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Reversibility of Binding of Cisplatin-Methionine in Proteins by Diethyldithiocarbamate or Thiourea: A Study with Model Adducts

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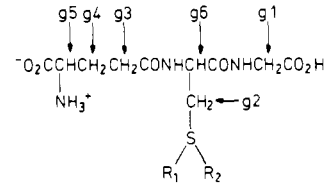
Model adducts for platinum-protein binding, i.e. at cysteine and methionine sites, have been synthesized by starting from [PtCl(dien)]Cl, *cis*-$Pt(NH_3)_2Cl_2$, *trans*-$Pt(NH_3)_2Cl_2$, and $[PtCl(NH_3)_3]Cl$. Glutathione (GSH) and *S*-methylglutathione (GS-Me) were used to mimic the sulfur atoms in the proteins. At pH 11 both *trans*-$Pt(NH_3)_2Cl_2$ and $[PtCl(NH_3)_3]Cl$ form *trans*-$Pt(NH_3)_2(GS)_2$ upon reaction with 2 equiv of GS^-. Only the intermediate $[Pt(NH_3)_3GS]Cl$ was found to be relatively stable. The Pt-cysteine type bonds in $[Pt(dien)GS]^+$ and in *trans*-$Pt(NH_3)_2(GS)_2$ could not be reversed by sodium diethyldithiocarbamate (Na(ddtc)) and thiourea. On the other hand the Pt-methionine models $[Pt(dien)GS\text{-}Me]^{2+}$ and cis-Pt(GS-Me) react fast with Na(ddtc) ($t_{1/2}$ = <2 min) and more slowly with thiourea ($t_{1/2}$ = 30 min-2 h), thereby restoring the original structure of the thioether linkage. The complex formed between Na(ddtc) and the liberated $Pt(dien)^{2+}$ unit proved to be a complex in which the Pt-dien chelate ring is partially opened, leaving one NH_2 noncoordinated. The ability of the nephrotoxicity inhibitors Na(ddtc) and thiourea to reverse Pt-protein adducts—these adducts are supposed to be the origin of nephrotoxicity of platinum antitumor compounds—is interpreted in terms of removing the platinum from methionine sulfurs only.

Introduction

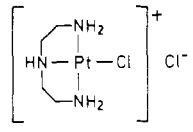
The mechanism of action of cisplatin (*cis*-$PtCl_2(NH_3)_2$, cis-Pt) as an antitumor agent is likely to be based on the interaction of the $Pt(NH_3)_2^{2+}$ moiety with the nucleobases of DNA.[1a,b] The main product of these interactions is the bifunctional intrastrand cross-link between two neighboring guanine bases through their N7 atoms.[2] A limitation of cis-Pt in its use as an antitumor drug is its concentration-dependent nephrotoxicity,[3,4] apart from a variety of other side effects.[5] The nephrotoxicity can be reduced by using the reagents sodium diethyldithiocarbamate (Na(ddtc)) or thiourea.[6-10] Borch et al. have suggested that nephrotoxicity is the result of inactivation of certain enzymes due to the binding of cis-Pt to the sulfhydryl groups of cysteine residues and that Naddtc and thiourea are able to reduce the nephrotoxicity by removing the platinum from the sulfur atoms, thereby restoring the original structure of the enzyme.[6] Recently, evidence for the Na(ddtc)- and thiourea-induced dissociation of Pt-enzyme adducts has been reported.[11-14] Except for the, probably antitumor irrelevant, cis-Pt-adenosine 1:1 and 1:2 complexes and the cis-Pt-guanosine 1:1 complex, Na(ddtc) cannot degrade cis-Pt-DNA adducts.[13] Thiourea appears to be less useful as an inhibitor of nephrotoxicity, because it reacts quite rapidly with platinum-DNA cross-links.[15]

As a result of the intermediate reactivity of Na(ddtc) (i.e. only the Pt-protein interactions are reversed), Na(ddtc) is a promising agent for reducing the nephrotoxicity of platinum antitumor compounds, and it is important, therefore, to understand its detailed mode of action. Until now no detailed working mechanism concerning the Na(ddtc)- and thiourea-induced dissociation of Pt-protein interactions has been proposed. Therefore, a study has been undertaken using complexes of glutathione (GSH, γ-L-glutamyl-L-cysteinylglycine) and *S*-methylglutathione (GS-Me) with cis-Pt and with the monofunctional platinum compound [PtCl(dien)]Cl (**1**; dien = for diethylenetriamine). These interactions can be considered to mimic Pt-cysteine and Pt-methionine adducts within a protein (see Chart I).[16] The first results with [PtCl(dien)]Cl have recently been reported.[17]

Chart I



Model Adduct	R_1	R_2
Pt-methionine	CH_3	$Pt(dien)^{2+}$
	$Pt(dien)^{2+}$	$Pt(dien)^{2+}$
Pt-cysteine	$Pt(dien)^{2+}$	-------



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Complexes of GSH and GS-Me with cis-Pt as such are relatively unstable, as shown by the results of Appleton et al.,[18] and therefore are not very well suited as model adducts. On the other hand, complexes of cis-Pt are biologically more relevant than the [PtCl(dien)]Cl complexes. Therefore, we have now studied the platinum-exchange reactions of the cis-Pt(GSH) and cis-Pt(GS-Me) adducts, with the aim to find out how the results obtained with [PtCl(dien)]Cl can be extrapolated. The exchange reactions

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(16) Theoretically, one could imagine that a metal compound could also react with a disulfide bond in a protein, generating e.g. a M-$^-$SR species, like in a metal-cysteine complex. In fact [PtCl(dien)]Cl reacts with the thioether linkage in GSSG through such a disproportionation reaction resulting in cleavage of the disulfide bridge, forming the same complex as with free GSH.[17] One could assume that disulfides can also be attacked by cis-Pt. Since the adducts formed are the same as those from a free cysteine, this possibility is not discussed separately.
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of the complexes of *trans*-$PtCl_2(NH_3)_2$ (trans-Pt) and $[PtCl(NH_3)_3]Cl$ with GSH were also investigated. Especially trans-Pt compounds are interesting, because only they can be expected to form stable bis(glutathione) complexes.[14]

Experimental Section

Chemicals. Glutathione, *S*-methylglutathione, sodium diethyldithiocarbamate, thiourea, and $^{15}NH_4Cl$ were obtained from Sigma Chemical Co. and used without further purification.

