Cisplatin Complexes with Phosphatidylserine in Membranes†

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ABSTRACT: Upon incubation of the anticancer drug cisplatin [*cis*-diamminedichloroplatinum(II)] with model membranes composed of phosphatidylserine (PS), a stable product is formed that has been isolated after chloroform/methanol extraction of the sample. The product formation is specific for PS and does not occur with other major membrane phospholipids. The rate and extent of product formation is dependent on the pH, chloride ion concentration, and temperature, with the highest rate at pH 6.0, in the absence of Cl^- and at 37 °C, indicating that positively charged aquated cisplatin is the reactive species. Over 80% of PS is converted within 15 h under these conditions with a halftime of 5 h. PS can be regenerated by an excess of glutathione. Mass spectrometry experiments demonstrate that interaction of cisplatin with PS involves a loss of two chloride ions and coordination of platinum to the amine and carboxyl group of the serine moiety. Cisplatin forms complexes specifically with PS not only in model membranes but also in the plasma membrane of human erythrocytes. Since PS is essential in several cellular processes, its interaction with cisplatin may have important physiological implications.

cis-Diamminedichloroplatinum(II) (cisplatin)[1] is a commonly used anticancer drug. It is a reactive compound that can occur in various species in aqueous solution depending on the pH and chloride ion concentration (Berners-Price et al., 1992; Miller & House, 1991; Reedijk, 1992, 1996). It is well-established that cisplatin interacts with DNA and thereby causes inhibition of DNA synthesis, which might be a primary therapeutic action (Garner et al., 1993; Bloemink & Reedijk, 1996; Yang et al., 1995). In addition the molecule interacts with proteins (Howe-Grant & Lippard, 1980; Courjault-Gautier et al., 1995). Whether cisplatin has an affinity for cellular lipids is not known. Knowledge about cisplatin−lipid interactions might contribute to a better understanding of the antitumor activity of cisplatin but might also shed light on the severe side effects of the drug, of which nephrotoxicity is the most serious (Reedijk, 1996; Tay et al., 1988; Daugaard & Abilgaard, 1989). A relationship between nephrotoxicity and interaction of positively charged aminoglucosides with negatively charged phospholipids (Mingeot-leClerq et al., 1992; Todd & Hottendorf, 1995) has been suggested.

Recently, it was observed that the positively charged aquated species of cisplatin have an affinity for negatively charged surfaces such as membranes composed of anionic phospholipids (Speelmans et al., 1996; Taylor et al., 1996). In the present study the formation of a stable coordination complex with PS is described involving a loss of two chloride ions (or in the case of aquated cisplatin two water molecules) and coordination of platinum to the amine and carboxylate group of the serine moiety. Because PS is involved in many cellular functions such as transmembrane signaling (Rando, 1988; de Kruijff, 1994) this implies that cisplatin−PS complex formation may exert an effect on these functions.

EXPERIMENTAL PROCEDURES

Materials. Cisplatin was purchased from Sigma (St. Louis, MO). Phospholipids were purchased from Avanti Polar Lipids, Inc. (Birmingham, AL) and contained two oleoyl fatty acyl chains, except cardiolipin derived from bovine heart, sphingomyelin derived from egg, and phosphatidylinositol derived from soybean.

Cisplatin Incubations with Phospholipid Dispersions. Lipid dispersions (2 mM phospholipid) were prepared by adding buffer containing 0 or 5 mM cisplatin to a dry lipid film followed by 10 times freeze-thawing and agitation with a vortex mixer. In all cases cisplatin solutions were freshly prepared by dissolving the compound in the appropriate buffer, which was facilitated by warming to 40−50 °C. Buffers employed were 10 mM Pipes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 7.4; 10 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 6.0; 10 mM Pipes, 100 mM NaCl, and 1 mM EGTA, pH 7.4; and 10 mM Mes, 100 mM NaCl, and

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[1] Abbreviations: cisplatin, *cis*-diamminedichloroplatinum(II); DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPS, 1,2-dioleoyl-*sn*-glycero-3-phosphoserine; DOPE, 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; Mes, 2-morpholinoethanesulfonic acid; MS, mass spectrometry; MS-MS, tandem mass spectrometry; Pipes, piperazine-1,4-diethanesulfonic acid; PC, phosphatidylcholine; PE, phospatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; TLC, thin-layer chromatography.

