Diamine Ligand Release from the Cisplatin Analogue [*meso*-1,2-Bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) in Cell Culture Medium

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The stability of the five-membered chelate ring of the cisplatin analogue [*meso*-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II) was investigated under typical cell culture conditions (IMEM-Richter's medium with 10% fetal calf serum, 37 °C). For this purpose, the platinum compound was radiolabeled with tritium in the meta position of the aromatic ring by an acid-catalyzed tritium-exchange reaction, and a reversed-phase HPLC assay with radiochemical detection was developed to monitor for the presence of the free diamine ligand in the cell culture medium. A gradual increase in radioactivity attributed to the free diamine was found in medium containing the dichloroplatinum(II) complex (ca. 25% after 24 h), indicating that the diamine ligand was being released from the metal atom. When 1 mM glutathione (GSH) was included in the incubation medium, the amount of free diamine nearly doubled after 24 h, while the amount of radioactivity attributed to serum protein–platinum adducts decreased relative to incubations without GSH. On the other hand, the omission of serum from the incubations resulted in a dramatic decrease in the amount of radioactivity eluting under the diamine peak, while the concentrations of the two methionine–Pt adducts, which formed in a 1:1 ratio, rose. Through the use of liquid secondary ion mass spectroscopy, the two methionine–Pt adducts were identified as monomethionine metabolites of the title compound, whereby the two chloride ligands have been replaced by the amino acid. These compounds are probably diastereomers since the sulfur of methionine can coordinate to platinum with equal probability either cis or trans to the *R*-configured benzylamine carbon. On the basis of the chemical shifts of the MeS groups in the 250-MHz ^{1}H NMR, it is concluded that a S,N-five-membered chelate ring is present in these methionine–Pt adducts.

Introduction

The clinical usefulness of inorganic compounds for the treatment of human cancer has been best demonstrated by cisplatin.[1] The need to reduce the systemic toxicity of this valuable chemotherapeutic, as well as to broaden the spectrum of antitumor activity, has led medicinal chemists to replace the NH_3 groups of cisplatin for a wide variety of "nonleaving" amine ligands.[2-4] Recently, interest has been directed toward developing cisplatin analogues that have pharmacologically active amines coordinated to the cytotoxic platinum(II) moiety.[5-15] Amines with either DNA intercalating, estrogenic, or antiestrogenic properties have been investigated most thoroughly. The rational behind these drug-design approaches is that such amines might alter either the interaction of the platinum atom with the supposed target molecule DNA or the organ distribution of the platinum complex. In this way, a more selective therapy with a new spectrum of antitumor activity might be achieved. For example, in an effort to exploit the presence of the estrogen receptor in several tumor types (i.e. breast and prostate cancers) and thereby facilitate the tumor-selective uptake of the cytotoxic platinum moiety, several groups including our own have

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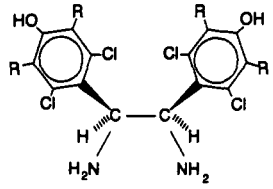
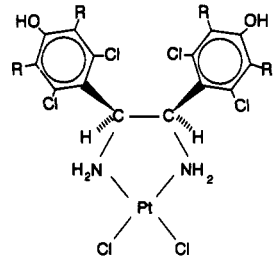


R:	Compound
H_4	1
D_4	[^{2}H]1
T_4	[^{3}H]1



R:	Compound
H_4	[$PtCl_2$(1)]
T_4	[$PtCl_2$([^{3}H]1)]

attempted to develop cisplatin analogues which have an affinity to that steroid hormone receptor.[9-17]

Since the amine ligands used in such a drug-design approach possess biological activity and could themselves be antineoplastic agents, it is important to be aware of the chemical stability of the coordinated Pt–N bonds before the pharmacological results can be accurately evaluated. Conflicting reports in the literature[18-21] regarding the stability of the Pt–N bonds of ethylenediamine-based analogues of cisplatin under biologically relevant conditions led us to reinvestigate this question with the platinum complex, [*meso*-1,2-bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine]dichloroplatinum(II)([$PtCl_2$(1)]). This compound has been shown to be a weak estrogen and to have potent antitumor activity in several rodent mammary[11] and prostate tumor models.[17] However, the diamine ligand 1 by itself is also estrogenic, and it possesses some, although considerably less, growth inhibitory activity toward the same estrogen-sensitive tumors.[11,17]

Cell culture methods are routinely used for the in vitro screening of cisplatin analogues for cytotoxic activity, and cell cultures of estrogen receptor positive neoplasms (the MCF-7 human breast cancer cell line) have recently been used to estimate the estrogenic activity of platinum complexes.[22] Described herein is the synthesis of tritium-labeled [$PtCl_2$(1)] and the reversed-phase HPLC analysis that was developed to monitor for the possible release of the free diamine under typical cell culture conditions. The kinetics and conditions of diamine release from [$PtCl_2$(1)] are described, and the pharmacological importance of these results is discussed.