Preparation of Pt Compounds. *cis*-$PtCl_2(NH_3)_2$, *cis*-$Pt(ONO_2)_2(^{15}NH_3)_2$, *trans*-$PtCl_2(NH_3)_2$, [PtCl(dien)]Cl, and $[PtCl(NH_3)_3]Cl$ were prepared from K_2PtCl_4 according to literature procedures.[19-23] The identity of these starting compounds was controlled by infrared spectroscopy (Perkin-Elmer 580 spectrometer) and elemental analysis (Microanalytical Laboratory, University College, Dublin). The complexes $[Pt(dien)GS]^+$, $[\{Pt(dien)\}_2GS]^{3+}$, $[Pt(dien)GS\text{-}Me]^{2+}$ and $[Pt(GS)_2]_n$ were prepared as previously described.[14,17] The synthesis of *trans*-$Pt(NH_3)_2(GS)_2$ went on straightforward by mixing *trans*-$PtCl_2(NH_3)_2$ (0.1 g, 0.33 mmol) and GSH (0.203 g, 0.67 mmol) at pH 11 in 100 mL of H_2O. After 3 h the sample was lyophilized.

$[Pt(dien)ddtc]^+$ was prepared by mixing Na(ddtc) (0.062 g, 0.36 mmol) with [PtCl(dien)]Cl (0.122 g, 0.33 mmol) at pH 7 in 100 mL of H_2O. After 15 min the pH was reduced (pH 2) with HCl to destroy the excess of Na(ddtc). After that the pH was raised immediately (pH 7) with NaOH. The solution was filtered ($Pt(ddtc)_2$), vacuum-evaporated, and finally lyophilized. The products of GS-Me with cis-Pt were prepared in situ by mixing 2 equiv of GS-Me with 1 or 2 equiv of cis-Pt at pH* 7.5 in the NMR tube and letting the mixture stand for 2 days. The product is called "Pt-GSMe".

pH Measurements. All pH measurements were performed at 298 K. The pH meter was calibrated with Fisher certified buffer solutions of pH 4.00, 7.00, and 11.00.

^1H and ^{195}Pt NMR Measurements. Experimental Conditions. The ^1H NMR measurements were recorded with a Bruker WM 300 spectrometer. D_2O or $CDCl_3$ was used as a solvent. Chemical shifts (δ) are reported in ppm relative to TMA (D_2O) or TMS ($CDCl_3$). The ^{195}Pt NMR spectra were recorded with a Bruker WM 300 spectrometer (at 64.4 MHz) and with a JEOL 200 spectrometer (at 42.8 MHz) with a 10-mm tunable probe (concentrations of 150 mM were used). A 10% D_2O/H_2O mixture was used as a solvent. The reference was Na_2PtCl_6 (external). For the titration of the ^{195}Pt signal of $[Pt(dien)ddtc]^+$ a spectral width of 5 kHz was used with a total of 8K data points. Routine spectra were collected by using a spectral width of 50 and 100 kHz for respectively the 200- and 300-MHz spectrometers. For monitoring the pH-dependent chemical shift behavior of the ^1H signals and the ^{195}Pt signals of the Pt compounds the pH was adjusted with 0.1–1 M solutions of NaOD and DCl. Meter readings, reported as pH*, were not corrected for deuterium isotope effects.

Reactions Followed by ^1H NMR. All reactions between platinated GSH or GS-Me with Na(ddtc) and thiourea were carried out in the NMR tube at a concentration of 5 mM substrate at pH* 7.5 with and without phosphate buffer (50 mM). Because of instability problems at pH* 7.5 the reactions of *trans*-$Pt(NH_3)_2(GS)_2$ with Na(ddtc) and thiourea were also carried out at pH* 11. Concentrations of Na(ddtc) or thiourea were adjusted to abstract all platinum. So 1 equiv of Na(ddtc) for $[Pt(dien)GS]^+$ and 2 equiv for $[Pt(GS)_2]_n$ were used. For the Pt–cysteine model adducts $[Pt(dien)GS]^+$ and *trans*-$Pt(NH_3)_2(GS)_2$ in addition the effect of an excess (10 equiv) of Na(ddtc) or thiourea was tested. The effect of the pH on the formation of free GSH was investigated by carrying out the reaction of $[Pt(GS)_2]_n$ with Na(ddtc) at pH* 11. All the reported $t_{1/2}$ values were measured by relative integration (estimated error is 10–15%) during reaction of suitable proton signals of both reaction products and starting compounds. All reactions were followed as a function of time by using ^1H NMR spectroscopy at 294 K.

