Alterations in the Binding of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to DNA by Glutathione: Reduction, Autoxidation, Coordination, and Decomposition

Dominic R. Frasca and M. J. Clarke*

Contribution from the Merkert Chemistry Center, Boston College, Chestnut Hill, Massachusetts 02467

Received April 8, 1999

Abstract: The autoxidation of glutathione (GSH) is catalyzed by $[Cl(NH_3)_5Ru^{III}]^{2+}$ yielding only $[OH(NH_3)_5Ru^{III}]^{2+}$ and GSSG according to the rate law $d[GSSG]/dt = k[Ru][GSH]$, where $k = 3$ M^{-1} s^{-1}. The anaerobic reaction of GSH with $[Cl(NH_3)_5Ru^{III}]^{2+}$ yields first $[OH(NH_3)_5Ru^{III}]^{2+}$ and then $[GS(NH_3)_5Ru^{III}]^+$ at neutral pH, both through redox catalysis. The reaction appears to proceed through reduction of Ru^{III} by GSH to give $[H_2O(NH_3)_5Ru^{II}]^{2+}$, followed by coordination to produce $[GSH(NH_3)_5Ru^{II}]^{2+}$ and then oxidation of the latter ion by $[OH(NH_3)_5Ru^{III}]^{2+}$ or GSSG to yield $[GS(NH_3)_5Ru^{III}]^+$. $[GS(NH_3)_5Ru^{III}]^+$ is also produced by the reaction of GSH with $[(NH_3)_6Ru]^{3+}$ or $[py(NH_3)_5Ru]^{3+}$. Glutathione reduces $[OH(NH_3)_5Ru^{III}]^{2+}$ through a pre-equilibrium mechanism according to the following rate law: $d[Ru^{II}]/dt = k[Ru^{III}][GSH]/(K_i + [GSH])$, where $K_i = 2.0 \times 10^{-3}$ M^{-1} and $k = 2.3 \times 10^{-3}$ s^{-1}. The reduction potential of $[(GS)(NH_3)_5Ru^{III}]$ is pH-dependent according to the Nernstian equation: $E = E° - 0.59\log\{K_a/([H^+] + K_a)\}$, where $E° = -440$ mV, $pK_a = 7.1$. While $[GS(NH_3)_5Ru^{III}]$ is stable for extended periods under inert atmosphere, it changes in air, eventually yielding $[HO(NH_3)_5Ru^{III}]$ among other products at high pH with k_{obs} $(s^{-1}) = (k_1K_a + k_2[H^+])/([H^+] + K_a)$, where $k_1 = 9 \times 10^{-6}$ s^{-1}, $k_2 = 1.2 \times 10^{-4}$ s^{-1} M^{-1}, and $pK_a = 12$. At $[GSH]/[Ru^{III}] \leq 1$, the coordination of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to DNA is facilitated by GSH reduction to the more substitution-labile $[H_2O(NH_3)_5Ru^{II}]^{2+}$. However, at $[GSH]/[Ru^{III}] \geq 1$, guanine binding on DNA is inhibited by GSH, which coordinates Ru^{II} and facilitates oxidation back to Ru^{III} because of the low $E°$ of $[GS(NH_3)_5Ru^{III}]^+$. Consistent with this is the increased toxicity of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to Jurkat T-cells, when GSH levels are suppressed. High $[GSH]/[Ru]$ alters the DNA binding of $[H_2O(NH_3)_5Ru^{II}]^{2+}$ to essentially eliminate G^7 coordination and lower C^4 binding, but leaving A^6 binding relatively unaffected, which may have implications for the mechanism of ruthenium antitumor agents.

Introduction

Since some ruthenium anticancer compounds appear to be activated by cellular reduction to bind to DNA,[1,2] it is important to determine how these complexes may be reduced in vivo and which species may scavenge the metal ions. Glutathione (γ-glutamate-cysteine-glycine) is the most common cellular non-protein thiol.[3] In cells, it exists predominately in the reduced form (GSH) at concentrations of 0.1−10 mM and is readily oxidized to the disulfide (GSSG, $E°' = -0.246$ V vs NHE).[4] Among glutathione's roles are to protect cells from reactive oxygen intermediates, UV radiation, and heavy metal toxicity.[5] In the latter case, GSH scavenges and sequesters heavy metal ions by coordinating them through its sulfhydryl, thereby inhibiting their binding to proteins and nucleic acids.[3,6−9]

Coordination by GSH may also facilitate transfer to metal binding proteins, such as metallothionein.

In some cases, GSH reduces metal ions, such as Pt^{IV} anticancer drugs, to species that coordinate or otherwise react with DNA.[9,10−12] On the other hand, coordination of Pt^{II} by GSH is thought to be a contributing factor to cisplatin resistance by tumor cells.[13,14] Since some ruthenium antitumor complexes are more active in hypoxic environments,[1,2] which obtain in many tumors, it is particularly important to understand the interactions of GSH with ruthenium ions under anaerobic conditions. GSH rapidly reduces the antitumor agent, *trans*-$[Cl_4(Im)_2Ru]^-$ ($E° = -0.24$ V), possibly through an inner-sphere mechanism, thereby facilitating the dissociation of the imidazole ligands.[15]

Simple ruthenium complexes coordinated by ammine and heterocyclic nitrogen ligands, such as $[(4MePy)(NH_3)_5Ru]Cl_2$ (4MePy = 4-methylpyridine = 4-picoline), have recently been discovered to possess remarkable immunosuppressant activity, which greatly exceeds that of the clinically used cyclosporin

(1) Frasca, D.; Ciampa, J.; Emerson, J.; Umans, R. S.; Clarke, M. J. *Metal-Based Drugs* **1996**, *3*, 197−209.

(2) Clarke, M. J.; Bitler, S.; Rennert, D.; Buchbinder, M.; Kelman, A. D. *J. Inorg. Biochem.* **1980**, *12*, 79−87.

(3) Corazza, A.; Harvey, I.; Sadler, P. *Eur. J. Biochem.* **1996**, *236*, 697−705.

(4) Rabenstein, D. L.; Millis, K. K.; Weaver, K. H. *J. Org. Chem.* **1993**, *58*, 4144−4146.

(5) Buttke, T. M.; Sandstrom, P. A. *Immunol. Today* **1994**, *15*, 7−9.

(6) Bose, R. N.; Mohhaddas, S.; Weaver, E. L.; Cox, E. H. *Inorg. Chem.* **1995**, *34*, 5878−5883.

(7) Djuran, M. I.; Lempers, E. L. M.; Reedijk, J. *Inorg. Chem.* **1991**, *30*, 2648−2652.

(8) Corden, B. J. *Inorg. Chim. Acta* **1987**, *137*, 125−130.

(9) Wetterhahn, K. E.; Brauer, S. L. *J. Am. Chem. Soc.* **1991**, *113*, 3001−3007.

(10) Chen, L.; Lee, P. F.; Wong, S. Y.; Ranford, J. D.; Vittal, J. J. *J. Chem. Soc.* **1999**, 1209.

(11) Berkovits, H. J.; Floyd, R. A.; Wetterhahn, K. E.; Aiyar, J. *Environ. Health Perspect.* **1991**, *EI0*, 53−62.

(12) Eastman, A. *Biochem. Pharmacol.* **1987**, *36*, 4177.

(13) Ishikawa, T.; Ali-Osman, F. *J. Biol. Chem.* **1993**, *268*, 20116−20125.

(14) Chen, Y.; Guo, Z.; Parkinson, J. A.; Sadler, P. J. *J. Chem. Soc., Dalton Trans.* **1998**, 3577−3585.

(15) Hartmann, M.; Lipponer, K.-G.; Keppler, B. K. *Inorg. Chim. Acta* **1998**, *267*, 137−141.

8524 *J. Am. Chem. Soc., Vol. 121, No. 37, 1999*

Frasca and Clarke

and exhibits an electrochemical window of activity between 100 and 400 mV.[16,17] Consequently, biological reductants may also be involved in the immunosuppressant activity of this new class of ruthenium pharmaceuticals.

In this study, $[Cl(NH_3)_5Ru]Cl_2$, $[(NH_3)_6Ru]Cl_3$, and $[(4MePy)(NH_3)_5Ru]Cl_2$ were used to model the chemical and biological interactions between potential ruthenium pharmaceuticals and glutathione. All yield $[GS(NH_3)_5Ru^{III}]$, whose characterization is consistent with earlier studies of thiolatopentaammineruthenium(III) complexes.[18] Glutathione significantly alters the type of DNA binding exhibited by $[Cl(NH_3)_5Ru]Cl_2$, which could have profound implications for interpreting the mechanism of ruthenium antitumor agents.

Experimental Section

Chemicals and Reagents. Glutathione, GSSG, glutathione reductase, 5′-guanosine monophosphate, Ellman's reagent (5,5′-dithiobis(2-nitrobenzoic acid), calf thymus DNA (10 mg/mL), NADPH, D,L Buthionine-[*S,R*]-sulfoxime (BSO), phenazine methosulfate, 3′-[1{(phenylamino)carbonyl}3,4-tetrazolium]-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate) (XTT), and ammonium hexaflurophosphate were purchased from Sigma. Pyridine, hydrogen peroxide (30%), copper (II) sulfate pentahydrate, and deuterium oxide (>99%) were purchased from Aldrich. Argon and oxygen were purchased from Wesco. Trichloroacetic acid, acetic acid, 4-picoline, and mossy zinc were purchased from Fisher. Mercury(II) chloride and ammonium iodide were purchased from Baker. Diethyl ether was purchased from Mallinckrodt. Propanoic acid was purchased from Acros and $[(NH_3)_6Ru]Cl_3$ from Strem Chemical.

$[Cl(NH_3)_5Ru]Cl_2$ and $[(4MePy)(NH_3)_5Ru]Cl_3$ were prepared according to literature procedures.[19,20] The trifluoroacetate salt was prepared by stirring a slight deficiency of Ag_2O in neat CF_3COOH over moderate heat until the acid was nearly evaporated, dissolving the residue in a minimum amount of water followed by $[Cl(NH_3)_5Ru]Cl_2$, which was stirred with heat until a clear yellow solution resulted. The solution was filtered to remove AgCl and taken to dryness at 40−50 °C under vacuum and centrifugation (Speedvac). $[(H_2O)(NH_3)_5Ru]^{2+}$ was prepared by dissolving 50 mg/mL of $[Cl(NH_3)_5Ru](CF_3CO_2)_2$ or $[Cl(NH_3)_5Ru]Cl_2$ in Tris acetate buffer (pH 7) and reducing it over Zn/Hg amalgam under argon.

Equipment. Spectra were taken at pH 7.5 in 0.1 M ammonium formate or 0.1 M Tris acetate on a Cary 1E or a Hewlett-Packard 8453 diode array spectrophotometer. Electrochemistry was performed on a BAS 100 W electrochemical analyzer interfaced to a Gateway computer. Differential pulse voltammetry and cyclic voltammetry were performed in water using 0.1 M Tris acetate as the supporting electrolyte with $[(NH_3)_6Ru]Cl_3$ as an external standard. ^{1}H NMR spectra were taken in D_2O on a Varian Unity 300 MHz NMR spectrometer. EPR experiments were performed by Peter Doan at Northwestern University, Evanston, IL.[21]

HPLC analysis was performed on a Rainin HPLC system, which was interfaced to a MacIntosh computer through Dynamax software. Complexes were separated on a Microsorb C_8 column (5 μM, 4.6 × 150 mm) eluted with 1 M ammonium propionate (pH 5.2) at 1 mL/min. HPLC retention factors (k'): GSH, 1.53; GSSG, 2.81; $[Cl(NH_3)_5Ru]^{2+}$, 2.56; $[GS(NH_3)_5Ru]^+$, 1.48.

