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HPLC and ^{31}P NMR CHARACTERIZATION OF THE REACTION BETWEEN ANTITUMOR PLATINUM AGENTS AND THE PHOSPHOROTHIOATE CHEMOPROTECTIVE AGENT *S*-2-(3-AMINOPROPYLAMINO)ETHYLPHOSPHOROTHIOIC ACID (WR-2721)

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Abstract—In prior studies, we examined the effects of the radioprotective and chemoprotective agent WR-2721 [*S*-2-(3-aminopropylamino)ethylphosphorothioic acid] on the *in vivo* biotransformation of the cisplatin [*cis*-diamminedichloroplatinum(II)] analog ormaplatin [(*d,l*)*trans*-1,2-diaminocyclohexanetetrachloroplatinum(IV), Pt(dach)Cl_4, (formerly called tetraplatin)]. Those data suggested that a direct interaction between WR-2721 and ormaplatin and/or the corresponding Pt(II) drug, Pt(dach)Cl_2, may be occurring *in vivo*. This would be in contrast to the generally accepted hypothesis that WR-2721 is a prodrug that must first be converted by alkaline phosphatase to a free thiol compound, WR-1065, before any appreciable reactivity would be evident. However, the major biotransformation product observed in the peritoneal fluid, plasma, and all tissues was Pt(dach)(WR-1065). We report here on further investigations into the *in vitro* reactivity of Pt(dach) compounds with WR-2721 and WR-1065. Separation of reaction products resulting from incubation of Pt(dach)(malonato) with either WR-2721 or WR-1065 under physiological conditions gave profiles that were indistinguishable by reverse phase HPLC and cation exchange HPLC at two different pHs. ^{31}P NMR characterization of the dephosphorylation of WR-2721 revealed essentially no loss of inorganic phosphate for up to 24 hr when incubated in unbuffered water at 30°. In contrast, when incubated with a 1:1 molar ratio of cisplatin under the same conditions, the WR-2721 signal was decreased markedly in the first 5 min, and had disappeared almost completely by 1 hr. The signal corresponding to inorganic phosphate increased in parallel to the decrease in the WR-2721 signal. No intermediate formation of a complex containing both platinum and phosphate could be detected at any time. These data suggest that the reaction between WR-2721 and platinum complexes results in rapid dephosphorylation of WR-2721, and, consequently, that the reaction products formed with either WR-2721 or WR-1065 and Pt(II) complexes are identical.

Key words: platinum(II); WR-2721; chemoprotection; biotransformation; HPLC; NMR

Antitumor platinum coordination complexes containing the dach† carrier ligand continue to receive considerable attention with respect to their ability to overcome inherent or acquired resistance of certain tumors to the prototypical platinum agent cisplatin [1]. There have also been reports demonstrating decreased toxicity to the kidney in comparison with cisplatin for one of these agents, ormaplatin (formerly called tetraplatin) [2–4]. Development of this agent had progressed to recent involvement in Phase I clinical trials, but has been halted at present due to the development of troubling neurotoxicity in those trials [5, 6]. As a result of the interest in this and other Pt(dach) compounds, this laboratory has examined the biotransformation of ormaplatin in cultured cells [7, 8], *in vitro* [9] and *in vivo* [10–13] by means of an HPLC system specifically designed for this purpose [14]. The underlying hypothesis throughout all of these studies has been that elucidation of the pathways of ormaplatin biotransformation may possibly explain the differential toxicity/cytotoxicity of Pt(dach) agents. Most recently, the effects of a phosphorothioate chemoprotective agent, WR-2721, on the disposition and biotransformation of ormaplatin were examined in the tumor-bearing Fischer 344 rat [15].

WR-2721 is a product of the Department of Defense research effort of the 1950s to find a radiation protective agent for use in the event of nuclear attack [16]. As a result, there exists a large body of research with respect to the mechanism whereby WR-2721, as a representative of the larger class of aminothiol compounds, is able to protect tissues from the effects of ionizing radiation [17, 18]. In the late 1970s, this research effort was expanded

