# Bismuth(III) Complexes of the Tripeptide Glutathione (γ-L-Glu–L-Cys–Gly)

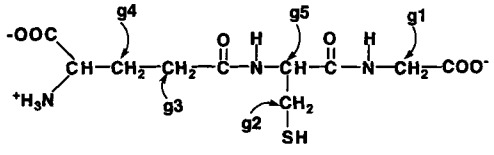
**Peter J. Sadler,* Hongzhe Sun and Hongyan Li**

**Abstract**: The tripeptide glutathione (γ-L-Glu–L-Cys–Gly, GSH) is thought to play an important role in the pharmacology of bismuth drugs, but to our knowledge no chemical studies of bismuth glutathione complexes have been reported. We report here studies of interactions of the antiulcer compound ranitidine bismuth citrate (**1**) and $[Bi(edta)]^-$ with glutathione in aqueous solution and in intact red blood cells by NMR spectroscopy. The deprotonated thiol group is shown to be the strongest binding site for $Bi^{III}$, and a complex with the stoichiometry $[Bi(GS)_3]$ is formed, as determined by $^{13}C$ NMR titrations. A remarkably large low-field shift of approximately 1.37 ppm for the $\beta$-$CH_2$ $^1H$ NMR resonances of GSH was observed on binding to $Bi^{III}$. The complex $[Bi(GS)_3]$ is stable over the pH* range 2–10 (pH* = pH meter reading in $D_2O$ solution). A formation constant log $K$ of $29.6 \pm 0.4$ ($I$ = 0.1 M, 298 K) for $[Bi(GS)_3]$ was determined by displacement of edta by GSH. The rate of exchange of GSH between free and bound forms is pH-dependent, ranging from slow exchange (on the $^1H$ NMR timescale) at low pH (ca. 3 $s^{-1}$ at pH 4.0) to intermediate exchange at biological pH (ca. 1500 $s^{-1}$). Such facile exchange may be important in the transport and delivery of $Bi^{III}$ in vivo. Spin-echo $^1H$ NMR showed that **1** reacts with GSH in red cells both in vivo and in vitro. A first-order reaction of **1** with red blood cells was observed in vitro ($k = 0.20 \pm 0.04$ $h^{-1}$, $t_{1/2}$ = 3 h, 310 K), and the rate-determining step appeared to involve the passage of $Bi^{III}$ through the cell membrane.

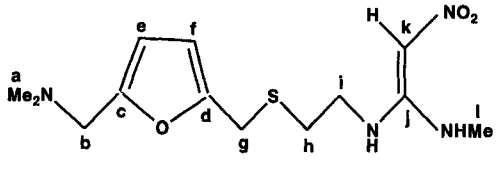
**Keywords**
bismuth complexes · drugs · glutathione · NMR spectroscopy · red blood cells

## Introduction

For more than two centuries, various bismuth(III) compounds have been used as therapeutic agents for the treatment of gastrointestinal disorders. These include bicarbonate, nitrate and subsalicylate salts and colloidal bismuth subcitrate (CBS, "Del-Nol") which is currently widely used in many countries to treat peptic ulcers.[1, 2] Recently, a new 1:1 adduct of ranitidine with bismuth citrate (Glaxo Wellcome, complex **1**), which combines the antisecretory action of ranitidine with the mucosal protectant and bactericidal properties of bismuth, has entered clinical trials.[3, 4] Complex **1** appears to be a polymer with $Bi^{III}$ ions bridged by citrate$^{4-}$ anions stabilized by second coordination sphere interactions with ranitidine.[4, 5]




**Glutathione**




**Ranitidine**

Glutathione (γ-L-Glu–L-Cys–Gly) is a potentially polydentate ligand, and is widely used as a model system for the binding of metal ions by larger peptides and proteins. It is present in many cells at a relatively high concentration (ca. 2 mM) and generally is the most abundant nonprotein thiol.[6] It can bind to a variety of metal ions and, in particular, its thiolate sulfur atom has a high affinity for "soft" metal ions, resulting in the formation of GS–M complexes; these metals include $Cu^I$, $Au^I$ and $Hg^{II}$.[7–10] $Bi^{III}$ is also a "soft" metal ion, but little is known about its mode of binding to glutathione. Such interactions could play a crucial role in the pharmacology of bismuth. For example, it has been demonstrated that a glutathione-dependent hepatobiliary transport system exists for $Bi^{III}$.[11]

In 1977, Williams found that cysteine and glutathione could prevent precipitation of colloidal bismuth subcitrate even at pH 2.0.[12] Later Chaleil et al. reported that the oral absorption of bismuth salts was increased by the presence of sulfhydryl ligands.[13] Recently, Rao and Feldman found that most of the bismuth added to whole blood entered the red blood cells, with less than 10% remaining in the plasma,[14] while Garner et al. have reported that when red cells were treated with colloidal bismuth subcitrate in vitro, most of the bismuth was adsorbed onto the outside of the cells.[15]

In general both the kinetics and thermodynamics of solution equilibria for $Bi^{III}$ are poorly understood. There are few stability constants for $Bi^{III}$ complexes in databases, for example ref. [16]. However, it is clear that hydrolysis can readily dominate aqueous equilibria of $Bi^{III}$, and that the insolubility of simple

[*] Prof. Dr. P. J. Sadler, H. Sun, H. Li
Department of Chemistry, Birkbeck College, University of London
Gordon House, 29 Gordon Square, WC1H 0PP, London (UK)
Fax: Int. code +(171)380-7464
e-mail: ubca424@ccs.bbk.ac.uk

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salts except in highly acidic media makes these processes difficult to study. Recent polarographic work by Hancock et al. has demonstrated the high affinity of $Bi^{III}$ for chelating N- and O-containing ligands.[17] One of the aims of the present paper is to compare the relative affinities of N, O and S ligands.

In this work we used NMR spectroscopy to investigate reactions between the new antiulcer compound ranitidine bismuth citrate (**1**) and glutathione both in aqueous solution and in intact red blood cells, and the competitive binding of glutathione and ethylenediaminetetraacetate (edta).[18] Unfortunately $^{209}Bi$ ($I = 9/2$, 100% natural abundance) gives rise to very broad NMR resonances on account of its large quadrupole moment, although its receptivity is relatively high ($819 \times {}^{13}C$).[19] Therefore we have confined our studies to $^{1}H$ and $^{13}C$ nuclei in the ligands.