^{195}Pt NMR. All ^{195}Pt chemical shifts of the complexes of [PtCl(dien)]Cl, $[PtCl(NH_3)_3]Cl$, and *trans*-$PtCl_2(NH_3)_2$ were measured on the 300-MHz spectrometer. $[Pt(dien)ddtc]^+$ and *trans*-$Pt(NH_3)_2(GS)_2$ were prepared separately and checked by ^1H NMR spectroscopy before measuring the ^{195}Pt chemical shift. The complexes between [PtCl(dien)]Cl and thiourea (pH 7) and between $[PtCl(NH_3)_3]Cl$ with 2 equiv of GS^- (pH 11) were prepared in the NMR tube. These complexes were afterward checked by ^1H NMR spectroscopy. The ^{195}Pt chemical shifts

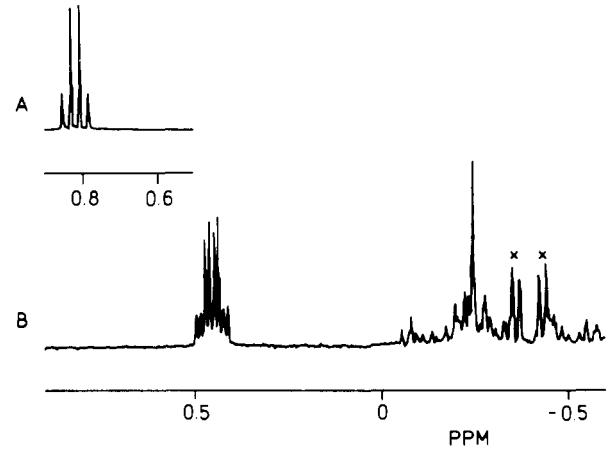


Figure 1. Part of the 300-MHz ^1H NMR spectra of (A) Na(ddtc) and (B) $[Pt(dien)ddtc]^+$ at pH* 10.4. Chemical shifts are in ppm relative to TMA. × represents free diethylenetriamine.

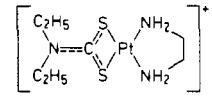
of the complexes of *cis*-$[Pt(H_2O)_2(^{15}NH_3)_2]^{2+}$ with GSH and GS-Me were measured on the 200-MHz spectrometer. *cis*-$[Pt(H_2O)_2(^{15}NH_3)_2]^{2+}$ was prepared by dissolving *cis*-$Pt(ONO_2)_2(^{15}NH_3)_2$ in 10% D_2O/H_2O. A 1-equiv amount of GS-Me or 1 or 2 equiv of GSH was added (pH values of the reaction mixtures were approximately 3). The 1:2 mixture was allowed to react for 1 day before measuring (i.e. in order to form $[Pt(GS)_2]_n$). For the 1:1 mixtures scanning commenced within 30 min. After 2 h the pH was raised to 11 with NaOH for the 1:1 mixture of *cis*-$[Pt(H_2O)_2(^{15}NH_3)_2]^{2+}$ and GSH. A 2-equiv amount of Naddtc was added, and scanning commenced after 30 min.

Results and Discussion

Reaction Products of [PtCl(dien)]Cl with Na(ddtc) and Thiourea. To find out how the starting platinum products, i.e. before binding to GSH and GS-Me, react with the rescue agents, [PtCl(dien)]Cl was reacted with Na(ddtc) and thiourea. The changes in time of the proton resonance intensities of free [PtCl(dien)]Cl in the reaction mixture were used as an indication whether the reaction was completed or not. Na(ddtc) reacted with [PtCl(dien)]Cl in a 1:1 ratio ($t_{1/2}$ = 5 min). Parts of the ^1H NMR spectra of $[Pt(dien)ddtc]^+$ and free Na(ddtc) are redrawn in Figure 1.

The original triplet (not shown) and quartet (see Figure 1B) of respectively the methyl and methylene protons of Na(ddtc) are doubled in the $[Pt(dien)ddtc]^+$ complex. This observation agrees with either of the following possibilities: (1) the formation of two products in equal abundance; (2) the formation of one product with two inequivalent $ddtc^-$ units; (3) the formation of one product with two inequivalent methyl and methylene groups.

The chemical shift of the methyl protons of $[Pt(dien)ddtc]^+$ is downfield (0.04 ppm), whereas the chemical shift of the methylene protons of the $ddtc^-$ unit is upfield (0.37 ppm) compared to free Na(ddtc). These chemical shifts can be explained in terms of coordination of the sulfur atom to Pt and an increase in the C=N double-bond character in $[Pt(dien)ddtc]^+$ compared to that in free Naddtc. The increased C=N double-bond character is the result of an increase in the nitrogen electronegativity on going from the sp^3 hybrid to the sp^2 hybrid.[24,25] Similar observations were found in some bis(amine)platinum(II) complexes of Na(ddtc), as redrawn in structure **2**.[26,27] The restricted rotation about



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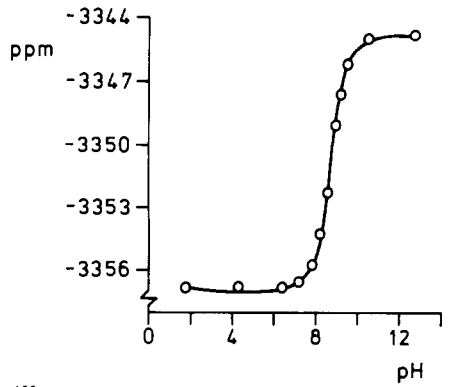
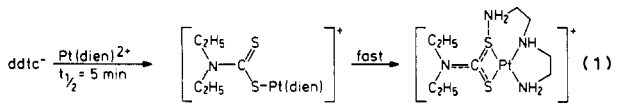


Figure 2. ^{195}Pt chemical shift of $[Pt(dien)ddtc]^+$ as a function of pH (10% D_2O/H_2O). Chemical shifts are in ppm relative to Na_2PtCl_6.

the C=N bond would nicely explain—for an asymmetrical $[Pt(dien)ddtc]^+$ complex—the inequivalence of the two methyl and methylene groups. Experiments with temperature variations, to show coalescence of the methyl and methylene resonances, were not successful, because of decomposition of $[Pt(dien)ddtc]^+$ above 350 K.