Elemental (C, H, N, S) analyses were performed by Robertson Microlit Laboratories. Ruthenium analyses were determined on a Perkin-Elmer graphite furnace atomic absorption instrument with an autosampler and an HGA 600 furnace, which was programmed as to [time (s) and temperature (°C)] as follows: [4, 70; 5, 200; 4, 800; 4, 1500; 1, 2800; 2, 2800].

Electrospray ionization mass spectra of glutathione, oxidized glutathione, and the GSH adducts of $[Cl(NH_3)_5Ru]Cl_2$ and $[(NH_3)_6Ru]Cl_3$ were performed at the University of Illinois Mass Spectroscopy Laboratory. All ions and fragments are formulated as having a monopositive charge.

Synthesis of $[(GS)(NH_3)_5Ru][X]$, X = PF_6^- and I^-. Small preparations were done with up to 50 mg of $[Cl(NH_3)_5Ru]Cl_2$ dissolved in a minimum of water. Larger syntheses required the more soluble $[Cl(NH_3)_5Ru](CF_3COO)_2$. An excess of glutathione was dissolved in a minimum amount of water and combined with the ruthenium solution, and the pH was adjusted to 7−8 with dilute KOH. After the mixture was allowed to react for 30 min at 37 °C under argon, the solution was loaded onto a Bio-Rex-70 column under an argon atmosphere. The product was eluted with cold, argon-purged 0.1 M NH_4I and rotary evaporated under vacuum to dryness. Excess ammonium iodide was removed by dissolving the residue in a minimum amount of water, filtering, washing the sample with large volumes of ethanol, precipitating with diethyl ether, and filtering through a medium porosity fritted glass filter. The complex was then dried under vacuum. Anal. calcd for $(C_{10}H_{15}N_3O_6S)(NH_3)_5RuI\cdot 2H_2O$: C, 18.35; H, 5.70; N, 17.13; S, 4.89; Ru, 15.44. Found: C, 18.66; H, 5.62; N, 17.08; S, 4.82; Ru, 14.83.

Adding $(NH_4)PF_6$ in ethanol to the reaction solution also precipitated the complex. Excess $(NH_4)PF_6$ was removed by washing the sample with 250 mL of hot ethanol. Spectroscopic yield: 87%. Mass spectra m/z, fragment: 630, GSSG + H_2O; 613, GSSG; 491, $[GS(NH_3)_5Ru]^+$; 474, $[GS(NH_3)_4Ru]^+$; 457, $[GS(NH_3)_3Ru]^+$; 440, $[GS(NH_3)_2Ru]^+$. ^{1}H NMR (δ, ppm): 1.25, Glu β CH_2; 2.4, Glu γ CH_2; 3.0, Cys CH_2; 3.55, Cys CH_2 (diasteriomer); 3.67, Gly CH_2; 3.78, Cys CH. UV−Vis, 505 nm; $\epsilon = 2250 \pm 170$ M^{-1} cm^{-1}; HPLC, $k' = 1.48$. The same material could also be prepared from $[(NH_3)_6Ru]Cl_3$ with a yield of 53%. No ^{1}H NMR signals that could be attributed to the paramagnetic $[GS(NH_3)_5Ru^{III}]^+$ were observed in D_2O solution, even at high salt concentration.

To determine whether glutathione disulfide formed an adduct with $[(H_2O)(NH_3)_5Ru]^{2+}$, we combined GSSG (60 mM) with $[(H_2O)(NH_3)_5Ru]^{2+}$ (300 mM). After the reactant mixture was allowed to stand under argon for 3 h at room temperature, it was cooled on ice and its UV−vis spectra were recorded.

To determine whether GSSG was formed by oxidation of GSH with Ru^{III}, we allowed a solution of $[Cl(NH_3)_5Ru]^{2-}$ (20 mM) and GSH (120 mM) to stand in air for 2 h before determining the organic products by ^{1}H NMR.

Kinetics Measurements. The formation of $[(GS)(NH_3)_5Ru^{III}]^+$ from either $[Cl(NH_3)_5Ru^{III}]^{2+}$ (326 nm, $\epsilon = 1900$ M^{-1} cm^{-1}) or $[OH(NH_3)_5Ru^{III}]^{2+}$ (296 nm, $\epsilon = 1500$ M^{-1} cm^{-1}) and GSH was monitored spectrophotometrically under pseudo-first-order conditions with excess GSH in 0.1 M ammonium formate (pH 7.5) or 0.1 M Tris acetate (TA) buffer (pH 7.5). Observed rate constants were determined from the decrease in absorbance of $[(OH)(NH_3)_5Ru]^+$ at 296 nm (A_{296}) and the increase in the absorbance of $[(GS)(NH_3)_5Ru^{III}]^{2+}$ at 505 nm (A_{505}). All solutions were adjusted to pH 7.6 with HCl or NaOH and purged with argon for 20 min before each experiment.

The formation of $[(GS)(NH_3)_5Ru^{III}]^+$ from $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ under argon, which was obtained by prior reduction of $[Cl(NH_3)_5Ru^{III}]^{2+}$ over Zn/Hg amalgam, was similarly monitored at 505 nm.

The reduction of $[Cl(NH_3)_5Ru^{III}]^{2+}$ (0.125 mM in 0.1 M TA buffer, pH 7.5) or $[(NH_3)_6Ru]^{3+}$ (0.125 mM in 0.1 M TA buffer, pH 7.5) by GSH (1 to 25 mM in TA buffer) in 0.75 M isonicotinamide was followed by monitoring the formation of $[Isn(NH_3)_5Ru^{II}]^{2+}$ at 480 nm ($\epsilon = 11\,100$ M^{-1} cm^{-1}), which presumably forms from the intermediate $[H_2O(NH_3)_5Ru^{III}]^{2+}$.[22]

(16) Franolic, J. D.; Bastos, C. M.; Ocain, T. D.; Gordon, K. A. in *Abstracts of Papers.* 212th National Meeting of the American Chemical Society, Orlando, FL, Fall 1996; American Chemical Society: Washington, D.C., 1996; pp INOR 327.

(17) Clarke, M.; Bailey, V. M.; Doan, P.; Hiller, C.; LaChance-Galang, K. J.; Daghlian, H.; Mandal, S.; Bastos, C.; Lang, D. *Inorg. Chem.* **1996**, *35*, 4896−4903.

(18) Kuehn, C. G.; Taube, H. *J. Am. Chem. Soc.* **1976**, *98*, 689−702.

(19) Allen, A. D.; Bottomley, F.; Harris, R. O.; Reinslau, V. P.; Senoff, C. V. *Inorg. Synth.* **1969**, *12*, 1−7.

(20) Ford, P.; Rudd, d. F. P.; Gaunder, R.; Taube, H. *J. Am. Chem. Soc.* **1968**, *90*, 1187−1194.

(21) LaChance-Galang, K. J.; Doan, P. E.; Clarke, M. J.; Rao, U.; Yamano, A.; Hoffman, B. *J. Am. Chem. Soc.* **1995**, *117*, 3529−3538.

(22) Gaunder, R. G.; Taube, H. *Inorg. Chem.* **1970**, *9*, 2627−2639.

The reduction of $[(4MePy)(NH_3)_5Ru^{III}]^{3+}$ (0.125 mM) by glutathione (1.25 mM to 25 mM) was followed by monitoring the following: (1) the decrease in absorbance of $[(4MePy)(NH_3)_5Ru^{III}]^{3+}$ at 299 nm, (2) the increase of $[(4MePy)(NH_3)_5Ru^{II}]^{2+}$ at 410 nm ($\lambda_{max}= 407$ nm),[20] and (3) the increase in $[(GS)(NH_3)_5Ru^{III}]^+$ at 510 nm. Reactions were performed with glutathione in a 10-fold or higher excess over the ruthenium concentrations in 0.1 M Tris acetate buffer at pH 7.5 and 22 °C.

The decomposition kinetics in air were on solutions of $[(GS)(NH_3)_5Ru^{III}]^+$ freshly eluted from a SP C-25 Sephadex column in either 0.1 M ammonium formate or ammonium acetate. Samples were diluted until $A_{505} = 1.0$ (4.4×10^{-4} M) and exposed to air at 25 °C. Reactions were followed with and without Cu^{2+} (4.9 mM) at pH 1−10 by monitoring the decrease in absorbance at 505 nm (A_{505}).

The ruthenium-catalyzed air oxidation of glutathione was monitored by following the appearance of glutathione disulfide by HPLC. Glutathione (0−20 mM) and $[Cl(NH_3)_5Ru]Cl_2$ (0−25 mM) were combined in 1 M ammonium propionate, pH 5.2, and left exposed to air at 25 °C. At 7−15 min intervals, aliquots were withdrawn and analyzed by HPLC on a C8 reverse-phase column (4.6×150 mm). The mobile phase was 1 M ammonium propionate, pH 5.2.

Equilibriuim Binding. Solutions (5 mL) of 0.25 mM $[Cl(NH_3)_5Ru]Cl_2$ in 0.1 M Tris acetate buffer, pH 7.5, were combined with the same volume of 2.5 mM glutathione. Pyridine was added so that [py] ranged from 12.5 to 500 mM in a total volume of 10 mL. After the mixture stood for 3 h at room temperature under argon, A_{405} ($[py(NH_3)_5Ru]^{2+}$, $\epsilon_{405} = 7780$ M^{-1} cm^{-1})[22] and A_{505} ($[(GS)(NH_3)_5Ru^{III}]^+$, $\epsilon_{505} = 2250$ M^{-1} cm^{-1}) were determined to quantify the ruthenium bound to pyridine and glutathione, respectively. The equation given in the results section was then used to calculate K_{eq}. Alternatively, $[Cl(NH_3)_5Ru]Cl_2$ (0.1 or 1 mM) was combined with an equivalent to 20-fold excess of GSH in 0.1 M TA buffer at pH 7.5 under argon for 5 h. The concentration of $[(GS)(NH_3)_5Ru^{III}]^+$ was determined spectrophotometrically and used to calculate K_{eq}.

DNA Binding Assays. To determine ruthenium binding by 5′GMP in the presence of GSH, we incubated $[Cl(NH_3)_5Ru]Cl_3$ (0.49 mM) with 5′GMP (2.7 mM) and GSH (25.2 mM) for 30 min in 0.1 M Tris acetate buffer at pH 7 under argon. The solution was diluted 1:1 with Tris acetate buffer and the UV−vis spectrum recorded. Spectra were recorded after oxidation by bubbling O_2 gas through the solution for 30 min.

To determine ruthenium binding to CT DNA, a DNA solution was quantified by absorption at 260 nm ($\epsilon = 6600$ M^{-1} cm^{-1} per phosphate) and diluted with 0.1 M Tris acetate buffer (pH 7) to a concentration of 5 mM. This was incubated with 0.8 equiv of $[Cl(NH_3)_5Ru]Cl_2$ in both the presence and absence of GSH, which was in the physiological concentration range (0.1−10 mM). Reactions were allowed to proceed for 24 h at room temperature under argon. The solutions were then dialyzed against 0.1 M Tris acetate (pH 7) in air in dialysis tubing with a molecular weight cut off of 20 000, to remove reagents not bound to DNA. To ensure that excess Ru was quantitatively removed by dialysis, the [Ru] in the dialysis baths was determined by graphite furnace atomic absorption. The third dialysis bath showed no significant [Ru]. DNA was quantified again by A_{260}, and dilutions were made to bring all solutions to the same [P$_{DNA}$]. Ruthenium concentrations were then determined by graphite furnace AA, and the ratio of Ru bound to the DNA per DNA phosphate, [Ru$_{DNA}$/P$_{DNA}$], was determined. The procedure was repeated with [P$_{DNA}$] = 50 μM, [Ru] = 20 μM, and GSH at physiological concentrations (0.1−10 mM), with a reaction time of 1.5 h. The reaction was also run under aerobic conditions for 1.5 h at 22 C, with [GSH] 10 mM, [P$_{DNA}$] = 100 μM, and [Ru] = 20 μM. A similar set of experiments was carried out at higher concentrations ([P$_{DNA}$] = 2.0 mM, [Ru] = 1.6 mM), and the formation of $[(NH_3)_5Ru^{III}]_n$-DNA was quantified by the guanine → Ru^{III} LMCT band at 570 nm ($\epsilon_{570} = 441$ M^{-1} cm^{-1}).[23,24]

To determine whether glutathione could remove the metal ion from $[(NH_3)_5Ru^{III}]_n$-DNA, we dissolved calf thymus DNA in 0.1 M TA buffer under argon and added a solution of $[H_2O(NH_3)_5Ru]^{2+}$. Final concentra-

tions were 2 mM in P$_{DNA}$ and 1 mM in $[Cl(NH_3)_5Ru]^{2+}$. After the mixture was allowed to react for 30 min, it was oxidized by sparging with O_2 for 60 min. After oxidation, the solution was dialyzed against 0.1 M NaCl several times to remove any unreacted ruthenium. [P$_{DNA}$] and [G$_{DNA}$-Ru$_{DNA}$] were quantified by absorbance at 260 and 570 nm, respectively.