Results

Synthesis of Tritium-Labeled [$PtCl_2$(1)]. The acid-catalyzed exchange of the meta aromatic protons is the most convenient means of introducing isotopic hydrogen into the diamine 1. The time-dependent exchange of the aromatic protons for deuterium in 70% D_2SO_4/D_2O can be followed by proton NMR and is completed within 3.5 h at 68 °C. Radioisotopic labeling of 1 is done under similar conditions to the deuterium labeling studies and results in a product of low to moderate specific activity (286 Ci/mol). Coordination of [^{3}H]1 with $K_2[PtCl_4]$ is done in DMF at 56 °C followed by the isolation and purification of [$PtCl_2$([^{3}H]1)] by means of reversed-phase HPLC. The meta position of the aromatic ring is chemically stable under the conditions of the in vitro experiments.

Assay Development. A reversed-phase HPLC assay with radiochemical detection was used to monitor for the presence of the free diamine in cell culture medium. For the best chromatographic separation of the diamine 1 from the dichloroplatinum complex, it is necessary to acidify the phosphate buffer to a pH of 3.3. An attempt was made to anticipate other metabolites of [$PtCl_2$(1)] that might be formed in measurable quantities. Of the amino acids present in Richter's medium, L-methionine, L-histidine, L-tryptophan, and L-cystine were investigated for their reactivity toward [$PtCl_2$(1)]; these amino acids represent the four most nucleophilic compounds in this medium that could react irreversibly with platinum(II) compounds.[21] Only L-methionine reacts fast enough with the dichloroplatinum complex (in phosphate-buffered saline (PBS) at 37 °C) to be of any kinetic relevance in the forthcoming investigations. Two methionine–Pt adducts are formed in a 1:1 ratio when a 10-fold excess of L-methionine is incubated in the presence of [$PtCl_2$(1)] (Figure 1). By using a linear gradient from 25 to 55% methanol, the separation of the diamine from the two methionine–Pt adducts is possible; the pair of metabolites elute approximately halfway between the diamine 1 and [$PtCl_2$(1)].

Several analytical difficulties were encountered in the assay of the cell medium. A time-dependent loss of 1 from the medium was attributed to the binding of the diamine to the cell culture plates. (Diamine bound to the plates can be recovered by rinsing the plates with a 0.05 M HCl solution.) To prevent the adhesion of the cold carrier to the surface of the plastic, it was necessary to preincubate the culture dishes overnight with medium. When this was done, the concentration of 1 in medium remained

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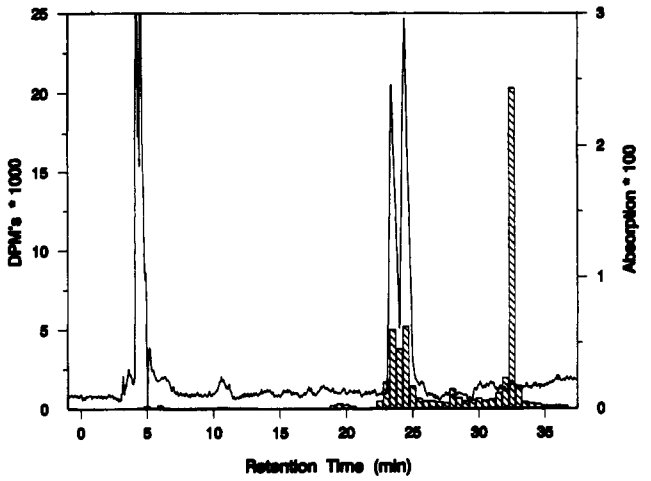


Figure 1. HPLC product analysis of the reactions of $[PtCl_2(1)]$ with L-methionine. The solid line is the UV absorption at 281 nm for the HPLC chromatogram obtained from the incubation of 10 μM $[PtCl_2(1)]$ with 0.1 mM L-methionine in PBS for 48 h at 37 °C. Hatched bars represent the radioactivity in each 30-s HPLC fractions collected from a 500-μL aliquot of Richter's medium (without FCS) incubated with 10 μM $[PtCl_2([^3H]1)]$ for 24 h at 37 °C.

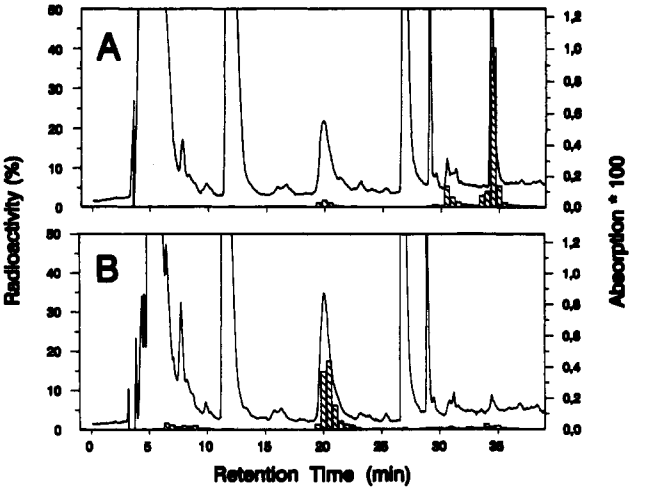


Figure 2. UV versus radiochemical detection of the reversed-phase HPLC analysis of 10 μM 1 and 10 μM $[PtCl_2([^3H]1)]$ 1.5 h following the addition to IMEM-Richter's cell culture medium (A) and 24 h later (B). Medium contained 10% FCS and 1 mM GSH, and incubations were done at 37 °C in a humidified atmosphere of 5% CO_2. The solid line is the UV absorption at 281 nm. The hatched bars represent the percent radioactivity collected in each of the 30-s fractions. The retention times of 1 and $[PtCl_2(1)]$ were ca. 20 and 34.5 min, respectively.

constant over the course of the incubation. Another problem was the appearance of giant "ghost" peaks in the HPLC chromatograms of the cell medium. These peaks were attributed to gaseous CO_2 that evolved under the acid conditions of the chromatography. To avoid this problem, the medium was acidified prior to HPLC analysis to convert HCO_3^- to CO_2, which could then be removed by bubbling helium through the solution.