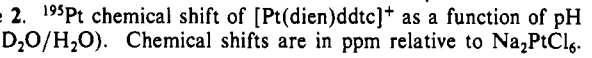
The chemical shifts of protons of intact tridentate dien ligands in $Pt(dien)^{2+}$ complexes are independent of pH. However, careful examination of the chemical shift titration behavior of the 8 protons (Figure 1B, −0.6 to 0 ppm) show that 3–4 of them are dependent on pH (pK_a = ~8.7). This would imply that the dien ligand has changed its coordination characteristics and that chelate ring opening has occurred. The ^1H NMR spectrum in the region −0.6 to 0 ppm is rather complex, which is most likely due to magnetically inequivalent dien protons and not to multiple products, as can also be concluded from the ^{195}Pt NMR results (vide infra). The observations from the ^1H NMR experiments lead to the structures depicted in eq 1.



Platinum initially coordinates to the sulfur atom with a half-life of 5 min. This intermediate will be very unstable due to the vicinity of a second nucleophilic sulfur donor atom. Therefore, a fast chelating step, and dien ring opening as a result, will occur leading to the complex in eq 1, which is similar to structure **2**. The rather low pK_a value of the noncoordinated amino group may be the result of hydrogen bonding to the sulfur atom. The above-shown final product is a more likely one than its symmetric isomer (platinum coordinated to the two sulfur atoms and the two NH_2 groups), because its asymmetric nature is in line with the observed inequivalence of the proton resonances in Figure 1B. A similar ring-opened diethylenetriamine complex of Pt(II) has recently been observed by Mahal et al.[28] (i.e. in $[PtCl_2\{(dien)HCl\}]$).

The ^{195}Pt NMR spectrum of $[Pt(dien)ddtc]^+$ shows a singlet at −3355 ppm. No other signals are observed, in agreement with the formation of only one product. The pH dependence of the ^{195}Pt chemical shift is depicted in Figure 2. As can be seen, the chemical shift is dependent of pH (pK_a = 8.7). The chemical shift differences are rather small but significant (estimated error for δ_{Pt} is 3 ppm). This pH dependence confirms the existence of a noncoordinated amino group of dien. The position of this signal can be ascribed to a PtN_2S_2 complex, i.e. for a bidentate N_2 ligand, although its position is a little to lower shielding than expected. Probably this is due to the incorporation of platinum in a four-membered ring, as reported for $[Pt(NH_3)_2OH]_2^{2+}$.[23] Finally, the IR spectrum shows a strong C=N band at 1545 cm^{-1}, which is in agreement with the IR data reported for $[Pt(en)ddtc]^+$.[26]

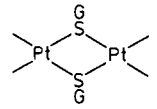
A precipitate appears when a solution of $[PtCl(dien)]Cl$ is added slowly to Na(ddtc). The ^1H NMR spectrum of the remaining reaction mixture shows the presence of free diethylenetriamine; small amounts of free diethylenetriamine can also be seen in the NMR spectrum shown in Figure 1B. The collected precipitate, redissolved in $CDCl_3$, shows ^1H NMR data (1.30 ppm (t), 3.60 ppm (q) relative to TMS) in agreement with bis(diethyldithiocarbamato)platinum, $(Pt(ddtc)_2)$.[29] The formation of some free diethylenetriamine and $Pt(ddtc)_2$ can be explained by assuming a nucleophilic attack of an excess of Na(ddtc) at the platinum in $[Pt(dien)ddtc]^+$ with simultaneous release of the already labilized diethylenetriamine ligand, resulting from the large trans-labilizing effect of the coordinated sulfurs.[30] This side reaction, with a half-life of <2 min, can also be observed separately, as represented by eq 2.



Thiourea was found to react with $[PtCl(dien)]Cl$ only in a 1:1 ratio, yielding $[Pt(dien)tu]^{2+}$. The downfield shift of the dien protons agrees with coordination to the sulfur atom. The ^{195}Pt NMR spectrum of $[Pt(dien)tu]^{2+}$ shows a singlet at −3239 ppm, which is invariable over the pH range 2–11, indicating no formation of a ring-opened adduct. The position of the signal is in the region expected for a PtN_3S complex (i.e. for a tridentate N_3 ligand).[17] The upfield shift of 522 ppm in relation to free $[PtCl(dien)]Cl$ can be compared with the 609 ppm upfield shift resulting from the coordination of thiourea to $[PtCl(^{15}NH_3)_3]Cl$.[31]

Reaction Products of Platinum Compounds with GSH and GS-Me. The reaction products of GSH and GS-Me with $[PtCl(dien)]Cl$ have been[17] well identified and appear as ideally suited for use as model adducts in ligand-exchange reactions (for a summary see Chart I). In all complexes, coordination to the sulfur atom is observed. No detectable side reactions have been observed.

The coordination chemistry of GSH with cis-Pt has already been studied.[14,18,32-34] There is some discussion about which complexes are formed, although there is general agreement that coordination to the ionized sulfhydryl group occurs. With combination of the results of Dedon et al.[14] and Appleton et al.,[18] most likely a polymeric species $[Pt(GS)_2]_n$ is formed, which contains two μ-GS units (**3**). Recent results of Appleton et al.[35] indicate



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that initially **3** is formed, in which the remaining coordination sites are still occupied by ammine groups, i.e. $[Pt_2(NH_3)_4(GS)_2]^{2+}$.

Both for $[Pt(GS)_2]_n$ and for the monomeric species, attempts were made to obtain ^{195}Pt NMR data. Since complicated spectra were expected, *cis*-$[Pt(H_2O)_2(^{15}NH_3)_2]^{2+}$ was used as a starting compound (δ = −1580 ppm, $^1J(^{195}Pt-^{15}N)$ = 390 Hz), to prevent severe broadening of the NMR signal due to the nuclear quadrupole moment of ^{14}N. No signals ascribed to $[Pt(GS)_2]_n$ could be detected. The ^{195}Pt NMR spectrum of the monomeric species shows two broad signals at −2771 ppm and at −2845 ppm. These results are in good agreement with those of Appleton et al.,[35] although no coupling to ^{15}N could be detected in our compound.

