Gadolinium(III) *in vivo* Speciation. Part 1. A Potentiometric and Spectroscopic Study of Gadolinium(III) Citrate Complexes

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Gadolinium(III) complexes of citric (3-carboxy-3-hydroxypentane-1,5-dioic) and 3-carboxypentane-1,5-dioic acid have been studied potentiometrically at 25 °C and an ionic strength of 0.15 mol dm^{-3} NaCl. Both [GdL] (H_3L = citric acid) and $[GdL(OH)]^-$ were detected. From potentiometric and spectroscopic evidence citrate is postulated to co-ordinate *via* the hydroxyl group and two carboxylate groups. The formation of the $[GdL(OH)]^-$ species results from loss of the hydroxyl proton rather than co-ordination of hydroxide.

In recent years magnetic resonance imaging (MRI) has rapidly evolved from an experimental to a very useful clinical modality. More recently paramagnetic contrast agents have been developed which allow images with improved tissue delineation to be obtained. The MRI monitoring of tissue function also necessitates the use of contrast material. For the most part these contrast agents have centred around Gd^{III} as the paramagnetic source, with $[Gd(dtpa)]^{2-}$ (dtpa = diethylenetriamine-*NNN'N''N''*-pentaacetate) being introduced into clinical trials in Europe[1-3] and the United States.[4] The usefulness of metal complexes as contrast agents of course depends on their speciation *in vivo*. Recently we have published a thermodynamic computer model of Gd^{III} in blood plasma.[5] Using this model we were able to calculate that initial binding of Gd^{III} *in vivo* is to transferrin but that at higher concentrations (10^{-5} mol dm^{-3}) the metal ion binds almost exclusively to citrate.

Citrate complexes (citric acid = 3-carboxy-3-hydroxypentane-1,5-dioic acid) of gadolinium have been studied before but at non-physiological pH values.[6,7] Sal'nikov and Zhuravieva[8] studied the complexation of Gd^{III} by citrate over a wide pH range, reporting constants for some 13 species. Our studies on the chelation of Al^{III} to citrate[9] indicate that the formation of hydroxy species is likely to predominate in neutral solution. For this reason and because citrate is so important to our computer model, we have reinvestigated citrate complexation of Gd^{III}.

Theoretical

The theory of the titration analysis has been presented before,[10] but for convenience the relevant functions used in this paper are reproduced here. The deprotonation function $\bar{Q}$ is the average number of protons released per metal ion, as a result of complexation and is defined according to equation (1), where T_H and T_M are the total proton and metal concentrations

$$\bar{Q} = (T_H^* - T_H)/T_M \quad (1)$$

respectively; T_H^*, given by equation (2), is the calculated total

$$T_H^* = [H] - [OH] + \Sigma r\beta_{0qr}[L]^q[H]^r \quad (2)$$

concentration of protons that would be necessary to give rise to the observed pH if no complexation took place. The summation is over all protonated ligand species. A formation function for the ligand subsystem is defined by equation (3) where T_L is the total ligand concentration, while the average number of ligands bound per metal ion, $\bar{Z}$, is given by equation (4).

$$\bar{n} = (T_H^* - [H] + [OH])/T_L \quad (3)$$

$$\bar{Z} = (T_L - [L])/T_M \quad (4)$$

Results and Discussion

Protonation constants for citrate and 3-carboxypentane-1,5-dioate are given in Table 1. The results are in good agreement with the literature.[11]