To determine whether coordination of ruthenium was occurring on A and C sites, we incubated CT-DNA ([P$_{DNA}$] = 4 mM) for 3 h under argon with 1 equiv of ruthenium ([Ru$_{DNA}$]/[P$_{DNA}$] = 1), with either Zn/Hg amalgam or one equivalent of GSH as the reductant. After the samples were oxidized by molecular oxygen for 6 h, they were dialyzed against 0.1 M TA buffer in air to remove any unbound species, and the UV−vis spectra were recorded. Absorbances at 570, 384, and 356 nm together with the molar absorptivities of $[L(NH_3)_5Ru^{III}]$, L = Guo, Ado, and Cyt, at these wavelengths, using A (384 nm, $\epsilon = 8.0 \times 10^3$ M^{-1} cm^{-1}) and C (356 nm, $\epsilon = 6.4 \times 10^3$ M^{-1} cm^{-1}) were used to quantify the concentrations of Ru^{III} bound to these three residues.[25] The sample that had been reduced by Zn/Hg amalgam was then incubated with a 60-fold excess of GSH under argon overnight, oxidized by molecular oxygen, and dialyzed as before. Spectra were recorded to determine any change in the relative amount of Ru binding. The solution was then allowed to react in the presence of 100 mM glutathione for 16 h under argon, before dialysis against several changes of 0.1 M NaCl, followed by quantitation of [Ru$_{DNA}$] and [P$_{DNA}$].

Toxicology Assay. Jurkat cells (a gift from the lab of Dr. Marc Snapper, Department of Chemistry, Boston College) were maintained in 1640 RPMI media, supplemented with 10% fetal bovine serum, 1% L-glutamine, and 0.5% penicillin−streptomycin solution (Bio-Whittaker). Cells were maintained at 37 °C with stirring in an incubator. Assaying the supplemented RPMI 1640 media for [GSH] by the glutathione reductase method described below yielded [GSH] = 58 nM, which was only 0.55% of the GSH present in the control cell culture (cells cultured with no GSH modifiers). Consequently, unless GSH was added, the measured [GSH] levels derive from the cultured cells alone.

In a typical experiment to determine the toxicity (IC$_{50}$) of a ruthenium compound under normal and suppressed cellular GSH levels, a cell culture (20 mL, 2×10^6 cells/mL) was incubated with the GSH suppressor, BSO (50 μM), for 48 h. The cells were centrifuged and resuspended to a concentration of 2×10^6 cells/mL with media supplemented with either no inhibitor or BSO and then plated in a 96 well plate against $[Cl(NH_3)_5Ru]Cl_2$. [Ru] ranged from 0 (control) to 5 mM. All data points were run in quadruplicate. Cell viability was assayed after 24 h of exposure to the ruthenium complex by measuring the activity of cellular dehydrogenase enzymes.[26] XTT was dissolved in warm culture media to obtain a concentration of 0.5 mg/mL. An aliquot of a 100 mM stock solution of phenazine methosulfate was then added to give a final concentration of 67 mM. Fifty microliters of XTT/phenazine methosulfate solution was added to each well, including blank wells that contained no cells. The final volume in each well was 450 μL. After 4 h, the reaction was stopped by the addition of 550 μL of cold water. The absorbance at 450 nm of each well was read. Data were expressed as a percentage of the control wells, where the absorbance of the control wells was expressed as 100% growth. IC$_{50}$ was determined from a plot of cell viability versus [Ru].

For the determination of the cellular [GSH], the cells were centrifuged, washed with 0.1 M Tris (pH 7), and homogenized with a glass homogenizer. After proteins were precipitated with a 10% TCA solution, the mixture was centrifuged and the supernatant stored at −93 C until needed. A glutathione reductase assay was used to measure [GSH].[27] Ellman's reagent (5,5′-dithio bisnitrobenzoic acid, 6 mM (100 μL), NADPH, 0.3 mM (700 μL)) and the glutathione solution were added to a cuvette. An aliquot of glutathione reductase (50 units/mL; 10 μL) was added immediately, and ΔA_{412} was measured over 6 min. The amount of glutathione in the cell suspension sample was determined

(23) Clarke, M. J.; Taube, H. *J. Am. Chem. Soc.* **1974**, *96*, 5413−5419.

(24) Clarke, M. J.; Jansen, B.; Marx, K. A.; Kruger, R. *Inorg. Chim. Acta* **1986**, *124*, 13−28.
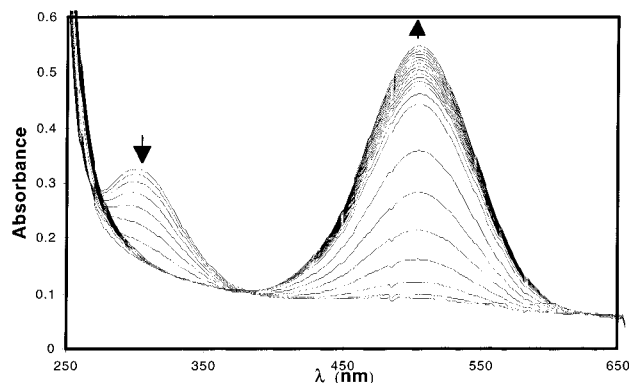
(25) McNamara, M.; Clarke, M. J. *Inorg. Chim. Acta* **1992**, *195*, 175−185.

(26) Roehm, N. W. R.; G. H.; Hatfield, S. M. *J. Immunol. Methods* **1991**, *142*, 257−265.

(27) Tietze, F. *Anal. Biochem.* **1969**, *27*, 502−522.

Figure 1. Spectroscopic changes during the reaction of GSH (2.5 mM) and $[Cl(NH_3)_5Ru]^{2+}$ (0.25 mM) in 0.1 M TA buffer, pH 7.5, at room temperature under argon.

from a calibration curve of [GSH] (3.2−320 nM) by plotting [GSH] versus ΔA_{412} over the 6 min interval.

Results

Reactivity of Glutathione with $[Cl(NH_3)_5Ru]Cl_2$. When allowed to react in the atmosphere at pH 8 at 23 and 40 °C, $[Cl(NH_3)_5Ru]Cl_2$ (0.3 mM) and GSH (2.5 mM) yielded no absorption indicative of complexation by GSH. However, the LMCT (Cl^- → d_π Ru^{III}) absorption at 326 nm[28] decreased as an absorption at 296 nm increased, which is consistent with the redox catalytic conversion of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to $[OH-(NH_3)_5Ru^{III}]^{2+}$. To determine whether GSSG was formed during this reaction, we dissolved $[Cl(NH_3)_5Ru]^{2+}$ (1 mM) and glutathione (3.5 mM) in D_2O and exposed them to air for 2 h at 22 °C. ^1H NMR showed that the single glutathione Cys-CH_2 (δ 2.92) resonance evolved into two resonances at 3.0 and 3.8 δ, which correspond to those of Cys-CH_2 in GSSG. Since no observable GSH−Ru complex occurs under these conditions, $[OH(NH_3)_5Ru^{III}]^{2+}$ appeared to catalyze the autoxidation of the GSH to GSSG. When the reaction between GSH (5 mM) and $[Cl(NH_3)_5Ru^{III}]^{2+}$ (1.5 mM) was run under air in D_2O and quantified by ^1H NMR (500 MHz), nearly all of the glutathione was converted to GSSG after 4.5 h, while there was only a 25% conversion under argon. When monitored by HPLC, the ruthenium-catalyzed autoxidation of GSH to GSSG by $[OH-(NH_3)_5Ru]^{2+}$ proceeded according to the rate law: d[GSSG] = k[GSH][Ru], with $k = 3 \pm 1$ M^{-1} s^{-1} (see Figure 7S).

When the reaction was run under an inert atmosphere, UV−vis monitoring of the reaction between $[Cl(NH_3)_5Ru]Cl_2$ and GSH revealed complex formation (see Figures 1 and 1S). The starting material, $[Cl(NH_3)_5Ru^{III}]^+$, disappeared rapidly in concert with the appearance of a strong absorbance at 296 nm (A_{296}) due to $[OH(NH_3)_5Ru^{III}]^+$, so that the reaction between $[OH(NH_3)_5Ru^{III}]^+$ and GSH was normally followed. A delay was usually observed before the product peak at 505 nm due to $[(GS)(NH_3)_5Ru^{III}]^{2+}$ appeared (see below and Figure 1S). This incubation time varied approximately with $[GSH]^{2-}$. Since complexes of the type $[(RS)(NH_3)_5Ru^{III}]^{2+}$ absorb strongly around 508 nm ($\epsilon = 1900$ M^{-1} cm^{-1}), while their Ru^{II} analogues do not,[18] the initial phase of the reaction appears to be due to the GSH reduction of Ru^{III} to Ru^{II} with the latter phase(s) arising from the subsequent binding of GSH followed by oxidation to yield $[(GS)(NH_3)_5Ru^{III}]^{2+}$ and related adducts.

Electrospray mass spectrometry of the solid product yielded a series of peaks at z/m of 491, 474, 457, and 450, which correspond to $[(GS)(NH_3)_5Ru^{III}]^+$ and sequential loss of NH_3,
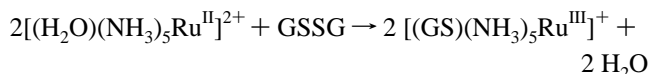
with good matches to the calculated isotopic peaks (see Figures 2S and 3S). At neutral pH, the coordinated glutathione is dianionic, owing to protonation of the ammine, ionization of the two carboxylate protons, and displacement of the thiol proton by the Ru^{III}.

In contrast with the broad line spectra usually seen for amminerruthenium(III) complexes,[17,21,29,30] EPR of $[(GS)(NH_3)_5$-$Ru^{III,}]^+$ in ethylene glycol/water (1:1) at 4 K yielded fairly narrow lines with $g_\perp = 2.3$ and $g_{||} = 1.88$ (Figure 5s). Quenching of spin−orbit coupling by the strong π-donor sulfur probably accounts for the relatively narrow EPR line widths. The lack of proton NMR signals probably also derives from the altered electron relaxation times relative to ammine or imine complexes as well as the movement of the flexible ligand through the paramagnetic field.

An attempt to measure the equilibrium binding constant for the formation of $[(GS)(NH_3)_5Ru^{III}]^+$ from GSH and $[(OH)-(NH_3)_5Ru^{III}]^{2+}$ by varying the reactant concentrations of these two compounds and spectrophotometrically monitoring the concentrations of the product at pH 7.5 yielded $K_{eq} = (3.8 \pm 1) \times 10^2$ M^{-1} at pH 7.5. Expressing the equilibrium in terms of the aqua complex ($pK_a([(H_2O)(NH_3)_5Ru]^{3+}) = 4.1$) reacting with GS^- ($pK_a(GSH) = 8.74$)[31] yields the following: $K_1 = [GS-Ru^{III}]/([GS^-][Ru^{III}]) = (1.7 \pm 0.5) \times 10^7$ M^{-1}.