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† Abbreviations: dach, (*d,l*)*trans*-1,2-diaminocyclohexane; WR-2721, *S*-2-(3-aminopropylamino)ethylphosphorothioic acid (Ethiofos, Amifostine, Ethyol); WR-1065, *S*-2-(3-aminopropylamino)ethanethiol, the thiol metabolite of WR-2721; cisplatin, *cis*-[$PtCl_2(NH_3)_2$], *cis*-diamminedichloroplatinum(II) (cDDP); ormaplatin (formerly called tetraplatin), (*d,l*)*trans*-1,2-diaminocyclohexanetetrachloroplatinum(IV); Pt(dach)Cl_2, (*d,l*)*trans*-1,2-diaminocyclohexanedichloroplatinum(II); RP, reverse phase; SCX, strong cation exchange; AAS, flameless atomic absorption spectroscopy; LSC, liquid scintillation counting; ODS, octadecyl silica. Platinum(II) complexes with various ligands are indicated as follows: Pt(dach)(ligand) or Pt(ligand)$_2$; in most cases the exact stoichiometry or charge of the complex is not known.

to include investigation of whether WR-2721 might selectively protect normal tissues from the toxicities of radiation and alkylating agent therapy of malignancy [19]. Results have been promising, and development has progressed to Phase III clinical trials [20, 21]. The most commonly described explanation for this purportedly selective protection of normal tissues is that the prodrug, WR-2721, requires conversion *in vivo* to the more active free thiol metabolite, WR-1065, by alkaline phosphatase, which is relatively lacking in tumor tissue [16, 17]. There have, however, been other mechanisms proposed [22, 23].

In consideration of the use of WR-2721 to protect against Pt-induced toxicities, predictions based upon the commonly proposed mechanism of action of WR-2721 would include: (a) minimal direct reaction between WR-2721 and Pt complexes; and (b) minimal formation of Pt(WR-1065) complex in tumor tissue. In results reported elsewhere [15], these predictions do not appear to have been born out in the case of the fibrosarcoma-bearing F344 rat receiving WR-2721 prior to ormaplatin. Thus, we have undertaken these *in vitro* studies to characterize more precisely the reactivity between WR-2721 and Pt coordination complexes.

MATERIALS AND METHODS

Drugs and chemicals

[4,5-$^3H_2$(n)]-(*d,l-trans*)-1,2-Diaminocyclohexane and [4,5-$^3H_2$(n)]-(*d,l-trans*)-1,2-diaminocyclohexanemalonatoplatinum(II) were prepared in the Radiosynthesis Laboratory, Division of Medicinal Chemistry and Natural Products, of the School of Pharmacy, University of North Carolina at Chapel Hill. The synthesis and purity of these compounds have been described elsewhere [24]. Reductive tritiation of the cyclohexene ring yields a radiolabel that is not chemically exchangeable. The specific activity of the final malonato product was 0.481 Ci/mmol by the $SnCl_2$ assay [24]. WR-2721 and its corresponding free thiol, WR-1065, were obtained from US Bioscience (West Conshohocken, PA). This WR-2721 is different from the clinical formulation available through the NCI, which contains a 1:1 mix by weight of WR-2721 and mannitol. Cisplatin was obtained from the Sigma Chemical Co. (St. Louis, MO). Guanosine-5′-*O*-(3-thiotriphosphate) (GTP-γ-S) was obtained as the tetralithium salt from Boehringer Mannheim (Indianapolis, IN). Deuterium oxide (D_2O) (99.9 atom% D) was obtained from the Aldrich Chemical Co. (Milwaukee, WI). HPLC grade reagents were obtained from commercial sources, and solutions were filtered and degassed prior to use. A Barnstead Nanopure water purification system with an organics filter provided the water used with solvents and for dissolution. All other general laboratory reagents were commercial reagent grade or better and were used without further purification.

HPLC

Standards of Pt(dach)(WR-2721) and Pt(dach)(WR-1065) were prepared essentially as previously described [14] by incubation of each compound (1.0 mM) with tritium-labeled Pt(dach)(malonato) (0.05 mM) at 37° in the dark overnight. Pt(dach)(malonato) was used as a precursor for the formation of these platinum standards because our previous studies have shown that it is much less prone to side-reactions than Pt(dach)Cl_2 or aquated platinum complexes [14]. Reactions of this compound with strong nucleophiles such as WR-2721 and WR-1065 occur primarily by direct displacement (S_N2) of the malonate ligand. Standards were characterized with a two-column HPLC system developed by Mauldin *et al.* [14], which has been used previously to resolve the various low molecular weight Pt(dach) biotransformation products of ormaplatin formed under a variety of conditions [8–14]. Briefly, an initial separation on a Partisil ODS-3 reverse phase column with heptane sulfonate as the ion pairing reagent provided resolution of species as neutral paired ions on the basis of polarity. Peak fractions of interest from this separation were pooled and then injected onto a Partisil 10 SCX column for further resolution of the positively charged species. Fractions from both separations were collected and analyzed for radioactivity by LSC with an LKB model 1215 scintillation counter. Radioactivity in each fraction was expressed as a percentage of the total radioactivity recovered from the column. Aliquots of fractions from RP HPLC were also stored at −20° and later analyzed for Pt by AAS. AAS was performed on a Perkin Elmer Zeeman 5100 atomic absorption spectrophotometer with an HGA-600 graphite furnace. Samples for AAS were analyzed without further preparation except for dilution where appropriate [13].

