Biochemical Pharmacology, Vol. 37, No. 17, pp. 3321–3333, 1988.
Printed in Great Britain.

0006-2952/88 $3.00 + 0.00

DISPLACEMENT OF THE BIDENTATE MALONATE LIGAND FROM (*d,l-trans*-1,2-DIAMINOCYCLOHEXANE)MALONATOPLATINUM(II) BY PHYSIOLOGICALLY IMPORTANT COMPOUNDS *IN VITRO*

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(*Received* 17 *June* 1987; *accepted* 26 *February* 1988)

Abstract—Previous studies of platinum(II) compounds with bidentate leaving ligands have emphasized the contrast between the stability of the bidentate leaving ligand *in vitro* ($T_{\frac{1}{2}} > 11$ days in water) and the apparent reactivity of these bidentate platinum compounds *in vivo*. However, none of these studies actually measured the stability of these compounds in tissue culture medium (or in any other reaction mixture resembling *in vivo* conditions). The experiments described in this paper were designed to measure the stability and fate of (*d,l-trans*-1,2-diaminocyclohexane)malonatoplatinum(II) [Pt(mal)(*trans*-dach)] in RPMI-1640 tissue culture medium. The $T_{\frac{1}{2}}$ for displacement of the malonate ligand in this medium was 9.5 hr at 37°. Of the inorganic anions present in the medium, chloride accounted for the greatest displacement of the malonate ligand. However, at the concentrations with which it is found in tissue culture medium and in blood, bicarbonate was nearly as effective as chloride at displacing the malonate ligand. This observation is of particular significance because the bicarbonato–platinum complex is unstable and the bicarbonate displacement reaction appears to represent a major non-enzymatic pathway for the formation of the biologically active aquated platinum complexes. At the concentrations with which they occur inside the cell, phosphates may play a similar role. Of the amino acids present in the medium, glutathione and the sulfur-containing amino acids were 50- to 400-fold more effective at displacing the malonate ligand than the other amino acids in RPMI-1640 medium. In the case of methionine, the reaction with Pt(mal)(*trans*-dach) was shown to be a direct displacement (S_N2) reaction at physiological methionine concentrations. When Pt(mal)(*trans*-dach) was incubated at 37° for 24 hr in RPMI-1640 medium, the major transformation products formed were (*d,l-trans*-1,2-diaminocyclohexane)methionineplatinum(II) (38%), other amino acid-platinum complexes (19%), and (*d,l,-trans*-1,2-diaminocyclohexane)dichloroplatinum(II) (14%). Eleven percent of the Pt(mal)(*trans*-dach) remained intact. Mass spectrometry and ^{1}H-NMR indicated that the (*d,l-trans*-1,2-diamino-cyclohexane)methionineplatinum(II) complexes that formed in RPMI-1640 medium consisted of approximately 60% of the bidentate mono-methionine complex coordinated to platinum at the sulfur and α-amino positions and 40% of the bis-methionine complex, presumably coordinated at the sulfurs. The (*d,l-trans*-1,2-diaminocyclohexane)methionine-platinum(II) complexes appeared to be essentially inactive, as judged by an extremely low rate of uptake and little or no binding to the cell membrane of L1210 cells.

Platinum(II) complexes containing bidentate leaving ligands such as malonate may offer therapeutic advantage to platinum complexes with monodentate leaving ligands because of low renal toxicity and improved therapeutic indices [1–3]. However, relatively little is known about the chemical reactivity of these ligands. It is clear that bidentate ligands are much more stable than chloride as leaving ligands. While the half-life for displacement of the chloride ligand in water is approximately 2 hr at 37° [4], the half-life for displacement of the malonate ligand is approximately 11 days [5]. The oxalate, 2-ethyl-malonate, and 1,1-cyclobutane dicarboxylate ligands have similar stabilities [6, 7]. The half-life for the displacement of bidentate ligands is decreased to around 1 day in 0.1 M NaCl [5, 7], but that is still very long from a physiological point of view. As might be expected from their stability in aqueous solution, platinum compounds containing bidentate ligands also react slowly with DNA *in vitro* [8, 9]. In fact, the displacement of the bidentate ligand to form the monoaquated species appears to be rate-limiting for the reaction of platinum with DNA [9–11].

However, all available evidence points to a much more rapid displacement of bidentate ligands *in vivo*. Platinum compounds containing bidentate ligands

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are only slightly less effective than *cis*-diamminedichloroplatinum(II) [cisplatin]against tumor cells in culture [1, 3, 9], and are often much more effective in the *in vivo* tumor screens due to lower toxicity [3, 9]. In addition, Gale *et al.* [12] have shown that a platinum drug containing the 4-carboxyphthalate ligand inhibits DNA synthesis in Ehrlich ascites tumor cells more than 60% at 2 hr and more than 90% by 4 hr. Thus, the relatively rapid inhibition of DNA synthesis and the high cytotoxicity of platinum compounds containing bidentate ligands *in vivo* contrast dramatically with their unreactive nature *in vitro*. This contrast has lead Cleare and Hoeschele [6] to suggest that bidentate ligands are rapidly removed enzymatically in the cell, although no direct proof for this hypothesis exists.

Part of the discrepancy between the *in vivo* and *in vitro* data may result from the fact that *in vitro* data are available only for the displacement of bidentate ligands by water or NaCl, whereas there are a number of other potential ligands *in vivo* that could cause more rapid displacement of the bidentate ligands. Thus, in this study we have measured the displacement of the bidentate malonate ligand by compounds of physiological interest. We chose (1,2-diaminocyclohexane)malonatoplatinum(II) [Pt(mal)-(dach)] as the parent compound because the 1,2-iaminocyclohexane (dach) ligand can be labeled to a very high specific activity by reductive tritiation and we have previously developed a two-column HPLC* system capable of separating platinum complexes with the diaminocyclohexane carrier ligand [13]. Thus, it is possible to quantitate even minor transformation products of Pt(mal)(dach).

The 1,2-diaminocyclohexane ligand can exist in three different conformations which have slightly different therapeutic effectiveness in most cell lines [14, 15]. We have chosen to use the *d,l-trans*-1,2-diaminocyclohexane (*trans*-dach) isomers because they are easier to prepare in radiolabeled form and they are usually more effective than the *cis* isomer in the L1210 cell line [14]. The studies described in this paper were designed to quantitate the rate and extent of the displacement of the malonate ligand in RPMI-1640 tissue culture medium and to identify the major transformation products formed. However, the information obtained from these studies is also applicable to our understanding of likely biotransformation products of Pt(mal)(*trans*-dach) and similar compounds in a number of *in vivo* situations.

* Abbreviations: HPLC, high performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; cisplatin, *cis*-diamminedichloroplatinum-(II); dach, 1,2-diaminocyclohexane; *trans*-dach, *d*,l-*trans*-1,2-diaminocyclohexane; *cis*-dach, *cis*-1,2-diaminocyclohexane; Pt(mal)(*trans*-dach), (*d,l-trans*-1,2-diaminocyclohexane)malonatoplatinum(II); $PtCl_2$(*trans*-dach), (*d,l-trans* - 1 , 2-diaminocyclohexane) dichloroplatinum (II); $[Pt(H_2O)(Cl)$(*trans*-dach)$]^+$, .(*d,l-trans*-1,2-diaminocyclohexane) aquachloro-platinum(II); $[Pt(H_2O)_2$(*trans*-dach)$]^{2+}$, (*d*,-*trans*-1,2-diaminocyclohexane) diaqua-platinum(II). Platinum(II) complexes with various amino acids are indicated as Pt(amino acid)(*trans*-dach); in most cases the exact stoichiometry or charge of the complex is not known.

