 1:1 (Figure 2).

Figure 3 displays ^{71}Ga spectra of 1.00 *M* $Ga(NO_3)_3$ in the presence of various proportions of trisodium citrate and citric acid (a total of 1.00 equiv of citrate added). The increase in the intensity of the $Ga(D_2O)_6^{3+}$ resonance as the acid/salt ratio increases reflects dissociation of the gallium citrate complex in more acidic media. However, even in 1.00 *M* citric acid (pD <0) 36% of the metal is still complexed (Figure 3b).

In highly alkaline aqueous solution the tetrahedral $Ga(OD)_4^-$ complex forms.[18] The ^{71}Ga resonance of this species occurs 700 Hz to high field of external $GaCl_4^-$ (Figure 4). Upon addition of 1 equiv of trisodium citrate the half-width of this resonance increases from 385 to 660 Hz, but its intensity is unaltered within experimental error. This indicates that very little of the citrate complex is formed. Broadening of the $Ga(OD)_4^-$ resonance may result from increased viscosity of the solution accompanying addition of citrate or from rapid chemical exchange between $Ga(OD)_4^-$ and some other less symmetrical complex of Ga present in trace amounts.

^{1}H NMR. Figure 5 displays ^{1}H nmr spectra of a solution of 1.00 *M* $Ga(NO_3)_3$ and 1.00 *M* citrate at various pD_c's between 0.70 and 8.62. For comparison stick figure representations of spectra of free citrate at corresponding pD_c's have also been included (spectral parameters in Table I). The methylene protons of the Ga citrate solution at pD_c 0.70 yield an AB spectrum (spectral parameters in Table II), which is quite similar to the corresponding spectrum of

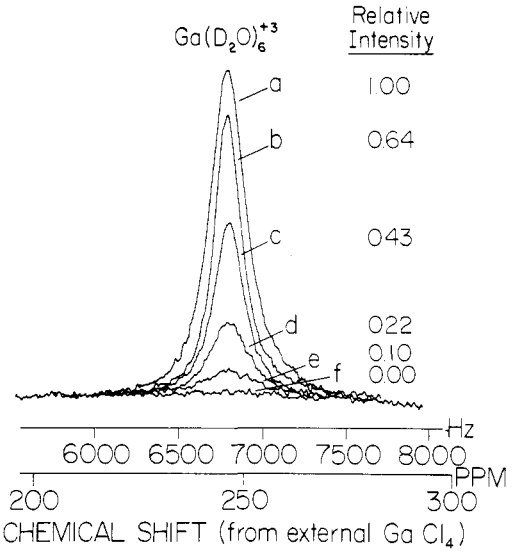

Figure 3. (a) ^{71}Ga NMR spectrum at pD_c 1.22 of 1.00 *M* $Ga(NO_3)_3$, which dissociates completely in D_2O to yield $Ga(D_2O)_6^{3+}$. To this solution were added mixtures of citric acid and trisodium citrate monohydrate (a total of 1 mmol of citrate was added per ml of $Ga(NO_3)_3$ solution). The fraction of citrate in the acid form (*A*), the intensity (corrected for volume change) of the residual $Ga(D_2O)_6^{3+}$ peak relative to spectrum (a) (*I*), and the pD_c (if measurable) were respectively: (b) $A = 1.00$, $I = 0.64$; (c) $A = 0.75$, $I = 0.43$; (d) $A = 0.50$, $I = 0.22$, pD_c 0.85; (e) $A = 0.25$, $I = 0.10$, pD_c 1.30; and (f) $A = 0.00$, $I = 0.00$, pD_c 1.53.