## Results

**$Bi^{III}$ binding to GSH**: GSH has several potential binding sites including two carboxylate groups, an amino group and a thiol group. To identify which of these are involved in $Bi^{III}$ binding, we investigated first $^{1}H$ NMR spectra of aqueous solutions containing ranitidine bismuth citrate and GSH at 298 K, pH* 4.5 (the pH* of aqueous solutions of complex **1** alone). At 1:1 and 2:1 GSH:Bi molar ratios the major changes in the spectrum were the disappearance of the g2 and g5 resonances of GSH, appearance of new very broad resonances for these protons at lower field, and the sharpening and high-field shift of the citrate resonances. Only when a ratio of 3:1 was reached were the new broad peaks more clearly defined.

Figure 1 compares $^{1}H$ NMR spectra of GSH, GSH in the presence of ranitidine bismuth citrate at a mol ratio of 3:1, and ranitidine bismuth citrate, pH* 4.5. It can be seen that reaction with $Bi^{III}$ leads to the appearance of new GSH peaks at $\delta = 4.28$, 4.35 and 4.70, while the Cys $\beta$-$CH_2$ and Cys $\alpha$-CH resonances of free GSH at $\delta = 2.92$, 2.97 and $\delta = 4.57$, respectively, have disappeared. Also the $\alpha$-$CH_2$ singlet of Gly at $\delta = 3.82$ becomes a quartet with $^{2}J = 17.5$ Hz, and the citrate $^{1}H$ chemical shifts are the same as those for free citrate at this pH*. None of the other resonances of GSH and ranitidine are much affected by the reaction. A 2D TOCSY spectrum (not shown) confirmed that the three new broad peaks at $\delta = 4.70$ and 4.28, 4.35 are coupled and can therefore be assigned to the $\alpha$ and $\beta$ protons of the Cys residue in a $Bi^{III}$ glutathione complex. The NMR data for the Cys residue of GSH are summarized in Table 1.

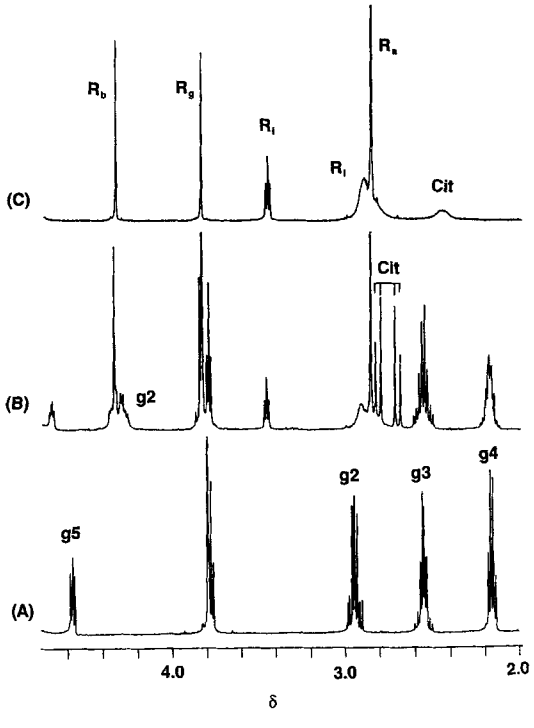


Fig. 1. 500 MHz $^{1}H$ NMR spectra of: A) glutathione; B) glutathione after addition of ranitidine bismuth citrate (mol ratio 3:1); C) ranitidine bismuth citrate, all at pH* 4.5. Glutathione assignments: g2 Cys $\beta$-$CH_2$, g4 Glu $\beta$-$CH_2$, g3 Glu $\gamma$-$CH_2$ and g5 Cys $\alpha$-CH. Resonances for ranitidine are labelled R (for lettering system see structure), and for citrate are labelled Cit. Note the large low-field shifts of the peaks for Cys $\beta$-$CH_2$ (g2) on complexation of GSH to $Bi^{III}$.

Table 1. 500 MHz $^{1}H$ chemical shifts ($\delta$) and $J$ coupling constants ($\pm 0.15$ Hz) for the Cys $\beta$-$CH_2$ (g2) and Cys $\alpha$-CH (g5) resonances of glutathione and $[Bi(GS)_3]$ at pH* 4.5 and 7.0.

| | pH* = 4.5 | | | pH* = 7.0 | | |
|---|---|---|---|---|---|---|
| | GSH | $Bi(GS)_3$ | $\Delta\delta$ [a] | GSH | $Bi(GS)_3$ | $\Delta\delta$ |
| $\alpha$-CH (g5) | 4.571 | 4.695 | 0.124 | 4.571 | 4.698 | 0.127 |
| $\beta$-$CH_2$ (g2) | 2.923 | 4.284 | 1.361 | 2.923 | 4.262 | 1.339 |
| $\beta$-$CH_2$ (g2′) | 2.968 | 4.348 | 1.380 | 2.968 | 4.326 | 1.358 |
| $\delta g2 - \delta g2'$ | 0.045 | 0.064 | | 0.045 | 0.064 | |
| $^{2}J$(g2,g2′) | −14.25 | −13.80 | | −14.3 | −13.69 | |
| $^{3}J$(g5,g2) | 7.02 | 6.57 | | 7.02 | 6.64 | |
| $^{3}J$(g5,g2′) | 5.19 | 5.03 | | 5.19 | 5.03 | |

[a] $\Delta\delta$ (ppm) is the coordination shift $\delta[Bi(GS)_3] - \delta$GSH.

In order to determine the stoichiometry of the $Bi^{III}$ glutathione complex, a $^{13}C$ NMR titration was performed. On addition of GSH to **1** at pH* 4.7, all the citrate $^{13}C$ resonances shifted gradually to high field with increasing GSH concentration, attaining the shifts of free citrate at a 3:1 mol ratio of GSH:$Bi^{III}$ (vide infra). The set of resonances for GSH which appeared in the spectrum had similar shifts to free GSH except for the Cys $\alpha$-C and $\beta$-C peaks, which shifted to lower field by 1.08 and 4.40 ppm, respectively (Fig. 2). The latter resonances increased in intensity until a ratio of 3:1 was reached, at which point peaks for free GSH appeared (in slow exchange with bound GSH on the NMR timescale). The $^{13}C$ NMR chemical shifts of GSH and the $Bi^{III}$ GSH complex are summarized in Table 2.

Plots of chemical shifts versus GSH:Bi mol ratio for citrate $^{13}C$ resonances showed abrupt discontinuities at GSH:Bi = 3:1, as illustrated in Figure 3 for citrate C6, indicative of strong GSH binding to $Bi^{III}$ and complete displacement of bound citrate to form a $[Bi(GS)_3]$ complex.