MATERIALS AND METHODS

Chemicals. HPLC grade methanol, acetonitrile, 1-heptanesulfonate, and KH_2PO_4 were obtained from Fisher Scientific (Raleigh, NC). HPLC grade water was obtained with a Barnstead Nanopure water purification system with an organics filter. Reagent grade KNO_3 was also obtained from Fisher Scientific. All HPLC solutions were filtered through a 0.22 micron hydrophilic Durapore filter (GWP, Millipore Corp., Bedford, MA) before use. Amino acids and glutathione were obtained from the Sigma Chemical Co. (St. Louis, MO). $[^{14}C]HCO_3^-$ and $[^{32}P]H_3PO_4$ were obtained from ICN Radiochemicals (Irvine, CA).

Medium. RPMI-1640 medium (GIBCO Laboratories, Grand Island, NY) has the following composition: 103 mM NaCl, 5.4 mM KCl, 23.8 mM $NaHCO_3$, 5.4 mM Na_2HPO_4, 0.8 mM $Ca(NO_3)_2$, 0.4 mM $MgSO_4$, 11 mM glucose, 3.2 μM glutathione, 1.1 mM arginine, 0.38 mM asparagine, 0.15 mM aspartic acid, 0.21 mM cysteine, 2.0 mM glutamine, 0.14 mM glutamic acid, 0.13 mM glycine, 0.10 mM histidine, 0.38 mM isoleucine, 0.38 mM leucine, 0.27 mM lysine, 0.1 mM methionine, 0.1 mM phenylalanine, 0.28 mM serine, 0.19 mM threonine, 0.02 mM tryptophan, 0.11 mM tyrosine, and 0.18 mM valine.

Synthesis and characterization of (d,l-trans-1,2-*diaminocyclohexane)methionineplatinum(II).* (*d,l-trans* - 1 , 2 - Diaminocyclohexane) methionineplati - num(II) [Pt(methionine)(*trans*-dach)] was prepared by stirring 9.0 mmol methionine and 9.0 mmol (*d,l-trans*-1,2-diaminocyclohexane)dinitratoplatinum(II) [16] in 50 ml water under reflux conditions for 30 min, and then at room temperature overnight. The water was evaporated *in vacuo*. The residual gum was dissolved in hot absolute ethanol, filtered, and crystallized at −10°. The precipitated solid was filtered, washed with ethanol, and air dried to give 270 mg of colorless solid. This solid contained an unidentified contaminant and required further purification by HPLC. One hundred milligrams of the solid was dissolved in water at 6 mg/ml. Aliquots (2.5 ml) were injected onto a 9.4 × 250 mm Whatman Partasil 10 ODS-3 semipreparative HPLC column and eluted isocratically with 0.1 M ammonium acetate, pH 5.5, at a flow rate of 4 ml/min. The major contaminant eluted in the void volume, and the Pt(methionine)(*trans*-dach) complex eluted as a single peak between 9 and 12 min. Combined fractions from this column were evaporated *in vacuo* at 50° and redissolved in water. Residual ammonium acetate was removed by two passes through a low pressure C-8 reverse phase column (Liquoprep RP-8, size A; E. Merck Darmstadt; available from Curtin-Matheson) at a flow rate of 1 ml/min. The column was washed with one void volume (14 ml) of water and the Pt(methionine)(*trans*-dach) complex eluted with one void volume of methanol. This procedure yielded 49 mg of a colorless solid; m.p., 242–245 dec. Elemental analysis was carried out by Galbraith Laboratories (Knoxville, TN). Calculated for $C_{11}H_{24}N_3O_2SPt$; C, 28.83; H, 5.25; N, 9.17; Pt, 42.57; S, 7.00. Found: C, 27.9; H, 5.02; N, 8.41; Pt, 42.07; S, 6.67.

For positive ion fast atom bombardment mass spectrometry (FAB-MS) the sample was dissolved in water at 1 μg/μl and 1 μg was applied to the probe tip. The spectrum was obtained on a VG ZAB E mass spectrometer (VG Analytical, Manchester, UK) using a xenon atom beam (ion current = 1 mA, 8 kV). The mass spectrometer scanned from m/z 180 to 1000 in 20 sec. The positive FAB-MS spectrum showed a strong ion cluster centered at 458 as expected for the $[M + H]^+$ ion. Weaker clusters were observed at 475 $[M + H_2O]^+$, 624 [M + methionine + H_2O + H]$^+$, and at several smaller fragments derived from $[M + H]^+$. The $[M + H_2O]^+$ and [M + methionine + H_2O + H]$^+$ ions most likely resulted from addition or substitution reactions occurring during the ionization process [17]. Elemental analysis precluded the presence of any significant levels of the bis-methionine complex or aquated species in the crystalline solid. In addition, the fact that the Pt(methionine)(*trans*-dach) complex was virtually unreactive to DNA (see Table 3) makes it highly unlikely that significant levels of the aquated species exist in solution.

For ^{1}H-NMR, 20 mg of the sample was dissolved in 0.5 ml D_2O. The analysis was carried out on a Bruker 250 MHz instrument. Chemical shifts were recorded in ppm (δ) relative to H_2O. Downfield shifts of 0.4 ppm were found for S-CH_3 and S-CH_2, indicating the presence of the Pt–S bond. Since not enough material was available to carry out the ^{1}H-NMR at more than one pH, the NMR data did not allow us to distinguish between coordination at the α-amino or α-carboxyl group. However, our previous studies [13] have shown that both the mono- and bis-methionine complexes are more positively charged at pH 2.3 than at pH 4, indicating that the α-carboxyl group is free rather than coordinated to platinum. Thus, the synthesized Pt(methionine)(*trans*-dach) complex most likely contains a single bidentate methionine ligand complexed to platinum at the sulfur and α-amino positions, as previously proposed [13]. There does not appear to be any significant *trans*-labilization of the diaminocyclohexane carrier ligand.

Synthesis of ^{3}H-*labeled* (d,l-trans-1,2-*diaminocyclohexane)malonatoplatinum(II)*. [4,5-^{3}H]-*d-l-trans*-1,2-Diaminocyclohexane was obtained from the Radiosynthesis Laboratory of the Research Triangle Institute (Research Triangle Park, NC). The compound was prepared by catalytic tritiation of *trans*-Δ^4-1,2-diaminocyclohexene. The preparation of *trans*-Δ-1,2,diaminocyclohexene and ^{3}H-labeled (*d,l-trans*-1,2-diaminocyclohexane)malonatoplatinum(II) is described elsewhere [16].

Stock solutions (200 μg/ml) of Pt(mal)(*trans*-dach) were prepared by dissolving the drug in water for 30 min at 60°. Pt(methionine)(*trans*-dach) was readily soluble in water at room temperature at concentrations of up to 10 mg/ml. When stored in the dark at 4°, either compound was stable for up to a month. At −80° they were stable for 4–6 months.

High performance liquid chromatography. The instrument configuration consisted of two LKB model 2150 dual piston pumps, an LKB model 2152 controller, and a Rheodyne model 7125 sample injector with either a 100 μl, 500 μl, or 5000 μl loop. Reverse phase HPLC was carried out as described previously [13]. In those cases where complete analysis of the sample was desired, the sample was concentrated approximately 10-fold by centrifugation under vacuum (SpeedVac, Savant Corp.) and injected into a cation exchange column. Cation exchange HPLC was carried out as described previously [13]. The elution profiles were analyzed and graphed using the SPECTRODATA software package (Spectrofuge Corp., Carrboro, NC). This two column separation method allows separation and identification of most possible transformation products of Pt(mal)(*trans*-dach).