To disrupt reversible ligand–protein binding that might prevent a fraction of the free diamine from passing through the ultrafiltration membrane, an 6 M urea solution was added to the medium prior to ultrafiltration. The fraction of radioactivity that remained on the membrane filter following ultrafiltration was attributed mostly to covalently bound protein–platinum adducts. (When solutions of 1 and $[PtCl_2(1)]$ in PBS only were filtered through the membrane filter, approximately 5% of these compounds were retained by the filter.)

Quantification of Diamine Release under Varying Experimental Conditions. Typical HPLC chromatograms of the ultrafiltrate from medium with 10% fetal calf serum (FCS), which contained 10 μM of both 1 and $[PtCl_2([^3H]1)]$ and 1 mM glutathione (GSH), are shown 1.5 h following the addition of tritiated platinum complex (Figure 2A) and one day later (Figure 2B). It is apparent that under these in vitro conditions a significant fraction of the total radioactivity coelutes with the cold carrier diamine 1 after 24 h; evidence that the diamine ligand is released from the platinum complex.

The kinetics of diamine release were investigated under several different experimental conditions. The incubation of $[PtCl_2(1)]$ with cell culture medium without FCS led to very little diamine release (Figure 3A). The two main products of these incubations coelute in approximately equal amounts with the two L-methionine adducts of $[PtCl_2(1)]$ (Figure 1). The addition of 10% FCS to cell culture medium increases significantly the amount of free 1 found in the incubations at the later time points (>7 h), while decreasing the amount of radioactivity attributed to the methionine–Pt adducts (Figure 3B). After 24 h approximately 25% of the administered radioactivity elutes as ligand. This fraction represents the maximum amount of ligand that is released under these conditions

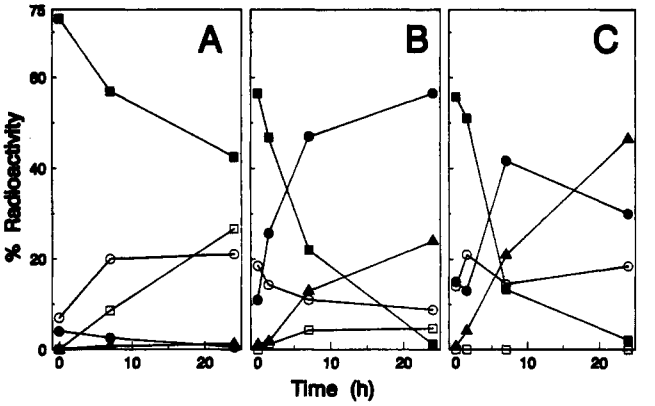


Figure 3. Reactions of 10 μM $[PtCl_2([^3H]1)]$ with (A) IMEM-Richter's cell culture medium without FCS, (B) Richter's medium supplemented with 10% FCS, and (C) same as B but with 1 mM GSH. Incubations were done at 37 °C in a humidified atmosphere of 5% CO_2. At the times indicated, a 1-mL aliquot was removed, mixed with 0.9 mL of 6 M urea solution and 0.1 mL of 0.5 M H_3PO_4, incubated 5 min at 37 °C, and filtered through an Amicon YMT membrane filter (30 000 mol wt cutoff). $[PtCl_2(1)]$ and transformation products were determined by reverse-phase HPLC as described in the Experimental Section: ■, $[PtCl_2(1)]$; ▲, 1; ●, nonfilterable; □, $[Pt(1)Met]^+$; and ○, other transformation products. Data points are the average of two independent experiments.

since incubating an additional week brings about no further increase in the concentration of free ligand (data not shown). At the earliest time point (1.5 h) the amount of free diamine detected is only slightly above the control level (Figure 3B). Recently, we have obtained the same quantitative results for diamine release when the addition of urea and acid as well as the ultrafiltration step are omitted and serum-containing medium is instead loaded directly onto the HPLC column.

An even greater release of the diamine 1 is observed when 1 mM GSH (approximate intracellular concentration) is included in the incubations (Figure 3C). In the presence of GSH, the amount of radioactivity remaining covalently bound to serum proteins after 24 h decreases by about one-half relative to incubations without GSH

(compare Figure 2, parts B and C). This could be due either to the trapping of electrophilic platinum species by GSH before they covalently bind to serum proteins or to the removal of Pt–protein adducts by GSH. The data in Figure 3, parts B and C, indicate that these differences in protein binding are due in part to the latter explanation. In the absence of GSH, the percent of Pt–protein adducts increases from 48 to 58% between 7 and 24 h. On the other hand, in the presence of GSH, the percent of covalently bound drug decreases from 41 to 29% between 7 and 24 h; suggestive that protein adducts are being removed by GSH.

