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cis-PLATINUM: SUBCELLULAR DISTRIBUTION AND BINDING TO CYTOSOLIC LIGANDS

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Abstract—In the present experiments the tissue uptake and subcellular distribution of Pt were measured in the liver and kidney of rats injected intraperitonially with 5 mg *cis*-diamminedichloroplatinum (*cis*-DPP)/kg. The binding of Pt to cytosolic ligands was also investigated. In both the liver and kidney Pt concentration was maximal within 24 hr following *cis*-DDP injection. Thereafter the clearance was bimodal with a rapid phase of about 48 hr (with 60–70% clearance) followed by a slow incomplete phase. In the kidney relatively high concentrations of Pt were measured in the microsomal, lysosomal and mitochondrial fractions. But in the liver the higher levels were found in the microsomal and nuclear fractions. On average 62% (kidney) and 89% (liver) of the cellular Pt were localized in the cytosol. At 24 hr following *cis*-DDP injection, about 52% of cytosolic Pt was present as low molecular weight species (molecular weight < 1000) and the remainder as protein-bound. About 25% was bound to a metallothionein-like protein in the liver as well as the kidneys. It is suggested that the high localization of cellular Pt in the cytosol and the presence of a high proportion as non-protein bound species may be important factors in relation to the therapeutic as well as the toxic effects of *cis*-DDP.

Cis-diamminedichloroplatinum (*cis*-DDP) is a platinum(II) coordinated complex that has demonstrated clinical activity in a broad spectrum of malignancies as a single agent and in combination chemotherapy [1, 2]. However, renal toxicity presently limits the dose that can be administered [3]. Toxicological studies in humans and animals indicate that lethal doses produce enzyme inhibition and histological damage in the gastrointestinal tract, bone marrow, lymph nodes, liver and kidney [4]. Clinically, even a single dose of *cis*-DDP exceeding 1.95 mg/kg is likely to produce renal impairment [5].

Pharmacokinetic studies in humans and animals have demonstrated that *cis*-DDP is eliminated from serum with a short initial half-life of less than 1 hr, and a slower terminal half-life of 1–3 days [6, 7]. The highest tissue levels of platinum are found in the kidneys, with detectable amounts present for as long as 4 months after *cis*-DDP administration [8, 9]. On a subcellular level, Choie *et al.* (10) have shown in rat kidney and liver that 70% of cellular Pt was in the cytosol and that only 40% was acid-precipitable as compared to 100% in the organelles.

Recently, it has been shown *in vitro* [11] that in cell lines exposed to *cis*-DDP the cytosolic Pt was to a large extent bound to metallothionein-like protein. Metallothioneins (MT) are inducible, sulfhydryl-rich intracellular proteins which are believed to play an important role in providing a protective mechanism against the toxic effects of Cd, Hg and perhaps other heavy metals [12–15]. Bakka *et al.* [11] have also demonstrated that the sensitivity to *cis*-DDP is significantly higher for human epithelial and mouse fibroblast cell lines without MT than that for corresponding MT-containing strains. However, the presence of Pt itself in the purified MT fraction was not demonstrated.

To understand the toxic or therapeutic effects of an agent it is useful to know its sites of localization and concentration within the cell and the molecular species with which it interacts. In this context, and in view of any role that MT might play in the detoxification of Pt, we have investigated the subcellular kinetics and binding of Pt to cytosolic ligands in the rat kidney and liver following a single i.p. injection of *cis*-DDP.

MATERIALS AND METHODS

Animal studies. Male Wistar rats weighing about 150 g were kept in separate cages in groups of four with access to food and water *ad libitum*. The rats were given a single i.p. injection of 5 mg *cis*-DDP/kg body wt as Cisplatin (Bristol Laboratories, Syracuse, NY) and subsequently sacrificed in pairs by exsanguination at 4 hr, 1 day, 2, 3, 4, 6 and 8 days later. The livers and kidneys were removed and placed in 0.25 M sucrose at 4°. Six control rats injected with 0.5 ml normal saline were similarly sacrificed at the end of the experimental period and the livers and kidneys removed as above. The dosing was repeated to obtain results in triplicate.