Pyridine displaced GSH from $[(GS)(NH_3)_5Ru^{III}]^+$ in the presence of GSH ([GS−Ru] = 15 mM, [GSH] = 30 mM, [py] = 2 M, under argon) to form $[(py)(NH_3)_5Ru^{II}]^{2+}$. GSH also displaced 4-picoline from $[(4MePy)(NH_3)_5Ru^{III}]^+$ ([Ru] = 0.125 mM, [GSH] = 1−25 mM, 22 °C), possibly following reduction to Ru^{II}. Consequently, K_{eq} was also estimated by running the complexation reaction in the presence of varying concentrations of pyridine to scavenge Ru^{II} (0 < [py] < 0.5 M, [GSH] = 2.5 $\times 10^{-3}$ M, $[Cl(NH_3)_5Ru]^{2+} = 2.50 \times 10^{-4}$ M, pH 7.5). The spectrophometrically determined values of the ratio $R = [GS-Ru^{III}][py])/([Ru^{II}-py][GSH])$ were between 6 and 60. When calculated by the equation $K_{eq} = K_{py}R[Ru^{II}]/[Ru^{III}]$, where K_{py} is the equilibrium binding constant for pyridine with $[(H_2O)-(NH_3)_5Ru]^{2+}$ (2.4×10^7 M^{-1}) and $[Ru^{II}]/[Ru^{III}]$ is the Nernstian ratio for the aquapentammine complexes calculated at the $E°$ of GSH (-0.246 V), K_{eq} is estimated to be between 10^{14} and 10^{15} M^{-1}. Chromatographic separation of the synthetic reaction mixtures always revealed some material that bound strongly to the column, which was counted as free ruthenium in the first type of determination of K_{eq}. On the other hand, in the competitive binding method, K_{eq} is calculated from the ratio, $[GS-Ru^{III}]/[py-Ru^{II}]$, which is experimentally determined, and the calculated Nernstian ratio, $[Ru^{II}]/[Ru^{III}]$.

Combining $[Cl(NH_3)_5Ru^{III}]^{2+}$ (5 mM) with GSSG (50 mM) at pH 8 in 0.1 M NaCl resulted in no observable reaction after 5 h under argon at room temperature. However, combining GSSG (17 mM) with $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ (133 mM) under argon at room temperature at pH 8 led to the formation of observable $[(GS)(NH_3)_5Ru^{III}]^+$ within 3 h. This suggests some reduction of GSSG to GSH by Ru^{II} ($\Delta E° = -0.35$ V) with the formation of $[(GS)(NH_3)_5Ru^{III}]^+$ ($E° = -0.44$ V) providing the driving force for the net reaction:

$$2[(H_2O)(NH_3)_5Ru^{II}]^{2+} + GSSG \rightarrow 2[(GS)(NH_3)_5Ru^{III}]^+ + 2 H_2O$$

where $\Delta E = 0.09$ V.

(28) Stritar, J. A.; Taube, H. *Inorg. Chem.* **1969**, *8*, 2281.

(29) Bailey, V. M.; LaChance-Galang, K. J.; Doan, P. E.; Clarke, M. J. *Inorg. Chem.* **1997**, *36*, 1873−1883.

(30) Carlin, R. L. *Magnetochemistry*; Springer-Verlag: New York, 1986.

(31) Rabenstein, D. L. *J. Am. Chem. Soc.* **1973**, *95*, 2797−2803.
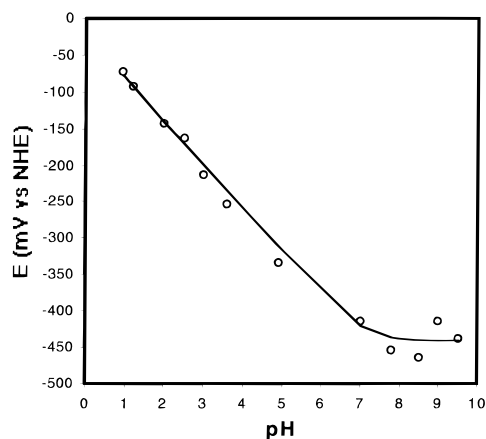
Figure 2. Pourbaix plot for [(GS)(NH₃)₅Ru^{III,II}]. Data were collected by differential pulse voltammetry at 23 °C, in 0.1 M Tris acetate buffer (see Figure 7S for a representative scan). Scan rate was normally 100 mV/s. The working electrodes used were either platinum or carbon paste; the reference electrode was a Ag/AgCl, and the auxiliary electrode was a platinum wire. The data were fit to the expression $E = E° - 0.59\log\{K_a/([H^+] + K_a)\}$, where $E° = -440 \pm 0.8$ mV, $pK_a = 7.1 \pm 0.2$.

Redox Chemistry. Cyclic voltammetry of [(GS)(NH₃)₅Ru^{III,II}] revealed an irreversible reduction process around −0.4 V in 0.1 M NaCl in both the presence and absence and of free glutathione. Anodic and cathodic peak separations (Δ$E_{a,c}$) were 25% larger than those of the reversible standard, [(NH₃)₆Ru^{III,II}], at each scan rate. Peak current ratios ($i_{p,a}/i_{p,a}$) were 0.90 compared with 1.02 for [(NH₃)₆Ru^{III,II}]. Plots of $i_{p,a}$ versus the square root of the scan rate were linear for both [(NH₃)₆Ru^{III,II}] and [(GS)(NH₃)₅Ru^{III,II}]. Since [(GS)(NH₃)₅Ru^{III}] decomposes in air (see below), a pH-dependent, reversible couple attributed to [(H₂O)(NH₃)₅Ru^{III,II}] was also evident. An irreversible oxidation peak at 614 ± 9 mV was unaffected by pH in the pH range of 1−9.

Reduction potentials as a function of pH were obtained by differential pulse voltammetry (Figure 8s). The Pourbaix plot shown in Figure 2 yielded the following: $E = E° - 0.59\log\{K_a/([H^+] + K_a)\}$, where $E° = -440 \pm 8$ mV, $pK_a = 7.1 \pm 0.2$. The pK_a value is significantly more acidic than that for free GSH (8.66)[32] and [(CH₃CH₂SH)(NH₃)₅Ru^{II}] (9.2).[18] The pK_a value for [(GSH)(NH₃)₅Ru^{III}] is sufficiently acidic that it could not be measured; however, assuming the reduction potential for [(GSH)(NH₃)₅Ru^{III}]$^{3+}$ to be similar to that of [((CH₃)₂S)(NH₃)₅Ru^{III}]$^{3+}$ ($E° = 0.50$ V),[18] its pK_{a1} can be estimated as −8.8.

Reduction of Ru^{III} by GSH. Mixtures containing GSH (2.5 mM), [Cl(NH₃)₅Ru^{III}]$^{2+}$ (0.25 mM), and pyridine (0.75 M) at pH 7.6 and 22 °C rapidly developed a peak at 405 nm (only), which corresponds to the d$_π$(Ru^{II}) → π*(py) MLCT band of [py(NH₃)₅Ru^{II}]$^{2+}$.[20] In the absence of GSH, argon purged solutions of pyridine and [Cl(NH₃)₅Ru]$^{2+}$ reacted only very slowly to produce the 405 nm peak.

The reductions of [Cl(NH₃)₅Ru^{III}]$^{2+}$ and [Ru(NH₃)₆]$^{3+}$ by GSH were quantitatively followed by monitoring the formation of [Isn(NH₃)₅Ru^{II}]$^{2+}$. Reduction of [Cl(NH₃)₅Ru^{III}]$^{2+}$ in water appears to rapidly yield [H₂O(NH₃)₅Ru^{II}]$^{2+}$, which substitutes Isn with a second-order rate constant of 0.105 M^{-1} s^{-1}.[33] Formation of the isonicotinamide complex occurred most rapidly
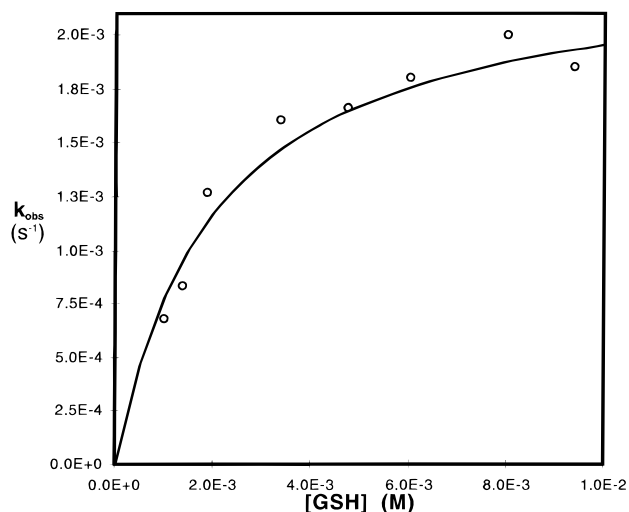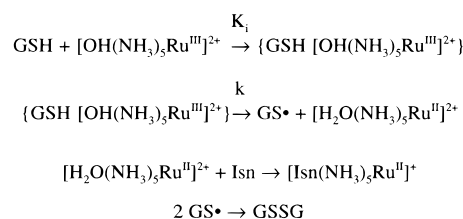
(32) Budavari, S., O'Neil, M., Smith, A., Heckleman, P., Kinnerary, J., Eds. *The Merck Index*, 12th ed.; Merck & Co: Whithouse Station, NJ, 1996; pp 761, ref# 4483.
(33) Shepherd, R. E.; Taube, H. *Inorg. Chem.* **1973**, *12*, 1392−1401.

Figure 3. k_{obs} versus [GSH] for the reduction of [Cl(NH₃)₅Ru^{III}]$^{2+}$ by GSH at pH 7.5. Fit is to the equation $k_{obs} = k[GSH]/(K_{ip} + [GSH])$, where $k = 2.34 \times 10^{-3}$ s^{-1} and $K_{ip} = 1.98 \times 10^{-3}$ M^{-1}; [Ru] = 1.25 × 10^{-4} M, [Isn] = 0.75 M; $T = 37$ °C; $μ = 0.1$ M TA buffer.

Scheme 1

$$GSH + [OH(NH_3)_5Ru^{III}]^{2+} \xrightarrow{K_i} \{GSH\,[OH(NH_3)_5Ru^{III}]^{2+}\}$$

$$\{GSH\,[OH(NH_3)_5Ru^{III}]^{2+}\} \xrightarrow{k} GS\bullet + [H_2O(NH_3)_5Ru^{II}]^{2+}$$

$$[H_2O(NH_3)_5Ru^{II}]^{2+} + Isn \rightarrow [Isn(NH_3)_5Ru^{II}]^+$$

$$2\,GS\bullet \rightarrow GSSG$$

between pH 7 and 8. Above pH 8, deprotonation of the glutamate NH₃$^+$ may lead to competitive ligand binding. At neutral pH, the carboxylic protons of GSH are ionized ($pK_{a1} = 2.12$, $pK_{a2} = 3.53$)[32] to yield the glutathione monoanion, which would be expected to ion-pair with [Cl(NH₃)₅Ru^{III}]$^{2+}$ or [HO-(NH₃)₅Ru^{III}]$^{2+}$. Pseudo-first-order rate constants as a function of [GSH] at pH 7.5 were fit to the relation $k_{obs} = k[GSH]/(K_i + [GSH])$, where $K_i = (2.0 \pm 0.4) \times 10^{-3}$ M^{-1} and $k = (2.3 \pm 0.2) \times 10^{-3}$ s^{-1} (see Figure 3). At low [GSH] this yields a second-order rate constant ($k' = k/K_i$) of 0.87 M^{-1} s^{-1}. The rate law is consistent with a pre-equilibrium mechanism at neutral pH (Scheme 1), which may involve ion-pairing, coordination, or hydrogen atom transfer. Activation parameters extracted from the Eyring plot shown in Figure 4S are Δ$H^‡ = 58.4 \pm 4$ kJ/mol and Δ$S^‡ = -116 \pm 18$ J/(mol K).