The formation and deprotonation curves for the gadolinium(III)–citrate system are shown in Fig. 1. The formation curves at different metal:ligand concentration ratios are not superimposable indicating the formation of protonated and/or polynuclear species. As the ligand:metal ratio is increased the formation function increases, indicating the presence of protonated species. Between pH 4 and 5 the $\bar{Q}$ and $\bar{n}$ curves [Fig. 1(*b*)] are parallel showing that in this pH range no change in complexation occurs. This is borne out by the gadolinium(III)–citrate species distribution curves [Fig. 1(*c*)]. As the pH is raised further the $\bar{n}$ and $\bar{Q}$ curves diverge. The $\bar{Q}$ curve rises indicating that more protons are lost from the ligand than were initially present *i.e.* an MLH_{-1} species is formed. Formation of a hydroxy species generally causes 'fanning back' of the formation function. This, however, is often curtailed by the function becoming ill defined. The formation function is strictly only defined for single stepwise mononuclear complex formation. The formation of hydroxy species causes the calculated free-ligand concentration to decrease while the formation function continues to increase, *i.e.* the curve fans back. Ultimately the free-ligand concentration is calculated to be negative and so pA (−log[free-ligand concentration]) is undefined. In Fig. 1(*a*) the pH at which this occurs is indicated. As the $\bar{Q}$ function is plotted against pH it does not suffer from this limitation. The final set of constants refined for this system are given in Table 1. The solid lines in Fig. 1(*a*) and (*b*) were calculated using these constants, together with the experimental concentrations, and indicate the 'goodness of fit' between the model and the experimental data.

The formation and deprotonation curves for the gadolinium(III)–3-carboxypentane-1,5-dioate system are similar to those for citrate. The formation of an MLH_{-1} species, however, only occurs at a much higher pH (> 6.6). At such pH values hydrolysis of the metal ion begins to occur making the refinement of β_{11-1} difficult. The final set of equilibrium constants refined for this system are given in Table 1.

It is of interest to know the structure of any complexes formed

Table 1 Formation constants determined in this study at 25 °C, I = 150 mmol dm^{-3} NaCl; n is the number of experimental observations used as data for the least-squares calculations, R the crystallographic R factor. The general formula of a complex is expressed by $M_pL_qH_r$

Ligand	p	q	r	$\log \beta_{pqr}$	n	R
Citrate	0	1	1	5.540 ± 0.005	87	0.009
	0	1	2	9.780 ± 0.006		
	0	1	3	12.647 ± 0.008		
	1	1	0	6.86 ± 0.01	244	0.009
	1	1	1	10.12 ± 0.05		
	1	2	0	10.53 ± 0.04		
	1	1	−1	0.77 ± 0.02		
	1	1	−2	−7.08 ± 0.03		
3-Carboxypentane-1,5-dioate	0	1	1	5.726 ± 0.004	166	0.003
	0	1	2	10.199 ± 0.006		
	0	1	3	13.655 ± 0.009		
	1	1	0	4.20 ± 0.01	97	0.003
	1	1	1	8.48 ± 0.01		
	1	1	−1	−4.47 ± 0.04		

during a potentiometric investigation. In this study it was possible to infer the structure of the citrate complex by comparison with ligands of related structure. Citrate has four possible co-ordination sites, with a maximum denticity of three. Molecular models show that it is not possible for all four groups to co-ordinate simultaneously to the same metal ion. If citrate were to act as a bidentate ligand (the stability constants are too high for monodentate co-ordination) it could form succinate (log β_{110} = 3.0)[11] or 2-hydroxyethanoate (log β_{110} = 2.79)[11] type complexes. However citrate forms more stable complexes than either of these two ligands and hence we conclude that it co-ordinates to Gd^{III} in a tridentate manner.

If citrate behaves as a tridentate ligand there are three possible geometric isomers for the complex (Fig. 2). In deciding which of these three structures is most likely, we can compare the stability of the citrate and 3-carboxypentane-1,5-dioate complexes. The citrate complexes are 100-fold more stable. Since 3-carboxypentane-1,5-dioic acid can only form complexes similar to structure (*a*), we conclude that this is not the preferred mode of co-ordination for citrate. On the potentiometric evidence it is not possible to decide between the remaining two isomers. However, since structure (*b*) gives rise to a five- and a six-membered chelate ring while (*c*) gives rise to two six-membered chelate rings we would expect (*b*) to be the more stable. The crystal structures of nickel(II) citrate[12] and manganese(II) citrate[13] reveal that these two metal ions are co-ordinated through the hydroxyl group, the central carboxylate group and a terminal carboxylate group, *i.e.* have structure (*b*). On the other hand isocitrate (3-carboxy-2-hydroxypentane-1,5-dioate) can form similar metal complexes to citrate with structures (*a*) and (*b*), but (*c*) leads to the formation of a five- and a seven-membered ring. Comparison of the metal(II) citrate and isocitrate stability constants reveals that the citrate complexes are always more stable than the isocitrate complexes (Mn^{II} 3.7, 2.55; Ca^{II} 3.5, 2.47; Mg^{II} 3.37, 2.32 for log β_{110} of citrate and isocitrate respectively).[11] This leads to the conclusion that structure (*c*) is preferred for citrate complexes.