Further experimentation showed that when Richter's medium is supplemented with 3.4 g/L bovine serum albumin (BSA) (approximate amount found in a 10% FCS solution; G. Bernhardt, personal communication) and incubated under the same conditions as with serum, no measurable diamine is released from [$PtCl_2$(1)] although the dichloroplatinum complex reacts irreversibly with the protein. Both bovine transferrin (0.4 g/L Richter's medium) and α-globulins (1.1 g/L medium) cause the release of some diamine but not enough to account for all of the ligand found in the incubations of culture medium. GSH (1 mM) is efficient at causing the release of the diamine 1 from the complex when this nucleophile is incubated with 10 μM [$PtCl_2$(1)] in PBC at 37 °C for 48 h, but because of the very low concentrations of GSH in serum it is unlikely that this molecule is responsible for the release of ligand under cell culture conditions.

Characterization of the L-Methionine Adducts from [$PtCl_2$(1)]. Work was done at elucidating the structures of the two methionine adducts from [$PtCl_2$(1)]. Semi-preparative reversed-phase HPLC is used to separate the two compounds that form when 50 μM [$PtCl_2$(1)] is incubated with 500 μM L-methionine for 48 h at 37 °C in PBS. The compounds are isolated by lyophilization of the elution solvent. The liquid secondary ion mass spectra (LISIMS) of the two compounds are identical; each compound gives rise to the same molecular ion cluster. The masses and intensities of this ion cluster correspond to monomethionine–Pt adducts with a molecular formula of $C_{19}H_{22}N_3O_4PtS$ (Figure 4). This suggests two configurational diastereomers present in a 1:1 ratio. The 250-MHz ^{1}H NMR spectra for the two compounds are very similar. For each of the compounds a pair of singlets are observed; with the more rapidly eluting compound these appear at 2.56 and 2.53 ppm in a ratio of 5:3, respectively, and with the later eluting compound they are at 2.55 and 2.54 ppm in a ratio of 1:1. Owing to the slow rate of sulfur inversion, these signals are attributed to the MeS absorptions from the two inversional diastereomers that would be expected with each pair of configurational diastereomers.[23,24] Similar ratios of inversional diastereomers have been reported for the monomethionine adduct of cisplatin.[23] The chemical shifts of the MeS groups indicate the presence of a *S,N*-five-membered chelated ring; the chemical shifts of the MeS groups from the [Pt(ND_3)$_2$(MetD-*S,N*)]$^{2+}$ were reported to be 2.54 and 2.53 ppm while for the analogous groups in [Pt(ND_3)$_2$(MetD-*S,O*)]$^{2+}$ the chemical shifts were 2.47 and 2.45 ppm.[23] Although the signals of the other aliphatic protons coming from the L-methionine moiety in [Pt(1)Met]$^+$ are too weak

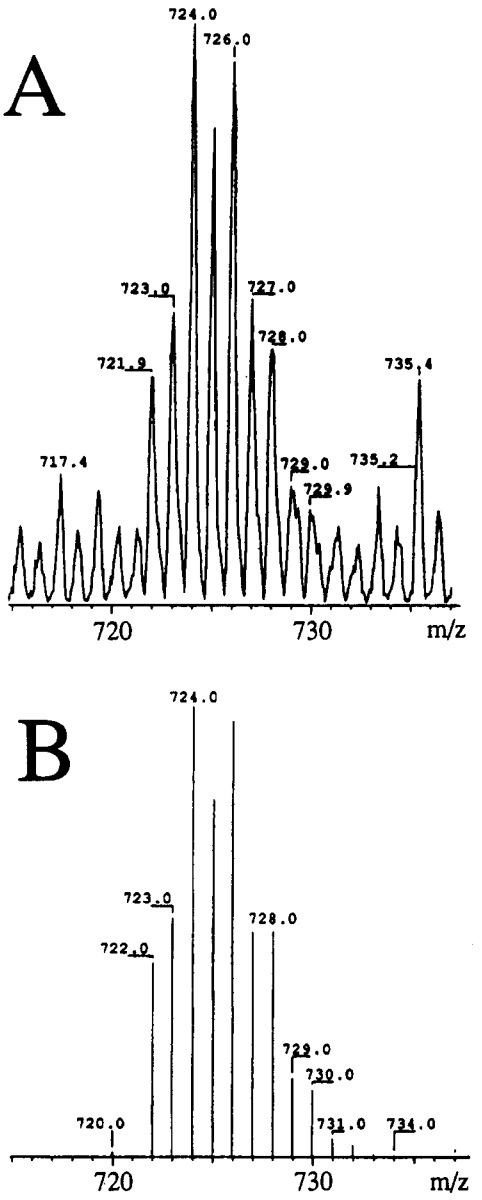
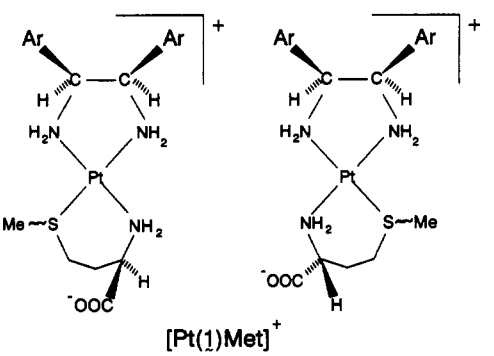