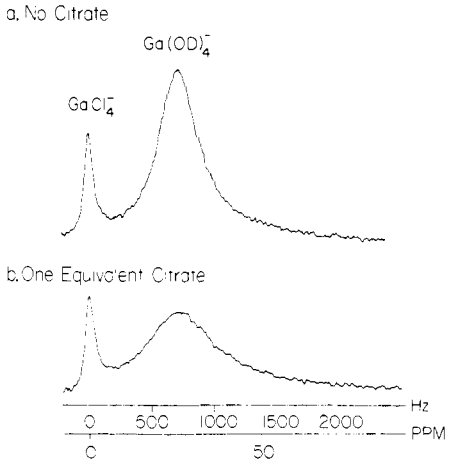

Figure 4. ^{71}Ga NMR spectra of 1.00 *M* $Ga(NO_3)_3$ and 4.87 *N* NaOH in D_2O (a) before and (b) after addition of 1.00 equiv of trisodium citrate monohydrate.

free citrate. Comparison with ^{71}Ga spectra in Figure 3 indicates that a significant amount of complex is present even under these conditions. The intensity of spectrum 3d (pD_c 0.85), which roughly corresponds to the pD_c 0.70 spectrum in Figure 5, indicates that 22% of the metal is complexed. Citrate methylene resonances of the gallium citrate mixture

Table I. NMR Spectral Parameters of 1.00 *M* Trisodium Citrate in D_2O (28°)

	Citrate[a]			
pD_c	$\nu(1)$, Hz	$\nu(2)$, Hz	$J(1,2)$, Hz	RMS error, Hz
0.75	148.7	164.2	−16.2	5.3×10^{-7}
1.48	148.6	164.3	−15.9	6.3×10^{-2}
2.59	146.5	161.3	−15.9	3.8×10^{-2}
5.80	121.7	134.3	−15.6	7.5×10^{-2}
7.45	112.7	128.4	−15.5	5.0×10^{-2}
8.71	112.0	128.1	−15.4	3.8×10^{-2}

[a] Least-squares best-fit parameters.

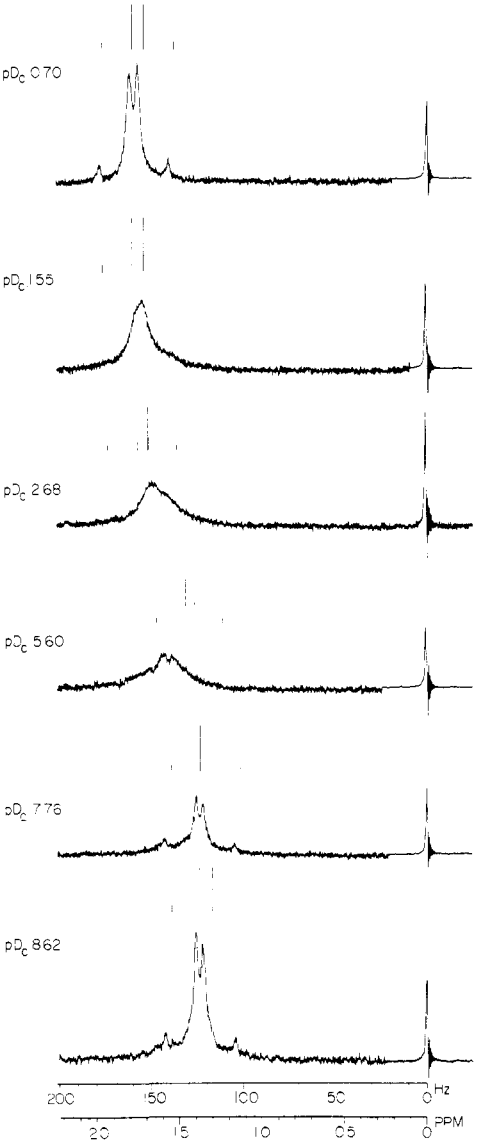

Figure 5. ^{1}H NMR spectra of 1.00 *M* $Ga(NO_3)_3$ and 1.00 *M* citrate in D_2O at various pD_c's. Simulated spectra of free citrate at the same pD_c are shown for comparison.

are very broad in moderately acidic solution (pD_c 1.55–5.60 in Figure 5). At pD_c 7.76 and 8.62 sharp AB spectra again appear, which differ significantly from those of free citrate (compare Tables I and II).