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1 mM EGTA, pH 6.0. The samples were incubated in the dark at room temperature for the indicated times, after which the lipids were extracted (Bligh & Dyer, 1959) and analyzed by TLC on silica gel, eluting with either chloroform/methanol/acetic acid (7/2/1 v/v/v), chloroform/methanol/ammonium/water (34/14/1/1 v/v/v/v), or chloroform/methanol/water (35/13/2 v/v/v). Lipids were identified by spraying with either 10% H_2SO_4 followed by charring, phosphorus, or ninhydrin spray reagents. For quantitative analysis lipid spots were scraped off, after which the material was destroyed by perchloric acid and the amount of phosphorus was determined (Rouser et al., 1970).

For the experiments in which it was tested whether the formed complex of cisplatin with DOPS could be reversed with glutathione, cisplatin was incubated overnight at 37 °C with lipid dispersions containing DOPS and DOPC (1/1) in a buffer composed of 10 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 6.0. The lipids were washed and resuspended, to a final concentration of 2 mM P_i, in a buffer composed of 10 mM Pipes, 100 mM NaCl, and 1 mM EGTA, pH 7.4, containing either 0, 15, or 100 mM glutathione. The samples were incubated at 37 °C, and at different time intervals samples were taken to analyze the amount of DOPS and cisplatin−DOPS complex as described above.

Cisplatin Incubations with Red Blood Cell Ghosts. Human red blood cell ghosts were isolated according to Auland et al. (1994). Cisplatin (5 mM final concentration) was added to the membranes (2 mM phospholipid P_i) and incubation was performed at 37 °C, in 10 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 6.0. Since these ghosts are open structures, cisplatin is able to reach both the inner and outer leaflet of the membrane. Samples were taken at different time points and phospholipids were extracted (Bligh & Dyer, 1959). TLC experiments were performed in one dimension with chloroform/methanol/acetic acid (7/2/1 v/v/v) as eluent and sprayed with ninhydrin or in two dimensions, eluting in the first dimension with chloroform/methanol/ammonium/water (34/14/1/1 v/v/v/v) and in the second dimension with chloroform/methanol/acetic acid (7/2/1 v/v/v), after which lipids were identified by spraying with phosphorus spray reagent.

Mass Spectrometry. Cisplatin was incubated with DOPS as described above after which the lipids were extracted (Bligh & Dyer, 1959). The chloroform phase, containing the complex, was used for analysis. Positive fast atom bombardment mass spectra and collision-induced dissociation MS-MS spectra were obtained using a Jeol JMS-SX102/102A four-sector instrument. Methylnitrobenzyl alcohol was used as a matrix for the fast atom bombardment mass spectra. The MS-MS spectra were acquired using nitrogen as collision gas. The gun was operated at 6 kV and at 5 mA current.

NMR Spectroscopy. Cisplatin was incubated with DOPS, after which the lipids were extracted as described above. ^1H, ^{13}C, ^{31}P, and ^{195}Pt NMR spectra were recorded in saturated solutions of $CDCl_3$ (^1H, ^{13}C, and ^{31}P) and in 9/1 $CDCl_3$/MeOH (^{13}C, ^{31}P, and ^{195}Pt); spectra were referenced to TMS, TMP (trimethyl phosphate), and K_2PtCl_4 (−1600 ppm), respectively.

RESULTS AND DISCUSSION

Cisplatin Specifically Complexes with Phosphatidylserine in Model Membranes. When DOPS is dispersed in a chloride-ion free buffer of pH 6.0 (10 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA) containing 5 mM cisplatin and incubated at room temperature for 3 days a new species (X) can be observed by TLC (Figure 1A). X has a lower R_f value then DOPS in an acidic eluent, contains phosphate, and in contrast to DOPS it does not stain purple (but brownish) when stained with the amino-group-specific ninhydrin reagent (data not shown). Dispersions of DOPE, DOPC, and sphingomyelin, representatives of the other major mammalian plasma membrane phospholipids, do not show the appearance of new reaction products upon incubation with cisplatin under these conditions (Figure 1A). This is also not observed for a DOPE/DOPC (1:1 molar) mixed dispersion (data not shown), which is organized in a lamellar phase at room temperature [pure DOPE forms a H_{II} phase (Cullis & de Kruijff, 1978)]. Furthermore, new reaction products are also absent when the negatively charged