^{31}P *NMR*

Phosphorus NMR spectra were acquired using a Bruker AMX500 NMR and computer equipped with an Oxford magnet (11.7 Tesla). Spectra were obtained at 202.46 MHz, with a sweep width of 20 kHz, with 32K data points. A one-pulse sequence, consisting of a 90° pulse, repeated every 5.8 sec for WR-2721 alone (every 10.8 sec for WR-2721 + cisplatin), was time-averaged for 16 acquisitions (1.5 min). For processing of the NMR data, a 3-Hz line broadening was applied. Each spectrum was Fourier transformed, phased and integrated using the UXNMR software supplied with the instrument. Relative concentrations of WR-2721 and inorganic phosphate in each spectrum were determined by computer integration of peak areas. Arrangement of the spectral data into an appropriate graphical format was accomplished using standard UNIX-based software on a Silicon Graphics workstation. A solution of GTP-γ-S and potassium phosphate buffer (4.6 mM) in 10% D_2O/H_2O was used as the external ^{31}P-chemical shift standard. The control experiment followed the hydrolysis of WR-2721 alone (16.25 mM) in 10% D_2O/H_2O at 30°. Spectral acquisitions for this experiment were continued for approximately 24 hr. The second experiment utilized the same conditions as above, but cisplatin (also 16.25 mM) was added to the WR-2721 at time zero, and the subsequent hydrolysis of the WR-2721 was followed. Spectral acquisitions in this case were continued for 1.5 hr, or until it was evident that hydrolysis was essentially complete.

RESULTS

The preparation of standards for the present investigations was carried out by displacement of the malonate ligand from Pt(dach)(malonato) [14]. The HPLC separations for the standard prepared by incubation of Pt(dach)(malonato) with the free thiol, WR-1065, are shown in Fig. 1. Figure 1A shows the initial RP sepa-

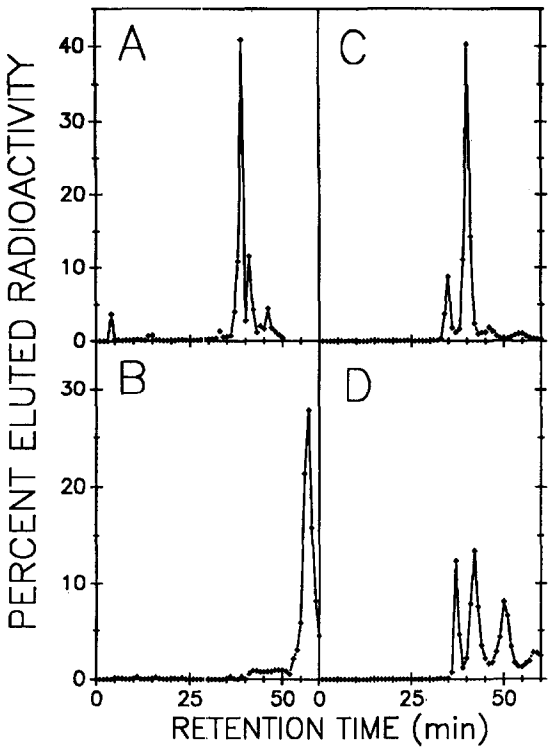

Fig. 1. Resolution of products of the reaction between Pt-(dach)(malonato) and WR-1065 by RP and SCX HPLC. Pt-(dach)(malonato) (0.05 mM) and WR-1065 (1.0 mM) were incubated at 37° overnight as described in Materials and Methods. The mixture was filtered (0.22 μm) following incubation and placed immediately on ice until injection onto the RP HPLC column that same day (A). HPLC separation was as described. Select fractions from RP HPLC were sealed and stored in RP buffer at −20° until subsequent resolution by SCX HPLC. Shown are: (B) RP fractions No. 41–42 following SCX HPLC, pH 4.0; (C) RP fractions No. 38–39 following SCX HPLC, pH 4.0; and (D) RP fractions No. 38–39 following SCX HPLC, pH 2.3. Note that the y-axes for panels A and C are on a scale different from those for panels B and D.