For the description of the reaction of GS-Me with cis-Pt, we first consider a related study of *S*-methylcysteine, in which coordination to the sulfur atom has been proven.[36] From the very

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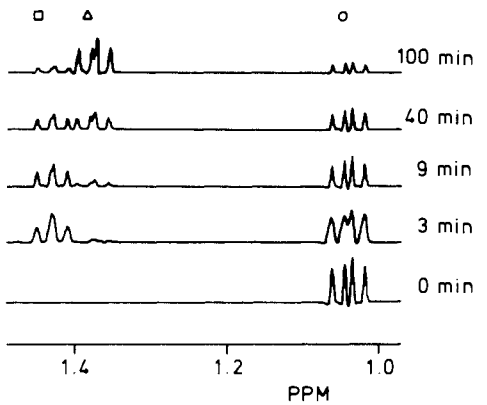

Figure 3. Part of the 300-MHz ^{1}H NMR spectra of the reaction between GS^- and 0.5 equiv of $[PtCl(NH_3)_3]Cl$ as a function of time at pH* 11. Chemical shifts are in ppm relative to TMA. ○ represents the g6 proton of GS^-, □ represents the g6 proton of $[Pt(NH_3)_3GS]^+$, and △ represents the g6 proton of *trans*-$Pt(NH_3)_2(GS)_2$.

complicated ^{1}H NMR spectrum of the GS-Me–cis-Pt system (about eight CH_3 signals, results not shown; from now on abbreviated as Pt–GS-Me) at least the ~0.5 ppm downfield shift for the CH_3 signal also indicates coordination to the sulfur atom. The ^{195}Pt NMR data show at least three broad signals, i.e. at −3205, −3387, and −3408 ppm. These signals are in the range expected for a PtN_2S_2 complex. However, no further identification was possible due to the complexicity of the system.

The product formed between trans-Pt and 2 equiv of GS^- (pH 11) proved to be the same as observed by Dedon et al.[14] The chemical shifts of the protons g2 and g6 of the complex *trans*-$Pt(NH_3)_2(GS)_2$ are independent of pH and located at −0.43 and 1.37 ppm, respectively. These results are comparable with the ^{1}H NMR results of $[Pt(dien)GS]^+$ and clearly indicate coordination of platinum at the ionized sulfhydryl group of the two equivalent GS^- units.[17] The intermediate *trans*-$Pt(NH_3)_2(GS)Cl$ could not be detected ($t_{1/2}$ for the formation of *trans*-$Pt(NH_3)_2(GS)_2$ is 15 min). Experiments with an excess of NH_4HCO_3 (up to a ratio of 25 to 1) and a small amount of GS^- (pH 11) with the aim to trap the intermediate were not successful. The ^{195}Pt chemical shift of *trans*-$Pt(NH_3)_2(GS)_2$ is located at −3173 ppm, which can be compared with that of *trans*-$[Pt(NH_3)_2(tu)_2]^{2+}$ (δ = −3239 ppm).[37] *trans*-$Pt(NH_3)_2(GS)_2$ is unstable at pH <7, yielding several decomposition products, including free GSH.

The reaction of $[PtCl(NH_3)_3]Cl$ with 2 equiv of GS^- (pH 11) yields the same product as the one formed from trans-Pt and 2 equiv of GS^- (parts of the ^{1}H NMR spectra of this reaction as a function of time are depicted in Figure 3). This observation can be explained by assuming that the initial product is $[Pt(NH_3)_3GS]^+$; this intermediate is more stable compared to *trans*-$Pt(NH_3)_2(GS)Cl$ and could quite easily be detected ($t_{1/2}$ for the formation of $[Pt(NH_3)_3GS]^+$ is <2 min). The chemical shifts of the protons g2 and g6 of $[Pt(NH_3)_3GS]^+$ are independent of pH and located at −0.65 and 1.43 ppm, respectively, proving coordination at the ionized sulfhydryl group. The amine ligand trans to the coordinated sulfur will still be labile, as a result of the trans-labilizing effect of the coordinated sulfur[30] and the presence of a second nucleophilic GS^- molecule. Therefore, this amine ligand is easily substituted by a second GS^- unit, resulting in the final product *trans*-$Pt(NH_3)_2(GS)_2$ ($t_{1/2}$ for the formation of *trans*-$Pt(NH_3)_2(GS)_2$ = 40 min). In the absence of a second equivalent of GS^-, $[Pt(NH_3)_3GS]^+$ is stable for about 10 h; this illustrates the lability of the amino group coordinated trans to the sulfur atom. The reason that the intermediate $[Pt(NH_3)_3GS]^+$ is more stable compared to *trans*-$Pt(NH_3)_2(GS)Cl$ is more likely the result of the more labile chloride ligand. The reaction of $[PtCl(NH_3)_3]Cl$ (δ = −2356 ppm) with 2 equiv of GS^- (pH 11) was also followed by ^{195}Pt NMR spectroscopy. $[Pt(NH_3)_3GS]^+$ was formed again quickly (δ = −2932 ppm). This value is characteristic for a PtN_3S complex (cf. $[Pt(^{15}NH_3)_3tu]^{2+}$: −2962 ppm[31]). This intermediate slowly reacts further with a second GS^-, forming *trans*-$Pt(NH_3)_2(GS)_2$ (δ = −3173 ppm, vide supra).