**Stability of $[Bi(GS)_3]$ as a function of pH***: To determine the pH* stability of $[Bi(GS)_3]$, $^{1}H$ NMR spectra were recorded over the pH* range 2–13. The pH* dependences of the chemical shifts of nonexchangeable protons are shown in Figure 4. The curves for Cys $\alpha$-CH and $\beta$-$CH_2$ are almost independent of pH* up to pH* 10, in contrast to those for free GSH, which show characteristic high-field shifts between pH* 8 and 10 caused by Cys thiol deprotonation. At pH* values < 2, the Cys peaks are too broad to observe. At pH* values > 10, resonances for both Cys $\alpha$-CH and $\beta$-$CH_2$ broaden and shift upfield (towards the shifts expected for unbound GSH). The pH* dependences of the chemical shifts of the peaks for $\gamma$-Glu and Gly residues of $[Bi(GS)_3]$ are almost the same as those of free GSH, suggesting that little binding of $Bi^{III}$ to the carboxylate or amino groups occurs.



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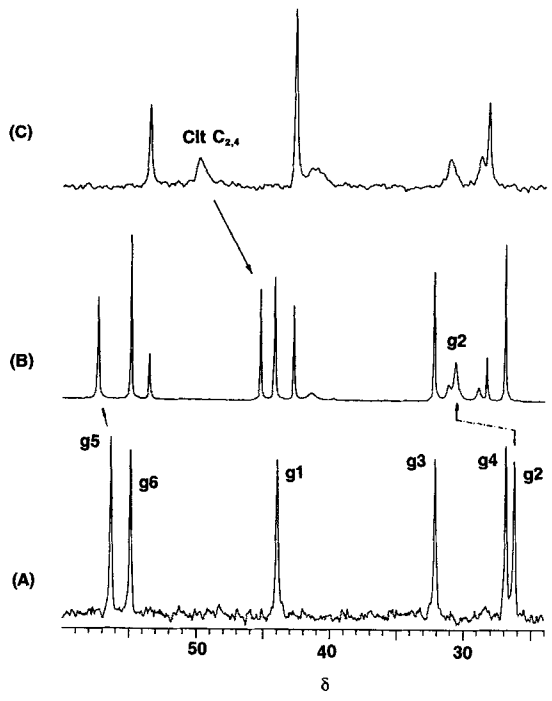
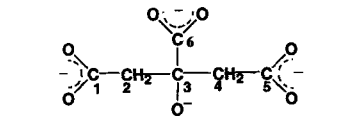



Fig. 2. 67.5 MHz $^{13}C$ NMR spectra of: A) glutathione; B) glutathione after addition of **1** (mol ratio 3:1); C) **1**, all at pH* 4.7. Note the large low-field shift of g2 on complexation of GSH to $Bi^{III}$, and displacement of citrate (high-field shift and sharpening of citrate peaks).

Table 2. $^{13}C\{^1H\}$ NMR chemical shifts ($\delta$) for glutathione and $[Bi(GS)_3]$ at pH* 4.70, 298 K.

| Carbon atoms | δGSH | δ[Bi(GS)$_3$] | Δδ [a] |
|---|---|---|---|
| Cys α-CH | 56.45 | 57.53 | 1.08 |
| Cys β-$CH_2$ | 26.31 | 30.73 | 4.40 |
| Cys CONH | 172.52 | 172.52 | 0.00 |
| Gly α-$CH_2$ | 44.03 | 44.25 | 0.22 |
| Gly COOH | 176.83 | 176.70 | −0.13 |
| Glu α-CH | 55.00 | 54.96 | −0.04 |
| Glu β-$CH_2$ | 27.00 | 27.04 | 0.04 |
| Glu γ-$CH_2$ | 32.24 | 32.32 | 0.08 |
| Glu COOH | 174.76 | 174.73 | −0.03 |
| Glu CONH | 175.83 | 175.57 | −0.26 |

[a] Δδ (ppm) is the coordination shift: δ[Bi(GS)$_3$] − δGSH.



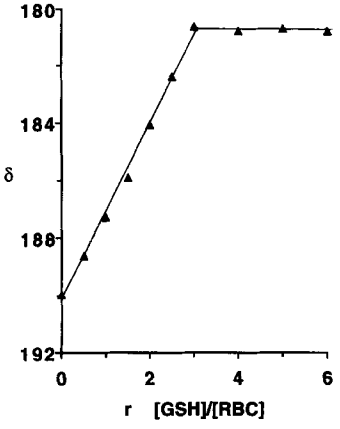




Fig. 3. Plot of the $^{13}C$ NMR chemical shift of citrate C6 versus the mol ratio of [GSH]/[RBC] at pH* 4.70; citrate is totally displaced from binding to $Bi^{III}$ at a mol ratio of 3:1, indicating the formation of the adduct $[Bi(GS)_3]$. RBC = ranitidine bismuth citrate (complex **1**).

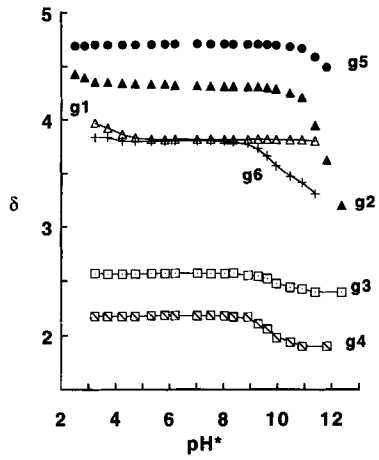



Fig. 4. Variation of the $^1H$ NMR chemical shifts of $[Bi(GS)_3]$ with pH*. The characteristic shift changes near pH* 8.60 seen for free GSH are absent for $[Bi(GS)_3]$, indicative of $Bi^{III}$ binding to the thiolate group of GSH. The shift changes observed at pH* > ca. 10 can be attributed to competitive binding of hydroxide and displacement of GSH.