Purification of the Pt(methionine)(trans-*dach) complex from medium*. RPMI-1640 medium (20 ml) without added fetal calf serum was incubated for 24 hr at 37° with 20 μg/ml (0.049 mM) ^{3}H-labeled Pt(mal)(*trans*-dach), 1.7 mCi/mmol. Following ultrafiltration, 2.5-ml aliquots of the reaction mixture were injected onto a 9.4 × 250 mm Whatman Partasil 10 ODS-3 semipreparative HPLC column and eluted as described previously for the ODS-3 column [13] except that a flow rate of 4 ml/min was used and 4-ml fractions were collected. Peak g (see Fig. 4) was collected and concentrated 50-fold by lyophilization. Aliquots (500 μl) of this sample were then purified by cation exchange HPLC as described previously. The Pt(methionine)(*trans*-dach) fraction (peak g_6, Fig. 4) was desalted in two steps. The sample was first repurified by semipreparative reverse phase HPLC on the Whatman ODS-3 column as described above. Residual 1-heptanesulfonate was removed by passing the sample through a low pressure C-8 reverse phase column as described earlier for purification of the synthetic Pt(methionine)(*trans*-dach) complex. The methanol was removed by flash evaporation at 50°. Based on recovery of radioactivity, the final yield of the Pt(methionine)(*trans*-dach) complex was 17 μg which represented an overall recovery of approximately 23%.

DNA binding studies. The reactivity of the isolated platinum transformation products towards DNA was carried out essentially as described by Johnson *et al.* [11] except that the incorporation of the [^{3}H-dach]platinum into the DNA was measured as follows: Carrier calf thymus DNA (100 μg) was added to each sample and the DNA was precipitated with cold 10% TCA (trichloroacetic acid). The samples were then dissolved in 0.1 N NaOH, reprecipitated with cold 10% TCA, and washed with cold 5% TCA. The resulting pellets were dissolved in 0.5 ml NCS Tissue Solubilizer (Amersham) at 37° for 1–2 hr. Finally, 5 ml of Neutralizer Scintillation Fluid (RPI) was added and the samples were counted. By avoiding filtration and adding the NaOH step, this procedure eliminates the high background reported by Johnson *et al.* [11]. The complete procedure is described elsewhere.*

RESULTS

Since many of the mechanistic studies of bidentate platinum compounds such as Pt(mal)(*trans*-dach) are

* S. K. Mauldin, G. Gibbons, S. D. Wyrick and S. G. Chaney, *Cancer Res.*, in press.

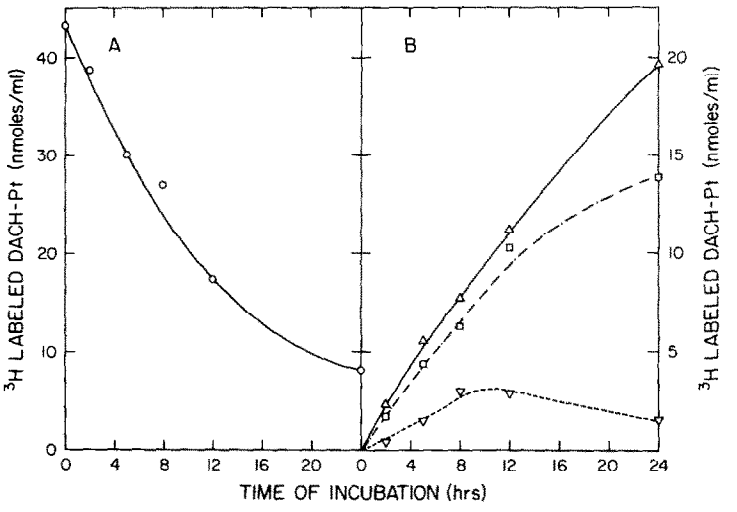

Fig. 1. Reaction of Pt(mal)(*trans*-dach) in RPMI-1640 medium. [^{3}H-*trans*-dach]Pt(mal)(*trans*-dach), (20 μg/ml, 141 mCi/mmol), was incubated at 37° with RPMI-1640 medium + 15% fetal calf serum in an atmosphere of 5% CO_2. At the times indicated, an aliquot was removed and filtered through an Amicon YMT membrane filter (30,000 mol. wt cut-off). Pt(mal)(*trans*-dach) and its transformation products were determined by reverse phase HPLC as described in Materials and Methods. (A) Pt(mal)(*trans*-dach) remaining; and (B) accumulation of transformation products: (▽—▽) $PtCl_2$(*trans*-dach); (□-·-□) Pt-protein and (△—△) other transformation products (primarily Pt–amino acid complexes).

carried out in tissue culture, it was obviously important to know the stability of these compounds in tissue culture medium. For that reason, we measured the displacement of the malonate ligand from Pt(mal)(*trans*-dach) in RPMI-1640 medium. Platinum binding to fetal calf serum was measured using an Amicon YMT membrane filter (30,000 mol. wt. cut-off). Pt(mal)(*trans*-dach), $PtCl_2$(*trans*-dach), and the other low molecular weight platinum complexes were quantitated by reverse phase HPLC as described previously [13]. The results are summarized in Fig. 1. The half-life for displacement of the malonate ligand (Fig. 1A) was 9.5 hr in RPMI-1640 medium at 37°. Control experiments showed that the half-life for the displacement reaction in 0.15 M NaCl was 18 hr, which is comparable to literature values [5, 7]. The accumulation of various transformation products in the medium is shown in Fig. 1B. Platinum bound to serum proteins represented a relatively constant 30–35% of the transformation products formed throughout the incubation period. $PtCl_2$(*trans*-dach) accumulated gradually over the first 8 hr and then decreased as the incubation continued. A number of other low molecular weight transformation products were also evident. They had retention times between 25 and 40 min (see Fig. 4A for reverse phase elution profile), accumulated more rapidly than $PtCl_2$(*trans*-dach), and were persistent in the medium.

Obviously, some components of the RPMI-1640 medium were at least as effective as serum proteins and chloride at displacing the malonate ligand from platinum(II). To determine which of these components were most effective at displacing malonate, they were tested individually at the concentrations with which they occur in the RPMI-1640 medium. The displacement of malonate by each of these components of the medium in 24 hr at 37° is summarized in Table 1. Chloride is the major anion in the RPMI-1640 medium, being present at a concentration of 108 mM. At this concentration, chloride alone displaced 60% of the malonate ligand in a 24-hr period. When the other components were tested individually, it was clear that phosphate, nitrate, sulfate, glucose, and most amino acids had little effect on the displacement of the malonate ligand at the concentrations present in the medium, whereas bicarbonate and the sulfur-containing amino acids were very effective at displacing the malonate ligand. The displacement of the malonate ligand by chloride, bicarbonate, and the sulfur containing amino acids appeared to be sufficiently rapid to account for the observed rate of displacement of the malonate ligand in the complete medium (Fig. 1).