ration of the two-column HPLC procedure [14]. The miniscule peak with a retention time of 14–15 min corresponds to the Pt(dach)(malonato) starting material [14] and confirms that almost complete displacement of the malonate ligand has been obtained. The RP profile (Fig. 1A) suggests the formation of at least two major Pt-(dach)(WR-1065) complexes, one comprising about 60% of the eluted radioactivity with a retention time of 39 min, and one comprising about 20% of the eluted radioactivity with a retention time of 41 min. The minor peak with a retention time of 46 min is thought to be artifactual, since it did not routinely appear. The peak with a retention time of 39 min was resolved further by SCX HPLC at pH 4.0 (Fig. 1C) and pH 2.3 (Fig. 1D). The peak with a retention time of 35 min at pH 4.0 and 37 min at pH 2.3 was characteristic of the free dach carrier ligand (see below). The peak with a retention time of 40 min at pH 4.0 appeared to be resolved into two discrete peaks at pH 2.3. These elution profiles (i.e. 39 min on RP HPLC, 40 min on SCX HPLC at pH 4.0, and 42 and 52 min on SCX HPLC at pH 2.3) do not match those of any of the over 40 Pt(II)(dach) complexes previously characterized in this laboratory [8, 14]. Similarly, the RP peak with a retention time of 41 min was characterized further by SCX HPLC at pH 4.0 (Fig. 1B). This elution profile also appears to be unique. Therefore, we consider it likely that at least three distinct Pt-(dach)(WR-1065) complexes are formed in this reaction.

The reaction of Pt complexes with sulfur-containing nucleophiles often leads to *trans*-labilization of the carrier ligand [25]. As mentioned above, the peak with a retention time of 35 min (Fig. 1C) and 37 min (Fig. 1D) was characteristic of the free dach carrier ligand and could have arisen from *trans*-labilization of the dach carrier ligand, either during the original reaction or during storage between RP and SCX HPLC. However, available data suggest that most of the *trans*-labilization occurs between the RP and SCX HPLC steps. Figure 2 shows a parallel analysis by LSC (Fig. 2B) and AAS (Fig. 2C) of the RP HPLC fractions from a freshly prepared Pt(dach)(WR-1065) standard, along with the RP HPLC profile of the free dach carrier ligand for comparison (Fig. 2A). The free dach carrier ligand reproducibly eluted 1–2 fractions earlier than the main Pt-

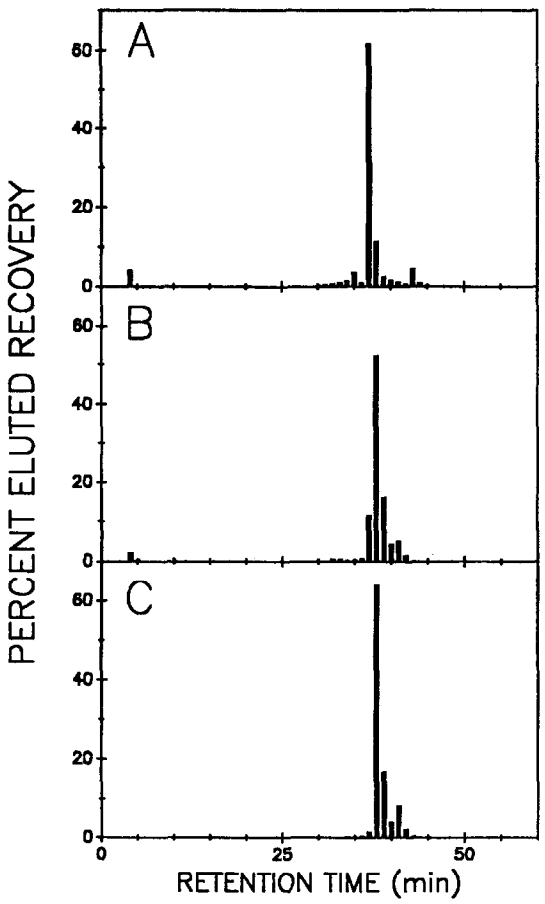

Fig. 2. Comparison of LSC and AAS analysis of RP HPLC fractions for dach carrier ligand and Pt(dach)(WR-1065) standard. Preparation of Pt(dach)(WR-1065) standard and RP HPLC separation were as described in Materials and Methods. Data are plotted as the percent of total eluted radioactivity (A and B) or Pt (C) in each fraction as a function of the retention time of the fraction. Shown are: (A) LSC analysis of dach carrier ligand profile; (B) LSC analysis of Pt(dach)(WR-1065) standard profile; and (C) AAS analysis of Pt(dach)(WR-1065) standard profile (same sample as for B).