**Determination of the stability constant for $[Bi(GS)_3]$**: A reversible reaction was observed between [Bi(Hedta)] and GSH. At low pH* values, $Bi^{III}$ binds strongly to edta, whereas at higher pH* values, GSH gradually displaces edta. Figure 5 shows $^1H$ NMR spectra of GSH and [Bi(Hedta)] in a 3:1 mol ratio at different pH* values. At pH* > 4.5, the Cys β-$CH_2$ multiplet (ca. $\delta$ = 2.9) broadens, and by pH* 6.5 has disappeared. Also within this pH* range, two new singlets appear at $\delta$ = 3.86 and 3.67. The

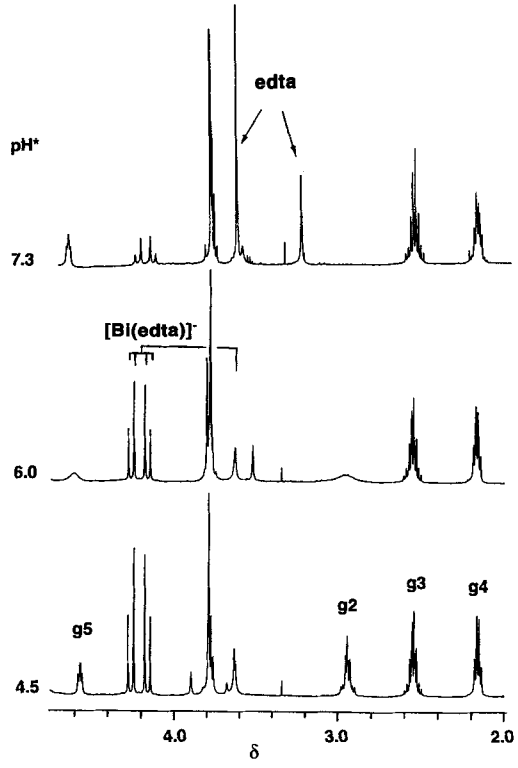



Fig. 5. 500 MHz $^1H$ NMR spectra of a solution containing [Bi(Hedta)] (10 mM) and glutathione (30 mM), $I$ = 0.1 M, $NaNO_3$, at different pH* values. GSH displaces edta from Bi as the pH* is increased: the AB pattern at $\delta$ = 4.227 and singlet at 3.635 for the $CH_2COO^-$ and $N(CH_2)_2$ protons of bound edta gradually decrease in intensity and resonances for free edta appear (as singlets), the Cys β-$CH_2$ g2 resonance for free GSH at $\delta$ = 2.9 broadens and finally disappears at high pH (g2 of bound GSH is too broad to be observed under these conditions).

latter have the same shifts as free edta, which is in slow exchange with Bi-bound edta (quartet at $\delta = 4.23$ and singlet at $\delta = 3.64$), and the amount of free edta present increases with increase in pH*.

The stability constant for $[Bi(GS)_3]$ was determined by integration of the peaks for free and bound edta (Fig. 5) with the following procedure. Firstly the conditional stability constant ($K$) for reaction (1) was determined at different pH* values by

$$[Bi(edta)]^- + 3GS^- \longrightarrow [Bi(GS)_3] + edta^{4-}$$
$$K = \{[Bi(GS)_3][edta^{4-}]\}/\{[[Bi(edta)]^-][GS^-]^3\} \quad (1)$$

integration of the appropriate $^1$H NMR peaks. Since the stability constant for $[Bi(edta)]^-$ [Eq. (2)], $K_{edta}$, is known[16]

$$Bi^{3+} + edta^{4-} \longrightarrow [Bi(edta)]^-$$
$$K_{edta} = [[Bi(edta)]^-]/\{[Bi^{3+}][edta^{4-}]\} \quad (2)$$

($\log K_{edta} = 27.8$ at 298 K, $I = 0.1$ M), we were able to calculate the conditional stability constant ($K'_{GS}$) for $[Bi(GS)_3]$ from Equation (3), by means of the relationship $K'_{GS} = K \times K'_{edta}$,

$$Bi^{3+} + 3GS^- \longrightarrow [Bi(GS)_3]$$
$$K'_{GS} = [Bi(GS)_3]/\{[Bi^{3+}][GS^-]^3\} \quad (3)$$

and hence obtain $K'_{GS}$ by assuming $pK_a$ values of 8.46 for GSH and 1.99, 2.67, 6.16 and 10.26 for $H_4$edta.[16] An average value of $\log K_{GS}$ of $29.6 \pm 0.4$ was obtained (Table 3).

Table 3. Equilibrium constants for reaction between $[Bi(edta)]^-$ and 3 mol equiv of GSH at various pH* values ($K$), conditional stability constants for $[Bi(GS)_3]$ ($K'_{GS}$) and derived stability constants ($K_{GS}$) at 298 K.

| pH* | log $K$ | log $K'_{GS}$ | log $K_{GS}$ |
|---|---|---|---|
| 4.5 | 1.86 | | |
| 5.0 | 1.94 | 23.3 | |
| 6.0 | 2.97 | 26.6 | 29.2 |
| 6.5 | 3.17 | 27.1 | 29.2 |
| 7.0 | 3.49 | 28.0 | 29.6 |
| 7.3 | 4.00 | 28.8 | 30.1 |

**Exchange behaviour**: When further GSH was added to the solution containing $[Bi(GS)_3]$ for which the $^1$H NMR spectrum is shown in Figure 1, broad peaks for free GSH appeared and the peaks for $[Bi(GS)_3]$ also broadened. However, interpretation was complicated by the presence of overlapping peaks for ranitidine. Therefore we investigated the exchange process further by means of $^1$H 2D exchange spectroscopy (EXSY). A 2D EXSY spectrum of a solution containing a GSH:ranitidine bismuth citrate mol ratio of 6:1 is shown in Figure 6 (top). The positive cross-peak at $\delta = 4.31/2.94$ is assignable to Cys $\beta$-$CH_2$ exchange between bound and free GSH, and this confirms that there is a broad g2 peak for free GSH beneath the ranitidine resonances at $\delta = 2.8-3.0$. Another cross-peak near the water signal ($\delta = 4.62/4.72$) is also clearly seen and is assignable to Cys $\alpha$-CH exchange, and weaker cross-peaks are also present for Glu $\beta$- and $\gamma$-$CH_2$.

By comparing the integrals of the diagonal peak ($I_{AA}$) and the cross-peak ($I_{AB}$), the exchange rate ($k$) was calculated with the relationship (i), in which $\tau_m$ is the EXSY mixing time.