Titration of 1.00 *M* citrate with $Ga(NO_3)_3$ at neutral pD_c was monitored by ^{1}H nmr (Figure 6). A transition occurs from an AB pattern associated with free citrate (Ga/citrate = 0) to a distinct AB pattern associated with gallium citrate complex (Ga/citrate = 0.75 and 1.00). Spectral overlap and pD_c drift make difficult the determination of the exact end point of the titration and the stoichiometry

Table II. NMR Spectral Parameters of 1.00 *M* Gallium Citrate[a] in D_2O (28°)

	Gallium Citrate[b]			
pD_c	$\nu(1)$, Hz	$\nu(2)$, Hz	$J(1,2)$, Hz	RMS error, Hz
0.70	152.2	165.8	−16.7	6.3×10^{-2}
1.55	*c*	*c*	*c*	*c*
2.68	*c*	*c*	*c*	*c*
5.60	*c*	*c*	*c*	*c*
7.76	118.06	129.84	−17.5	8.8×10^{-2}
8.62	117.9	129.5	−17.0	8.8×10^{-2}

[a] A solution of 1.00 *M* $Ga(NO_3)_3$ and 1.00 *M* trisodium citrate. [b] Least-squares best-fit parameters. [c] Too broad to analyze spectrum.

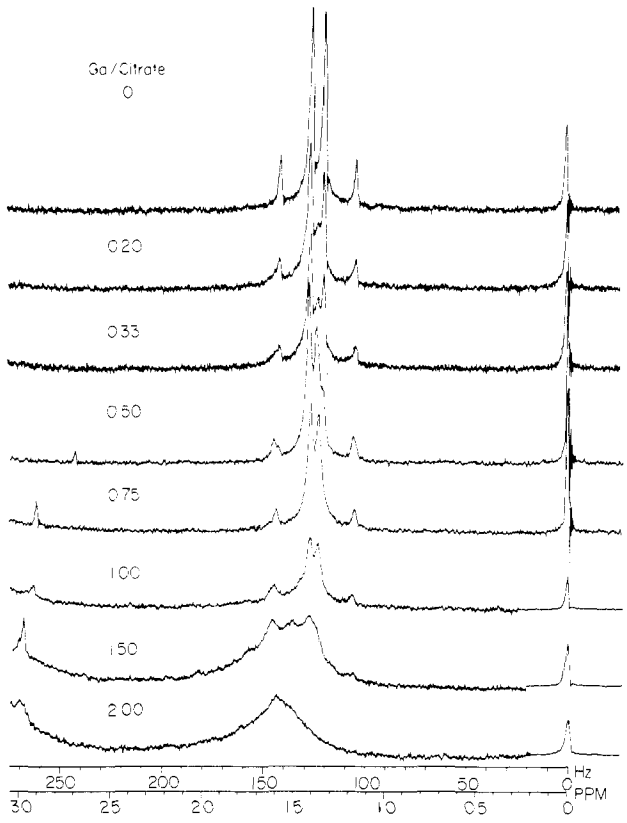

Figure 6. ^{1}H NMR spectra of 1.00 M trisodium citrate at pD_c 7.4 ± 0.4 after addition of various quantities of $Ga(NO_3)_3$.

of the complex. However, a narrow line width indicates a relatively small complex. At intermediate Ga/citrate ratios the methylene spectrum consists of a weighted superposition of the two AB patterns. Exchange between free and bound citrate at neutral pD_c is therefore slow on the nmr time scale. The difference in chemical shifts between methylene resonances of free and bound citrate indicates an upper limit of 3-4 sec^{-1} for the pseudo-first-order rate constant for this process. In the presence of excess Ga spectral broadening is observed.