While the reaction of GSH with [(NH₃)₆Ru]$^{3+}$ under argon provides [GS(NH₃)₅Ru]$^+$ in 53% yield after 3 h, when the reaction is run in the presence of high concentrations of isonicotinamide or pyridine, [L(NH₃)₅Ru]$^{2+}$ (L = Isn or py) is cleanly formed. Under such conditions (e.g., 37 °C, 0.1 M TA buffer, [Ru] = 0.125 mM, [GSH] = 1.2 mM, [Isn] = 0.75 M; or 25 °C, [Ru] = 0.25 mM, [GSH] = 7.7 mM and [py] = 1 M), no [GS(NH₃)₅Ru^{III}]$^+$ was evident. The appearance of [L(NH₃)₅Ru]$^{2+}$ ($t_{1/2} \cong 10^{-5}$ s) from [(NH₃)₆Ru]$^{3+}$ was approximately 100 times slower than that from [OH(NH₃)₅Ru]$^{2+}$ ($t_{1/2} \cong 10^{-3}$ s) at pH 7.5 ([Ru^{III}] = 0.125 mM, 37 °C), which is likely due to the much slower rate-limiting aquation rate of [(NH₃)Ru]$^{2+}$ ($k = 9.3 \times 10^{-6}$ s^{-1}, 25 °C).[33] Indeed, the rate of appearance of the pyridine complex from [(NH₃)₆Ru]$^{2+}$ was the same, regardless of whether the metal ion was initially Ru^{II} (with or without GSH) or generated by the GSH reduction of Ru^{III}.

The reduction of [(4MePy)(NH₃)₅Ru^{III}]Cl₃ by glutathione (1.25−25 mM) under argon at 22 °C exhibited the expected

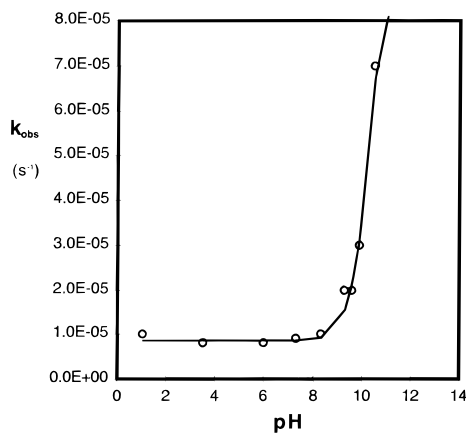**Figure 4.** k_{obs} versus pH for the decomposition of $[GS(NH_3)_5Ru^{III}]^{n+}$ in air. Data were fit to the equation $k_{obs} = (k_1[H^+] + k_oK_{ip})/(K_{ip} + [H]^+)$, where $k_o = (9 \pm 1) \times 10^{-6}\ s^{-1}$, $k_1 = (1.3 \pm 0.7) \times 10^{-4}\ s^{-1}$, and $pK_{ip} = 10.5 \pm 0.2$; $[Ru] = 5.3 \times 10^{-4}$ M in 0.1 M ammonium formate or ammonium acetate, $T = 25\ °C$.

absorbance at 410 nm for the MLCT of $[(4MePy)(NH_3)_5Ru^{II}]^{2+}$ but also revealed a minor absorbance centered at 510 nm, which suggested the formation of a glutathione complex of Ru^{III}. Indeed, HPLC analysis of a reactant mixture of $[(4MePy)(NH_3)_5-Ru]Cl_3$ (0.125 mM) and GSH (0.25 mM), which was allowed to stand under argon for 24 h, revealed peaks (k') for the following: $[(4MePy)(NH_3)_5Ru]Cl_3$ (16.5), GSH (1.54), GSSG (3.47), and $[GS(NH_3)_5Ru]^+$ (1.42) with approximately 9% of the $[(4MePy)(NH_3)_5Ru]^{3+}$ being converted to $[GS(NH_3)_5Ru]^+$. HPLC analysis of the stock solution of $[(4MePy)(NH_3)_5Ru]Cl_3$ verified that no $[Cl(NH_3)_5Ru]^{2+}$ or $[OH(NH_3)_5Ru]^{+2}$ was initially present in the reactant mixture. At $[GSH] \leq 12.5$ mM, the disappearance of Ru^{III} (monitored spectrophotometrically at 299 nm) followed the rate law $-d[Ru^{III}]/dt = k[Ru][GSH]$, where $k = 1.7 \pm 0.2\ M^{-1}\ s^{-1}$. However, rate saturation appeared to occur at $[GSH] = 25$ mM. Since $[GS(NH_3)_5Ru]^+$ continued to form after reaction times when conversion to Ru^{II} should be quantitative, the glutathione complex appears to be possible from $[(4MePy)(NH_3)_5Ru^{II}]^{2+}$.

Autoxidation Decomposition Products and Kinetics. Solutions of $[(GS)(NH_3)_5Ru^{III}]^+$ exhibited a decrease in the absorption at 505 nm concomitant with a new peak appearing at 485 nm upon prolonged standing in air, on an ion-exchange column in air, or following the addition of a few drops of 30% H_2O_2. Solutions of $[GS(NH_3)_5Ru^{III}]^+$ maintained at room temperature (pH 2−10) under argon showed no change in spectra over periods of 1−2 days. However at pH > 12, a product absorbing at 485 nm was evident within 48 h.

When $[GS(NH_3)_5Ru^{III}]^+$ was exposed directly to air at 25 °C in 0.1 M ammonium formate, the peak at 505 nm decreased with a shift toward higher energy and eventually disappeared. New absorbances arose around 260 and 310 nm with an isosbestic point at 405 nm (see Figure 6S). Decomposition proceeded according to the rate law $-d[GS-Ru^{III}]/dt = k_{obs}-[GS-Ru^{III}]$, with $t_{1/2} = 21$ h between pH 1 and 8. Above pH 8, the decomposition was markedly more rapid with $k_{obs} = (k_1-[H^+] + k_oK_a)/(K_a + [H]^+)$, and $k_o = (9 \pm 1) \times 10^{-6}\ s^{-1}$, $k_1 = (1.3 \pm 0.7) \times 10^{-4}\ s^{-1}$, and $pK_a = 10.5 \pm 0.2$ (Figure 4). When the effect of Cu^{II} (4.9 mM) on $[GS(NH_3)_5Ru^{III}]^+$ (490 μM) in 0.15 M NaCl at pH 8.1 in air was determined, the decomposition of $[GS(NH_3)_5Ru^{III}]^+$ accelerated by approximately 500-fold with the rate being second-order in $[GS(NH_3)_5Ru^{III}]^+$.

The 485 nm species was separated from $[(GS)(NH_3)_5Ru^{III}]^+$ by elution from a SP-C25 Sephadex column with water or water

followed by 0.05 M HCl. Assuming a single ruthenium species, ϵ_{485} was estimated as $\sim$500 $M^{-1}\ cm^{-1}$ by quantifying [Ru] by atomic absorption spectrophotometry from chromatographically purified solutions. Hygroscopic materials were obtained following vacuum evaporation of the eluted band. ESI-MS revealed substantial peaks attributable to GSSG and its fragmentation products. The relative intensities of the ruthenium-containing peaks of the 485 nm decomposition product were inverted relative to $[(GS)(NH_3)_5Ru^{III}]^+$ (m/e): $440 \gg 457 > 474 > 491$ (cf. Figures 2S and 3S). In particular the Ru-isotope pattern centered at 440 was prominent in the MS spectrum of the decomposition product and barely discernible in that of $[(GS)-(NH_3)_5Ru^{III}]^+$. Some samples showed a possible oxo-GSH peak, but no strong absorption attributable to an $S=O$ stretch was evident in the infrared. Upon further standing in air or in the presence of H_2O_2, the material absorbing at 485 nm yielded a colorless product that eluted from an SP-C25 column with water. Elemental analysis of this second hygroscopic material indicated that it did not contain ruthenium nor did ESI-MS reveal peaks with characteristic ruthenium isotope patterns. Major peaks occurred at m/z 711, 654, 582, and 356.

GSH Effects on Ru Binding to DNA. Binding to 5′GMP was assayed by allowing a 10-fold excess of 5′GMP (10 mM) to react with $[Cl(NH_3)_5Ru^{III}]^{2+}$ (1 mM) at room temperature under argon. When no GSH was present, $\sim$9% of the ruthenium coordinated 5′GMP after 1 h as determined from the broad absorbance of $[(5′GMP^{\kappa7})(NH_3)_5Ru^{III}]^{2+}$ centered at 570 nm.[23,24] When 10 mM GSH was also present, only the absorbance of $[(GS)(NH_3)_5Ru^{III}]^{2+}$ at 505 nm was observed. After this solution was subjected to a stream of oxygen gas for 1 h, there was still no evidence of nucleotide binding.

As shown in Figure 5 at $[GSH]/[Ru^{III}] < 1$, GSH facilitates the binding of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to CT-DNA under argon by enabling the formation of $[(G_{DNA})(NH_3)_5Ru^{III}]$ at concentrations that are only slightly lower than those expected by equilibrium binding.[24] However, at $[GSH]/[Ru] > 1$, GSH inhibits the binding of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to DNA through the formation of $[GS(NH_3)_5Ru^{III}]^+$, which was evident from its absorption at 505 nm. When GSH was in 10−100-fold excess, only a peak at 505 nm was observed. Similar results were obtained whether the reactions were run at sufficiently high concentrations to spectroscopically quantify the formation of $[(G_{DNA})(NH_3)_5Ru^{III}]$ (570 nm) (Figure 5a) or at lower concentrations, where the total $[Ru_{DNA}]$ was determined by graphite furnace atomic absorption spectrophotometry (Figure 5b).

Glutathione also removed Ru^{III} from G_{DNA}. When glutathione (100 fold excess) was incubated with ruthenated calf thymus DNA ($[P_{DNA}] = 2$ mM; $[Ru_{DNA}]/[P_{DNA}] = 0.48$) for 14 h under argon, an absorbance appeared at 505 nm, indicative of $[(GS)-(NH_3)_5Ru^{III}]^{2+}$. After dialyzing in air against 0.1 M NaCl to remove excess GSH and $[(GS)(NH_3)_5Ru^{III}]^{2+}$, spectrophotometry at 570 nm indicated that the $[Ru-G_{DNA}]/[P_{DNA}]$ had decreased by 80% to 0.1. Similarly, when $[(H_2O)(NH_3)_5Ru^{III}]^{2+}$ (1mM) was incubated with CT DNA (3 mM) over Zn/Hg amalgam under argon (16 h at 23 °C in 0.1 M TA buffer, pH 7.5) followed by air oxidation (3 h at 23 °C), the spectrophotometrically determined amount of binding to each of the bases[24,34,35] was $[Ru-G_{DNA}]/[P_{DNA}] = 0.833$; $[Ru−C_{DNA}]/[P_{DNA}] = 0.008$; and $[Ru-A_{DNA}]/[P_{DNA}] = 0.020$. For an analogous sample with one equivalent of glutathione as the reductant, $[Ru-G_{DNA}]/[P_{DNA}] = 0.236$, $[Ru−C_{DNA}]/[P_{DNA}] = 0.004$, and $[Ru-A_{DNA}]/[P_{DNA}] = 0.0056$. When the sample of Ru-DNA that had been prepared with Zn/Hg amalgam was later incubated with a 100-fold excess of glutathione (50 mM) for 24 h under argon,
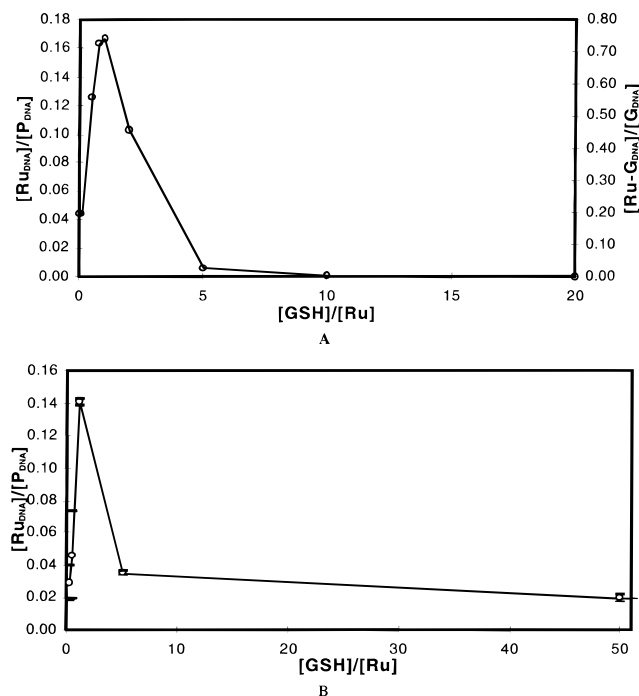
Binding of [Cl(NH₃)₅Ru^{III}]^{2+} to DNA by Glutathione