Figure 4. A positive ion liquid secondary ion mass spectrum of one of the methionine adducts of [$PtCl_2$(1)], in this case the compound that eluted later from the reverse-phase column (see Experimental Section for details): (A) the observed molecular ion cluster of [Pt(1)Met]$^+$, and (B) the theoretical isotope pattern that would be expected for a compound with a molecular formula of $C_{19}H_{22}N_3O_4PtS$.

to resolve the multiplicity of each peak, the chemical shifts of these protons are consistent with what has been reported for other monomethionine–Pt(II) adducts.[24] On the basis of this data, the most likely structures for the two monomethionine adducts of [$PtCl_2$(1)] are shown below.



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Discussion

These findings indicate that the chelating diamine of the cisplatin analogue [$PtCl_2$(1)] is lost from the platinum atom under typical cell culture conditions (maximum 25%). As in all chromatographic assays that rely on synthetic standards for the qualitative identification of metabolites, the possibility that some other radioactive compound could be coeluting with the diamine standard cannot be completely ruled out. However, this is unlikely for several reasons. Firstly, the L-methionine adducts of [$PtCl_2$(1)], which would be expected to be the main ultrafilterable platinum-containing metabolites in cell culture medium,[21] are well separated form the diamine ligand. In fact, the two L-methionine adducts are the main metabolites when serum is omitted from the incubations. Secondly, had a platinum-containing metabolite of [$PtCl_2$(1)] eluted at the same time as 1, then the addition of a competing nucleophile, such as GSH, should have reduced the radioactivity found in those fractions. However, quite the opposite occurred; the addition of GSH to incubations increases the amount of radioactivity eluting at the time of the diamine. Thirdly, 24 h after the addition of [$PtCl_2$(1)] to cell culture medium +10% FCS, no measurable platinum in the HPLC eluant that was collected at the retention time of 1 could be detected by atomic absorption spectroscopy (AAS).

While there is ample evidence that sulfur-containing bionucleophiles such as L-methionine, L-cysteine, and glutathione can cause the loss of an ammine group from cisplatin,[20,23-28] the question concerning the release of chelating ethylenediamines from cisplatin analogues has been an area of uncertainty in the literature for many years. Early experiments by Robins and co-workers gave no evidence for the in vivo release of ethyenediamine from a dual radiolabeled cisplatin analogue, [$^{195m}PtCl_2$([^{14}C]-en)].[18] Ten years later the same group used another dual label, [$^{191}PtCl_2$([^{14}C]en)], and reported the opposite finding that diamine was indeed released.[19] However, these methods are indirect; the time-dependent changes in the ratio of radioactivity of the two radioisotopes are used as an indication that the isotopes are becoming separated. Sadler and co-workers showed by ^{15}N NMR methods that the Pt–N bonds were cleaved when [$PtCl_2$(en)] was incubated in the presence of either *N*-acetyl-L-methionine at pH 2.2 or RNase A at pH 6.5 and reported that ethylenediamine was the product of these reactions.[20] By means of the same spectroscopic method, they also showed that ethylenediamine was released from [$PtCl_2$(en)] in human serum containing 0.2 mM L-methionine.[27] Unfortunately, no quantitative data for this ^{15}N NMR work was reported, and so it is impossible to judge how much and how fast the diamine was being released. Mauldin et al. reported *no* release of the diamine ligand, *d,l*-1,2-diaminocyclohexane, from (*d,l*-1,2-diaminocyclohexane)malonatoplatin(II) under cell culture conditions (RPMI-1640 medium + 15% FCS).[21] As in our work, a reversed-phase HPLC assay with radiochemical detection was used to monitor for the loss of diamine. It could be that the increased stability of malonate versus chloride as the anionic "leaving" group is the explanation why no diamine release was observed with the malonatoplatinum complex. However, modest amounts of the dichloroplatinum complex formed in cell culture medium incubated with the malonatoplatinum complex.[21] It could also be argued that the stability of the ethylenediamine chelated ring is dependent on the substituents present on the ethane portion of that five-membered ring (i.e. 1,2-diarylethane versus 1,2-(1,4-butanediyl)ethane); that is the 1,2-diaryl substituents destabilize the five-membered chelate ring. However, the mechanism by which the diamine 1 is lost from the platinum atom is not clear and, until this question is resolved, speculation will be avoided as to the reason(s) for the varying stability of diamine chelate rings from different cisplatin analogues. Ongoing work is aimed at answering this question.