Polymer Formation. The dialysis experiment shown in Figure 7 demonstrates that formation of Ga citrate polymers is at least in part the cause of spectral broadening observed between pD_c 1.55 and 5.60 in Figure 5. Since, in the presence of 10^{-3} M gallium citrate, diffusion of trace quantities of ^{67}Ga through the membrane does not significantly alter the concentration of Ga citrate, the extent of dialysis is a measure of the self-diffusion rate of the gallium citrate complexes. After 3 hr of dialysis, the pH profile of radioactive tracer retained by the membrane is that shown in Figure 7. The region of maximum radioisotope retention corresponds approximately to the pD_c range in which broadened citrate resonances are observed (Figure 5). Decreased membrane permeability in this pH range reflects formation of gallium citrate polymers. Gallium perchlorate polymers have been detected in approximately the same pH range by light scattering[10,11] and melting point depression[9] experiments.

Formation of gallium citrate polymers is also reflected by a decrease in the spin-lattice relaxation time (T_1) of methylene protons (Table III). For a spherical aggregate $1/\eta T_1$ (where η is the viscosity of the solution) is proportional to the hydrodynamic volume of the sphere if relaxation occurs by a dipole-dipole mechanism in the "extreme narrowing" limit).[26] Even if these conditions are not completely satisfied, $1/\eta T_1$ still serves as a semiquantitative measure of the

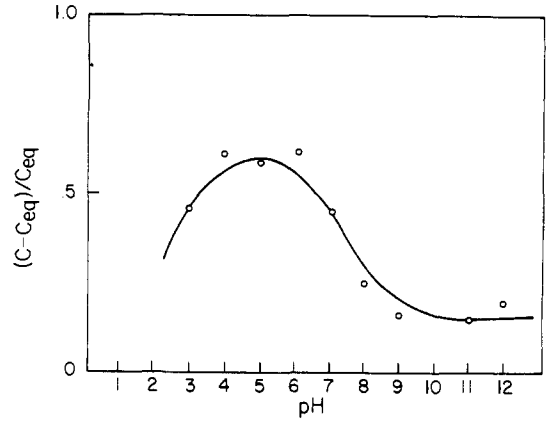

Figure 7. The fraction of trace ^{67}Ga retained after 3 hr of dialysis in 10^{-3} M gallium citrate at various pH's (see text). C is the radioactivity of the retentate sample after 3 hr of dialysis, and C_{eq} is the radioactivity at equilibrium (one-half the original radioactivity). Corrections for decay of ^{67}Ga ($t_{1/2}$ = 78.1 hr) have been made.

size of polymeric species of gallium citrate. Table III shows that $1/\eta T_1$ reaches a maximum between pD_c 2.05 and 4.05. The maximum degree of polymerization indicated by the dialysis experiment (Figure 7) occurs in somewhat less acidic solution. The difference may reflect the concentration dependence of the degree of polymerization (dialysis experiments were performed on 10^{-3} M solutions, whereas T_1 measurements were made on 1.00 M solutions).

The ^{1}H NMR spectrum of a 1.00 M citrate and 0.50 M $Ga(NO_3)_3$ solution at pD_c 4.0 is a superposition of spectra of free citrate (sharp AB pattern) and polymeric gallium citrate (broad complex pattern). Chemical exchange between free and polymeric citrate is therefore slow on the nmr time scale. Craig and Tyree[11] have demonstrated that gallium perchlorate polymers are metastable, decomposing slowly to monomeric Ga_{aq}^{3+} ions. At least 2 years are required to attain equilibrium at 25° (at 75° this time is reduced to 3-4 weeks). Heating the gallium citrate polymer for 1 week at 100° (1.00 M $Ga(NO_3)_3$, 1.00 M citrate, pD_c 3.73) results in the generation of no detectable free citrate.