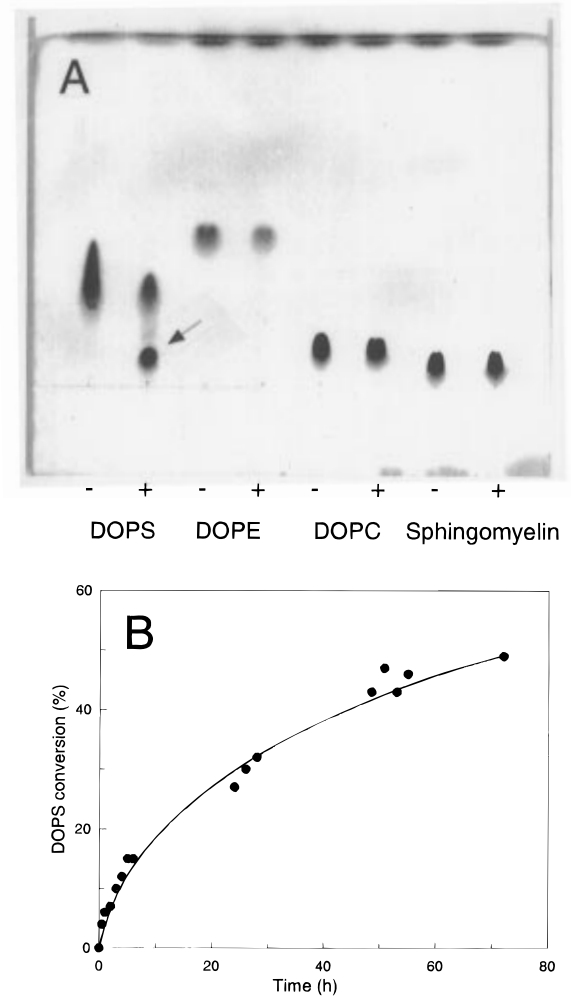

FIGURE 1: Formation of a product of DOPS upon incubation with cisplatin. (A) Thin-layer chromatography of DOPS, DOPE, DOPC, and sphingomyelin (SM). LUVET composed of these lipids were incubated in buffer composed of 10 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 6.0, for 72 h at room temperature in the presence or absence of 5 mM cisplatin. Lipids were extracted, subjected to TLC with chloroform/methanol/acetic acid (7/2/1 v/v/v) as eluent, and visualized with phosphorus spray. The arrow indicates the new product formed (X). (B) Conversion of DOPS in time. DOPS was incubated with 5 mM cisplatin at room temperature in the buffer described above. Samples were taken, extracted, and subjected to TLC. The increase in the DOPS−cisplatin reaction product was based on the phosphor content of the product spot on the TLC plate. One hundred percent is the sum of the amount of phosphor in the product spot and in the DOPS spot.

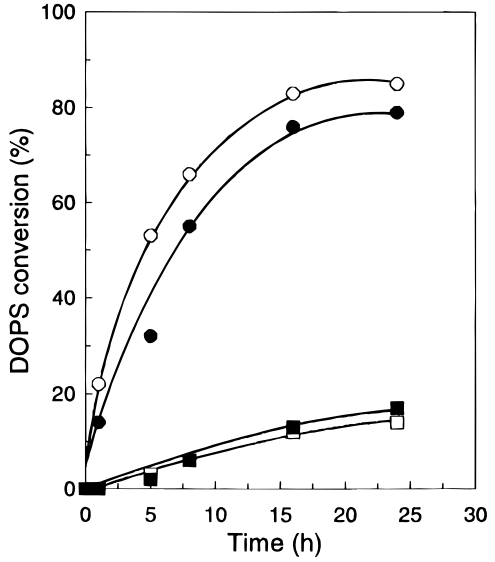

FIGURE 2: Conversion of DOPS at 37 °C in buffers of various composition. DOPS was incubated in the presence of 5 mM cisplatin. Samples were taken and analyzed as described in the legend to Figure 1. (●) Incubation in buffer composed of 10 mM Pipes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 7.4; (○) incubation in buffer composed of 50 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 6.0; (■) incubation in buffer composed of 10 mM Pipes, 100 mM NaCl, and 1 mM EGTA, pH 7.4; (□) incubation in buffer composed of 10 mM Mes, 100 mM NaCl, and 1 mM EGTA, pH 6.0.