Above pH 5 [GdL](H_3L = citric acid) loses a proton to form $[GdLH_{-1}]^-$. This complex may result from the loss of a proton from either a co-ordinated water molecule or from the ligand. From the stability constants of MLH_{-1} and ML, we can calculate the equilibrium constants for the loss of this proton, *i.e.* $\beta[ML \rightleftharpoons MLH_{-1} + H^+] = \beta_{11-1}/\beta_{110} = 8.1 \times 10^{-7}$ mol dm^{-3}. If this proton were lost from a co-ordinated water molecule, we would not expect a value for the equilibrium constant very different from that for the formation of gadolinium(III) hydroxide. The literature[14] gives log β_{10-1} as −8.39. Similarly log(β_{11-1}/β_{110}) for 3-carboxypentane-1,5-dionate is −8.7 which is in very good agreement with the literature value for β_{10-1}. Thus we conclude that the proton is lost from the hydroxyl group which must be co-ordinated for this to occur at such a low pH.

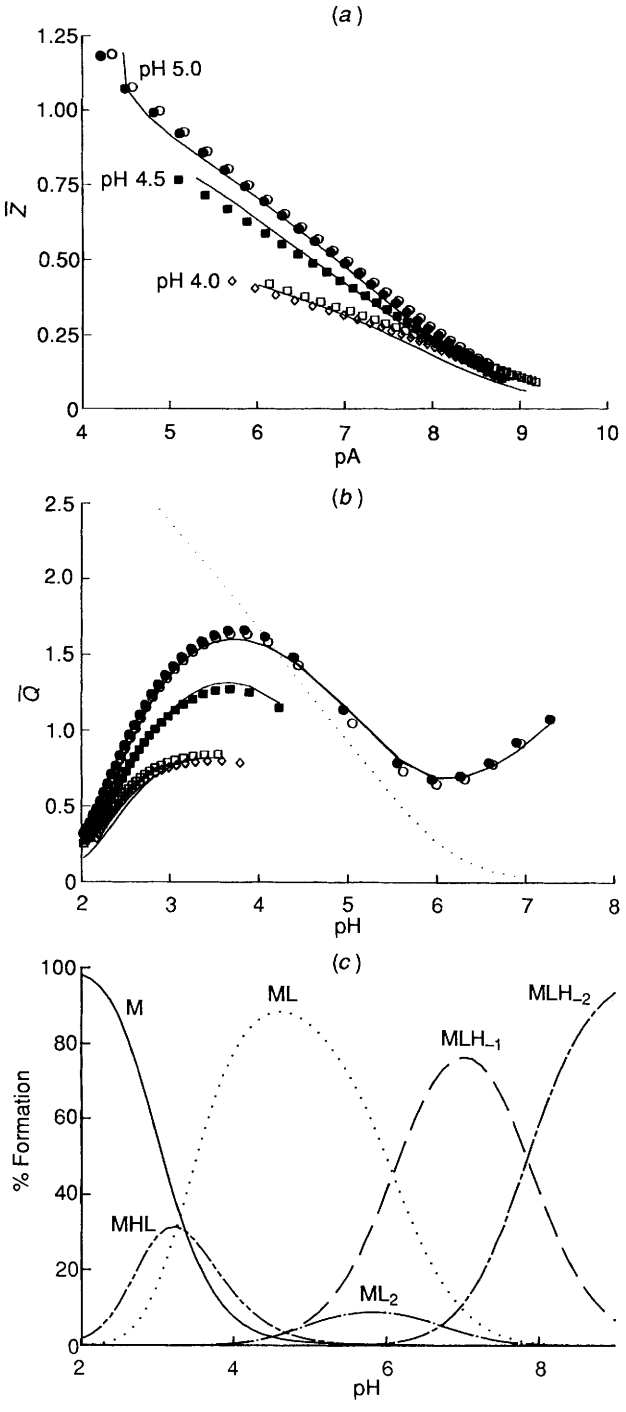