Subcellular fractionation. The livers and kidneys from each group above (4 hr, 1 day, etc.) were pooled and homogenized in 10% (w/v) 0.25 M sucrose at 4° as described previously [16]. The homogenates were initially subjected to differential centrifugation and the crude nuclear (N), mitochondrial (M), lysosomal (L) and microsomal (P) pellets obtained were then purified as shown schematically in Fig. 1. All steps

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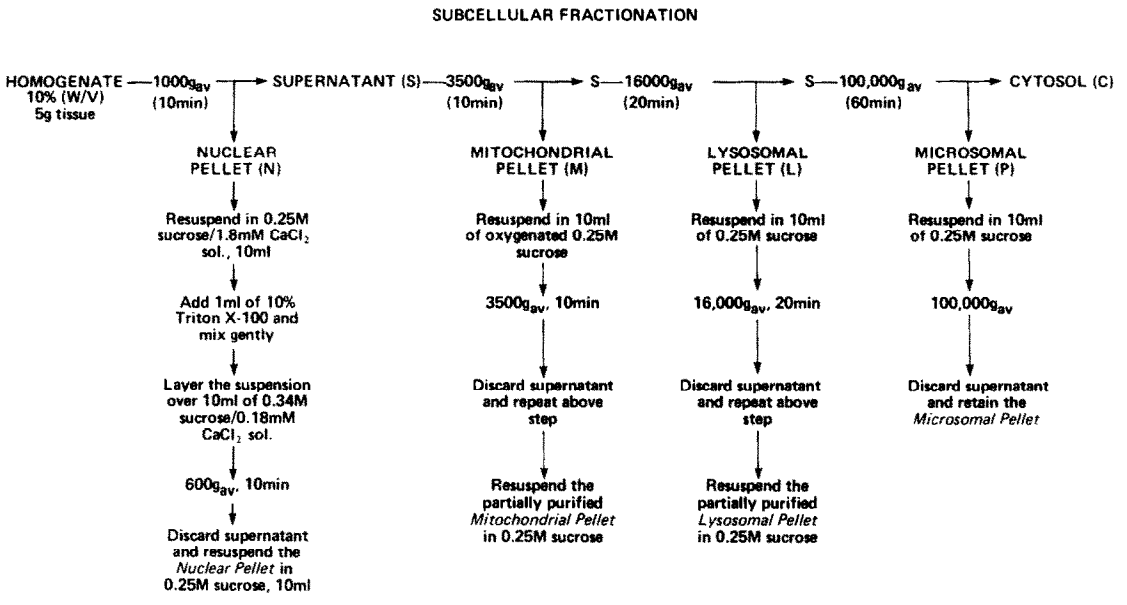

Fig. 1. A schematic representation of the procedure for cell fractionation.

in subcellular fractionations were carried out at 4° using a Beckman L-5 ultracentrifuge and a Sorvall RC-2B refrigerated centrifuge. The subcellular fractionation procedure is an adaption of a method described by Griffiths [17].

Electron microscopic examination of the subcellular fractions, and assays of protein, deoxyribonucleic acid (DNA), acid phosphatase (EC 3.1.3.2), and glucose-6-phosphatase (EC 3.1.3.9) were carried out to evaluate the degree of cross-contamination in the fractions. Protein concentrations were measured by the Biuret Method [18]. DNA was used as the marker for nuclear fraction and the concentrations in the isolated subcellular fractions were determined by a spectrophotometric method [19] in which *p*-nitrophenyl-hydrazine was used to quantitate the deoxyribose of DNA. The activities of acid phosphatase (lysosomal marker) and glucose-6-phosphatase (microsomal marker) in the subcellular fractions were estimated by the methods described by Ignarro [20] and Harper [21], respectively.

Sephadex chromatography. Aliquots of 5 ml cytosol were chromatographed on Sephadex G-75 columns equilibrated and eluted with 0.1 M ammonium formate/8 mM Tris–HCl buffer at pH 8.0. Fractions of 5 ml were collected at a flow rate of 45 ml/hr. The columns were calibrated for mol. wt estimations as described previously [22]. The cytosol eluate fractions corresponding to metallothioneins (mol. wt 6000–12,000) were pooled, heated at 75° for 3 min and centrifuged at 1000 *g*(av) for 5 min. The supernatants were analysed for Pt, and the absorbance at wavelengths 250 and 280 nm were also measured.

Determination of metals. The subsamples of tissue homogenates, subcellular fractions, cytosol and various supernatants were digested in concentrated nitric acid and analysed for Pt by graphite furnace atomic absorption spectroscopy as described elsewhere [23]. Analysis of Pt in the chromatographed fractions was carried out without any acid pretreatment and the concentrations of Pt were read against the corresponding standard addition calibration curves.