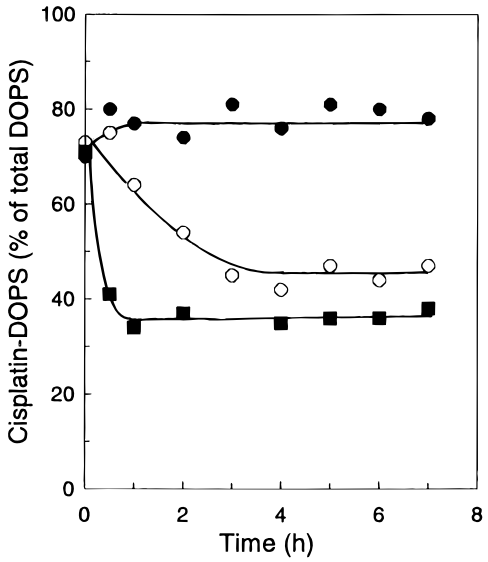

FIGURE 3: Conversion of the cisplatin−DOPS complex at 37 °C by glutathione. DOPS/DOPC lipid dispersions (1/1) were incubated overnight in the presence of 5 mM cisplatin in 10 mM Mes, 50 mM Na_2SO_4, and 1 mM EGTA, pH 6.0, at 37 °C. Lipids were washed and resuspended at 10 mM Pipes, 100 mM NaCl, and 1 mM EGTA, pH 7.4, containing either 0 (●), 15 (○), or 100 mM (■) glutathione. DOPS and cisplatin−DOPS were analyzed as described under Experimental Procedures.

phospholipids cardiolipin, phosphatidylglycerol, and phosphatidylinositol are incubated with cisplatin (data not shown).

Formation of X at room temperature is a slow process with an estimated half-time of 70 h (Figure 1B) and is paralleled by a reduction in free DOPS, suggesting that X is the product of the DOPS−cisplatin interaction.

The most reactive species of cisplatin is not the native drug but the positively charged, aquated species (Reedijk, 1996). When cisplatin comes in contact with water, chloride ions are stepwise replaced by water molecules resulting in monoaquated $[(NH_3)_2PtCl(H_2O)]^+$ and diaquated $[(NH_3)_2Pt(H_2O)_2]^{2+}$ species. This process is inhibited by Cl^-. The mono- and diaquated species are weak acids with pK_as of 6.41 and 5.37, respectively (Berners-Price et al., 1992; Miller & House, 1991). To test which species is responsible for the reaction with DOPS, the lipid was dispersed and incubated at 37 °C in buffers with 0 or 100 mM NaCl and at pH values of 6.0 and 7.4. Because the properties of anionic lipids like DOPS depend on the nature and amount of cations in the buffer, the chloride-free buffer was supplemented with 50 mM Na_2SO_4. After certain time intervals the samples were analyzed by TLC and the percentage of conversion of DOPS was determined (Figure 2). Comparing the incubation of $[Cl^-] = 0$, pH 6.0, in Figure 1B (room temperature incubation) with that in Figure 2 immediately reveals that at the clinically relevant temperature of 37 °C the conversion of DOPS is greatly increased, with a half-time of the reaction of 5 h. In the absence of Cl^- the reaction rate is slightly lower at the higher pH value. Furthermore, it is clear from Figure 2 that also in the Cl^--containing buffers the reaction occurs to a significant extent, but at a 20−30-fold reduced rate for the two pH values tested.

Interaction of Cisplatin with Phosphatidylserine Can Be Reversed with Glutathione. To examine whether the complex formed between cisplatin and DOPS can be reversed, experiments were performed in which this complex was incubated with glutathione. In Figure 3 it is shown that a concentration-and time-dependent disappearance of the complex and concomitant reappearance of DOPS occurs upon incubation with glutathione. This phenomenon was observed even under conditions of high $[Cl^-]$ and pH 7.4 (Figure 3). Glutathione was chosen because it is known to compete for cisplatin complexation with other (macro)molecules in the cell, such as DNA. Intracellular glutathione therefore plays an important role in the anticancer activity of cisplatin (Timmer-Bosscha et al., 1992). Furthermore, cisplatin−glutathione complexes can be extruded by (cancer) cells by a GS-X pump (Ishakawa et al., 1994), thereby also decreasing the anticancer activity of the drug.