$$I_{AA}/I_{AB} = [1 + \exp(-2k\tau_m)]/[1 - \exp(-2k\tau_m)] \quad (i)$$

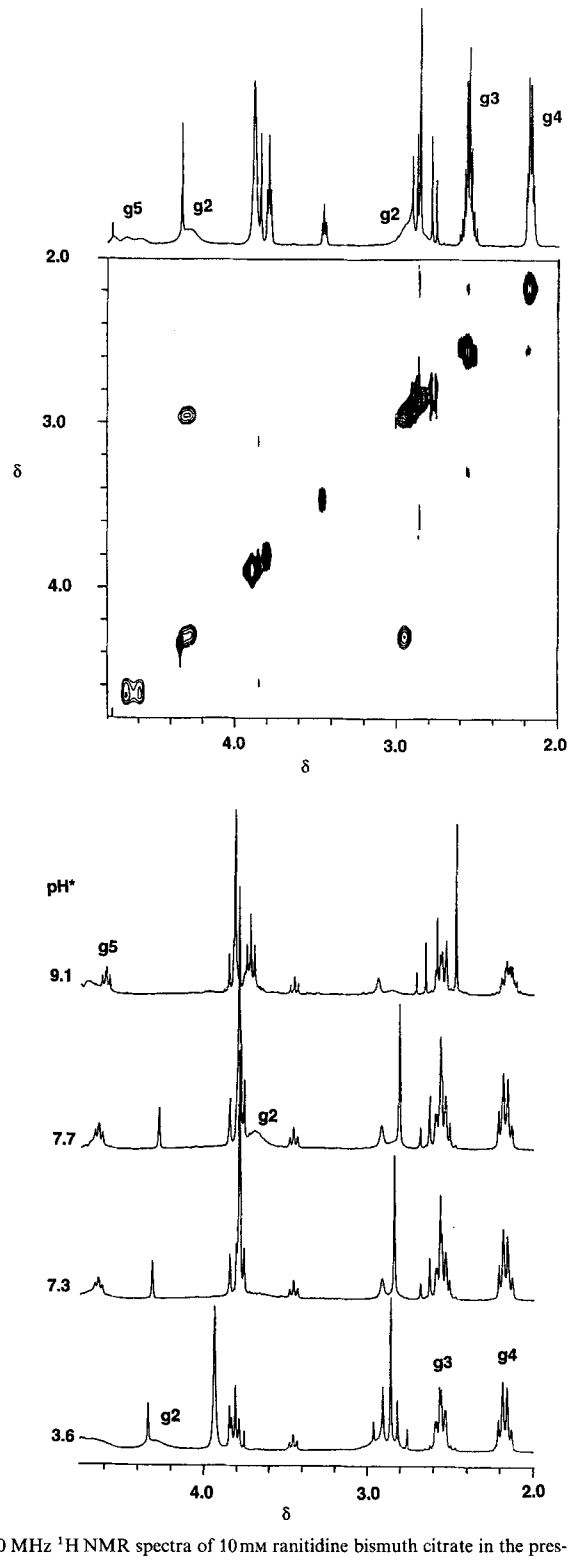



Fig. 6. 500 MHz $^1$H NMR spectra of 10 mM ranitidine bismuth citrate in the presence of 6 mol equiv of glutathione, 298 K. Top: 2D EXSY spectrum (mixing time 100 ms) at pH* 4.0, showing cross-peaks for exchange between bound and free glutathione: Cys $\beta$-$CH_2$ (g2) $\delta = 4.31/2.94$, and Cys $\alpha$-CH (g5) $\delta = 4.62/4.72$. Bottom: 1D $^1$H NMR spectra of the same solution at different pH* values. The broadened g2 signals for free and bound GSH are indicative of relatively slow exchange on the NMR timescale at low pH* values, whereas at biological pH* (ca. 7.4) exchange is more rapid and an averaged resonance is observed.

Equation (i) assumes two-site exchange and equal relaxation rates for free and bound GSH[20]. The exchange rate at pH* 4.0 was determined to be $3\ s^{-1}$ based on Cys $\beta$-$CH_2$ peaks, and $5\ s^{-1}$ based on Cys $\alpha$-CH.



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The exchange rate increases with increased pH*, such that around biological pH (7.4) the Cys $\beta$-$CH_2$ signal is too broad to observe (Fig. 6, bottom), and the exchange rate was estimated to be approximately 1500 $s^{-1}$,[21] while the Cys $\alpha$-CH peak, which has a much smaller difference between the chemical shift of free and bound forms, is still sharp. With further increase in pH* to 8.5, an averaged sharp multiplet at $\delta$ = ca. 3.8 was observed for Cys $\beta$-$CH_2$.

**Reaction of ranitidine bismuth citrate with red blood cells (ex vivo)**: In order to observe $^1$H NMR signals from GSH in intact red cells, the Hahn spin-echo technique was used. This method allows observation of cellular components such as GSH, ergothioneine, carnitine, choline, alanine and lactate, whereas peaks from proteins and membranes are largely eliminated from the spectrum by $T_2$ relaxation.[22, 23]

Figure 7 shows a comparison of $^1$H NMR Hahn spin-echo spectra of red blood cells obtained from a rat before and after oral administration of ranitidine bismuth citrate (**1**). The most

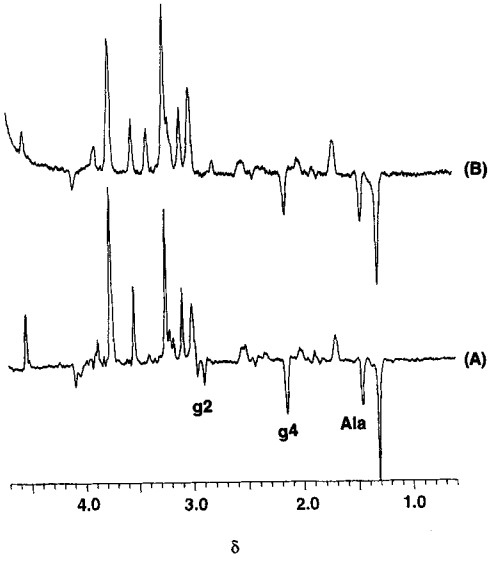



Fig. 7. The aliphatic region of 500 MHz $^1$H Hahn spin-echo NMR spectra ($\tau$ = 60 ms) at 310 K of red blood cells from a rat A) before, B) after in vivo dosing with ranitidine bismuth citrate. The disappearance of the inverted multiplet for Cys $\beta$-$CH_2$ (g2) of GSH can be explained by exchange between free GSH and Bi-bound GSH at an intermediate rate on the NMR timescale (see Fig. 6).

significant difference between these two spectra is for the g2 proton resonance, which is an inverted multiplet before administration of **1**, and almost undetectable for red cells from the dosed rat. The other resonances of GSH and those for intracellular glycine, creatine, alanine, ergothioneine and lactate are almost unchanged. Intriguingly, the spectrum of red cells from the dosed rat contains a new peak at $\delta$ = 3.40 that cannot be readily assigned.

**Reaction of ranitidine bismuth citrate with human red blood cells in vitro**: Incubation of human red blood cells with 2 mM of compound **1** at 310 K led to a gradual decrease in the intensity of the Cys $\beta$-$CH_2$ resonance (g2) of GSH over a period of 8 hours (Fig. 8), suggestive of complexation of $Bi^{III}$ by intracellular GSH. There were no apparent changes in the other resonances.