Since the displacement of the malonate ligand by bicarbonate and the sulfur-containing amino acids was not dependent on the presence of NaCl in the reaction mixture and since the rate of dissociation of the malonate ligand in water is very slow [5], it seemed likely that these reactions occurred by a direct displacement (S_N2) mechanism. This hypothesis was tested rigorously in the case of displacement by methionine. In previous studies of platinum(II) compounds with halide leaving ligands, Gray and coworkers [18, 19] have shown that the displacement reactions can be represented by the equation:

$$k_{obs} = k_1 + k_2[Y]$$

where k_{obs} = the pseudo first-order rate constant determined under conditions of excess Y, k_1 = the first-order rate constant for the dissociation of the leaving ligand in water, k_2 = the second-order rate

Table 1. Displacement of the malonate ligand from Pt(mal)(*trans*-dach) by various nucleophiles

Compounds tested	% Malonate displaced from Pt(mal)(*trans*-dach)*
Components of RPMI-1640 medium	
H_2O	<1
108 mM NaCl	60
23.8 mM $NaHCO_3$	42
1.6 mM $NaNO_3$	<1
0.4 mM Na_2SO_4	3
5.6 mM Na_2HPO_4/NaH_2PO_4, pH 7	1
11 mM Glucose	2
Sulfur-containing amino acids†	66
Amide amino acids†	12
Basic amino acids†	8
Acidic amino acids†	5
OH-containing amino acids†	4
Hydrophobic amino acids†	<1
Selected anions at intracellular levels	
10 mM $NaHCO_3$	19
3 mM NaCl	2
45 mM Na_2HOP_4/NaH_2PO_4, pH 7	21
90 mM Na_2HPO_4/NaH_2PO_4, pH 7	36

* Twenty-four-hour incubation at 37° with 20 μg/ml [^{3}H-*trans*-dach]Pt(mal)-(*trans*-dach). Amounts of Pt(mal)(*trans*-dach) remaining were determined by reverse phase HPLC as described in Materials and Methods.

† Sulfur-containing amino acids = glutathione, methionine, and cysteine; amide amino acids = asparagine and glutamine; basic amino acids = histidine, lysine, and arginine; acidic amino acids = aspartate and glutamate; OH-containing amino acids = serine, threonine, and tyrosine; hydrophobic amino acids = all other amino acids found in RPMI-1640 medium. The concentrations of the amino acids used were the same as those found in the medium (see Materials and Methods for a complete description of RPMI-1640 medium).

constant for the bimolecular (S_N2) reaction between the platinum complex and the incoming nucleophile, and Y = the incoming nucleophile. For platinum(II) compounds with halide leaving ligands, the hydrolysis step is kinetically significant and plots of k_{obs} versus Y show a positive intercept corresponding to k_1 [18–20]. However, if the dissociation rate in water were very slow compared to the bimolecular displacement reaction, then k_1 would become kinetically insignificant and one would expect a plot of k_{obs} versus Y to go through the origin. The data obtained by measuring the rate of disappearance of Pt(mal)(*trans*-dach) at various concentrations of methionine are shown in Fig. 2. Clearly the plot of k_{obs} versus methionine concentration goes through the origin, indicating that the direct displacement (S_N2) reaction predominates at physiological methionine concentrations. The non-linearity of the plot at high methionine concentration will be discussed later.

The preceding experiments identified glutathione and a group of twelve amino acids as being significant biological nucleophiles for the displacement of the malonate ligand. However, since each amino acid was tested at its concentration in the medium, these experiments did not give a good estimate of the relative reactivities of these amino acids. Thus, the abilities of gluthathione and these reactive amino

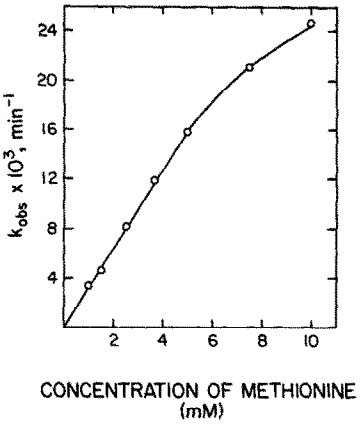

Fig. 2. Variation of the pseudo first-order rate constant with methionine concentration. [^{3}H-*trans*-dach]Pt(mal)-(*trans*-dach) (20 μg/ml, 0.05 mM) in water was reacted with methionine at the various concentrations shown. At appropriate time intervals, aliquots were withdrawn and diluted 1:10 in cold (4°) water, and the amount of ^{3}H-labeled Pt(mal)(*trans*-dach) remaining was determined by reverse phase HPLC (Materials and Methods). The pseudo first-order rate constants were determined from the slope of the line obtained by plotting $\log\left(\frac{a}{a-x}\right)$ versus time.

Table 2. Displacement of the malonate ligand from Pt(mal)(*trans*-dach) by amino acids*

Amino acid	% of Pt(mal)(*trans*-dach) converted to amino acid complexes at an amino acid concentration of†: 0.1 mM	1 mM	10 mM
Methionine	39	98	98
Glutathione	16	48	97
Cysteine	12	31	87
Aspartate	—	8	43
Glutamate	—	7	37
Serine	—	5	10
Threonine	—	5	10
Asparagine	—	4	10
Glutamine	—	4	10
Histidine	—	0	8
Arginine	—	0.67	4
Lysine	—	1	4
Alanine	—	—	3
Valine	—	—	2

* Twenty-four-hour incubation at 37° with 20 μg/ml of [^{3}H-*trans*-dach]Pt(mal)(*trans*-dach), 141 mCi/mmol.

† Conversion of Pt(mal)(*trans*-dach) to amino acid complexes was measured by reverse phase HPLC as described in Materials and Methods.

acids to displace the malonate ligand were tested at a similar range of concentrations (Table 2). Clearly, the sulfur-containing amino acids were the most reactive with methionine > glutathione > cysteine. The remaining amino acids were much less reactive, with the order of reactivity being: aspartate = glutamate > serine = threonine = asparagine = glutamine > arginine = lysine. The hydrophobic amino acids such as alanine and valine were the least reactive. The reactions reported in Table 2 were carried out in distilled water to minimize the appearance of extraneous reaction products between Pt(mal)(*trans*-dach) and buffers which complicate the identification and quantitation of the amino acid–platinum complexes. Since the comparable reactions would be buffered in the cell, the reactivity of several of these amino acids for displacement of the malonate ligand was also determined in 40 mM phosphate buffer, pH 7. Of the amino acids tested, only histidine showed slightly greater reactivity (12% instead of 8% displacement of malonate at 10 mM) in the buffered reaction mixture (data not shown).

The reactivities of several likely platinum transformation products are shown in Table 3. With respect to the platinum–amino acid complexes, those which formed the most readily (Table 2) appeared to have the least reactivity towards DNA (Table 3). Only the platinum–arginine and platinum–lysine complexes showed any significant reactivity. For comparsion, $PtCl_2$(*trans*-dach) itself was relatively unreactive towards DNA under our assay conditions. However, in water at 37° $PtCl_2$(*trans*-dach) dissociated to form the more reactive aquated species with a $T_{\frac{1}{2}}$ of approximately 2 hr at 37°, whereas the platinum–amino acid complexes showed little or no dissociation over a 24-hr period.