Structure of the Cisplatin−Phosphatidylserine Complex. The lower R_f value in an acidic eluent (Figure 1A) and higher R_f in an alkalic or neutral eluent (data not shown) indicate that the complex is less negatively charged than DOPS and probably neutral. In order to examine the structure of the cisplatin−DOPS complex, mass spectrometry was performed. The mass spectrum shows a $(M + H)^+$ cluster around *m*/*z* 1015 (Figure 4), the exact mass of the most abundant isotope being 1015.49. On the basis of the intensity ratio and the *m*/*z* values of the ions in this cluster, the molecule must contain platinum. A calculated isotope distribution of a molecule containing 42 C, 83 H, 3 N, 10 O, 1 P, and 1 Pt is inset in Figure 4. For this calculation nominal weights were used.

The $(M + H)^+$ ions at *m*/*z* 1014, 1015, and 1016 (exact masses 1014.49, 1015.49, and 1016.50, respectively) were subjected to MS-MS. In the MS-MS spectra two dominant fragment ion clusters are observed at *m*/*z* 412, 413, and 414 and *m*/*z* 314, 315, and 316 (Figure 5). In the product ion spectrum of *m*/*z* 1014 only *m*/*z* 412 and 314 are observed (Figure 5A). In the MS-MS spectrum of *m*/*z* 1015 the peak intensity ratios $I(m/z\ 412)/I(m/z\ 413)$ and $I(m/z\ 314)/I(m/z$

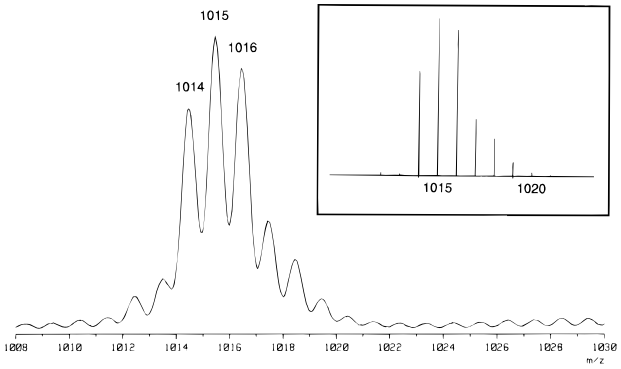

FIGURE 4: Mass spectrometry of the product formed upon incubation of DOPS with cisplatin. The inset shows a calculation of the isotope distribution as can be expected for a molecule with a composition of 42 C, 83 H, 3 N, 10 O, 1 P, and 1 Pt.

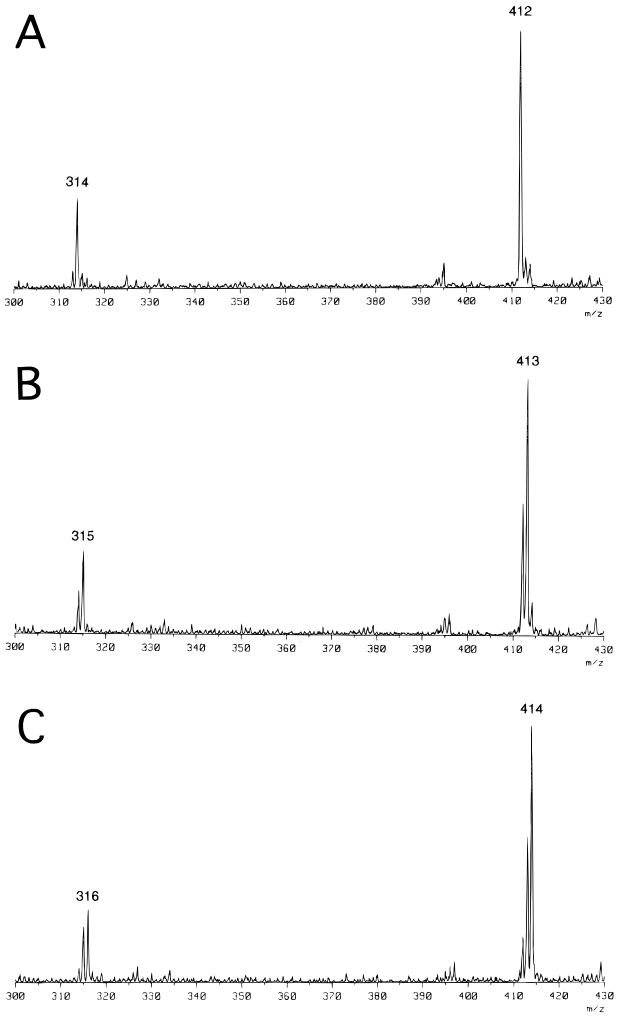

FIGURE 5: MS-MS spectrometry of fragment ions obtained from the ion of *m*/*z* 1014 (A), *m*/*z* 1015 (B), and *m*/*z* 1016 (C).