Discussion

Gallium citrate complexes have been characterized by ^{71}Ga (Figures 1 and 3) and ^{1}H NMR (Figures 5 and 6). In acidic solution a 1:1 Ga:citrate complex forms (Figure 2), but at neutral pD_c the stoichiometry of the complex is more difficult to determine. Citrate complexes dissociate in strongly alkaline solution, in which the $Ga(OD)_4^-$ complex predominates.

Formation of Ga citrate polymer in mild acid is indicated by a decrease in citrate proton T_1's (Table III), and by di-

Table III. Spin–Lattice Relaxation Data of 1.0 M Gallium Citrate Solutions[a] at 28°

pD_c	[OD]/[Ga]	T_1, sec	$1/\eta T_1$, cP^{-1} sec^{-1}
[b]	0	0.26_0	1.4
0.98		0.13_4	3.2
1.34	3.00	0.10_7	3.7
2.05	3.68	0.095	4.5
4.05	4.32	0.096	4.7
5.15	4.48	0.11_5	3.9
6.34	4.74	0.12_0	3.5
7.86	5.00	0.12_0	3.4
9.15		0.14_3	2.6
12.4[c]		0.18_0	

[a] 1.0 M $Ga(NO_3)_3 \cdot 9H_2O$, 1.0 M citrate (as acid or trisodium salt) in D_2O. [b] 1.0 M citric acid, 1.0 M $Ga(NO_3)_3 \cdot 9H_2O$; pD_c too low to measure. [c] No correction applied for sodium error of electrode.

alysis experiments (Figure 7). Thus, broadening of citrate resonances in this pD_c range probably results from formation of polymers. Furthermore, spectral broadening in the presence of excess Ga at neutral pD_c (Figure 6) is also probably due to polymer formation. Dialysis escape rates of Ga^{3+} citrate are much shorter than those reported for $iron^{3+}$ citrate,[13] indicating that the iron polymer (molecular weight about 200,000) is much larger than the gallium polymer. Unlike the iron citrate polymer, which is stable in alkaline solution, the gallium citrate polymer, like the gallium perchlorate polymer studied by Tyree and coworkers,[9-11] dissociates in strong acid and base. This behavior probably results from the amphoteric properties of Ga.

These experiments demonstrate the presence of molecular species of Ga which may influence cellular binding of the isotope if they persist at the low concentrations of this metal employed in ^{67}Ga cell binding experiments (10^{-12}–10^{-11} M). Formation of Ga citrate complexes may explain the inhibitory effect of citrate on in vitro localization of ^{67}Ga in L1210 cells.[5] These complexes may either be tumor impermeable, or they may be more stable than complexes of the metal with intracellular receptors. An alternate possibility is suggested by the observation that the extent of ^{67}Ga binding by L1210 cells diminishes as the pH increases from 6 to 8.[5] Figure 7 shows that depolymerization of Ga citrate occurs over this pH range. This suggests that the tumor cells may be accumulating polymeric species of Ga preferrentially, perhaps by pynocytosis. However, changes in pH may also influence cellular uptake of ^{67}Ga by altering the structure of the cell membrane. The inhibitory effect of citrate may result from formation of impermeable smaller molecular weight complexes when citrate is present in excess. Such a depolymerization effect of excess citrate is indicated in Figure 6, and has also been observed in iron citrate polymers.[14]

A choice between these and other possible mechanisms for this inhibitory effect requires additional data. In particular it is desirable to identify the molecular species of this metal which localizes in tumor cells. This is a difficult task because of the complexity of the aqueous chemistry of Ga, and because of the difficulty of performing molecular studies at the low concentrations of the metal employed in clinical and in vitro ^{67}Ga binding experiments. Structural characteristics of normal and malignant cells which determine their ability to localize ^{67}Ga are as yet poorly understood and require additional study. It is, however, clear that L1210 leukemic cells are highly specific with respect to the molecular state of ^{67}Ga which they bind.[4,5] Further investigation of the behavior of Ga salts in aqueous solution may help elucidate how this metal accumulates in normal and malignant cells.

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References and Notes

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