J. Am. Chem. Soc., Vol. 121, No. 37, 1999 8529

Figure 5. (A) DNA binding of $[Cl(NH_3)_5Ru]^{+2}$ as a function of [GSH] when allowed to react for 1.5 h under argon: [Ru] = 1.6 mM, [P_{DNA}] = 2.0 mM. Ru^{III}-G binding determined by absorption at 570 nm. Hollow, black circles represent $[Ru_{DNA}]/[P_{DNA}]$ (left axis) and $[Ru\text{-}G_{DNA}]/[G_{DNA}]$ (right axis) vs increasing GSH; T = 25 °C; μ = 0.1 M TA buffer, pH 7.5. (B) Same but [Ru] = 20 μM, [P_{DNA}] = 100 μM. Ru–DNA binding determined by graphite furnace atomic absorption. Points not shown ([GSH]/[Ru], $[Ru_{DNA}]/[P_{DNA}]$): 250, 0.032 ± 0.002; 500, 0.034 ± 0.002).

Table 1. Effects of GSH Inhibitors and Enhancers on IC$_{50}$ of $[Cl(NH_3)_5Ru^{III}]^{2+}$ on Jurkat Cells

modifier	mol GSH/10⁵ cells	IC$_{50}$
none	2.1×10^{-8}	9.7 mM
BSO	0.48×10^{-8}	1.6 mM

followed by oxidation and dialysis, the relative amounts of binding were $[Ru\text{-}G_{DNA}]/[P_{DNA}]$ = 0.029, $[Ru\text{-}C_{DNA}]/[P_{DNA}]$ = 0.0006, and $[Ru\text{-}A_{DNA}]/[P_{DNA}]$ = 0.004. These results suggest that GSH effectively removes Ru^{III} from G and possibly C sites, but not from A residues.

When identical reactions were run under air and argon ([GSH] = 10 mM, [Ru] = 20 μM, [P_{DNA}] = 200 μM, 1.5 h), total ruthenium binding to DNA was 41% in air relative to argon.

No binding to DNA was evident when $[GS(NH_3)_5Ru^{III}]^+$ (0.46 and 0.8 mM) was allowed to react with CT-DNA ([P_{DNA}] = 0.46 mM) in 0.1 M NaCl for 3 h under argon and then oxidized overnight.

Cell Studies. The toxicity of $[Cl(NH_3)_5Ru^{III}]^{2+}$ to Jurkat T-cells in the presence and absence of BSO, which inhibits GSH biosynthesis, is summarized in Table 1. UV-Vis spectrometry indicated that BSO, which also contains a sulfur atom, does not react with $[Cl(NH_3)_5Ru^{III}]^{2+}$ under argon for 3 h at 37 °C. While GSH suppression causes IC$_{50}$ to decrease by less than an order of magnitude, Figure 6 shows that the toxicty is greatly enhanced even for very low concentrations of $[Cl(NH_3)_5Ru^{III}]^{2+}$.
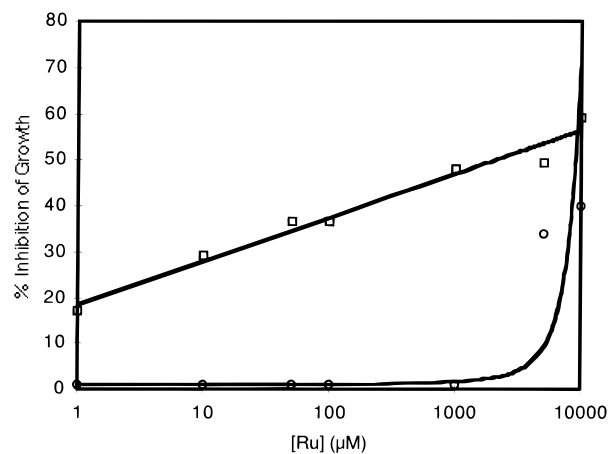

Figure 6. Plot of inhibition of Jurkat Cell growth versus [Cl(NH₃)₅-Ru]²⁺ for normal and glutathione suppressed cells: squares, BSO treated; circles, control.

Discussion

Ru^{III}-Catalyzed Autoxidation of GSH. Autoxidation of glutathione in a metal-free environment proceeds only very slowly;[36,37] however, it is catalytically autoxidized to GSSG by $[OH(NH_3)_5Ru]^2$ ($k = 3 \pm 1$ M^{-1} s^{-1}). The conversion of excess GSH to GSSG was close to 100% in air.

At neutral pH, GSH is thermodynamically capable of reducing $[Cl(NH_3)_5Ru]^{2+}$ ($E°$ = −0.1 V) and $[(HO)(NH_3)_5Ru]^{2+}$ ($E°'$ = −0.095 V). The rapid conversion of $[Cl(NH_3)_5Ru]^{2+}$ to $[(HO)(NH_3)_5Ru]^{2+}$ in the presence of GSH suggests that the chloride is lost through redox catalysis as a small amount of Ru^{III} is reduced to the more substitution-labile Ru^{II} ($k = \sim$5 s^{-1}),[38] which then exchanges electrons with other Ru^{III} ions. Since the second-order rate constant for autoxidation ($k = 3$ M^{-1} s^{-1}) is within experimental error of that for the reduction of $[(HO)(NH_3)_5Ru]^{2+}$ by GSH ($k_{obs} = k/K_i = 1.2 \pm 0.4$ M^{-1} s^{-1} at $K \gg$ [GSH]), reduction appears to be the rate-limiting step rather than oxidation of Ru^{II} by O$_2$.[39] Under conditions where [GSH] $\gg K_i$, a limiting first-order rate constant of 2.3×10^{-3} s^{-1} would be expected for the GSH reduction of Ru^{III}, which is again smaller than the expected autoxidation rate of Ru^{II}.[39] The low efficiency for GSH scavenging Ru in air is due to the oxidation of Ru^{II} by O$_2$ before significant coordination to glutathione occurs.

Glutathione Reduction of Ru^{III}. Of particular note is the loss of ammonia and picoline ligands in the GSH reduction and complexation of $[(NH_3)_6Ru]^{3+}$ ($E°$ = 0.06 V) and $[(4MePy)(NH_3)_5Ru]^{3+}$ ($E°$ = 0.3 V), which suggests a reduced intermediate capable of eliminating even fairly strongly bound nitrogen ligands. Since pyridine ligands substitute onto $[(NH_3)_6Ru]^{3+}$ in the presence of GSH, the intermediate appears to lead to $[(H_2O)(NH_3)_5Ru]^{2+}$, which is readily scavenged by high concentrations of pyridine or isonicotinamide. Given the relatively high concentrations of GSH in cells, GSH reduction and elimination of a nitrogen ligand via a redox mechanism may provide a means for $[(4MePy)(NH_3)_5Ru]^{2+}$ and related complexes to bind in vivo so as to affect their immunosuppressive activity. Alternatively, glutathione complexes of Ru^{III} may be involved.

(34) Clarke, M. J. *J. Am. Chem. Soc.* **1978**, *100*, 5068−5075.

(35) Gariepy, K. C.; Curtin, M. A.; Clarke, M. J. *J. Am. Chem. Soc.* **1989**, *111*, 4947−4952.

(36) Voegtlin, C. J.; J. M.; Sanford, M. *J. Biol. Chem.* **1931**, *93*, 435−452.

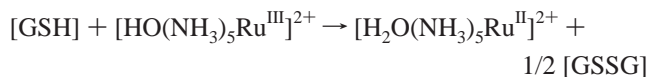(37) Lyman, C. B.; Guzman *J. Biol. Chem.* **1937**, *223*, 2754−284.

(38) Taube, H. *Surv. Prog. Chem.* **1973**, *6*, 1−46.

(39) Stanbury, D. M.; Haas, O.; Taube, H. *Inorg. Chem.* **1980**, *19*, 518−524.

On the basis of reduction potentials, the equilibrium constant for the reaction

$$[GSH] + [HO(NH_3)_5Ru^{III}]^{2+} \rightarrow [H_2O(NH_3)_5Ru^{II}]^{2+} +$$
$$1/2 [GSSG]$$

is calculated as 363 $M^{-1/2}$ at pH 7 ($\Delta E = 0.151$ V). When a ligand is present that coordinates and stabilizes the lower oxidation state, GSH can be expected to quantitatively reduce Ru^{III}. Since $[L(NH_3)_5Ru]^{2+}$ (L = py or Isn) forms quantitatively at [L] $\gg$ [Ru] and such complexes appear at a rate much less than that of ligand substitution on $[H_2O(NH_3)_5Ru^{II}]^{2+}$ ($\sim$0.1 s^{-1}),[33] GSH reduction of Ru^{III} is also the rate-limiting step in Scheme 1. Single electron reduction of metal ions by thiols generally proceeds through a thiyl intermediate (GS$\cdot$ in Scheme 1), which rapidly dimerizes to the disulfide.[40] Consequently, the rate-limiting step is expected to be the formation of the thiyl radical. Since the rate law for this reaction (Scheme 1) is in accord with a preassociation mechanism at neutral pH, the initial step probably involves an outer-sphere association between the monoanionic GSH and $[HO(NH_3)_5Ru^{III}]^{2+}$. While the electrostatic attraction is not large, hydrogen-bonding interactions between the carboxylates and ammine protons might also be expected. On the other hand, the small value of K_i may be indicative of the formation of an intermediate with an expanded coordination sphere.