Table 3. DNA binding activity of platinum(II) complexes*

Platinum(II) complex	Retention time on reverse phase (min)	% Binding to DNA	No. of determinations
Amino acid complexes			
Pt(methionine)(*trans*-dach)	35	0.2 ± 0.05	3
Pt(glutathione)(*trans*-dach)	31	0.29 ± 0.08	3
Pt(glutathione)(*trans*-dach)	33	0.06 ± 0.03	3
Pt(cysteine)(*trans*-dach)	27	1.2 ± 0.2	3
Pt(cysteine)(*trans*-dach)	33	1.3 ± 0.2	3
Pt(serine)(*trans*-dach)	29	2.5 ± 0.4	3
Pt(threonine)(*trans*-dach)	29	2.1 ± 0.6	3
Pt(aspartate)(*trans*-dach)	29	2.1 ± 0.5	3
Pt(glutamate)(*trans*-dach)	29	1.9 ± 0.2	3
Pt(asparagine)(*trans*-dach)	29	1.6 ± 0.2	2
Pt(glutamine)(*trans*-dach)	29	1.6 ± 0.4	2
Pt(arginine)(*trans*-dach)	36	10.8 ± 1.2	3
Pt(lysine)(*trans*-dach)	35	9.6 ± 0.2	3
Other platinum(II) complexes			
Pt(mal)(*trans*-dach)	15	<0.1	2
$PtCl_2$(*trans*-dach)	10	4.5 ± 0.5	3
[$Pt(H_2O)(Cl)$(*trans*-dach)]*	28	43.6 ± 5.9	10
[$Pt(H_2O)_2$(*trans*-dach)]$^{2+}$	32	73.8 ± 4.4	8

* Platinum–amino acid complexes and the aquated platinum complexes were prepared and separated by reverse phase HPLC as described previously [13]. The isolated peak fractions for each complex were pooled and tested immediately for DNA binding activity as described in Materials and Methods. In the case of the Pt–arginine and Pt–lysine complexes some aquated species could have formed since the amino acids were used as their chloride salts. Thus, they were first purified on cation exchange HPLC to separate them from any aquated species and then purified on reverse phase HPLC before testing for their reactivity towards DNA. The values reported are mean ± SEM.

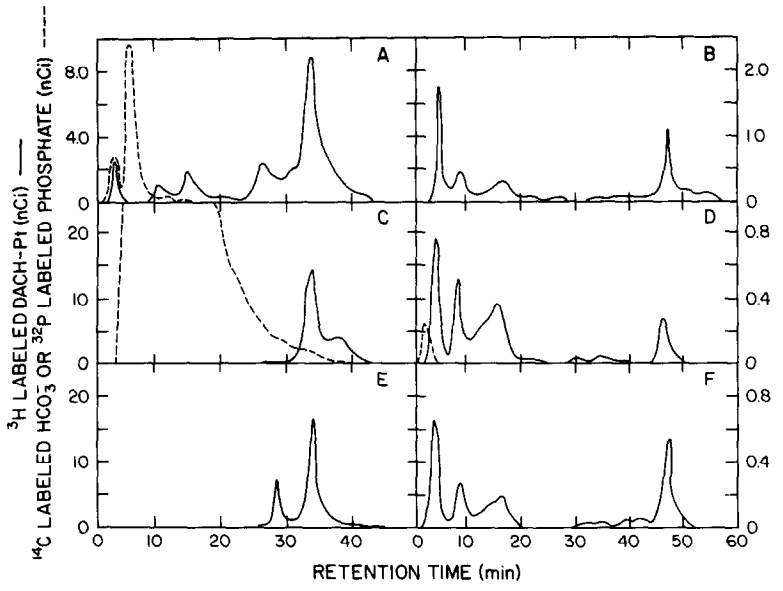

Fig. 3. Identification of compounds formed by incubation of Pt(mal)(*trans*-dach) with bicarbonate or phosphate. 20 μg/ml [^{3}H-*trans*-dach] Pt(mal)(*trans*-dach) (20 μg/ml, 141 mCi/mmol), was incubated with either 100 mM [^{14}C]$NaHCO_3$, 4.3 mCi/mmol, or 90 mM [^{32}P] potassium phosphate buffer, 1.7 mCi/mmol, pH 7, for 7 days at 37°. (A) reverse phase elution profile of the [^{14}C]$NaHCO_3$ reaction mixture; (B) cation exchange profile of the major peak (retention time = 32 min) from reverse phase separation of $NaHCO_3$ reaction mixture; (C) reverse phase separation of the [^{32}P]potassium phosphate reaction mixture; (D) cation exchange separation of the major peak from reverse phase separation of potassium phosphate reaction mixture; (E) reverse phase separation of $[Pt(H_2O)_2$(*trans*-dach)$]^{2+}$ standard; and (F) cation exchange separation of $[Pt(H_2O)_2$(*trans*-dach)$]^{2+}$ standard following the reverse phase step. All cation exchange separations were at pH 4 (note: 1 nCi = 2.2×10^3 dpm).

The effectiveness of bicarbonate at displacing the malonate ligand was surprising since bicarbonate is known to be a weak nucleophile. In fact, based on determinations of the pseudo first-order rate constants for displacement of the malonate ligand, bicarbonate was approximately 120-fold less reactive than methionine (data not shown) at equimolar concentrations. However, this difference in reactivity was overcome by the large difference in concentration of these two compounds in RPMI-1640 medium (see Materials and Methods). The bicarbonato displacement reaction was also surprising because no bicarbonato platinum(II) complexes have been described to date. To test for the formation of a bicarbonato platinum(II) complex, ^{3}H-labeled Pt(mal)(*trans*-dach) was incubated with ^{14}C-labeled bicarbonate. In this case, the Pt(mal)(*trans*-dach) was incubated with 100 mM bicarbonate for 7 days to maximize the formation of any bicarbonato complex which might exist. Following incubation, the reaction mixture was analyzed by reverse phase and cation exchange HPLC (Fig. 3, A and B). The major ^{3}H-labeled transformation product coeluted with the $[Pt(H_2O)_2$(*trans*-dach)$]^{2+}$ peaks on both reverse phase (Fig. 3A) and cation exchange (Fig. 3B, compare to the elution pattern for $[Pt(H_2O)_2$(*trans*-dach)$]^{2+}$ on cation exchange, Fig. 3F). No detectable ^{14}C coeluted with the ^{3}H peaks. Even when the fractions were collected directly into NaOH (final concentration = 0.1 N) to minimize dissociation of the bicarbonate, no coelution of ^{14}C with the ^{3}H peaks was observed (data not shown). One can only conclude that, at sufficiently high concentrations, bicarbonate can be an effective nucleophile and can facilitate the displacement of the malonate ligand, but that any bicarbonato complex that forms must be unstable and is rapidly displaced to give $[Pt(H_2O)_2$(*trans*-dach)$]^{2+}$ and other aquated species. Since bicarbonate levels in the blood are normally around 25 mM, bicarbonate may represent a major route for extracellular displacement of the malonate ligand *in vivo*.

We next asked if phosphates could play a similar role inside the cell. Total intracellular phosphate concentration (organic + inorganic) is approximately 140 mM. However, since some of the organic phosphates are less reactive than inorganic phosphate, the effective phosphate concentration is probably somewhat less. At concentrations ranging from 45 to 90 mM, inorganic phosphate was capable of causing significant displacement of the malonate ligand (Table 1). The displacement of the malonate ligand by 90 mM ^{32}P-labeled potassium phosphate, pH 7, as measured by reverse phase HPLC is shown in Fig. 3C. When the major peak was rechromatographed on cation exchange HPLC (Fig. 3D), no ^{32}P coeluted with the ^{3}H and the elution pattern was almost identical to that observed for $[Pt(H_2O)_2$-(*trans*-dach)$]^{2+}$ (Fig. 3F).