Table 1. Distribution of marker enzymes in subcellular fractions

Subcellular fraction	DNA* (Abs. unit/mg protein)	Acid phosphatase RSA†	Glucose-6-phosphatase RSA†
Liver			
Nuclear	0.34 ± 0.06	0.43 ± 0.08	0.38 ± 0.05
Mitochondrial	0.05 ± 0.01	1.58 ± 0.53	0.46 ± 0.08
Lysosomal	0.011 ± 0.002	5.53 ± 1.34	1.37 ± 0.54
Microsomal	0.018 ± 0.004	0.54 ± 0.08	3.08 ± 0.88
Cytosol	0.04 ± 0.01	0.38 ± 0.11	0.38 ± 0.06
Kidney			
Nuclear	0.30 ± 0.09	0.59 ± 0.16	0.26 ± 0.06
Mitochondrial	0.13 ± 0.04	1.96 ± 0.78	0.55 ± 0.15
Lysosomal	0.08 ± 0.03	3.60 ± 0.99	1.66 ± 0.72
Microsomal	0.07 ± 0.02	0.55 ± 0.13	3.67 ± 1.07
Cytosol	0.03 ± 0.01	0.33 ± 0.14	0.18 ± 0.04

* The concentration values of deoxyribonucleic acid (DNA) are expressed as absorbance units/mg protein, and are an average of four determinations ± S.D.

† The relative specific activity (RSA) values are an average of three determinations ± S.D.

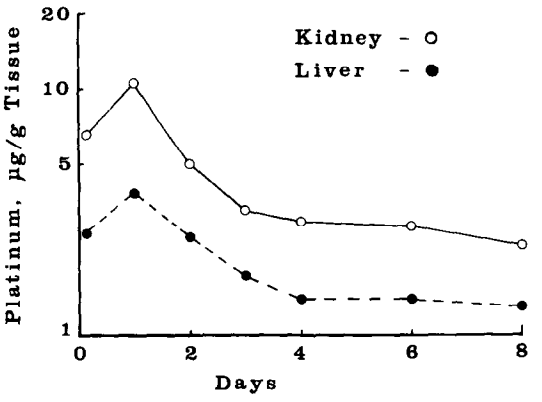

Fig. 2. Time course of Pt in the liver and kidney tissues following a single i.p. injection of *cis*-DDP.

RESULTS

Analysis of subcellular fractions

Electron microscopic examination of the purified subcellular fractions showed little visible cross-contamination. Table 1 shows the relative specific activity (% enzyme activity/% protein) of marker enzymes, acid phosphatase and glucose-6-phosphatase, and the concentration of DNA in the subcellular fractions. In both liver and kidney, the highest concentration of DNA was present in the nuclear fraction. Similarly the highest activities of acid phosphatase and glucose-6-phosphatase were in the lysosomal and microsomal fractions, respectively. The visible and quantitative measures of purity both indicated that the subcellular preparations were reasonably homogenous, although there were small degrees of cross-contaminations (this was mainly confined to the lysosomal fraction).

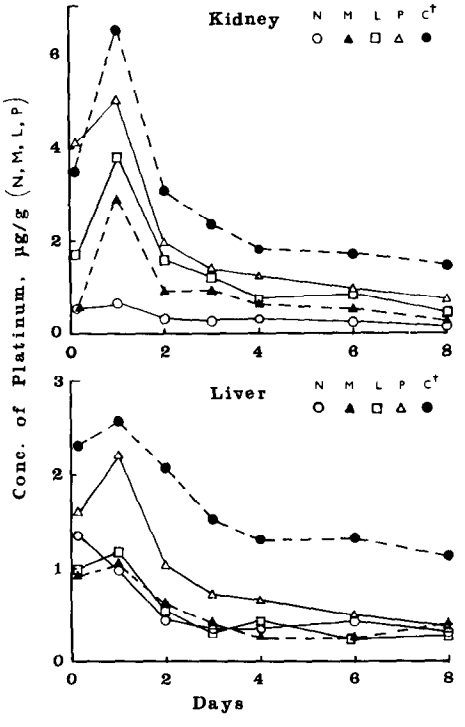

Fig. 3. Time course of Pt in the subcellular fractions of the liver and kidney tissues following *cis*-DDP injection. The symbols N, M, L, P and C denote the nuclear, mitochondrial, lysosomal, microsomal, and the cytosolic fractions, respectively. †The concentrations in the cytosol are expressed as μg/g tissue.

