TOXICITY OF CISPLATIN AND ITS METABOLITES ON OVARIAN CANCER CELLS

By

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ABSTRACT

Cisplatin (CP) is one of our most useful antineoplastic drugs. When CP is dissolved in human plasma, different metabolites are formed. We examined the interactions of CP and its metabolites, namely aquated CP (AP), monomethionine CP (MP),bismethionine CP (BP) and CP dissolved in plasma ultrafiltrate (UP) on the OV 2008 human ovarian cancer cell line. By clonogenic assays, cell survivals (%) with AP, CP, MP, BP and UP were 3.28±0.73, 9.84±0.99, 15.93±1.11 , 76.83±2.13 and 13.11±0.74, respectively. AP was the most cytotoxic species, and BP was the least cytotoxic species. Cellular platinum (ng/10^6 cells) accumulation after addition of 0.33(#1), 1.6(#2) and 2.5(#3) mM of each species was:

[Species]	CP	AP	MP	BP	UP
#1	289±26	1080±90	138±5	24±3	187±26
#2	607±31	4352±145	372±8	56±2	498±69
#3	713±41	6610±279	710±8	78±2	711±96

There is a strong correlation between cytotoxicity of the platinum species and their cellular uptake (r=0.997). Infrared spectroscopy (IR) was done on OV 2008 cells after one hour exposure to different concentrations of species. IR data suggest that MP induced more

cellular changes than the other species. While bands of all CP metabolites attributed to the phosphate and amide II stretching indicate a frequency shift and intensity variation, those of methyl and methylene stretching vibrations of the cell membrane seem to be hardly affected. In conclusion, CP and its metabolites have different effects on OV 2008 cells.

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In the name of God ...

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LIST OF ABBREVIATIONS:

ATCC	American Type Culture Collection
Avg	Average
δas	Asymmetric Bending Mode
δs	Symmetric Bending Mode
cm^{-1}	Wavenumber
DNA	Deoxyribonucleic acid
IR	Infrared Spectroscopy
Pt	platinum
RNA	Ribonucleic acid
SE	Standard Error
νas	Asymmetric Stretching Mode
νs	Symmetric Stretching Mode

* "Species", "Metabolites" and "platinum compounds under investigation" are terms used for all of following solutions interchangeably:
cisplatin, aquated cisplatin, monomethionine cisplatin, bismethionine cispaltin and plasma ultrafiltrate cisplatin.

1

Cisplatin and its application in cancer chemotherapy:

1.1 - History :

As has been the case in many other medical developments, the discovery of antitumour activity in platinum amine complexes was somewhat fortuitous (Cleare,1980). Professor Barnett Rosenberg, of Michigan State University, was fascinated by the appearance of spindle cell formation during cell mitosis as they appeared to him to resemble lines of magnetic force such as those seen with iron filings around a magnet. Thus, a study was initiated to see if an electromagnetic field would influence cell division. The setting up of these experiments involved two pieces of good fortune. Firstly, AC current was passed through *Escherichia coli* bacteria in a growth chamber via a set of platinum electrodes, and secondly, the bacteria were supported in a nutrient medium containing ammonium chloride as the nitrogenous source. *E. coli*, and prokaryotic cells in general, do not show mitotic figures in division and were only being used to

test the equipment; this was another piece of good fortune. Under the influence of the current, the bacteria underwent filamentous growth. Bacterial rods, normally some 2-5 μm in length, formed strands up to some 300 times that length. Thus cell division was inhibited while cell growth was unaffected. Further tests showed that gram-negative rods were most sensitive with gram positive rods much less so; spherical bacilli (cocci) were unaffected.

A long series of control experiments showed that the current was not causing the filamentous growth but was causing some 10 ppm of platinum to dissolve from the electrodes. The species formed was identified as $[PtCl_6]^{2-}$, which is present in part as the ammonium salt.

Fresh solutions of $(NH_4)_2[PtCl_6]$ were bacteriostatic and inhibited cell growth at these concentrations (approximately 10 ppm); however, aged solutions (2-3 days) were very effective in producing filaments at low platinum concentrations. Further studies confirmed a photochemical reaction (Rosenberg,1967).

The inhibition of cell division, but not cell growth, suggested that these compounds might have antitumour properties; this was emphasized by the fact that known antitumour agents caused elongation and lysis in lysogenic bacteria. Initially, four compounds,

including cis-$[PtCl_4(NH_3)_2]$ and cis-$[PtCl_2(NH_3)_2]$, were tested against Sarcoma 180 in the mice and were found to be effective in inhibiting the tumour growth (Rosenberg,1969). Neither of the trans isomers showed any appreciable antitumour activity. The two cis isomers of the platinum compounds were submitted to the U.S. National Cancer Institute and screened against L1210 leukaemia in mice. These compounds showed potent antitumour activity and affected several cures with single injections at the therapeutic dose of 8 mg/kg of body weight. Rosenberg and Van Camp went on to show that cis-$[PtCl_2(NH_3)_2]$ was capable of regressing large solid Sarcoma-180 tumours (8 days old) in Swiss white mice (Rosenberg,1970). Cis-$[PtCl_2(NH_3)_2]$ (figure 1-int) appeared to be the more potent of the original compounds and has since been tested against many transplanted animal tumours where it has proved to have a wide spectrum of activity. It has undergone extensive toxicological and clinical trials leading to governmental approval for certain human tumours (see Sec. 1.5).