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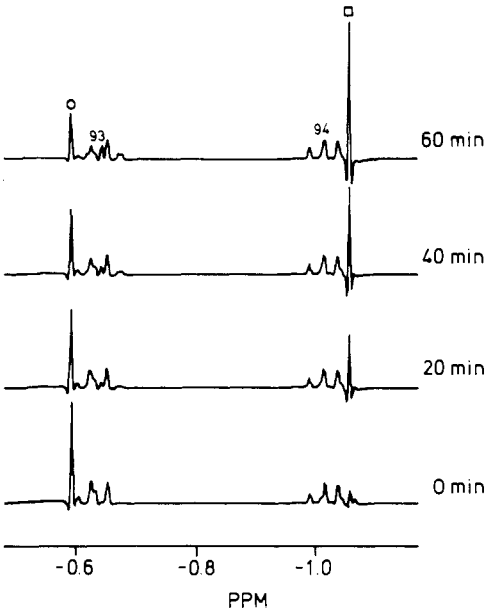

Figure 4. Part of the 300-MHz ^{1}H NMR spectra of the reaction between $[Pt(dien)GS\text{-}Me]^{2+}$ and thiourea as a function of time at pH* 7.5 (phosphate buffer). Chemical shifts are in ppm relative to TMA. ○ represents the CH_3 signal of $[Pt(dien)GS\text{-}Me]^{2+}$, and □ represents the CH_3 signal of GS-Me.

Table I. Half-Lives (Estimated Error 10–15%) and Products of Exchange Reactions between Na(ddtc) or Thiourea and Platinated Sulfur Compounds at pH* 7.5 (Phosphate Buffer)

	half-lives		
	Na(ddtc)	thiourea	products[d]
$[Pt(dien)GS]^+$	*b*	*b*	
$[Pt(dien)GS\text{-}Me]^{2+}$	<2 min	30 min	GS-Me
$[\{Pt(dien)\}_2GS]^{3+}$	40 min	7 h	$[Pt(dien)GS]^+$
$[Pt(GS)_2]_n$[a]	1–2 h	*b*	Pt-GS, GSH
Pt–GSMe	<2 min	<2 min; 1–2 h[c]	GS-Me
trans-$Pt(NH_3)_2(GS)_2$[a]	*b*	*b*	

[a] Reaction also carried out at pH* 11. [b] No reaction up to 12 h. [c] Two half-lives originating from at least two different adducts; see text. [d] All products are given except the complexes formed between liberated platinum with Na(ddtc) and thiourea: no side reactions occur.

Ligand-Exchange Reactions. The model adducts $[Pt(dien)GS]^+$, $[\{Pt(dien)\}_2GS]^{3+}$, $[Pt(dien)GS\text{-}Me]^{2+}$, $[Pt(GS)_2]_n$, *trans*-$Pt(NH_3)_2(GS)_2$, and "Pt-GS-Me" were incubated with Na(ddtc) or thiourea. As an example of the exchange reactions, parts of the ^{1}H NMR spectra of the reaction between $[Pt(dien)GS\text{-}Me]^{2+}$ with thiourea are depicted in Figure 4 as a function of time. The values of $t_{1/2}$ for the exchange reactions and the resulting reaction products are listed in Table I.

Not listed in Table I are the reaction products between Na(ddtc), or thiourea, and the eventually liberated platinum. Reaction products of Na(ddtc) with liberated $Pt(dien)^{2+}$, thiourea with liberated $Pt(dien)^{2+}$, and Na(ddtc) with platinum liberated from the original cis-Pt products (i.e. $Pt(ddtc)_2$) are all as described above.

$[Pt(dien)GS]^+$ did not react with Na(ddtc) or thiourea within 12 h. Both Na(ddtc) and thiourea do react with 1 equiv of $Pt(dien)^{2+}$ deriving from $[Pt(dien)GS\text{-}Me]^{2+}$ and $[\{Pt(dien)\}_2GS]^{3+}$, leaving respectively GS-Me and $[Pt(dien)GS]^+$ in the reaction mixture. The reaction product $[Pt(dien)GS]^+$ appeared to be identical with the model adduct $[Pt(dien)GS]^+$ and consequently did not react further with Na(ddtc) or thiourea. The product of the exchange reaction between $[Pt(dien)GS\text{-}Me]^{2+}$ with Na(ddtc), i.e. $[Pt(dien)ddtc]^+$, could quite easily be detected. Detection of $[Pt(dien)ddtc]^+$ resulting from the exchange reaction between $[\{Pt(dien)\}_2GS]^{3+}$ and Na(ddtc) appeared to be more

difficult. This is mainly the result of a competing reaction of free Na(ddtc) with the formed $[Pt(dien)ddtc]^+$ ($t_{1/2}$ = <2 min compared to 40 min for the exchange reaction). As a result, a precipitate of $Pt(ddtc)_2$ (see eq 2) appeared during the reaction. This side reaction could be avoided by using an excess of $[\{Pt(dien)\}_2GS]^{3+}$ ($[\{Pt(dien)\}_2GS]^{3+}$: Na(ddtc) = 10:1). Small amounts of $[Pt(dien)ddtc]^+$ could be detected, indeed, in the ^1H NMR spectrum.