The major transformation products formed by incubating Pt(mal)(*trans*-dach) for 24 hr with RPMI-1640 medium were separated by the two column

Table 4. Identification and quantitation of the major transformation products arising from the incubation of Pt(mal)(*trans*-dach) in RPMI-1640 medium*

Peaks from HPLC	Retention time on reverse phase† (min)	Fractional retention time on cation exchange‡ pH 4	pH 2.3	Identification	% of Total ^{3}H-*trans*-dach recovered in this form
Clear identification					
Peak b	10 (8–12)	0.11	0.10	$PtCl_2$(*trans*-dach)	14.4
Peak c	15 (13–17)	0.14	0.13	Pt(mal)(*trans*-dach)	10.8
Peak e_1	27 (24–27)	0.11	0.12	Pt(cysteine)(*trans*-dach)	1.4
Peak e_2	27 (24–27)	0.18	0.19	Pt(cysteine)(*trans*-dach)	2.8
Peak f_6	29 (28–30)	0.55	0.76	Pt(glutamine)(*trans*-dach)	1.3
Peak g_5	33 (31–34)	0.67	0.64	Free *trans*-dach	2.2
Peak g_6	33 (31–34)	0.76	0.90	Pt(methionine)(*trans*-dach)	37.6
Peak h_7	35 (35–38)	0.87	0.87	Pt(arginine)(*trans*-dach)	3.9
Tentative identification					
Peak f_3	29 (28–30)	0.23	—	Pt(glutamate)(*trans*-dach) and Pt(aspartate)(*trans*-dach)	3.6
Peak f_4	29 (28–30)	0.28	0.28	Pt(serine)(*trans*-dach) and Pt(threonine)(*trans*-dach)	4.9
Peak h_6	35 (35–38)	0.68	0.68	Pt(lysine)(*trans*-dach)	1.3

* The experimental conditions and elution profiles are shown in Fig. 4. Identification is based on co-migration with known standards under three different HPLC conditions.

† The retention time of the peak fraction is indicated on the left. The fractions actually pooled for further analysis on cation exchange are shown in parentheses.

‡ Fractional retention time = retention time of the unknown peak divided by the retention time of the $[Pt(H_2O)_2$(*trans*-dach)$]^{2+}$ standard on the same column [13].

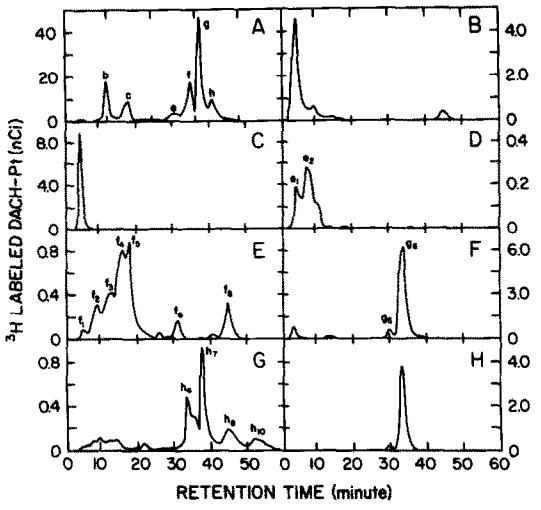

Fig. 4. Major transformation products formed from Pt(mal)(*trans*-dach) in RPMI-1640 medium. [^{3}H-*trans*-dach]Pt(mal)(*trans*-dach) (20 μg/ml), 141 mCi/mmol, was incubated at 37° for 24 hr with RPMI-1640 medium, and an aliquot of the reaction mixture was analyzed by the two column HPLC separation system described in Materials and Methods. The nomenclature used in labeling the peaks is based in part on the more complex elution profiles observed in studies of intracellular biotransformation products. Thus, not all peaks in our numbering system are observed in this elution profile. (A) Reverse phase elution profile; (B) cation exchange elution profile of peak b; (C) cation exchange elution profile of peak c; (D) cation exchange elution profile of peak e; (E) cation exchange elution profile of peak f; (F) cation exchange elution profile of peak g; (G) cation exchange elution profile peak of h; and (H) cation exchange elution profile of Pt(methionine)(*trans*-dach) standard. All cation exchange separations shown were at pH 4 (note: 1 nCi = 2.2×10^3 dpm).

HPLC separation method described in Materials and Methods. Identification of the major transformation products in RPMI-1640 medium was carried out by comparing their retention times under three different HPLC conditions (reverse phase, cation exchange at pH 4, and cation exchange at pH 2.3) with the retention times of a series of eighteen standards prepared *in vitro* [13]. The elution profiles on reverse phase HPLC and cation exchange HPLC at pH 4 are shown in Fig. 4. The identifications and quantitations are summarized in Table 4. The identification was most definitive for seven compounds: Pt(methionine)(*trans*-dach) (38% of the total), $PtCl_2$(*trans*-dach) (14%), Pt(mal)(*trans*-dach) (11%), Pt(cysteine)(*trans*-dach) (4.2%), Pt(arginine)(*trans*-dach) 3.9%), free *trans*-dach (2.2%), and Pt(glutamine)(*trans*-dach) (1.3%). Other platinum–amino acid complexes constituted approximately 11% of the total transformation products, but could not be unambiguously identified or quantitated due to overlapping peaks. Only 15% of the transformation products in RPMI-1640 medium remained unidentified.

In most cases the structures of the platinum–amino acid complexes are not known in detail, although the stoichiometry has been determined and tentative structures have been suggested for some of the complexes on the basis of double label experiments and the pH dependence of retention times on cation exchange HPLC [13]. Since the Pt(methionine)(*trans*-dach) complex was the major amino acid complex which accumulated in RPMI-1640 medium, it was characterized in some detail. The complex was purified from the medium and desalted as described in Materials and Methods. In spite of the extensive purification employed, the level of non-platinum-containing impurities was still too high to allow direct analysis by FAB-MS. Accordingly, the sample was analyzed by coupled HPLC-thermospray mass spectrometry [21]. Either 6.8 μg of the sample or 5 μg of the Pt(methionine)(*trans*-dach) standard dissolved in H_2O was injected onto a 4.6 × 250 mm RP-304 column (Biorad) and eluted isocratically with 0.1 M ammonium acetate in 40% H_2O/60% methanol at 1 ml/min. In both cases, the major platinum-containing material eluted with a retention time of 3.5 min. The on-line mass spectra obtained at 3.5 min for the standard and the sample from the medium are shown in Fig. 5. Both spectra showed a strong ion cluster at 458 (which is characteristic of the $[M + H]^+$ ion of the mono-methionine complex) and ion signals at 193 and 229 (which appear to represent solvent background). The sample from RPMI-1640 medium also showed an ion cluster at 606, which would be predicted for the $[M + H]^+$ ion of the bis-methionine complex. Our previous data have shown that both the mono-methionine and bis-methionine complexes co-migrate under the HPLC conditions employed [13]. The ion signal at 264 was unidentified, but did not contain the characteristic platinum ion cluster. Based on the relative intensities of the major ion clusters, we estimate that Pt(methionine)(*trans*-dach) obtained from the medium contained approximately 60% of the mono-methionine and 40% of the bis-methionine complex.

We next asked whether the Pt(methionine)(*trans*-dach) complexes could be taken up by the cell. ^{3}H-labeled Pt(methionine)(*trans*-dach) was prepared by reacting 0.2 mM [4,5-^{3}H](*d,l-trans*-1,2-diamino-cyclohexane)malonatoplatinum(II) (141 mCi/mmol) with 10 mM methionine for 24 hr at 37°. Under these conditions, the final reaction mixture contained greater than 93% Pt(methionine)(*trans*-dach), less than 1% Pt(mal)(*trans*-dach), approximately 3% free *trans*-dach, and just less than 3% of an unidentified impurity, as judged by cation exchange HPLC. The ratio of Pt(mal)(*trans*-dach) and methionine used in this reaction mixture would be expected to give approximately 70% of the mono-methione and 30% of the bis-methionine complexes [13], which is similar to the ratio of the two methionine complexes found in the medium. Further purification of this reaction mixture on the scale required for the uptake experiments was not feasible, so it was used directly as the source of Pt(methionine)(*trans*-dach). Uptake of this Pt(methionine)(*trans*-dach) was compared to the uptake of pure Pt(mal)(*trans*-dach) at a final concentration of 10 μg/ml (Fig. 6). Both total uptake (which includes platinum bound to macromolecules and the cell membrane as well as low molecular weight platinum biotransformation products) and the intracellular accumulation of filterable platinum (less than 30,000 molecular weight)