315) correspond to the ratio between the ^{13}C and ^{195}Pt contribution (35:65) in the selected precursor ion (Figure 5B). Three major components contribute to the *m*/*z* 1016 ion, namely, $(^{13}C)_2{}^{194}Pt$, $^{13}C^{195}Pt$, and ^{196}Pt in an intensity ratio of approximately 10:35:55. In the product ion spectrum of *m*/*z* 1016 we observed again two fragment ion clusters each containing three peaks with relative intensities corresponding to the component distribution in the precursor ion (Figure 5C). These observations allow us to conclude that the fragment ions mentioned above all contain a platinum atom.

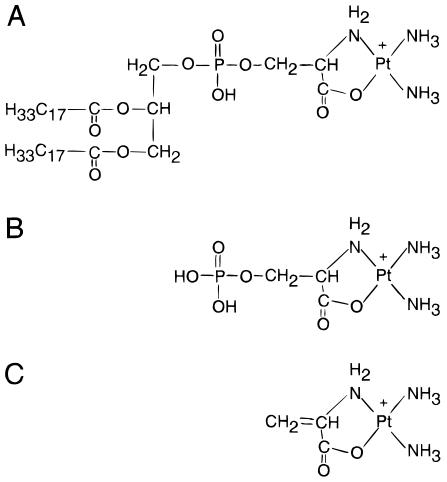

FIGURE 6: Proposed structure of the product formed upon incubation of DOPS with cisplatin with a molecular mass of 1015 Da (A) and the fragments with molecular masses of 412 (B) and 314 (C) Da.

The existence of these fragments can only be explained if the cisplatin−PS complex has a structure as shown in Figure 6, in which the platinum is coordinated to the carboxylate and amine group of the serine moiety. This molecule has a nominal molecular weight of 1015 in its protonated form, and upon fragmentation of this complex by MS-MS, platinum-containing fragment ions of *m*/*z* 412 and 314 are obtained. The former fragment indicates that platinum is not associated with the fatty acyl chain part or the glycerol backbone but with the headgroup. The latter fragment can be explained by a loss of H_3PO_4 from the serine moiety. The structure shown in Figure 6 is at a physiologically relevant pH zwitterionic, i.e., with a loss of a H^+ from the phosphate.

From ^{31}P spectroscopy a shift observed at 0.67 agrees with phosphate groups not coordinated to platinum. The signal observed in the ^{195}Pt spectrum at −2159 ppm is in excellent agreement with a chromophore [PtN_3O], although theoretically unreacted cisplatin also could be in this range. As a reaction has occurred because the ^{13}C signal at 184.6 ppm agrees with platinated carboxylate group, it can be concluded that all NMR data agree with the structure proposed in Figure 6.

Taylor et al. (1995) studied cisplatin−dimyristoyl-PS systems by infrared spectroscopy and they observed that cisplatin complexes to the carboxylate of the serine moiety, as also observed in this study. However, they proposed that the carboxylate is the only site on PS to which cisplatin coordinates. This is clearly different from our results. The difference could be due to differences in either samples or techniques. Dimyristoyl-PS was studied under gel-state conditions, whereas we studied more natural PS species under the more physiological liquid crystalline condition. The combination of mass spectroscopy and NMR as applied in our study gives an unambigious structure determination. Whether complexation to the amine function of PS would have been detected by infrared spectroscopy is not clear.

Interaction of Cisplatin with Phosphatidylserine in Erythrocyte Membranes. Subsequently, it was examined whether the formation of a cisplatin−phosphatidylserine coordination complex also occurs in biological membranes, where mixtures of phospholipids and proteins are present. Human