The reaction between Na(ddtc) and $[Pt(GS)_2]_n$ has already been investigated in some detail by Dedon et al.[14] They used the HPLC detection of $Pt(ddtc)_2$ to measure the exchange rate. From the formation of $Pt(ddtc)_2$ they concluded that Na(ddtc) is able to liberate platinum from platinated sulfhydryl groups and that the Na(ddtc)-induced dissociation of Pt–protein interactions may be based on this property of Na(ddtc). In the ^1H NMR study indeed the appearance of $Pt(ddtc)_2$ is observed (vide supra). However, the main product appears to be not free GSH but a GS-containing adduct instead; its chemical shifts of the g2 and g6 protons are located at –0.41 and at 1.22 ppm, respectively, and the positions of these signals are independent of pH. These observations strongly suggest that the main product still contains a platinated RS^- group.[17] Furthermore, a broad quartet (0.48 ppm) and triplet (–1.92 ppm) characteristic for a platinated $ddtc^-$ unit were observed.[26] The ^{195}Pt NMR study of the reaction of the monomeric species $[Pt_2(^{15}NH_3)_4(GS)_2]^{2+}$ at pH 11 with Na(ddtc) agrees with the rapid formation of a precipitate of $Pt(ddtc)_2$ and a soluble platinated compound (δ = –3733 ppm). The ^1H NMR spectrum of this filtrated and lyophilized sample showed—besides free Na(ddtc)—the presence of only one product, which proved to be the same as that formed between $[Pt(GS)_2]_n$ and Na(ddtc), although much more pure. No free GSH appeared to be formed. Most likely this Pt product has a GS^- to $ddtc^-$ ratio of 2:1 (based on the relative integration of the proton signals). The platinum chemical shift can be best compared with that of a PtS_4 complex. The combined ^1H and ^{195}Pt NMR results, therefore best agree with the formation of *cis*-$[Pt(GS)_2ddtc]^-$. Unfortunately, further verfication of this hypothesis was impossible, because during further purification by column chromatography the product, abbreviated as "Pt–GS", appeared to be unstable. After a prolonged reaction time of 2 h between Na(ddtc) and $[Pt(GS)_2]_n$ small amounts of free GSH could be detected. It appears that this is not the result of Na(ddtc) treatment but the result of the intrinsic instability of Pt–GS. Especially H^+ catalyzes this reaction. In fact when the pH of a solution of Pt–GS is lowered, the complex decomposes completely, leaving free GSH in the reaction mixture. This acid hydrolysis of a Pt–S(cysteine) bond was also observed for *trans*-$Pt(NH_3)_2(GS)_2$ (vide supra). The observation that the amount of free GSH formed during the reaction of $[Pt(GS)_2]_n$ with Na(ddtc) is strongly dependent on the pH is a further indication for the H^+-induced instability of Pt–GS. At pH 11 almost no free GSH could be detected, whereas at pH 7.5 significant amounts of free GSH were formed. No reaction could be detected between thiourea and $[Pt(GS)_2]_n$.

Within 2 min the complex Pt–GS-Me system was completely "restored" by Na(ddtc), leaving only free GS-Me and $Pt(ddtc)_2$ in the reaction mixture. A part of the Pt–GSMe system reacts very rapidly with thiourea, under formation of free GS-Me ($t_{1/2}$ = <2 min). The rest reacts more slowly with thiourea, eventually also forming free GS-Me ($t_{1/2}$ = 1–2 h). From this observation it can be concluded that at least two different adducts may be formed between GS-Me and cis-Pt. These adducts, unfortunately, could not be identified due to the complexicity of the system and the instability on the column.

Finally, *trans*-$Pt(NH_3)_2(GS)_2$ was found to be not reactive toward Na(ddtc) or thiourea treatment within 12 h.

Mechanistic Aspects. $[Pt(dien)GS]^+$ can be considered as a model for a monofunctional Pt–cysteine protein adduct. Neither Na(ddtc) nor thiourea is able to reverse the Pt–S binding in this adduct. $[Pt(dien)GS\text{-}Me]^{2+}$, a model for a monofunctional Pt–methionine protein adduct, on the other hand, reacts quite rapidly with both Na(ddtc) or thiourea. $[\{Pt(dien)\}_2GS]^{3+}$, which in a sense can also be considered as a model for Pt–methionine protein adducts, reacted also with Na(ddtc) or thiourea. The resulting reaction products strongly suggest that a nucleophilic attack at the Pt–S bond and not at the Pt–N bond has occurred. Especially, the observed formation of $[Pt(dien)tu]^{2+}$ containing the intact $Pt(dien)^{2+}$ unit justifies this assumption. The lability of the Pt–S bond in $[Pt(dien)GS\text{-}Me]^{2+}$ and in $[\{Pt(dien)\}_2GS]^{3+}$ was also surmised from our earlier study of these compounds.[17] The inert Pt–S bond in Pt–cysteine complexes under neutral conditions is probably the result of a very strong metal–S^- binding compared to rather weak neutral metal–S binding in Pt–methionine complexes.

At first sight the results of the exchange reactions between Na(ddtc) and $[Pt(GS)_2]_n$ appear to disagree with those found in the $Pt(dien)^{2+}$ system. Formation of $Pt(ddtc)_2$ indicates the ability of Na(ddtc) to liberate platinum from platinated GSH; in other words Na(ddtc) would reverse Pt–cysteine interactions in a protein, whereas the $Pt(dien)^{2+}$ system indicates only the dissociation of Pt–methionine adducts by Na(ddtc). However, careful examination of the ^1H NMR spectra of the reaction mixtures of $[Pt(GS)_2]_n$ and $[Pt_2(^{15}NH_3)_4(GS)_2]^{2+}$ with Na(ddtc) shows that at first instance no free GSH is formed at all. Most likely an intermediate product is formed which still contain platinated sulfur atoms (i.e. Pt–GS). This product is stable toward further Na(ddtc) treatment but sensitive to acid hydrolysis. The acid hydrolysis, in fact, is responsible for the formation of small amounts of free GSH.