At least two L-methionine adducts of [$PtCl_2$(1)] were detected in incubations with cell culture medium (Figure 1). The relative intensities of these two closely eluting species stay at an approximate 1:1 ratio throughout the course of the incubations. The reaction of (*d,l*-1,2-diaminocyclohexane)malonatoplatinum(II) (50 μM) with a 2-fold excess of L-methionine for 24 h was also reported to give two L-methionine adducts.[21] Through the use of HPLC–thermospray mass spectroscopy, it was estimated that approximately 60% of the reaction products were in the form of a monomethionine complex and 40% in the form of a bismethionine complex.[21] However, in our case the relative amounts of the two methionine adducts of [$PtCl_2$(1)] did not change during the course of the incubations in spite of a 10-fold excess of L-methionine. On the basis of LISIMS studies with the isolated methionine adducts of [$PtCl_2$(1)], it is concluded that these two compounds are diasteriomers. The *R,S* configuration of the 1,2-aryl-substituted diamine of [$PtCl_2$(1)] means that methionine can coordinate through the sulfur atom with platinum either cis or trans to the *R*-configured benzylamine carbon, and two diastereomeric monomethionine adducts would be expected to form in approximately equal amounts. NMR studies have shown that the reaction of one L-methionine with cisplatin at pH 7 leads to the loss of the two chloride ligands and a platinum complexes with either *S,O*- or *S,N*-chelated rings are formed; the compounds having a *S,N* ring being the thermodynamic products.[23] In the case of the monomethionine adducts of [$PtCl_2$(1)] both chloride ligands are lost as well, as shown by the LISIMS of the compounds (Figure 4). In addition, the 250-MHz ^{1}H NMRs of [Pt(1)Met]$^+$ show chemical shifts for the MeS protons consistent with the methionine forming a five-membered *S,N* chelate ring with platinum.

It has been reported that when L-methionine reacts with [$PtCl_2$(en)], ethylenediamine is released, although no quantitative data was given.[27] In contrast, we found *no* evidence for the release of diamine 1 when [$PtCl_2$(1)] was incubated for several days in the presence of a 10-fold excess of L-methionine. In fact, the monomethionine Pt adducts of [$PtCl_2$(1)] are stable enough to resist the addition of a second L-methionine at 37 °C. Evidently, the bulky aryl groups of [Pt(1)Met]$^+$ prevent the nucleo-

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philic attack of a second L-methionine on the central platinum atom. This is in contrast with recently reported NMR results for the reaction of L-methionine with cisplatin; a 2-fold excess of L-methionine led to the formation of various bismethionine–Pt adducts.[24]

The possibility that under cell culture conditions the neutral diamine ligand is released after prolonged incubation times should be considered when interpreting biological results obtained for cisplatin analogues containing pharmacologically active amine ligands. Judicially chosen experiments will be needed if one is to differentiate between pharmacological effects due to the parent platinum complex, the free amine ligand, and a combination of these two.

In the case of [$PtCl_2$(1)], the object of this study, the diamine 1 possessed only marginal cytotoxic activity on the MCF-7 cell line growing in culture.[22] Thus, the in vitro cytotoxic action of [$PtCl_2$(1)] appears to be due to the $PtCl_2$ group. It could be argued that the ligand disassociates from the platinum atom in cell culture medium, and that the free ligand is responsible for the estrogen receptor binding effects formally attributed to [$PtCl_2$(1)]. This appears unlikely in view that reversible binding of [$PtCl_2$(1)] to the estrogen receptor is measured 30 min after the addition of the platinum complex to the MCF-7 cells.[29] This time frame would be too short for appreciable amount of diamine ligand to have accumulate in the medium. In fact, if released diamine ligand was solely responsible for the observed estrogen receptor binding effects, incubation times of at least 7 h would be necessary before enough ligand was released into the medium to account for all of the estrogen receptor binding affinity of [$PtCl_2$(1)]. It could also be argued that the ability of [$PtCl_2$(1)] to induce the biosynthesis of the progesterone receptor in MCF-7 cells could also be a result of released diamine ligand. This does not appear to be the case since a 10-min pulse of MCF-7 cells with [$PtCl_2$(1)] is also sufficient to cause the induction of progesterone receptor biosynthesis. As argued above, this incubation time would be too short for enough free diamine to be released into medium to account for the estrogen-like activity of [$PtCl_2$(1)].[29]

Although it is tempting to speculate on the in vivo significance of these findings, the stability of the chelated diamine for [$PtCl_2$(1)] must first be determined under in vivo-like conditions before such discussions can be made.

In conclusion, it would appear that the release or nonrelease of a diamine ligand from the platinum(II) central atom is decided by a variety of factors such as the nature of the "nonleaving" diamine and the anionic "leaving" group, as well as the chemical environment in which the platinum complex is investigated. For these reasons, it is recommended that the stability of the Pt–N bonds should be determined for any new cisplatin analogues possessing pharmacologically active amines under relevant biological conditions.