(dach)(WR-1065) peak. When one compares the RP profiles of the Pt(dach)(WR-1065) complexes by LSC and AAS, it is evident that there is a leading shoulder in the elution position of the free dach carrier ligand in the profile obtained by LSC, but not in the profile obtained by AAS. This leading shoulder corresponds to about 10% of the eluted radioactivity. Thus, these data suggest that approximately 10% *trans*-labilization occurs during the initial reaction. Significant additional *trans*-labilization appears to occur during subsequent storage of the Pt(dach)(WR-1065) complexes in RP buffer at −20°. The SCX HPLC shown in Fig. 1C was performed 30 days after the RP HPLC, and the peak corresponding to the free dach carrier ligand only accounted for 10% of the eluted radioactivity. However, in another experiment this peak was observed to increase in size with longer storage of the RP sample, becoming the major peak following 90 days of storage (data not shown). Therefore, it

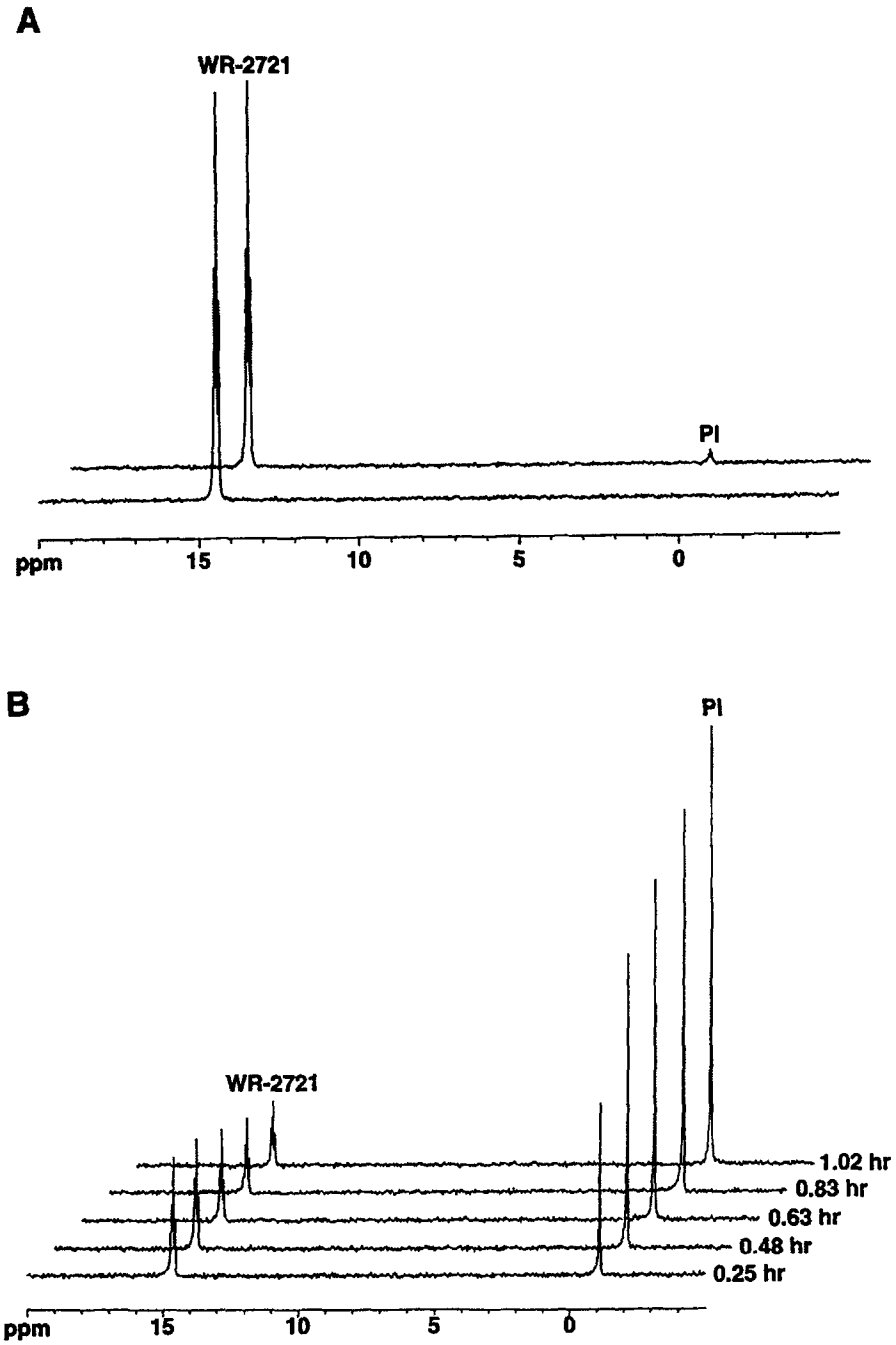

Fig. 3. ^{31}P NMR analysis of WR-2721 hydrolysis. Conditions were as described in Materials and Methods. Shown are: (A) WR-2721 alone (16.25 mM) in 10% D_2O/H_2O; and (B) WR-2721 + cisplatin (1:1 molar ratio). The triplet signal at 14.5 ppm designated WR-2721 corresponds to the intact parent compound; the singlet signal at −1.2 ppm designated P_i corresponds to liberated inorganic phosphate.

appears that continued *trans*labilization of the carrier ligand from the Pt(dach)(WR-1065) complexes can occur during storage in RP buffer at −20°. Unfortunately, it was not possible to confirm this by parallel AAS analysis of the SCX HPLC fractions due to the high salt concentrations of these fractions.

We next examined the reaction of WR-2721 with Pt(dach)(malonato). Since the phosphate group is negatively charged at the pH values employed in these separations, and since phosphate has an ionizable group with a pKa of approximately 2.1, one might expect that the HPLC profiles of the Pt(dach)(WR-2721) complexes would be distinct from those obtained from the Pt(dach)(WR-1065) complexes. However, the elution profiles were indistinguishable under all conditions of analysis (data not shown). These HPLC separations, obviously, do not allow one to delineate pathways of reaction, but it was clearly apparent that, even in an *in vitro* system without alkaline phosphatase activity, the Pt-containing reaction end products were the same whether the starting material was WR-2721 or WR-1065.