Figure 9 shows the decrease in intensity of resonance g2 relative to g4 (which did not change in intensity during the reaction) with time. A nonlinear least-squares fit of the data to the first-

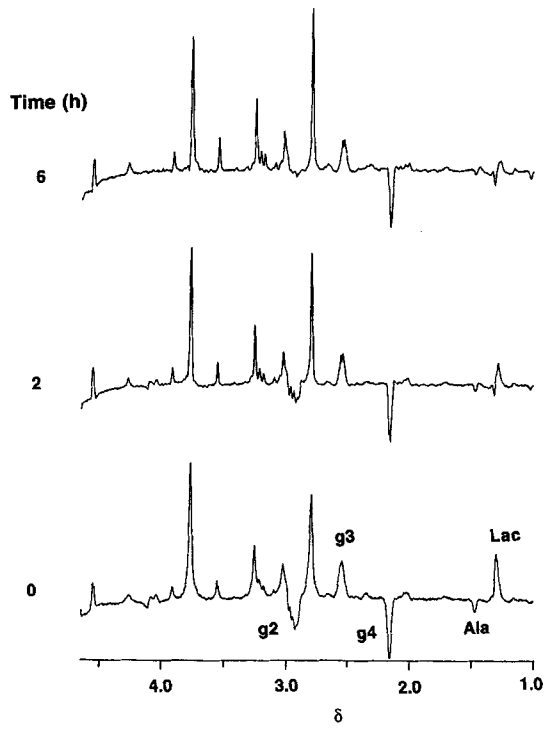



Fig. 8. The aliphatic region of 500 MHz $^1$H Hahn spin-echo NMR spectra ($\tau$ = 60 ms) at 310 K of human red blood cells before and at various times after addition of ranitidine bismuth citrate (2 mM). The intensity of the g2 resonance of intracellular GSH gradually decreases with time, indicative of slow passage of $Bi^{III}$ across the cell membrane. The lactate methyl resonance at $\delta$ = 1.29 consists of a negative doublet and a positive singlet and arises from molecules which are protonated ($CH_3CHODCO_2^-$) or deuterated ($CH_3CDODCO_2^-$), respectively, at CH.[23]

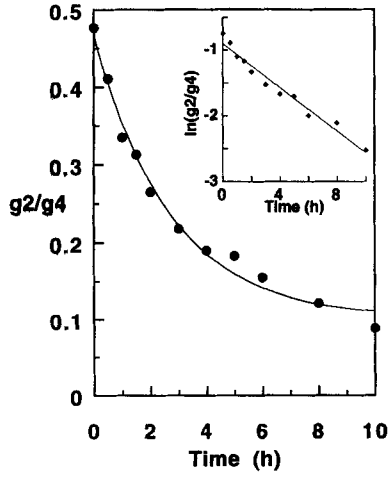



Fig. 9. Plot of the ratio of the intensities of GSH resonances g2/g4 in Hahn spin-echo spectra versus time after addition of ranitidine bismuth citrate (2 mM) to human red blood cells at 310 K. The exponential decay (see inset plot of ln(g2/g4) versus time) has a first-order rate constant of $0.20 \pm 0.04$ $h^{-1}$, and an associated half-life of ca. 3 h.

order expression $I = I_0 \exp(-kt)$, where $I_0$ and $I$ are the intensities of g2 at times zero and $t$, respectively, yielded a first-order rate constant of $0.20 \pm 0.04$ $h^{-1}$ at 310 K. When the concentration of **1** was doubled, a similar value of $k = 0.24 \pm 0.03$ $h^{-1}$ was obtained.

We also found that the spectra of red cells isolated from whole human blood which had been treated in vitro with either **1** or [Bi(Hedta)] (10 mM) for 1 h at 310 K contained no g2 peak for GSH.

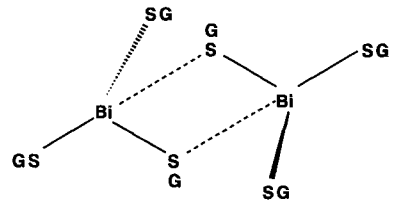
## Discussion

GSH is an abundant thiol-containing tripeptide that is present in most cells at concentrations in the range 1–10 mM. It is clear that it plays a major role in redox reactions of many metal ions in vivo and in the mobilization and transport of some of them across membranes.[24] For example, methylmercury is transported as a GSH complex through membranes into bile,[24] and metal binding to GSH by the thiolate sulfur alone, as in $Hg^{II}$–GSH complexes,[9, 10] probably allows metal–GSH complexes to be recognized by specific GSH transport proteins similarly to GSH itself.

Although bismuth compounds have been used as pharmaceutical agents for centuries, their molecular pharmacology is poorly understood. Currently, there is particular interest in the antiulcer activity of the $Bi^{III}$ compounds colloidal bismuth citrate[25, 26] (CBS, potassium ammonium bismuth citrate) and ranitidine bismuth citrate (**1**). Recently it has been shown that after administration of ammonium bismuth citrate, bismuth is transported from liver to bile as a GSH complex.[11] The apparent absence of previous chemical studies of the complexation of $Bi^{III}$ by GSH prompted us to investigate reactions of GSH with the antiulcer compound **1** and the competitive binding to $Bi^{III}$ of edta and GSH, both in aqueous solution and in intact red blood cells.

Our $^1H$ and $^{13}C$ NMR data show that $Bi^{III}$ forms a very strong complex with GSH with the stoichiometry $[Bi(GS)_3]$, and that glutathione is bound to bismuth by the thiolate only. This is clear from the $Bi^{III}$-induced coordination shifts which are about 1.37 and 0.12 ppm for the Cys $\beta$-$CH_2$ and $\alpha$-CH protons, respectively, and 4.40 and 1.08 ppm for the $^{13}C$ resonances, whereas the coordination shifts for other residues of GSH are $<0.02$ and $<0.30$ ppm for $^1H$ and $^{13}C$, respectively. Moreover, the characteristic pH-dependent shifts associated with thiol deprotonation[27] of GSH around pH 8.6 are absent for $[Bi(GS)_3]$. The $^3J(H,H)$ values for the Cys side-chain in $[Bi(GS)_3]$ are very similar to those of free GSH, as are those for the other side-chains of GSH (data not shown); this is consistent with S-only coordination and shows that $Bi^{III}$ binding has little effect on the overall conformation of GSH. This may allow $[Bi(GS)_3]$ to be recognized by cellular receptors (transport proteins) for GSH and provide a mechanism of $Bi^{III}$ transport. It is notable that Gyurasics et al. found that excretion of $Bi^{III}$ from the liver is accompanied by the cotransport of three molecules of GSH, consistent with the excretion of $[Bi(GS)_3]$.[11] Glutathione adducts also appear to be used to effect the cellular export of other metal ions,[24] including $Pt^{II}$ from cisplatin-treated tumor cells.[28]