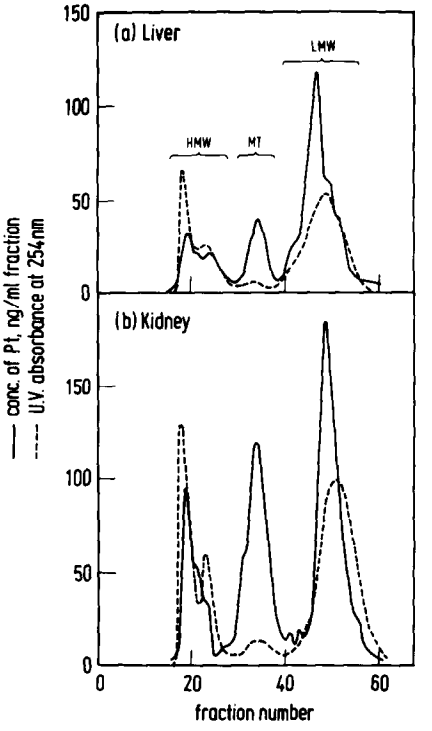

Fig. 4. The Sephadex G-75 elution profiles of cytosolic proteins and Pt-binding in the rat liver (a) and kidney (b) at 24 hr following *cis*-DDP injection. The eluate fractions corresponding to molecular weight > 20,000, 6000–12,000 and <1000 are defined as high molecular weight (HMW), 'metallothionein' (MT), and low molecular weight (LMW) fractions.

Tissue uptake and subcellular distribution of Pt

In both the liver and kidney the concentration of Pt was maximal within 24 hr after a single i.p. injection of *cis*-DDP (Fig. 2). The tissue clearance of Pt was bimodal with a rapid phase of about 48 hr followed by a slow, incomplete phase. Relative to the peak Pt concentrations in the liver (3.8 μg/g) and kidney (10.6 μg/g), about 55 and 70% of the tissue Pt were respectively eliminated during the initial clearance phase. In the terminal phase (days 3–8) the decrease in the liver Pt levels (33%) was greater than that in the kidney (23%).

The intracellular uptake and clearance of Pt followed a similar profile to that in the tissues as a whole (Fig. 3). It must be noted here that the cytosolic Pt concentrations are expressed as μg/g tissue, whereas those in the particulate fractions (N, M, L and P) are as μg/g fraction. The cytosol contained the highest amounts of cellular Pt in the liver (89%) as well as the kidneys (62%). In the kidney relatively high concentrations of Pt were measured in the

Table 2. Binding of Pt to cytosolic ligands in the kidney and liver of rats at 24 hr after a single *cis*-DDP injection

Cytosol	Concn of Pt, μg/ml cytosol ± S.D.		
	HMW fraction*	'MT' fraction*	LMW fraction*
Kidney	0.32 ± 0.04	0.34 ± 0.06	0.72 ± 0.09
Liver	0.21 ± 0.03	0.20 ± 0.03	0.41 ± 0.06

* The abbreviations HMW, 'MT' and LMW fraction denote the high molecular weight (mol. wt > 20,000), metallothionein (mol. wt 6000–12,000) and low molecular weight (mol. wt < 1000) fractions, respectively.

microsomal (5.0 μg/g), lysosomal (3.8 μg/g) and mitochondrial (2.9 μg/g) fractions at 24 hr after *cis*-DDP injection. The concentration in the nuclei was low (0.6 μg/g) and was even less than that in the liver nuclei (1.0 μg/g) at 24 hr. In contrast, the concentrations of Pt in the liver subcellular fractions were similar (average 1.1 ± 0.1 μg/g), except in the microsomes (2.2 μg/g).

Binding of Pt *to cytosolic ligands*

Typical elution profiles, obtained by chromatography of the liver and kidney cytosol on Sephadex G-75, are shown in Fig. 4. Eluate fractions corresponding to molecular weight > 20,000, 6000–12,000, and <1000 are defined as high molecular weight (HMW) 'metallothionein' (MT), and low molecular weight (LMW) fractions, respectively. The 'MT' fractions were heat-stable at 75° with an average recovery of 92 ± 4%. The heat-treated fraction had an $E_{250/280}$ ratio of 3.2. Since only supportive evidence is provided for the actual identity of the Pt-binding low molecular weight proteins, the term 'metallothionein', in inverted commas, is used to denote the Pt-binding protein.