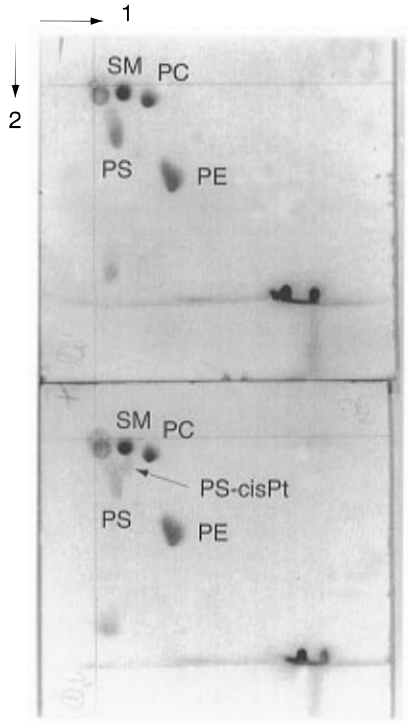

FIGURE 7: Formation of a cisplatin−PS complex in biological membranes. Ghosts of red blood cells were incubated with or without cisplatin as described in the legend to Figure 1. Lipids were analyzed with two-dimensional TLC, in the first dimension with chloroform/methanol/ammonium/water (34/14/1/1 v/v/v/v) and in the second dimension with chloroform/methanol/acetic acid (7/2/1 v/v/v) after which the lipids were identified by spraying with phosphorus reagent. The upper panel shows the incubation without cisplatin and the lower panel shows the incubation in the presence of cisplatin.

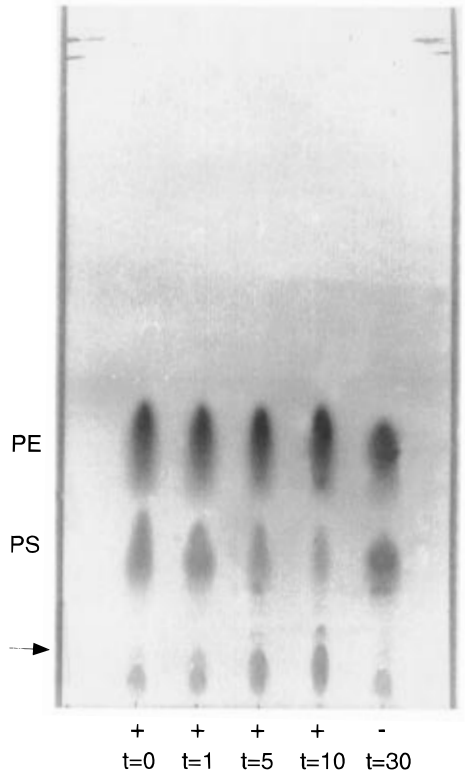

FIGURE 8: Formation of a cisplatin−PS complex in biological membranes. Ghosts of red blood cells were incubated with or without cisplatin as described in the legend to Figure 1. Lipids were analyzed after incubation with or without cisplatin for various periods of time and extraction with one-dimensional TLC with chloroform/methanol/acetic acid (7/2/1 v/v/v) as eluent, after which the plate was sprayed with ninhydrin. The arrow indicates the cisplatin−PS product.

erythrocyte ghosts were used to study the interaction of cisplatin. In a two-dimensional TLC an extra spot near the spot representing PS was observed (Figure 7). When we analyze the lipid fractions by ninhydrin staining it is clear that after incubation with cisplatin the amount of PS is significantly reduced in a time-dependent manner, concomitant with the appearance of a brownish spot with a lower retention time R_f, representing the cisplatin−PS product (Figure 8). The amount of phosphatidylethanolamine remains constant. Furthermore, in control experiments without cisplatin present during incubation the amount of PS remains constant in time (Figure 8). Therefore, the interaction of cisplatin with PS takes place not only in model membranes but also in biological membranes.

PS is exclusively located in the inner leaflet of the plasma membrane and contributes 20% to the phospholipids present in this leaflet (Op de Kamp, 1979). At this side of the membrane the pH and Cl^- concentration are lower than those of the extracellular compartment; i.e., the highest concentration of aquated, reactive cisplatin is formed at the side were PS is located. PS plays a crucial role in cellular processes such as signal transduction (Rando, 1988), cell proliferation and apoptosis (Sambrano, 1995), the blood clotting cascade (Krishnaswamy et al., 1992), and the biogenesis of mitochondria (Voelker, 1984) and is necessary for the activity of enzymes such as Na^+/K^+-ATPase (de Pont et al., 1978) and protein kinase C (Rando, 1988). For the latter enzyme the carboxyl acid moiety of PS is involved (Rando, 1988; Igarashi et al., 1995), which is obscured by the interaction with cisplatin. Indeed, effects of cisplatin on the activity of this enzyme have been reported (Kupper et al., 1994; Rubin et al., 1991) and now studies are underway in which the effect of the cisplatin−PS interaction on PS-dependent processes in biological membranes is investigated.

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