Fig. 1 Experimental formation (*a*) and deprotonation (*b*) curves obtained at 25 °C and I = 150 mmol dm^{-3} for the gadolinium(III)–citrate system. The solid lines represent the theoretical curves calculated using the formation constants given in Table 1. The broken line is an $\bar{n}$ curve. For clarity only selected results have been plotted: [Gd], [citrate] = 7.9, 4.0 (◇); 6.9, 3.4 (□); 5.7, 7.1 (●); 5.2, 6.3 (■); 5.7, 7.2 (○) mmol dm^{-3}. (*c*) Species distribution curves for [Gd] = [citrate] = 1 mmol dm^{-3}

Nuclear Magnetic Resonance Spectroscopy.—In order to obtain more direct evidence for the structure of [GdL] (L = citrate) in solution a ^{13}C NMR study of the complex was undertaken. Initially, the pH dependence of the citric acid ^{13}C chemical shifts was determined. Generally, this would allow assignments of pK_a values to different protonation sites or

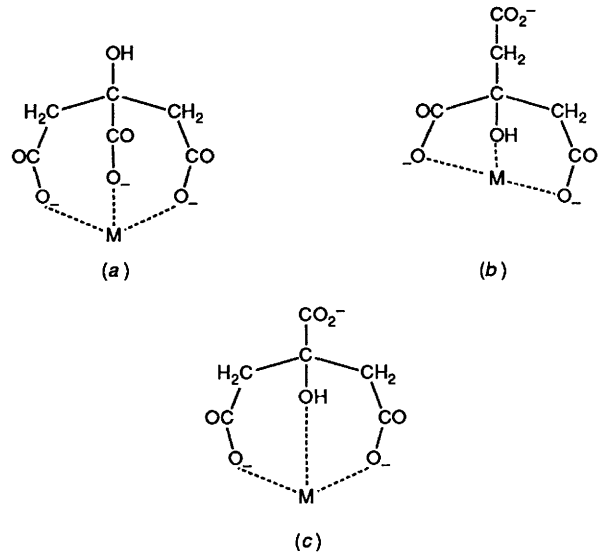

Fig. 2 Three possible structures for a tridentate citrate complex

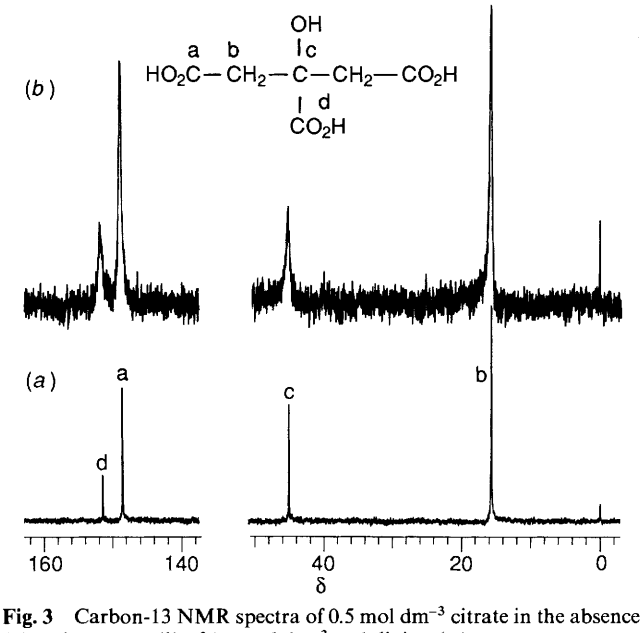

Fig. 3 Carbon-13 NMR spectra of 0.5 mol dm^{-3} citrate in the absence (*a*) and presence (*b*) of 1 mmol dm^{-3} gadolinium(III)