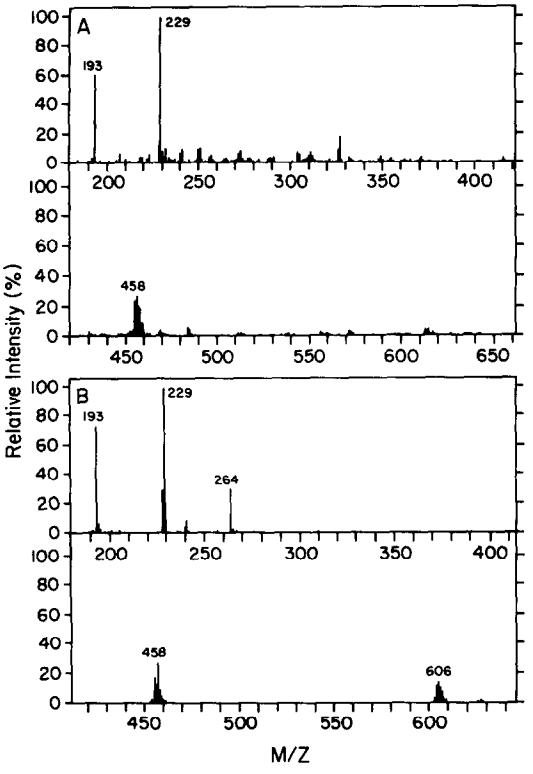

Fig. 5. Mass spectra of Pt(methionine)(*trans*-dach) standard and the Pt(methionine)(*trans*-dach) sample obtained from RPMI-1640 medium. The preparation of the Pt(methionine)(*trans*-dach) standard and the purification of Pt(methionine)(*trans*-dach) from the medium are described in Materials and Methods. Both samples were dissolved in water and analyzed by coupled HPLC-thermospray MS as described in the text. The thermospray MS analysis was performed on a Finnigan MAT 4600 quadrapole mass spectrometer with a Finnigan MAT interface. The thermospray interface was operated at a source temperature of 270° and a vaporizer temperature of 94°. Positive ion detection using ion evaporation ionization was employed. The mass spectrometer scanned from *m/z* 180 to 650 in 2 sec. The mass calibration of the quadrapole was verified with propylene glycol (average mol. wt 1000). (A) mass spectrum for the Pt(methionine)(*trans*-dach) standard; and (B) mass spectrum for Pt(methionine)(*trans*-dach) isolated from RPMI-1640 medium.

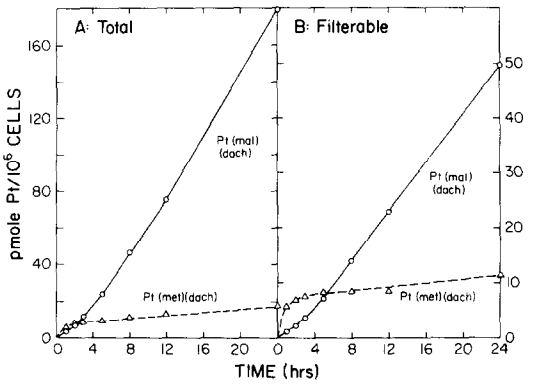

Fig. 6. Uptake of Pt(mal)(*trans*-dach) and Pt(methionine)(*trans*-dach) by L1210 cells. Pt(methionine)(*trans*-dach) was prepared by incubating 0.2 mM Pt(mal)(*trans*-dach) with 10 mM methionine for 24 hr at 37°. The uptake of Pt(mal)(*trans*-dach) and Pt(methionine)(*trans*-dach) at 10 μg/ml was measured as described previously [22]. Total uptake represents the platinum associated with the cells after thorough washing in phosphate-buffered saline (50 mM potassium phosphate, pH 7.4, and 0.15 M NaCl). Filterable platinum represents the platinum obtained following sonication and filtration of the sonicate through an Amicon YMT membrane filter (30,000 mol. wt cut-off).

were measured. The data showed very rapid uptake (< 2 hr) of an impurity representing approximately 2.5% of the original Pt(methionine)(*trans*-dach) reaction mixture. This likely represents either the free *trans*-dach or the other unidentified impurity present. Whatever this impurity was, it was almost totally unreactive, since the total and filterable counts associated with the cells were virtually identical at early times (0–4 hr). This was followed by a slow uptake of ^{3}H-labeled platinum at approximately 4.4% the rate at which Pt(mal)(*trans*-dach) was taken up by the cell. Since the Pt(methionine)(*trans*-dach) reaction mixture contained 1% Pt(mal)(*trans*-dach) and at least 3% of another impurity, it is not clear whether this 4.4% value accurately represents the uptake of Pt(methionine)(*trans*-dach). However, it does represent a maximum value for the rate of uptake, and, thus, it is clear that any uptake of Pt(methionine)(*trans*-dach) is insignificant compared to the rate of uptake of Pt(mal)(*trans*-dach). Of the small amount of platinum taken up by the cell, 50–60% was filterable (Fig. 6, Filterable). Since the non-filterable platinum represented platinum bound to macromolecules as well as platinum bound to the cell membrane, the maximum amount of platinum bound to the cell membrane was small indeed (≦ 12 pmol/10^{6} cells). Thus, both uptake of the Pt(methionine)(*trans*-dach) complexes and their binding to the cell membrane were very low, and one can consider these amino acid complexes to be essentially inert in the medium.

DISCUSSION

This report is the first study to detail the stability, and the major transformation products, of any platinum complex with a bidentate leaving ligand in tissue culture medium. The 9.5-hr half-life for Pt(mal)(*trans*-dach) in RPMI-1640 medium at 37% is considerably shorter than the half-life reported for bidentate platinum complexes in water or NaCl alone [5–7]. Considering that some biological nucleophiles are present at much higher concentrations inside the cell than in the medium, even shorter intracellular half-lives for the activation of platinum compounds with bidentate leaving ligands would be possible without invoking enzymatic activation.

The data in Table 1 and Fig. 2 suggest that biological nucleophiles can directly displace the malonate ligand at the concentrations which occur in the medium. The plateauing of the rate at which methionine reacts with Pt(mal)(*trans*-dach) at higher

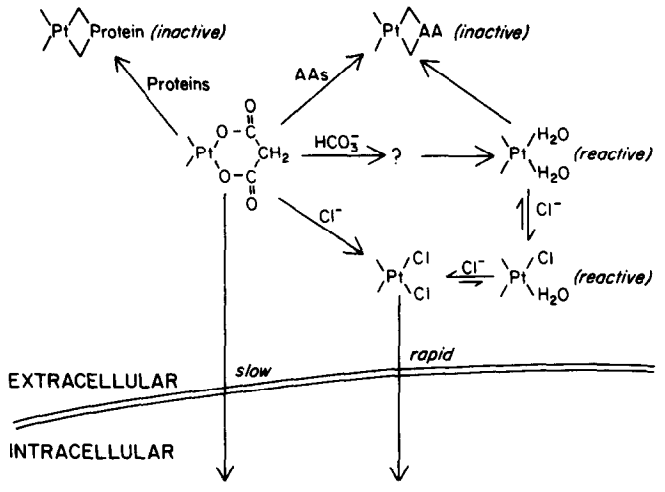

Fig. 7. Model for transformations of Pt(mal)(*trans*-dach) in RPMI-1640 medium.

methionine concentrations (Fig. 2) is similar to the kinetics previously reported for the reaction of *cis*-diamminedichloroplatinum(II) [cisplatin] with diadenosine 5′, 5‴-P^1,P^4-tetraphosphate (Ap_4A) [23] and may have a similar explanation. In both cases the incoming ligand has two nucleophilic centers, one which reacts rapidly with platinum(II) compounds and one which reacts more slowly. In the case of Ap_4A, it was proposed that the slower reaction with the phosphates of Ap_4A becomes kinetically significant at higher Ap_4A concentrations [23]. In our case, the plateauing would represent the slower reaction with the carboxylate moiety of methionine. Alternatively, the plateauing could simply represent an effect of ionic strength on the reaction, since the reaction of cisplatin with biological nucleophiles is known to be very sensitive to ionic strength.