Experimental Section

Materials. *meso*-1,2-Bis(2,6-dichloro-4-hydroxyphenyl)ethylenediamine (1) was synthesis as described.[11] $K_2[PtCl_4]$ was generously provided by the Degussa AG (Frankfurt A.M., FRG). D_2SO_4 and D_2O was purchased from the Fluka AG (Neu-Ulm, FRG). Reduced glutathione, BSA (catalog A-7906), bovine globulins (catalog G-8512), bovine transferrin (catalog T-5761), and L-methionine were obtained from Sigma (Deissenhofen, FRG). HPLC-grade methanol and HPLC-grade *N,N'*-dimethylformamide (DMF) were from Baker Chemicals (Deventer, Holland) and Aldrich (Steinheim A.A., FRG), respectively. Water was deionized by means of a Millipore Milli-Q Water System (Eschborn, FRG). Plastic cell culture plates (35 mm i.d. × 10 mm) were from Falcon (Nr. 3001) (Becton Dickinson, Heidelberg, FRG). IMEM-Richter's medium (without phenol red) was from Biochrom/Seromed (Berlin, FRG) and had the following composition: 116 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.1 mM $MgSO_4$, 1.0 mM $MgCl_2$, 1.0 mM NaH_2PO_4, 11.1 mM glucose, 0.6 mM L-arginine, 0.4 mM L-asparagine, 0.1 mM L-cystine, 1.8 mM L-glutamine, 0.2 mM L-histidine, 0.4 mM L-isoleucine, 1.0 mM L-leucine, 0.5 mM L-lysine, 0.1 mM L-methionine, 0.2 mM L-phenylalanine, 0.4 mM L-serine, 0.4 mM L-threonine, 0.05 L-tryptophan, 0.2 mM L-tyrosine, 0.4 mM L-valine, 0.1 mM pyruvate, 0.4 mM choline chloride, 0.2 mM *myo*-inositol, and trace amounts of vitamins. Fetal calf serum (FCS) was from Gibco (Paisley, Scotland, UK). Quickszint 212 scintillation cocktail was from the firm Zinser Analytik (Frankfurt A.M., FRG).

Equipment. HPLC analysis was done with a Beckman Altex 110A high-pressure mixing system (Fullerton, CA), equipped with a Rheodyne 7125 injector (Berkeley, CA) and having a 500-μL injection loop. Unless otherwise indicated, chromatography was done on a Macherey-Nagel (Düren, FRG) 30 × 4 mm Nucleosil 120-5C_{18} guard column preceded by a 250 × 4 mm Nucleosil 100-5C_{18} column, both of which were heated to 37 °C by a thermostatically controlled water jacket. The solvent flow rate was 0.7 mL/min. Detection was achieved by means of a Kontron Uvikon 720 LC variable-wavelength UV spectrophotometer (Eching b. München) set at 281 nm. HPLC fractions were collected in scintillation minivials by a BioRad 2110 Fraction Collector (Richmond, CA).

Proton NMR analysis was done with either a 60-MHz Varian EM 360L or a 250-MHz Brucher WM 250 spectrometer. Infrared spectroscopy (IR) was done with a Beckman AccuLab 7 spectrophotometer as Nujol mulls. LISIMS were recorded on a Finnigan MAT-95 instrument and samples were dissolved in a glycerol/H_2O matrix. Liquid scintillation counting was done through the use of a Beckman LS 5801 β-counter.

Synthesis of *meso*-1,2-Bis(2,6-dichloro-4-hydroxy[3,5-$^2H_2$]-phenyl)ethylenediamine ([^{2}H]1). In 1.0 mL of an 80% (v/v) solution of D_2SO_4/D_2O was dissolved 10 mg (26 μmol) of 1. The progress of the reaction was monitored by 60-MHz NMR. Since no exchange was observed at room temperature after 1.5 h, the solution was warmed to 68 °C. After 3.5 h the aromatic protons had fully exchanged with deuterium. The reaction was cooled and diluted with 2 mL of ice-cold water. The acidic solution was made neutral through the addition of a 20% (w/w) NaOH solution followed by the addition of 0.5 M phosphate buffer (pH 7.0). The precipitated product was collected by centrifugation and dried over P_2O_5 in vacuo to give a 45% yield of [^{2}H]1. The deuterated product displayed an identical HPLC retention time to that of undeuterated 1. The IR spectra of the undeuterated and deuterated compounds were comparable to one another except that the absorption bands at 3100 (weak, Ar–H stretching), 1060 (moderate, in-plane Ar–H bending), and 800 cm^{-1} (moderate, out-of-plane Ar–H bending) were absent in the deuterated product while a new absorption band at 2280 cm^{-1} (weak, Ar–D stretching) was present.

Synthesis of *meso*-1,2-Bis(2,6-dichloro-4-hydroxy[3,5-$^3H_2$]-phenyl)ethylenediamine ([^{3}H]1). The tritium labeling of 1 was done by Amersham Buchler (Cardiff, UK) in a similar manner described in the deuterium-labeling experiment. Briefly, 4.0 mg of 1 was dissolved in 100 μL of an 80% (v/v) H_2SO_4/HTO solution (1.5 Ci) at 70 °C for 4 h. The reaction was diluted with 10 mL of ice-cold water. The crude reaction mixture was made neutral with a 20% NaOH solution and then with a 1 M phosphate buffer solution (pH 7.0). After cooling in an ice bath for 1 h, the precipitated [^{3}H]1 was isolated by centrifugation at 4 °C. The supernate was pipetted off, and the precipitate was redissolved

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in 0.05 M HCl to a concentration of 0.53 mM and stored at −20 °C until later use.