If the end products of the two different incubation mixtures described above were the same by HPLC detection, then WR-2721 must have been dephosphorylated during the reaction. WR-2721 is known to be subject to acid hydrolysis [26]. Therefore, ^{31}P NMR studies were employed in order to more precisely determine whether the reaction of WR-2721 with Pt agents was occurring after the WR-2721 was first hydrolyzed to the free thiol, or if the Pt was attacking the sulfur of the intact phosphorothioate and labilizing the phosphate in the process. Figure 3 shows the ^{31}P NMR spectra for the hydrolysis of WR-2721 (16.25 mM) in H_2O alone (Fig. 3A) and in the presence of cisplatin (1:1 molar ratio) (Fig. 3B). The quantitative data obtained from these spectra are shown in Fig. 4. The triplet signal (≈14.5 ppm) labeled WR-2721 corresponds to the intact parent compound, and the singlet signal (≈−1.2 ppm) designated as P_i corresponds to inorganic phosphate liberated by hydrolysis. As shown in Fig. 3A, there was essentially no loss of P_i from WR-2721 for upwards of 24 hr when it was incubated alone in unbuffered 10% D_2O/H_2O at 30°. Conversely, when an equal concentration of cisplatin was added to the incubation mixture (Fig. 3B), the concentration of WR-2721 had decreased by over 40% by the time the first NMR spectra could be acquired at 15 min, and hydrolysis was essentially complete by 1.5 hr. The signal corresponding to P_i increased in parallel to the disappearance of the signal for WR-2721. The formation of a $Pt(NH_3)_2$(WR-2721) complex with a Pt-S-P_i linkage would have been expected to result in a 15–20 ppm upfield shift of the triplet WR-2721 ^{31}P NMR signal [27]. However, no ^{31}P NMR signal other than those for WR-2721 and P_i was detected in the range of −50 ppm to +50 ppm at any time during the reaction, suggesting that intermediate accumulation of the $Pt(NH_3)_2$(WR-2721) complex is undetectable under these reaction conditions. Cisplatin was used in this experiment due to its greater solubility than Pt(dach)Cl_2 and Pt(dach)(malonato). However, we expect the carrier ligand (*cis*-diammine vs 1,2-diaminocyclohexane) to have little influence on the mechanism of the reaction of platinum(II) complexes with WR-2721. Thus, taken together with the HPLC findings described above, these data suggest that direct reaction readily occurs between intact WR-2721 and Pt coordination complexes, and that this reaction leads to a rapid loss of phosphate from the WR-2721.