In the liver as well as the kidney, at 24 hr after *cis*-DDP injection about 52% of the cytosolic Pt was present either bound to LMW species or as complex ion (Table 2). On average, 23 and 25% of cytosolic Pt were bound to the HMW and 'metallothionein'-like proteins respectively in both organs.

DISCUSSION

Renal impairment is the main toxic complication of *cis*-DDP therapy. The adverse effects are dose-dependent and cumulative [24]. The extent of cellular uptake and retention of Pt, particularly in the renal tissues, is therefore critical. It is clear from the results (Fig. 2) that during the first 72 hr the rate of clearance per g tissue was faster in the kidney than in the liver, and the renal Pt concentration was reduced from three times that in the liver to two times. But during the terminal phase the renal clearance was longer than that in the liver. The much higher kidney concentrations of Pt during the early phase has also been measured in other experimental animals [25, 26]. However Choie *et al.* [10] have reported that in rats injected (i.p.) with 7 mg *cis*-DDP/kg, the kidney and liver Pt concentrations were similar during the first 24 hr, but the kidney levels increased gradually to about three times that in the liver at 72 hr after the dose. The tissue and subcellular kinetics of Pt observed in our study are similar to those reported for antiarthritic gold(I) complexes [22], which have a similar reactivity in the body as *cis*-DDP [27]. As for gold, the lower clearance of Pt during the terminal phase, particularly in the kidneys, may similarly be responsible for the cumulative retention of Pt following multiple doses of *cis*-DDP. It is suggested that the significantly higher concentration of Pt in the renal tissues immediately after *cis*-DDP injection may to some extent explain the dose-dependent renal toxicity of *cis*-DDP.

In the kidney relatively high concentrations of Pt were found in the microsomal, lysosomal and mitochondrial fractions. Choie *et al.* [10] reported similar high concentrations in the microsomal and nuclear fractions. Following exposure to *cis*-DDP, the major site of renal toxicity is the tubules [28, 29]. It has been demonstrated in isolated tubules [30] that *cis*-DDP significantly inhibits Na^+, K^+ as well as Ca^{2+} and Mg^{2+}-ATPase activity, thus affecting such vital cellular functions as the mitochondrial synthesis of the nucleotide ATP, the Na^+–K^+ pump and the Ca^{2+}-pump. In this context the high concentrations of Pt measured in the kidney mitochondrial and microsomal (which also contains plasma membranes) fractions suggest that there might be an association between the renal toxicity of *cis*-DDP and the inhibitory effect of the complex on cellular ATPase.

In plasma up to 90% of the injected Pt is protein-bound [31]. Only about 48% of the cytosolic Pt was protein-bound in the liver and kidney tissues. This is in agreement with the finding of Choie *et al.* [10]. About 52% of the cytosolic Pt was associated with LMW species. The LMW Pt species are diffusible and the high reactivity of *cis*-DDP with nucleophilic biomolecules such as histidine and methionine [32], including divalent sulphur-containing species such as cysteine and glutathione [33], suggests that it may interact non-specifically with enzymes and proteins which are essential for normal biochemical processes. Since a large proportion of the cellular Pt was present in the cytosol, the extent of Pt protein binding and the reversibility of the interactions in the cytosol may therefore be critical in relation to its anti-cancer activity as well as toxicity.

The results also showed incorporation of cytosolic Pt into a metallothionein-like protein. The stimulation of MT biosynthesis as a response to metals such as Cd^{2+} [34], Hg^{2+} [35] and Au^+ [36], and the immobilization of the metal ions by MT is believed to provide a protective mechanism against the toxic

cations. In this regard a stimulated (e.g. by Zn pretreatment) intracellular binding of Pt to MT may be important. *In vitro* studies [11] carried out with human epithelial and mouse fibroblast cells have demonstrated that the low molecular weight Pt-binding protein in the cytosol is MT. Whether this is true for the 'MT-like' protein found *in vivo* experiments described here is yet to be determined. Even if MT is involved in the sequestration of intracellular Pt its role appears to be limited.

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