Synthesis of [*meso*-1,2-Bis(2,6-dichloro-4-hydroxy[3,5-$^3H_2$]phenyl)ethylenediamine]dichloroplatinum(II) ([$PtCl_2$([^{3}H]1)]). From the HCl solution of [^{3}H]1, 1.5 mL (0.8 μmol) was neutralized with a 0.1 M phosphate buffer solution and cooled in an ice bath and the precipitate collected by centrifugation. The supernate was carefully decanted off and the precipitate dried over P_2O_5 in vacuo. The ligand was dissolved in 400 μL of DMF at 56 °C and 0.6 mg (1.5 μM) of $K_2[PtCl_4]$ in 100 μL of DMF was added. After 40 h at 56 °C, the reaction mixture was loaded onto a 10 × 250 mm LiChrosorb RP-18 column (Merck) and eluted at room temperature in the following manner: 2 min isocratic 80% (v/v) 100 mM NaCl solution/MeOH; 1 min linear gradient from 80% to 40% NaCl solution; 40% isocratic NaCl solution/MeOH. Solvent flow rate was 2.0 mL/min. Column eluant was collected when the UV absorption corresponding to [$PtCl_2$(1)] was detected. The chemical yield was 18% and the specific activity of the product was 286 Ci/mol. Radiochemical purity of the preparation was determined by HPLC and was found to be 94%, and no free [^{3}H]1 was detected. The radiolabeled compound was stored as a 0.1 mM solution in a 2:3 100 mM NaCl/MeOH solution at −20 °C until used. When stored in this way the radiochemical purity was stable for at least 6 months.

Incubations of Platinum Complex with Cell Culture Medium. Cell culture medium consisted of IMEM-Richter's Medium containing 10% (v/v) FCS and 2.2 g/L $NaHCO_3$. All incubations were done in plastic cell culture plates that were placed in a Narco 5300 incubator (Portland, OR) set at 37 °C, in a humidified atmosphere of 5% CO_2/air. Medium was preincubated in cell culture plates overnight at 37 °C. On the following day, 5 μL of a 2 mM solution of 1 in 0.1 N HCl (sterile filtered) and 10 μL of a 1 mM solution of [$PtCl_2$(1)] in DMF was added per milliliter of cell medium. In addition, a volume of the [$PtCl_2$([^{3}H]1) solution containing ~200 000 DPMs was added to each milliliter of medium. The medium was divided into two portions; to one portion a 0.1 M aqueous GSH solution was added, which gave a final GSH concentration of 1.0 mM, and to the other portion only sterile water was added. Immediately following these additions, a 1-mL probe was removed from each of the +GSH and −GSH incubations. Figure 3 shows the times at which additional samples were taken. No radioactivity was detected in a distilled fraction of cell culture medium, which had been incubated with [$PtCl_2$([^{3}H]1) for 24 h at 37 °C. This was an indication of the chemical stability of the radioisotope in the meta position of the aromatic ring.

HPLC Analysis. The 1.0-mL medium probes were diluted immediately with 0.9 mL of a freshly prepared 6 M urea solution and 0.1 mL of a 0.5 M H_3PO_4 solution and warmed at 37 °C for 5 min. Carbon dioxide gas was removed from the medium by bubbling helium through the solution for ca. 30 s. Ultrafiltration was done with an Amicon Micropartition System MPS-1 (filter membrane YMT 30) (Danvers, MA) in a refrigerated centrifuge run at 4 °C and 1000*g* for 20 min. Following the ultrafiltration of the sample, the ultrafiltrate (500 μL) was loaded onto the HPLC column and eluted as follows: 15 min isocratic at 25:75 MeOH/K_2PO_4 (20 mM, pH 3.3); 15 min linear gradient from 25% to 55% MeOH; 10 min isocratic at 55% MeOH. Fractions were collected every 30 s, mixed with 3.5 mL scintillation cocktail per fraction, and allowed to stand for several hours before the radioactivity was measured. The percent of radioactivity in each collected fraction was equal to the DPMs in that fraction divided by the DPMs in 500 μL of the sample before ultrafiltration and multiplying by 100. The fraction of platinum complex binding covalently to serum protein was estimated by dividing the amount of radioactivity in the ultrafiltrate by the amount of radioactivity in the cell medium before ultrafiltration.

Synthesis and Isolation of the Methionine–Pt Adducts [Pt(1)Met]$^+$. A solution of 50 μM [$PtCl_2$(1)] and 500 μM L-methionine in PBS was incubated 2 days at 37 °C. The crude reaction mixture (2.0 mL) was loaded onto a 30 × 4 mm Nucleosil 120-5C_{18} guard column preceded by a 250 × 4 mm Nucleosil 100-5C_{18} column and chromatographed with 85% mixture of HOAc (0.1%)/methanol. Eluant containing the leading edge of the first [Pt(1)Met]$^+$ peak and the trailing edge of the second [Pt(1)Met]$^+$ peak were collected separately. This separation procedure was repeated 20 times, with the respective fractions being pooled. The two combined fractions were then frozen and lyophilized. Subsequent HPLC of these two fractions showed each to be free of the other compound. For the NMR experiment, the lyophilized sample was dissolved in 0.1% CD_3COOD/D_2O, frozen, and lyophilized. The 250-MHz ^{1}H NMRs were done in D_2O and chemical shifts are reported relative to TPS.

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Registry No. 1, 111086-58-9; [^{2}H]1, 143707-46-4; [^{3}H]1, 143707-47-5; [$PtCl_2$(2)], 105856-23-3; [$PtCl_2$([^{3}H]1)], 143707-48-6; [Pt(1)Met]$^+$, 143707-49-7; $K_2[PtCl_4]$, 10025-99-7; Met, 63-68-3.