The relatively low second-order rate constant for 1e reduction ($k' = 1.2$ M^{-1} s^{-1}) observed here in comparison to that for $[(CN)_6Fe]^{3-}$ ($k = 294$ M^{-1} s^{-1}, $E° = 0.36$ V)[41] probably derives from the significantly lower thermodynamic driving force for the rate-limiting step. Similarly, the 2e transfer of GSH to *trans*-$[Cl_2(CN)_2Pt^{IV}]$ ($E° = 0.926$ V), which is thought to proceed through a halide-bridged inner-sphere mechanism, proceeds with $k = 655$ M^{-1} s^{-1} at neutral pH.[42] The outer-sphere rate constant for the 1e reduction of a putative thioester chromate/GSH complex by GSH is 0.89 M^{-1} s^{-1}. The thioester complex also exhibits an inner-sphere electron-transfer rate of 7.2 $\times$ 10^{-3} s^{-1}.[40] GSH reduction of $[(cdta)Mn^{III}]^{-}$ proceeds by inner-sphere electron transfer with a second-order rate constant of 24 M^{-1} s^{-1}.[43] Since the electron-transfer rates reported here are rapid relative to the hydroxide or chloride exchange rates on Ru^{III} ($\sim$10^{-6} s^{-1}),[44,45] direct inner-sphere electron transfer would normally be considered unlikely, at least in the classical sense of the ligand occupying an octahedral coordination position. Nevertheless, H-atom abstraction from GSH onto an octahedral face of the Ru^{III} and even a halide-bridged, inner-sphere electron transfer through the movement of Cl$\cdot$ to form a transient GS$-$Cl remain possibilities.[46] The slowness of the reaction is largely due to the appreciably negative $\Delta S^{\ddagger}$ indicative of an organized transition state. This is may be due to wrapping of the ruthenium ion by the anionic carboxylates on the GSH, which may also hydrogen bond to the coordinated ammines, so as to bring the thiol into closer proximity with the metal ion.
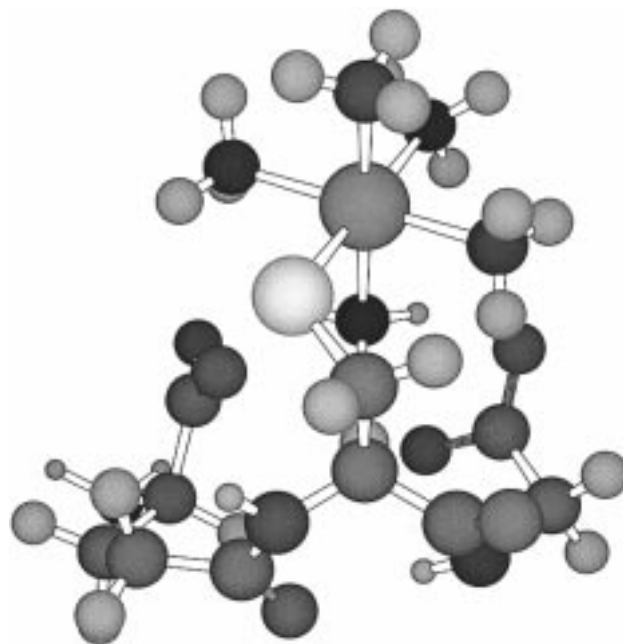
(40) Bose, R. N.; Moghaddas, S.; Gerlerinter, E. *Inorg. Chem.* **1992**, *31*, 1987−1994.

(41) Stochel, G.; Martinez, P.; van Eldik, R. *J. Inorg. Biochem.* **1994**, *54*, 131−40.

(42) Elding, L. I.; Shi, T. S.; Berglund, J. *Inorg. Chem.* **1996**, *35*, 3498−3503.

(43) Gangopadhyay, S.; Ali, M.; Dutta, A.; Banerjee, P. *J. Chem. Soc., Dalton Trans.* **1994**, 841−845.

(44) Broomhead, J. A.; Basolo, F.; Pearson, R. G. *Inorg. Chem.* **1964**, *3*, 832−836.

(45) Kane-Maguire, L. A. P.; Thomas, G. *J. Chem. Soc., Dalton Trans.* **1975**, 1324.

(46) Haukka, M.; Ahlgren, M.; Pakkanen, T. A. *J. Chem. Soc., Dalton Trans.* **1996**, 1927.

Figure 7. MM2 energy-minimized structure for the peptide portion of $[(GS)(NH_3)_5Ru^{III}]^+$ obtained from Chem3D calculations.[47,48] The metal ion was constrained to be octahedral with Ru−NH$_3$ bond distances of 2.11 Å. No solvent molecules were included.

The displacement of ammonia from $[(NH_3)_6Ru^{III}]^{3+}$ and the displacement of 4-picoline from $[(4MePy)(NH_3)_5Ru^{III}]^{3+}$ to form $[(GS)(NH_3)_5Ru^{III}]^{2+}$ support the formation of an inner-sphere complex. This could result from hydrogen atom abstraction to form a seven-coordinate hydrido complex, followed by ligand loss to yield $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$, which is then coordinated by a second GSH. However, $[(4MePy)(NH_3)_5Ru^{II}]^{2+}$ may also undergo picoline substitution by GSH.

Figure 7 illustrates a wrapping of the glutathione carboxylates around the ruthenium ion following an energy minimization (MM2)[47,48] of the peptide structure of $[(GS)(NH_3)_5Ru^{III}]^{2+}$. The low reduction potential of this complex (see below) is in accord with strong π-donation from the thiolato to the partially occupied d_{yz} orbital. Such strong π-donation is also commensurate with quenching of spin−orbital coupling in Ru^{III} resulting in a narrow-line ESR (Figure 5s) spectrum relative to those of more weakly π-interacting species.[17,29,30,49]

Equilibrium Binding. Since the estimations of K_{eq} by the ligand competition experiments are relatively independent of small amounts of rutheniium byproducts, the equilibrium binding constant for GS$^-$ coordinating to $[H_2O(NH_3)_5Ru^{III}]^{3+}$ is likely to be 10^{14}−10^{15} M^{-1}. This is similar to that for HS$^-$ (2.4 $\times$ 10^{13} M^{-1}) and the estimated minimum binding constant for $CH_3CH_2S^-$ (9.3 $\times$ 10^{13} M^{-1}) on the basis of the reduction potentials for $[(CH_3CH_2S)(NH_3)_5Ru]^{2+}$ and $[(CH_3)_2S)(NH_3)_5Ru]^{3+}$.[18] Considering the ion-pairing and hydrogen-bonding interactions possible in structures such as that shown in Figure 7, the affinity of GS$^-$ should be somewhat higher than that of HS$^-$ or $CH_3CH_2S^-$. Assuming $K_{eq} = 1 \times 10^{14}$ M^{-1}, the apparent binding constant under physiological conditions (pH 7.4) can be estimated as $K_{eq}' = K_{eq} \alpha_{GS}\alpha_{RuH2O} = 2 \times 10^9$ M^{-1}, where K_{eq}' is expresssed in terms of the total (protonated and ionized) concentrations of GSH and the analogous total aquaruthenium-(III) ion (α_{GS} = fraction of GSH as GS$^-$ at a given pH and

(47) Rubenstein, M.; Rubenstein, S. *Chem3D Pro;* 3.51 ed.; Cambridge Scientific Computing, Inc.: Cambridge, MA, 1997.

(48) Allinger, N. L. *J. Am. Chem. Soc.* **1977**, *99*, 8127.

(49) LaChance-Galang, K. J. Ph.D. Thesis, Boston College, 1995.

α_{RuH2O} = fraction of $[(H_2O)(NH_3)_5Ru]^{3+}$ as the aqua ion at the same pH). From $E°$ values, the corresponding values for Ru^{II} are approximately estimated as $K_{eq}(Ru^{II}) = 5 \times 10^6$ M^{-1} and $K_{eq}{'}(Ru^{II}) = K_1(Ru^{II})\alpha_{GS} = 2 \times 10^5$ M^{-1}.

Electrochemistry of [(GSH)(NH₃)₅Ru^III]²⁺. Reduction potentials of thiolatopentaammineruthenium(III) complexes are particularly low due to strong π-donation by the thiolato ligand into the d$_\pi$ orbitals of the Ru^{III}; for example, the $E°$ of $[(C_2H_5S)(NH_3)_5Ru^{III}]^{2+}$ is -0.43 V.[17] Consequently, the low $E°$ of $[(GS)(NH_3)_5Ru^{III}]^{2+}$ (-0.44 V) is expected for GSH coordination through the cysteinyl sulfur. On the other hand, the pK_a for $[(GSH)(NH_3)_5Ru^{II}]^+$ (7.1) is somewhat lower than expected in comparison with that of $[(CH_3CH_2SH)(NH_3)_5Ru^{II}]^+$ (9.2),[18] which may have to do with effects of the glutathione wrapping around the metal ion (Figure 7) or, perhaps, with the observed proton equilibria involving the organic amine.[31,50]
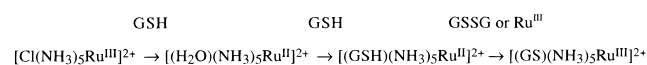
The irreversible oxidation process observed at 614 mV is tentatively attributed to a Ru^{III/IV} couple, but oxidation of the sulfur has not been ruled out. This couple is thermodynamically accessible by molecular oxygen over a wide range of pH and may be involved in the aerobic decomposition reaction, in which the metal ion appears to facilitate autoxidation of glutathione. Alternatively, the Ru^{III} may simply serve as a conduit for autoxidation as has previously been observed in complexes such as $[(Guo)(NH_3)_5Ru^{III}]^{3+}$, which yields 8-oxo-guanine products.[29,35]

The acceleration of the autoxidation reaction above pH 8 may derive from deprotonation of an ammine ligand, which would stabilize Ru^{IV} and thereby further facilitate Ru^{III} oxidation. While solutions of $[(GS)(NH_3)_5Ru^{III}]^+$ were stable under inert atmosphere for days, solutions in air decomposed over periods of hours at pH > 8. Electrospray mass spectra of the ruthenium intermediate absorbing at 485 nm suggest that a major component is $[(GS)(NH_3)_2Ru^{III}]$, in which the amine and carboxylate groups also coordinate the metal ion. However, since this material is neutral it may also contain thiolato- and amine-coordinated glutathione of lower dentacity, such as $[(GS)(NH_3)_3Ru^{III}]$ and $[(GS)(NH_3)_4Ru^{III}]$. Suppression of the 485 nm peak when reactions were run in ammonia buffers is in harmony with this array of products. That such species occur most readily in the presence of oxygen may be due to amine ionization of a Ru^{IV} autoxidation product with the resulting amide serving to labilize a *trans*-ammine. Consequently, the 485 nm material appears to be a substitution, rather than an oxidation product, and is probably not a necessary intermediate in the final oxidative loss of the thiolate.

The mechanism of Cu²⁺ severing the Pt−S bond in [GS-(terpy)Pt]²⁺ has been suggested to proceed through coordinating an ionized glutathione amide nitrogen followed by a Pt−S−Cu heteronuclear bridged species.[51] The substantial acceleration of the autoxidation of $[GS(NH_3)_5Ru^{III}]^+$ by Cu^{II} may result from the latter ion's facilitating electron transfer to oxygen through a heterodinuclear intermediate of the type postulated for $[(GS)-(terpy)Pt^{II}]^{51}$ or simply by facilitating the dissociation of GSH from Ru^{III}.

Formation of [(GS)(NH₃)₅Ru^III]⁺. GSH was not observed to coordinate to $[Cl(NH_3)_5Ru]^{2+}$ under atmospheric conditions, since oxygen oxidizes $[(H_2O)(NH_3)_5Ru]^{2+}$ before GSH can bind.

(50) Martin, R. B.; Edsall, J. T. *J. Am. Chem. Soc.* **1959**, *81*, 4044.
(51) Cheng, C.-C.; Pai, C.-H. *J. Inorg. Biochem.* **1998**, *71*, 109−113.
(52) Zhao, M.; Clarke, M. J. *J. Biol. Inorg. Chem.* **1999**, *4*, 325−340.
(53) Sava, G.; Alessio, E.; Bergamo, E.; Mestroni, G. *Top. Biol. Inorg. Chem.* **1999**, *1*, 143−170.
(54) Morgunova, E.; Tuuttila, A.; Bergmann, U.; Isupov, M.; Lindqvist, Y.; Schneider, G.; Tryggvason, K. *Science* **1999**, *284*, 1667−1670.

Scheme 2

GSH   GSH   GSSG or Ru^{III}

$[Cl(NH_3)_5Ru^{III}]^{2+} \rightarrow [(H_2O)(NH_3)_5Ru^{II}]^{2+} \rightarrow [(GSH)(NH_3)_5Ru^{II}]^{2+} \rightarrow [(GS)(NH_3)_5Ru^{III}]^{2+}$

However, under anaerobic conditions, the coordination reaction proceeds fairly readily (Scheme 2). Under argon, a less than stoichiometric amount of GSH was oxidized by $[Cl(NH_3)_5Ru^{III}]^{2+}$ to GSSG within 4.5 h. Monitoring the formation of the GSH adducts of either $[Cl(NH_3)_5Ru]^{2+}$ or $[(NH_3)_6Ru]^{3+}$ under argon suggested a multistep process, which was not readily amenable to study.