In general, previously reported thiolate complexes of $Bi^{III}$ have been poorly characterized, and the structures of only three complexes have been determined by X-ray crystallography: $[Bi(SC_6H_2\text{-}2,4,6\text{-}t\text{Bu}_3)_3]$ contains a highly sterically hindered thiolate and is monomeric,[29] whereas $[Bi(SC_6F_5)_3]$ is a dimer containing four-coordinate (trigonal bipyramidal) $Bi^{III}$ with three short Bi–S bonds of 2.5–2.6 Å, and one weak intermolecular Bi–S bond (3.3 Å) from a bridging thiolate.[30] The third complex $[Bi(SC_6F_5)_5]^{2-}$ is ionic and contains five-coordinate $Bi^{III}$ with a square-based pyramidal coordination geometry. Irregular structures of $Bi^{III}$ complexes often appear to arise from the need to accommodate the $6s^2$ lone pair of electrons in the coordination sphere. It seems likely that the structure of $[Bi(GS)_3]$ is related to $[Bi(SC_6F_5)_3]$ and is either a monomer in solution or a weak dimer such as **2**, in which there could be exchange between bridging and terminal glutathione ligands in solution. Although it is common for the coordination number of $Bi^{III}$ to be higher than four, which could be achieved by additional weak binding of carboxylate or water oxygen atoms, our preliminary EXAFS studies suggest that Bi in $[Bi(GS)_3]$ is bound to sulfur atoms only.[43]




Our attempts to crystallize $[Bi(GS)_3]$ were unsuccessful, and we were unable to obtain peaks for molecular ions of this complex in mass spectra (data not shown). For $As^{III}$, also a member of Group 15 of the periodic table, a complex with the same stoichiometry $[As(GS)_3]$ has also been characterized.[31, 32] The reported $^{13}C$ coordination chemical shifts of the latter complex are similar to those we observed for $Bi^{III}$, but curiously the $As^{III}$-induced $^1H$ coordination shifts for Cys $\beta$-$CH_2$ (0.25, 0.40 ppm) are much smaller than those for $Bi^{III}$ (1.37 ppm, Table 1). It would be interesting to study $[Sb(GS)_3]$, which has not been reported as far as we know.

Complexes of glutathione with several other "soft" metal ions have been characterized by NMR and EXAFS and likewise shown to contain S-only binding. Both $Cu^I$ and $Au^I$ readily form polymeric 1:1 complexes, and $Hg^{II}$ mainly forms linear two-coordinate 2:1 complexes.[7, 8] When the GSH:$Hg^{II}$ mol ratio is $>2$,[33] $Hg(GS)_3$ can form, but the binding of the third GSH is much weaker.[9, 10] Charge neutralization at the metal centre appears to be a major factor in determining the stoichiometry of these stable GSH complexes of non-transition metal ions.

Our competition studies show that $[Bi(GS)_3]$ (log $K$ 29.6) is stable over the pH* range 2–10 and is more stable than $Bi^{III}$ complexes with edta (log $K$ 27.8) or citrate (log $K$ 13.5),[16] suggesting that glutathione is indeed likely to be an important ligand for $Bi^{III}$ in vivo. Also the rate of displacement of bound edta or citrate by GSH within this pH* range is very fast (<1 min at 298 K) under the conditions used here. At high pH (>10) there was some evidence from $^1H$ NMR for the displacement of $GS^-$ from $Bi^{III}$ by hydroxide, as indicated by the broadening and high-field shift of the g2 $^1H$ NMR resonance. The observed $\beta$-$CH_2$ $^1H$ NMR chemical shift ($\delta_{obs}$) is the weighted average of the shifts for free ($\delta_{GSH}$) and bound ($\delta_{Bi}$) GSH: $\delta_{obs} = f_{GSH} \times \delta_{GSH} + f_{Bi} \times \delta_{Bi}$, where $f_{GSH}$ and $f_{Bi}$ are the fractions of free and bound GSH in solution ($f_{GSH} + f_{Bi} = 1$). From this equation, mol fractions of $[Bi(GS)_3]$ at various pH* values were calculated. Even at pH* 12, about 20% of the GSH is still bound to $Bi^{III}$.

$[Bi(GS)_3]$ seems more stable than $[Zn(GS)_2]$ and $[Cd(GS)_2]$, for which log $K$ is 10.9 and 13.3, respectively.[16] In consequence, edta can remove $Zn^{II}$ and $Cd^{II}$ from metallothionein, in which these metals are bound by cysteine thiolates,[34] whereas [Bi(Hedta)] can donate $Bi^{III}$ to metallothionein.[35] It would be interesting to investigate the possible transfer of $Bi^{III}$ from $[Bi(GS)_3]$ to metallothionein, since it has been shown that administration of Bi can protect against some of the toxic side-effects induced by the anticancer drug cisplatin.[36–38] The protection probably arises from induction of metallothionein synthesis by $Bi^{III}$.

The "soft" metal ion $Hg^{II}$ also forms a very strong complex with GSH, the formation constant for $[Hg(GS)_2]$ being log $K = 41.6$.[9] Despite this high thermodynamic stability, the complex is kinetically labile towards glutathione exchange; this readily allows the transfer of $Hg^{II}$ between thiol-containing molecules in different parts of biological systems. A similar situation appears to hold for $Bi^{III}$. The exchange rate of GSH on $Bi^{III}$ at biological pH (7.4) is approximately 1500 $s^{-1}$. Thus when $Bi^{III}$ enters red cells it can exchange between all the GSH molecules in the cells at an intermediate rate on the (500 MHz) NMR



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timescale. Hence we observe a broadening of the $\beta$-$CH_2$ $^1H$ NMR resonance (g2) of intracellular GSH and its gradual disappearance from spin-echo spectra as more $Bi^{III}$ enters the cells. The binding of $Bi^{III}$ to GSH inside red cells appears to be at the same site (thiolate only) as in aqueous solution according to the pattern of NMR chemical shift changes.