The formation of Pt–GS can be explained by assuming that $[Pt(GS)_2]_n$ is a polymeric species, which contains two μ-GS units.[14,18] The μ_2-GS species can in a sense be compared to $[\{Pt(dien)\}_2GS]^{3+}$, which also proved to be reactive toward Na(ddtc) treatment. The breaking of these Pt–methionine type bonds stops at the Pt–cysteine level. For $[Pt(GS)_2]_n$ this is Pt–GS and for $[\{Pt(dien)\}_2GS]^{3+}$ this is $[Pt(dien)GS]^+$. The formation of Pt-GS from the monomeric species $[Pt_2(^{15}NH_3)_2(GS)_2]^{2+}$ and Na(ddtc) can be explained in a similar way. Cleavage of first the Pt–methionine type bonds and second the Pt–N bonds or vice versa will form no free GSH. Especially for this cleavage reaction it is understandable to propose *cis*-$[Pt(GS)_2ddtc]^-$ as the identity of Pt-GS. From the detection of $Pt(ddtc)_2$, Dedon et al. concluded that Na(ddtc) could well reverse the platinum binding of Pt–cysteine adducts.[14] The present results demonstrate this only to be an indication of the dissociation of the Pt–methionine type bonds. Although $[\{Pt(dien)\}_2GS]^{3+}$ and $[Pt(GS)_2]_n$ resemble in a sense Pt–methionine adducts, their reactivity toward nucleophilic agents is at least a factor of 10 less compared to that of the actual Pt–methionine model adduct $[Pt(dien)GS\text{-}Me]^{2+}$ (see values of $t_{1/2}$ in Table I). It should be noted that the formation of $(\mu\text{-}GS)Pt_2$ products in proteins is not very likely, because of the low concentrations of platinum in the cytoplasm.

The exchange reactions of Na(ddtc) and thiourea with the system Pt–GS-Me are in agreement with $[Pt(dien)GS\text{-}Me]^{2+}$. Na(ddtc) reacts with a rate similar to that for $[Pt(dien)GS\text{-}Me]^{2+}$, leaving also free GS-Me in the reaction mixture. The Pt-exchange reactions of thiourea are at least a factor of 10 slower compared to the exchange reactions of Na(ddtc) (see also Table I). This is in agreement with an earlier study in which thiourea was found slightly less effective than Na(ddtc).[14] On the other hand, a part of the Pt–GS-Me system shows similar reaction rates for both nucleophilic agents. This unusual observation for thiourea should originate from coordinated H_2O or Cl^- ligands, which are known to increase the exchange reaction rate. The labile H_2O or Cl^- ligands are easily substituted by the thiourea S atom, thereby labilizing the coordinated GS-Me.

trans-$Pt(NH_3)_2(GS)_2$ is the only stable well-identified complex formed between a bifunctional Pt compound and GSH. Like $[Pt(dien)GS]^+$, it can be considered as a model adduct resembling and representing Pt–cysteine interactions in a protein. The inability of Na(ddtc) and thiourea to remove platinum from *trans*-$Pt(NH_3)_2(GS)_2$ again shows the stability of Pt–cysteine adducts toward nucleophilic agents.

The results shown in the present study strongly suggest that the working mechanism of Na(ddtc)- and thiourea-induced dis-

sociation of Pt–protein interactions is mainly based on the removal of the platinum from methionine type sulfurs and less or not at all from cysteine type sulfurs.

On the basis of a similarity of histopathology of the kidney after Pt(II) or Hg(II) exposure in the rat, Borch et al.[6] suggest that the same mechanism might play a role in the nephrotoxicity of these metals (i.e. inactivation of enzymes by the coordination of Pt^{2+} or Hg^{2+} to thiol residues). The overexpression of metallothionein in tumor cell lines resistant to cis-Pt indicates the reactivity of cysteines.[38] On the basis of this hypothesis, they suggest that the ability of Na(ddtc) and thiourea to reduce the nephrotoxicity is the result of removing platinum from these thiol residues.[6,13,14] However, as yet there is no actual evidence concerning the dissociation of Pt–cysteine type bonds. The platinated inactivated enzyme γ-glutamyl transpeptidase, which is reactivated by Na(ddtc) or thiourea, contain a sulfhydryl group essential for enzyme activity, but the detailed interactions are not known.[12-14,39] On the other hand, a few examples are known about the Na(ddtc)- or thiourea-induced dissociation of Pt–methionine type bonds.[11,41-43] Cytochrome *c* is cross-linked by $PtCl_4^{2-}$ at its Met-65 residues, although not inactivated, yielding *trans*-$[PtCl_2(cyt)_2]$. This complex can be cleaved by thiourea, after which the native cytochrome *c* is restored.[43] α_2-macroglobulin is cross-linked by cis-Pt through its methionine residues and can be reactivated by Na(ddtc).[11,41,42] The last example clearly suggests that proteins can be inactivated as a result of Pt coordination to methionine sulfurs and can be reactivated by Na(ddtc) or thiourea, in agreement with the present study. Furthermore, our study suggests that Na(ddtc) and thiourea will only reduce the nephrotoxity, when the inactivation of proteins would be caused by Pt coordination to methionine residues and not to cysteine residues. That Na(ddtc) reacts only with a part of the platinated proteins was also observed by Hegedus et al.[44]

The present study clearly proves that Na(ddtc) and thiourea are far more reactive toward Pt–S methionine type adducts than toward Pt–S cysteine type adducts. This observation can be important in the unraveling of the actual mechanism of the Na(ddtc)- and thiourea-induced dissociation of Pt–protein interactions and the related effect of counteracting the nephrotoxicity of platinum antitumor compounds.

Concluding Remarks

This investigation has made clear that therapeutic nucleophilic agents for cis-Pt, like Na(ddtc) and thiourea, may help to restore the original structure of certain proteins (most likely initially distorted by e.g. Pt–methionine binding). The mechanism of the reduction of the nephrotoxicity of platinum compounds by Na(ddtc) and thiourea may be based on the relatively easy dissociation of the Pt–methionine binding. Our results strongly indicate that the Pt–S cysteine bond cannot be reversed by Na(ddtc) or thiourea. Nephrotoxicity, as a result of Pt–cysteine adducts, can therefore not be suppressed by Na(ddtc) or thiourea treatment. It should be noted, however, that the present model study may not mimic the interactions of platinum antitumor drugs with all kinds of proteins.

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