Following the redox catalytic conversion of $[Cl(NH_3)_5Ru]^{2+}$ to $[HO(NH_3)_5Ru]^{2+}$, the first step appears to be reduction of the latter to $[(H_2O)(NH_3)_5Ru]^{2+}$, which accounts for the decrease in A_{296} during the initial phase of the reaction, with the concomitant oxidation of GSH to GSSG (Schemes 1 and 2). The strong donor and acceptor effects of the sulfur to Ru^{III} and Ru^{II}, respectively, which are evident in the electrochemistry,[18] suggest that a thiol may interact strongly with a t$_{2g}$ orbital protruding from the octahedral face of Ru^{III} to form an intermediate species that facilitates H-atom transfer. While the coordinating GSH probably initially attaches to the resulting Ru^{II}, the strong binding of thiolate to Ru^{III} and the low reduction potential of the resulting complex ($E°{'} = -0.44$ V) result in oxidation to the observed GS−Ru^{III} species.

As the reaction proceeds well under argon, the GSSG formed in the initial step may be one source of oxidant making the reaction catalytic in GSH. Thermodynamically, either GSSG ($E°′ = -0.246$ V), the starting material, $[HO(NH_3)_5Ru]^{2+}$ ($E°′ = -0.095$ V), or even H⁺ (pH < 7) may oxidize the GS−Ru^{II} adduct ($E° = -0.44$ V). Since added glutathione disulfide had no apparent effect on the reaction rate, it is most likely that the more rapidly electron-transferring agent, $[HO(NH_3)_5Ru^{III}]^{2+}$, effects the oxidation during the initial phases of GSH complexation. In later phases, the more slowly reacting GSSG or traces of oxygen or H⁺ may become the oxidant. The formation of the reduced intermediates $[(H_2O)(NH_3)_5Ru]^{2+}$ and $[(GSH)-(NH_3)_5Ru]^{2+}$ as indicated in Scheme 2 accounts for the delay in the appearance of the final product $[(GS)(NH_3)_5Ru]^{2+}$, which is evident in Figures 1 and 1S, after the initial decrease in the ruthenium starting material.

Effects on DNA Binding. DNA binding by $[X(NH_3)_5Ru^{III}]^{2+}$ (X = OH⁻ or Cl⁻) to DNA is a complex function of [GSH] (Figure 5). In the absence of GSH, some DNA binding was observed. No DNA binding was observed with $[(GS)(NH_3)_5Ru^{III}]^+$. At [GSH] < [Ru], Ru-DNA binding increased with [GSH]. This is attributed to the GSH reduction of $[X(NH_3)_5Ru^{III}]^{2+}$ to $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$, which more rapidly substitutes imine ligands, such as G⁷ on DNA. Since the $E°′$ of $[(G^7)_{DNA}-(NH_3)_5Ru^{II}]^{2+}$ is 48 mV,[24] redox catalysis does not play a major role in this reaction. Consequently, as more $[X(NH_3)_5Ru^{III}]^{2+}$ is reduced, more ruthenium coordinates to DNA and DNA binding increases with [GSH] up to a reactant [GSH]/[Ru^{III}] ratio of one. At [GSH]/[Ru] > 1, GSH serves as a competitive ligand for $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ to yield $[(GS)(NH_3)_5Ru^{III}]^+$. This ligand competition increases with increasing [GSH] until no Ru-G$_{DNA}$ binding is evident by UV−vis spectroscopy at [GSH]/[Ru] > 10. The presence of air inhibits the GSH-facilitated binding of Ru to DNA, probably owing to the autoxidation of Ru^{II} before it can coordinate to the nucleic acid.

In contrast to G$_{DNA}$ binding, coordination by adenine residues was little affected by [GSH]. Coordination to the exocyclic amines of A and C residues[24,34] is obscured by the absorption by $[GS(NH_3)_5Ru^{III}]^{2+}$; however the residual binding evident by

AA (Figure 5b) can be attributed to A and C binding following dialysis to remove free ruthenium complexes. A residual DNA binding of $[Ru_{DNA}]/[P_{DNA}] = 0.03$ was evident by atomic absorption even at $[GSH]/[Ru] \gg 10$, which is in accord with the small amount of A and C binding persisting on DNA after removal of Ru by GSH from the G sites. Such binding may be due to the single-stranded ends of the sonicated DNA, where coordination to the exocyclic amines of A and C residues could occur by redox catalysis.[24,34] Alternatively, it may result from the metal ion attacking transiently separated strands of DNA or distorted segments caused by G^7 binding. The lower reduction potentials of $[(Ado)(NH_3)_5Ru^{III}]^+$ ($E^{o\prime} = -0.16$ V) and $[(Cyd)-(NH_3)_5Ru^{III}]^+$ ($E^{o\prime} = -0.14$ V) should lead to less reduction and less complexation of the metal ion by GSH so that the ruthenium is less likely to be removed from these sites on DNA. Even when reduced, the Ru^{II} on the exocyclic amine of adenine can linkage isomerize to the adjacent pyrimidine nitrogen (N1), where it can form a much stronger π-back-bond than is possible on N7.[29,34] This route may account for the greater effectiveness in GSH removing Ru from C relative to A sites.

Since $[GS(NH_3)_5Ru^{III}]^{2+}$ was not observed to bind to DNA over a period of 3 h under argon followed by 12 h in air, the inhibitory effect of GSH appears to be complete upon coordinating the metal ion. While the estimated equilibrium binding constant for Ru^{II} coodinating to GS^- ($\sim 10^6$ M^{-1}) may allow DNA binding, both $GS-Ru^{II}$ and $GSH-Ru^{II}$ are likely to be rapidly oxidized. Consequently, it is more likely that a kinetically available quantity of $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ coordinates to DNA under physiological conditions. This is supported by the unusual GSH binding kinetics (Figures 1 and 1S), which are not easily treated, and the apparently low value of $K_{eq}\prime$ (380 M^{-1}) in an attempt to measure $K_{eq}\prime$ through the direct binding of GSH. On the other hand, the more active Ru-anticancer agents such as *cis*-$[Cl_2(NH_3)_4Ru]^+$ or *trans*-$[(Im)_2Cl_4Ru]^+$ [15] might accommodate a single glutathione (particularly in a trans position) and still bind to DNA.

Cell Toxicity. Previous studies revealed a direct correlation between the cellular toxicity of ruthenium complexes and the binding of Ru to nuclear DNA ($[Ru_{DNA}]/[P_{DNA}]$).[1] The increased toxicity of $[Cl(NH_3)_5Ru]^{2+}$ to Jurkat cells, whose GSH levels have been suppressed by BSO, is consistent with a net inhibition of ruthenium binding to DNA by GSH in vivo, so that GSH appears to protect the cells from ruthenium toxicity. Relative to other complexes such as *cis*-$[Cl_2(NH_3)_4Ru]^+$ or *trans*-$[Cl_4(Im)_2Ru]^-$,[1,15] relatively high levels of $[Cl(NH_3)_5Ru]^{2+}$ are needed to exhibit cell toxicity, so that effects other than DNA binding may be involved in its toxicity.

Conclusions. Under hypoxic conditions, GSH forms $[GS-(NH_3)_5Ru^{III}]^{2+}$ from $[L(NH_3)_5Ru^{III}]^{n+}$, where L = Cl^-, NH_3, or 4-picoline, which may be indicative of an H-atom abstraction process to yield a 7-coordinate hydrido intermediate. Since GSH can reduce Ru^{III}, thereby facilitating the elimination of a nitrogen ligand, and coordinate the metal ion in the types of ruthenium complexes that exhibit immunosuppressant activity, represented here by $[(4MePy)(NH_3)_5Ru^{III}]^{3+}$, it may be involved in their "redox-window" mechanism of activity.[17]

Since GSH only slowly reduces $[Cl(NH_3)_5Ru]^{2+}$ under physiological conditions ($t_{1/2} = \sim 10$ min) and the Ru^{II} product is readily oxidized by air, this mode of activating Ru to bind to biopolymers by reduction is probably unimportant in tissues under normal oxygen tensions. Since oxygen also effectively prevents GSH coordination, this may circumvent some thiol-based resistance to rutheniumammine anticancer agents.

The formation of a GSH complex under hypoxic conditions probably protects the cell from Ru binding to its DNA, which is consistent with the increased cytotoxicity of $[Cl(NH_3)_5Ru^{III}]^{2+}$ when cellular [GSH] is suppressed. While unlikely, a relatively low [GSH] in a hypoxic cell compartment could conceivably facilitate biopolymer binding and, therefore, cell toxicity. The equilibrium binding constant for GS^- coordinating to $[H_2O(NH_3)_5-Ru]^{3+}$ appears to be in the range $10^{14}-10^{15}$ M^{-1} and that for Ru^{II} is a factor of 2×10^7 less.

At $[GSH]/[Ru] < 1$, GSH facilitates the binding of $[Cl(NH_3)_5-Ru^{III}]^{2+}$ to DNA through reduction to $[H_2O(NH_3)_5Ru^{II}]^{2+}$; while, at $[GSH]/[Ru] > 1$, GSH inhibits the binding of $[Cl(NH_3)_5-Ru^{III}]^{2+}$ to DNA by forming $[GS(NH_3)_5Ru^{III}]^{2+}$. High [GSH]/[Ru] alters the DNA binding of $[H_2O(NH_3)_5Ru^{II}]^{2+}$ to essentially eliminate G^7 coordination and lower C^4 binding, leaving A^6 binding relatively unaffected. In concert with this, GSH removes most of the metal ion from G^7 sites on DNA but is less effective at removing the metal from A^6 and C^4 sites owing to the lower $Ru^{III,II}$ reduction potential, when the metal ion is attached to the exocyclic ammine of these ligands. The ability of adenine to provide strong π-binding sites for both Ru^{II} (N1) and Ru^{III} (ionized N6) may account for its maintaining ruthenium binding even at high [GSH].

The alteration in DNA binding by $[Cl(NH_3)_5Ru]Cl_2$ could have profound implications for interpreting the mechanism of ruthenium antitumor agents such as *cis*-$[Cl_2(NH_3)_5Ru]Cl$ and (ImH)*trans*-$[Cl_4(Im)_2Ru]$ (Im = imidazole).[1,15] It is now of considerable interest to determine whether GSH affects the type of DNA binding occurring in cells. Moreover, the release of ruthenium from imidazole nitrogens on the surface of DNA may presage a similar GSH-mediated release of this metal ion from histyl nitrogens of transferrin or other proteins within cells. Finally, glutathione complexes may transfer the metal to metal-sequestering proteins, such as metallothionein. These topics are now under study.

Note added in proof: While *trans*-$[(H_2O)(py)(NH_3)_4Ru^{II}]^{2+}$ binds only to G^7 sites on DNA,[52] $[(H_2O)(NH_3)_5Ru^{II}]^{2+}$ binds to G, A, and C residues but can be restricted to A and C in the presence of GSH. Consequently, the DNA binding of similar Ru^{II} and Ru^{III} ions can now be directed with high selectivity.

The antimetastatic agent, *trans*-$[(CH_3)_2SO(Im)(NH_3)_4Ru]^{2+}$, which is now in clinical trials, functions by inhibiting a type IV matrix metalloproteinase (MMP-2).[53] MMP-2 is controlled by an intraprotein cysteine switch that coordinates to Zn^{2+} in the active site.[54] Consequently, ruthenium coordination to the switching cysteine should prevent cysteine coordination to the active-site metal center, thereby activating, rather than inhibiting, MMP-2 and related collegenases.