A model of the Pt(mal)(*trans*-dach) transformations in RPMI-1640 medium is shown in Fig. 7. Based on the data in Fig. 4 and the identification summarized in Table 4, most of the "other" transformation products observed in Fig. 1 would appear to be platinum–amino acid complexes, as might be expected from the reactivities of these amino acids towards Pt(mal)(*trans*-dach) (Table 2) and their abundance in RPMI-1640 medium. Previous studies have shown that most platinum–protein complexes appear to be biologically inert. Our data suggest that the platinum–amino acid complexes which accumulate in the greatest abundance are also largely inert (Table 3).

It is clear that the platinum–methionine complexes are the most abundant transformation products in RPMI-1640 medium. Based on the combination of HPLC and MS data, we are quite confident in the identification of the mono-methionine complex. Our MS data cannot establish the exact structure of the mono-methionine complex formed in the medium, but it is likely to be the same as that obtained for the synthetically prepared mono-methionine complex. While the ion cluster at mass 606 could conceivably be derived from some platinum-containing complex other than the bis-methionine complex, we consider it unlikely. We have investigated all of the components of RPMI-1640 medium except the vitamins, which are present in only micromolar concentrations. In each case, the compounds tested either did not show significant reaction with Pt(mal)(*trans*-dach) or did not form complexes which co-migrate with the Pt(methionine)(*trans*-dach) complexes on both reverse phase and cation exchange HPLC ([13] and this paper).

The uptake and/or binding of the Pt(methionine)(*trans*-dach) complexes to the cell membrane (Fig. 6) was of particular interest because of their abundance in RPMI-1640 medium (and presumably most other media) and because of the observations of Scanlon *et al.* [24] that cisplatin interferes with methionine uptake by L1210 cells. Our data clearly show that both uptake of the Pt(methionine)(*trans*-dach) complexes and their binding to the cell membrane were minimal. This is in agreement with the data of Daley-Yates and McBrien [25] which showed that cisplatin methionine complexes have neither antitumor nor nephrotoxic properties.

Of all the components of the RPMI-1640 tissue culture medium, chloride, bicarbonate, and methionine were the most effective at displacing the malonate ligand. Both the chloride and bicarbonate displacement reactions appeared to represent activation pathways for compounds with bidentate leaving ligands. The chloride displacement reaction can be considered an activation pathway because $PtCl_2$(*trans*-dach) is taken up eight times more rapidly by the cell than Pt(mal)(*trans*-dach) [22]. Once inside the cell, $PtCl_2$(*trans*-dach) will be converted to the more reactive $[Pt(H_2O)(Cl)$(*trans*-dach)$]^+$ due to the low intracellular chloride concentrations [26, 27]. Since the displacement of the malonate ligand by chloride was minimal at intracellular chloride concentrations (Table 1), it appears likely that most of the intracellular $PtCl_2$(*trans*-dach) was formed in the medium, as shown in Fig. 7. In agreement with this prediction, we found that the pattern of accumulation of $PtCl_2$(*trans*-dach) inside the cell

was virtually identical to that observed in the medium.*

Bicarbonate was of particular interest since it does not form stable bicarbonato complexes, but appears to facilitate the formation of various aquated complexes. In the absence of NaCl, the major product formed appeared to be $[Pt(H_2O)_2(trans\text{-dach})]^{2+}$ (Fig. 3). However, at the high chloride concentration found in the medium, $PtCl_2(trans\text{-dach})$ and $[Pt(H_2O)(Cl)(trans\text{-dach})]^+$ would be the products accumulating in the greatest abundance [26]. Thus, the bicarbonate displacement reaction is a likely source of reactive aquated platinum complexes in the medium, and of $PtCl_2(trans\text{-dach})$ which will rapidly be taken up and converted to aquated species inside the cell. Since the bicarbonate concentration in plasma is approximately 25 mM, the bicarbonate displacement reaction may represent a major route for conversion of bidentate platinum compounds to the more reactive aquated species *in vivo*.

The other anions present in the tissue culture medium were clearly much less effective at displacing the malonate ligand at the concentrations found in the medium. However, at the concentration present inside the cell [28], phosphates could also diplace the malonate ligand (Table 1). As with the bicarbonato complex, the platinum–phosphato complex did not appear to be stable enough to be isolated by HPLC (Fig. 3). Several other laboratories have reported the formation of platinum–phosphato complexes [20, 23, 29], but none of them have measured the stability of the platinum–phosphato complex in water. While our data do not exclude the possibility that the platinum–phosphato complex might remain intact in the presence of the high phosphate concentrations found in the cell, Segal and Le Pecq [23] have reported that the phosphato complex formed under such conditions has comparable reactivity to the monoaqua-monochloro species. Thus, whether the final product is a phosphato complex or aquated species, our data and the data of Segal and Le Pecq [23] indicate that it is reactive. Hence, our data suggest that, at the concentrations which prevail inside the cell, phosphates could also play a significant role in the nonenzymatic activation of platinum compounds with bidentate leaving ligands.

Very little free *trans*-dach was observed even after a 24-hr incubation with the RPMI-1640 medium. This is significant because Ismail and Sadler [30] have reported that the reaction of cisplatin with sulfur-containing compounds results in the displacement of the carrier ligand. Obviously, if that had happened in these experiments, we would not have been able to follow the resulting platinum biotransformation products. However, control experiments showed that recovery of free *trans*-dach in the two column HPLC separation system was greater than 90%. Thus, clearly we would have been able to observe free *trans*-dach if any significant amount had been liberated. We conclude, therefore, that the dach carrier ligand remains stably attached to platinum during these incubations. Taylor *et al.* [31] have made similar observations for ethylenediaminedichloroplatinum(II).

In summary, this study is the first major application of the two column separation scheme for *trans*-dach platinum compounds that has been reported elsewhere [13] and clearly demonstrates the usefulness of the system. We have demonstrated relatively rapid displacement of the bidentate malonate ligand under conditions similar to those which exist in the blood and inside the cell. We have identified which amino-acids are most likely to displace bidentate leaving ligands. We have shown for the first time that bicarbonate and phosphates may play a major role in activation of bidentate platinum compounds. Finally, we have identified most of the major transformation products in the RPMI-1640 medium. Of course, those identifications based on HPLC separations alone must be considered tentative until confirmed by mass spectrometry or other methodology. We feel that this same technique should be equally applicable to identification of the major intracellular biotransformation products of *trans*-dach platinum compounds as well as the biotransformation products which form in the blood and urine.

Acknowledgements—This research was supported by U.S. PHS Grant CA 34082 awarded by the National Cancer Institute, DHHS. One of us (S. K. M.) was supported by a predoctoral traineeship (National Research Service Award 5T32ES-07126) from the National Institute of Environmental Health Sciences, National Institutes of Health. The authors gratefully acknowledge the scholarly discussions and helpful suggestions by Dr. Aziz Sancar.

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