DISCUSSION

The apparent selectivity of WR-2721 for normal versus tumor tissue generally is hypothesized to be due to selective removal of phosphate by alkaline phosphatase in the vicinity of normal tissue, leading to selective accumulation of the dephosphorylated WR-1065 in normal tissue [16, 17]. It has generally been assumed that extracellular WR-2721 is either too inactive or present at too low a concentration to react with platinum complexes, since such reactions would lead to non-selective protection of both normal and tumor tissue. Treskes *et al.* [28] have shown recently that the second order rate constant for the reaction of WR-2721 with platinum complexes was nearly 50% of that for WR-1065. These data clearly demonstrated that WR-2721 could display significant reactivity towards platinum complexes if *in vivo* concentrations are sufficiently high. In results reported elsewhere [15] on investigations of the effects of WR-2721 on the biotransformation of ormaplatin in the Fischer 344 rat, we obtained results which suggest that direct reaction is occurring when WR-2721 and ormaplatin are injected 30 min apart into the peritoneal cavities of rodents. However, the major biotransformation products detected in the peritoneal cavity, plasma, and all tissues were indistinguishable from the Pt(dach)(WR-1065) complexes by HPLC analysis. Thus, we undertook the present studies to further clarify the pathways and products of reaction between these compounds.

We have shown that the end products of the reactions between WR-2721 or WR-1065 and Pt(dach)(malonato) are indistinguishable by means of HPLC and, further,

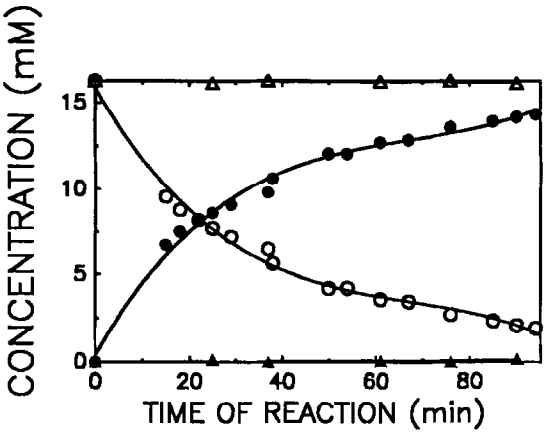

Fig. 4. Time course for reactions of WR-2721. Data obtained from ^{31}P NMR spectra, such as those shown in Fig. 3, were integrated as described and converted to molar concentrations based on the initial concentration of WR-2721. Since recovery times were used which allowed for full relaxation of the phosphorus signals, the area under each phosphorus signal was proportional to its concentration: (△) WR-2721 in 10% D_2O/H_2O; (○) WR-2721 in the presence of cisplatin; (●) P_i released from WR-2721 in the presence of cisplatin; and (▲) P_i released from WR-2721 in the presence of 10% D_2O/H_2O. The reaction between WR-2721 and cisplatin does not follow second order kinetics because cisplatin is being converted to more reactive aquated forms as the reaction progresses.