The rate of uptake of $Bi^{III}$ by red cells was much slower ($t_{1/2}$ ca. 3 h at 310 K) than those previously reported for $As^{III}$, $Cd^{II}$, $Au^{I}$, $Hg^{II}$, $Pb^{II}$ and $Pb^{IV}$, which appear to be complete within minutes.[9, 39] For $Bi^{III}$, the rate-determining step seemed to involve passage across the cell membrane. This may involve transfer of Bi onto thiol-containing membrane proteins such as the hexose transporter; such a shuttle mechanism has been proposed for $Au^{I}$.[40] Our data showing that Bi is readily taken up by red cells are in agreement with those of Rao and Feldman, who showed that after equilibration of bismuth (as nitrate) added to whole blood, most of the bismuth was present in red cells.[14] Garner et al. have studied the interaction of colloidal bismuth citrate with red cells by means of spin-echo $^1H$ NMR.[15] They attributed the spectral changes to oxidation of GSH caused by adsorption of the colloid onto the cell surface. However, our data on aqueous GSH solutions, which show that $Bi^{III}$ can readily cause exchange broadening of GSH peaks, allow a different interpretation in terms of Bi entry into the cell and formation of $[Bi(GS)_3]$.

## Conclusion

Although bismuth compounds have been widely used in medicine for many centuries, the chemistry and biochemistry of bismuth are poorly understood. In this paper we have investigated in detail the interaction of the new antiulcer drug ranitidine bismuth citrate (**1**) with GSH both in aqueous solution and in intact red blood cells by means of NMR spectroscopy. The deprotonated thiolate group is shown to be the only strong binding site for $Bi^{III}$, and the complex $[Bi(GS)_3]$ forms readily by citrate displacement. The formation constant for $[Bi(GS)_3]$ was determined as log $K$ 29.6 by studies of the displacement of edta from [Bi(Hedta)] with GSH.

In spite of the extremely high thermodynamic stability of $[Bi(GS)_3]$, the bound glutathione is kinetically labile with respect to exchange with free GSH, the exchange rate being 3 $s^{-1}$ at pH* 4.0, and about 1500 $s^{-1}$ at biological pH. This would allow facile transfer of $Bi^{III}$ amongst thiol ligands in biological systems, and hence we can expect a wide distribution of $Bi^{III}$ in the body.

We have shown that $Bi^{III}$ is readily transported into red blood cells where it forms an intracellular complex with GSH with similar characteristics to $[Bi(GS)_3]$. The relatively slow rate of uptake ($t_{1/2}$ ca. 3 h at 310 K) compared with other metal ions such as $Hg^{II}$ and $Zn^{II}$ may be related to slow formation of intermediates in which $Bi^{III}$ is bound to proteins in red cell membranes. The speciation of $Bi^{III}$ in blood plasma after administration of bismuth drugs is currently poorly understood but could include bismuth–protein complexes. The complexation of $Bi^{III}$ by proteins is currently under investigation in our laboratory.[41]

## Experimental Procedure

**Chemicals**: Ranitidine bismuth citrate (**1**) was supplied by Glaxo Wellcome as an amorphous off-white powder containing ranitidine, bismuth and citrate in approximately equal mol ratio, and was used directly without further purification. [Bi(Hedta)] was prepared according to the literature procedure [42] and had a satisfactory elemental analysis (C,H,N). Glutathione (GSH, Sigma) and $D_2O$ (Fluorochem) were used as received. NaCl and $NaNO_3$ (99.99% A. R. grade) were purchased from Aldrich.

**Sample preparation**: The NMR titration experiments were carried out with $D_2O$ solutions of complex **1** (0.2 M for $^{13}C$ and 10 mM for $^1H$) and various mol ratios of GSH. Adjustments of pH* were made with NaOD, and then solutions were degassed for 10 min by bubbling with argon to minimize oxidation of GSH. All solutions for the formation constant studies contained 10 mM [Bi(Hedta)] and 30 mM GSH in $D_2O$, and the ionic strength was adjusted to 0.1 M with $NaNO_3$. Measurements of pH* were made with a Corning 135 pH meter equipped with an Aldrich micro combination electrode, calibrated with standard buffer solutions (pH 4.00, 7.00 and 10.00). The pH meter readings for $D_2O$ solutions were recorded as pH* values without correction for isotope effects, and no corrections for the effects of $Na^+$ ions on the glass electrode were applied at high pH values.

**Red blood cells**: Fresh venous blood from a healthy volunteer was collected into heparinized anticoagulant tubes. The whole blood was centrifuged at 6000 rpm for 20 min at 277 K, and the plasma and buffy coat drawn off with a plastic pipette. The packed red cells were washed three times in an equal volume of isotonic saline (0.154 M NaCl in $D_2O$, 277 K). Immediately prior to the final centrifugation step, the dilute cell suspension was saturated for 10–15 min with carbon monoxide or dioxygen to ensure that Fe(II) hemoglobin was carbonylated or oxygenated and therefore in the low-spin, diamagnetic form. About 0.7 mL of packed red cells was transferred to a 5 mm NMR tube.

Red blood cells from rats dosed orally with ranitidine bismuth citrate (200 mg $kg^{-1}$ bodyweight) were supplied by Glaxo Wellcome. The blood samples were taken 1 hour after dosing. The red cells were separated as above and NMR spectra recorded about 3 h later.

**NMR measurements**: All $^1H$ NMR experiments were made at 500 MHz on JEOL GSX 500 and Varian unity 500 NMR spectrometers. 67.5 MHz $^{13}C$ NMR spectra were recorded on a JEOL GXS270 instrument with proton decoupling. Typical pulsing conditions for $^1H$ NMR: 5 μs (45°) pulsewidth, 32 k data points, 2 s recycle delay (5 s for determination of formation constants), 16 to 64 transients. The standard NOESY pulse sequence was used to perform the 2D EXSY experiment with a mixing time of 100 ms. For $^{13}C$ NMR the following conditions were used: 6 μs (50°) pulsewidth, relaxation delay 2 s, 16 k data points, broadband $^1H$ decoupling, and 800–2000 transients.

The $^1H$ NMR spectra of red blood cells were typically the result of 128 transients, 8 k data points. A value of $\tau = 60$ ms was used in the Hahn spin-echo sequence (90°-$\tau$-180°-$\tau$-acq) [22]. To suppress the solvent resonance, presaturation for a period of 2 s prior to the observation pulse was used. For the kinetic experiments, a micro-program was used to acquire spectra at different reaction times. Typically each spectral accumulation took about 10 min. $^1H$ NMR chemical shifts for aqueous solutions are referenced to TSP (sodium 2,2,3,3-[$D_4$]trimethylsilylpropionate), and to alanine $CH_3$ ($\delta = 1.47$) for suspensions of red blood cells; $^{13}C$ NMR chemical shifts are referenced to TSP. All NMR experiments on aqueous solutions were carried out at 298 K, and those on red blood cells at 310 K.

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