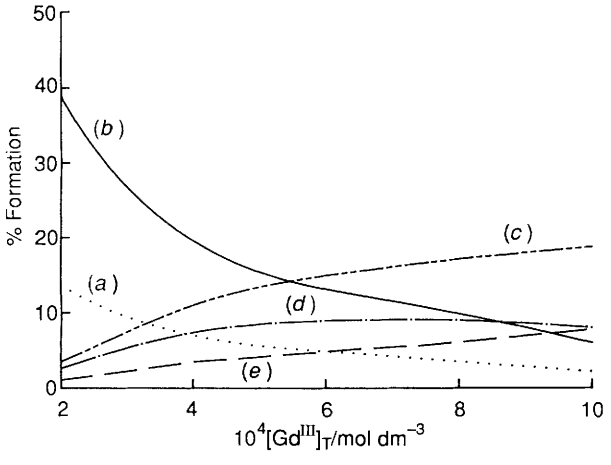

Fig. 4 Calculated distribution of low-molecular-weight species of gadolinium(III) in blood plasma as a function of the total metal concentration species: (*a*) $[GdLH_{-2}]^{2-}$, (*b*) $[GdLH_{-1}]^{-}$ (L = citrate); (*c*) $[Gd(GlnO)]^{2+}$ (GlnO = glutaminate); (*d*) $[Gd(ThrO)]^{2+}$ (ThrO = threoninate); (*e*) $[Gd(AlaO)]^{2+}$ (AlaO = alaninate)

the occurrence of microscopic dissociation processes to be studied.[10] However, in this case the three carboxylate carbon resonances were found to shift in unison, indicating no preferred site of protonation.

Fig. 3 shows the changes which occur in the ^{13}C NMR spectrum of citrate upon the addition of $GdCl_3$. Gadolinium(III) is a paramagnetic metal ion ($S = \frac{7}{2}$) with a long electronic relaxation time and is therefore suitable as an NMR relaxation reagent. Relaxation phenomena have been reviewed;[15] essentially the broadening of an NMR signal is proportional to r^{-6} where r is the internuclear distance between the metal ion and the nucleus in question. In this case it is clear that the citrate resonances are broadened relative to the reference and that the methylene carbon resonance is the least broadened. Also the central carbon resonance is as broad as those due to the carboxylate groups indicating that these four carbons are equidistant from the metal ion. The only way for this to occur is for the metal ion to co-ordinate to the central carboxylate group, the central hydroxyl group and one of the terminal carboxylate groups [structure (*b*)]. Fast exchange of the two terminal carboxylate groups would lead to the observed results. Similar results have been found by Vijverberg *et al.*[16]

Computer Simulation.—The primary objective in studying citrate complexation of gadolinium(III) was the inclusion of its species in our computer model of blood plasma.[5] This model attempts to simulate the co-ordination equilibria taking place in blood plasma and consists of some 40 ligands, 11 metal ions and *ca.* 5000 complexes. Details of the program and data base used to construct the model have been published.[5,17,18] Previous studies, using literature data, have shown citrate to be an important chelator of gadolinium(III),[5] while work on aluminium(III)[19] indicates that hydroxy species should be important at physiological pH.[9] Fig. 4 shows the calculated distribution of the low-molecular-weight species of gadolinium(III) in blood plasma as a function of the total metal concentration. At low concentrations of gadolinium(III) ($< 10^{-4}$ mol dm^{-3}) the metal ion is bound almost exclusively as the $[GdLH_{-1}]^{-}$ (65.1%) and the $[GdLH_{-2}]^{2-}$ (23.0%) complexes. As the concentration of gadolinium(III) increases the available amount of citrate (1.13×10^{-4} mol dm^{-3}) is exceeded and a new species distribution is established. At a concentration of 10^{-3} mol dm^{-3} gadolinium(III) (LD_{50} of $GdCl_3 \approx 6$ mmol dm^{-3})[5] the metal ion is more evenly distributed amongst several complexes, the most important of which are shown in Fig. 4.