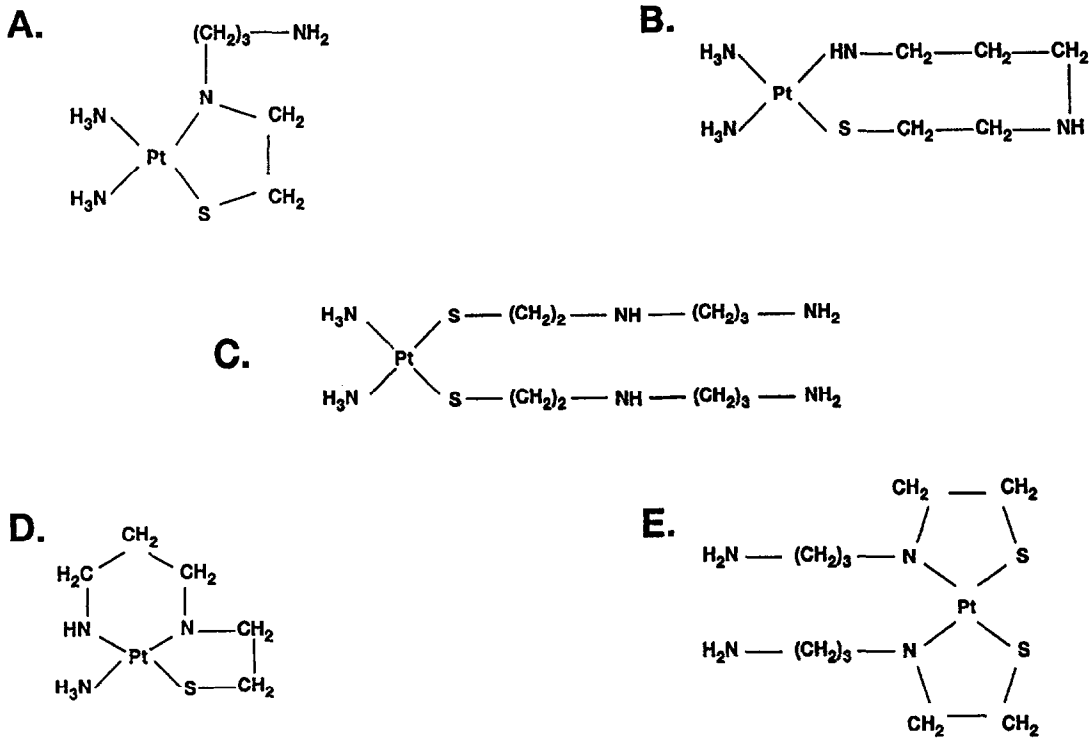

Fig. 5. Proposed structures for products of reaction between cisplatin and WR-2721 or WR-1065. Structures **A**, **B**, and **C** are the products most likely to form initially. Structures **D** and **E** are likely products of rearrangement following *trans*-labilization of the carrier ligands.

have shown by ^{31}P NMR that the disappearance of WR-2721 correlates precisely with the appearance of P_i in the reaction between cisplatin and WR-2721. These data clearly show that the reaction of platinum complexes with WR-2721 results in a very rapid loss of the phosphate group, and that the end products of the reaction are, therefore, identical to those that form when WR-1065 reacts with the same platinum complexes. This information may be important for more detailed studies of the chemistry and biology of Pt complex/WR-2721 interactions. For example, it resolves the apparent contradiction between the direct reaction of ormaplatin and WR-2721 in the peritoneal cavity and the subsequent accumulation of Pt(dach)(WR-1065) complexes in the *in vivo* experiments mentioned above. In contrast, clinical protocols and some preclinical studies have utilized intravenous administration of WR-2721 15 min prior to the administration of cisplatin. Since most of the parent drug is converted to the free thiol compound, WR-1065, in both the plasma [16] and tissue [29] within the first 15 min of administration, direct reaction of WR-2721 with platinum complexes would be unlikely under these conditions.

Our data also show that at least three major Pt(dach)(WR-1065) complexes form. Because both the sulfur and the nitrogens of WR-1065 are capable of displacing the malonate ligand and complexing with Pt, we propose structures such as **A**, **B**, and **C** in Fig. 5 for these complexes. However, it is important to note that these structures are based on theoretical considerations only and have not been confirmed by NMR or mass spectrometry. In data reported elsewhere [15], we have found that at least two of the same complexes appear to form in about the same relative abundance *in vivo*. Finally, our data suggest that significant *trans*-labilization of the carrier ligand can occur upon storage of the Pt(dach)(WR-1065) complexes. We propose structures such as **D** and **E** (Fig. 5) as likely products of rearrangement following *trans*-labilization. We have not examined the kinetics of this *trans*-labilization in detail, and it appears to vary in severity from experiment to experiment. However, significant *trans*-labilization during storage of the Pt(dach)(WR-1065) complexes is in direct contrast to our previous experience with dach-Pt amino acid complexes, such as Pt(dach)(methionine), which are stable for at least 3–6 months under identical storage conditions. This indicates the necessity for rapid HPLC analysis of plasma and/or tissue samples in future studies on the effects of WR-2721 and similar compounds on biotransformation of platinum complexes.

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