So far, computer simulations of biological fluids have, to a large extent, excluded specific protein binding. However, the co-ordination chemistry of gadolinium(III) is similar, in many respects, to that of iron(III) which is transported in blood plasma as the transferrin complex.[20] Gadolinium(III) has also been shown to have a high affinity for transferrin.[21] Since the *in vivo* concentration of transferrin (H_6L') exceeds that of iron(III) it is likely that there will be transferrin available to co-ordinate gadolinium(III).[20] The introduction of transferrin (3.1×10^{-5} mol dm^{-3}) into the blood model shows that at a total gadolinium(III) concentration less than 6×10^{-5} mol dm^{-3} the metal ion is bound almost exclusively as $[Gd_2L']$. Once all the available transferrin is co-ordinated the gadolinium(III) is distributed amongst the low-molecular-weight ligands. At 1.7×10^{-4} mol dm^{-3} gadolinium(III) is bound to citrate ($[GdLH_{-1}]^{-1}$, 37.9%) and transferrin ($[Gd_2L']$, 36.5%). However, the presence of transferrin does not affect the relative distribution of gadolinium(III) amongst the low-molecular-weight ligands.

Experimental

Solutions for potentiometry were prepared using degassed, double glass-distilled, deionized water. All titrations were carried out under an atmosphere of purified nitrogen. The ionic strength was maintained at 150 mmol dm^{-3} using NaCl.

Citric acid and 3-carboxypentane-1,5-dioic acid were recrystallized from water. All other reagents were of analytical grade and were used without further purification. Gadolinium chloride stock solutions were prepared by the action of HCl upon Gd_2O_3, and standardized by complexometric titration. The residual acid concentration in the gadolinium stock solution was determined using the Gran method.[22]

Potentiometric titrations were carried out in a double-walled vessel, thermostatted at 25.0 °C. Measurements were made on a Radiometer PHM84 research pH meter equipped with a Metrohm glass electrode and a Ag–AgCl reference electrode with a renewable liquid junction of 150 mmol dm^{-3} NaCl. Electrode calibration was according to the method of May *et al.*,[23] in which strong acid–strong base titrations were used to calibrate the electrode in terms of hydrogen-ion concentration. The Nernstian slope of the glass electrode was checked against standard buffers over the range pH 4–9. Protonation constants were determined over the concentration range 5–10 mmol dm^{-3}. Metal complexation was studied over the range pH 2–9 using different concentrations of ligand (citrate, 2–8; 3-carboxypentane-1,5-dioate, 6–9 mmol dm^{-3}) and metal (gadolinium chloride 2–7 mmol dm^{-3}). In each titration ≈ 25 cm^3 of solution were titrated against 0.1 mol dm^{-3} NaOH using a Metrohm 645 Dosimat. All titrations were repeated at least twice.

Data were analysed on a Vax 6330 computer using the ESTA suite of programs,[24] which operate on titration data to yield overall formation constants defined by equation (5). A Gauss–Newton method was used to minimize an objective function

$$\beta_{pqr} = [M_pL_qH_r]/[M]^p[L]^q[H]^r \quad (5)$$

calculated as a sum of residuals in the square of the electromotive force.

NMR spectra were recorded in D_2O solutions on a Varian VXR200 spectrometer.

Computer simulation was carried out using the ECCLES program.[17] The blood model data base, as described by May and co-workers[17,18] and modified by Jackson *et al.*[5] to include gadolinium(III) equilibria, was used. The total blood plasma phosphate concentration was also corrected to 1.6 mmol dm^{-3}.

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