1.2 - Chemistry:

Since the original discovery of anticancer effects of platinum^Xxomplexes, numerous studies have been undertaken to determine the relationships that exist

between chemical structure and antitumour activity with a view to finding more active, less toxic drugs . This work has been discussed in several reviews and a summary is given here and in the following sections (Cleare,1974-a).

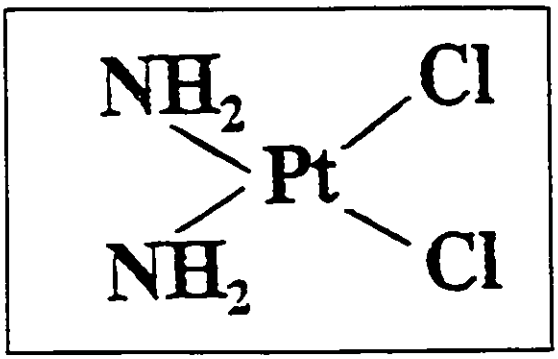

Figure 1-1: Chemical Structure of cisplatin

An obvious similarity between Rosenberg's original active complexes is the cis arrangement of the chloride ligands. The trans isomers have been found to be inactive (and usually non-toxic) in comparison to the corresponding active cis isomers. Thus, for platinum complexes, this had led to a concentration of effort on cis complexes of the general type cis-$[PtA_2X_2]$. Platinum complexes of the type $[PtX_2A_2]$ (X_2 = two monodentate anionic ligands or one bidentate anionic ligand; A2 = two monodentate amine ligands or one bidentate amine ligand) have attracted the most attention, while limited data on other systems have been reported. It has been established that antitumour activity is only found in neutral species and in cis rather than trans isomers. The X and A ligands have been systematically varied and the chemistry of active compounds has been investigated, particularly in kinetic

terms. Trans isomers are consistently more reactive in chemical reactions than their cis analogues. Thus, trans-$[Pt(NH_3)_2Cl_2]$ aquates approximately four times faster (Tucker,1964) than cis-$[Pt(NH_3)_2Cl_2]$, and undergoes ammonation some thirty times faster, although the corresponding cis equilibrium constants for these reactions are larger (Colvin,1968). Thus, trans compounds are likely to react more quickly and with a wider variety of body constituents and should be rather less specific in their action than cis compounds (Hoeschele,1971). Since only cis compounds have the potential to form chelates, this might imply that the anti-tumour activity is largely associated with a chelating interaction. Chemical studies on the $[Pt(NH_3)_2Cl_2]$ isomers have clearly established that the chlorides are the reactive ligands (See following sections). The Pt-NH_3 bond is very stable and ranks close to CN^- in affinity for PtII (Basolo,1967-a).

The chemistry of platinum(II) amine species of the type $[PtX_2A_2]$ is dominated by the high affinity of NH_3 for the Pt(II) centre. Affinities for common ligands vary (Basolo,1967-b):

$CN^- > NH3$ &/or $OH^- > I^- > SCN^- > Br^- > Cl^- > F^-$ &/or H_2O

The nature of the amine ligands (A) have a primary effect on the antitumour properties of the platinum compounds, while it is expected to have only a secondary

effect on reactivity via different steric. Regarding this group (A), the most interesting results have come from complexes with alicyclic amines. The sequence from cyclopropylamine through cyclooctylamine gives excellent results. These have the best therapeutic indices ever reported for the ADJ/PC6 tumour system (Cleare,1974-b).

The chemistry of cisplatin and its derivatives, which may lead to the discovery of new platinum compounds, has been reviewed extensively recently (Lippard,1982).

1.3 - Chemical Reactions:

The pharmacologic behaviour of cisplatin is in part determined by its reactions in water. These are described below:

1.3.1 - Stability and Hydrolysis:

The platinum coordination complexes have square planar geometry. As mentioned before, they have two "inert" ligands ($2NH_3$) in the two corners of the square and two "moderately labile" ligands ($2Cl^-$) in the other two corners of the square structure with the platinum in the centre.

The stability of cisplatin in 0.9% sodium chloride solution and the effects of pH and light on its degradation were studied by Zieske and his co-workers (Zieske,1991). Cisplatin was reconstituted with sterile water for injection to achieve a concentration of approximately 1 mg/mL in 0.9% sodium chloride solution. The solutions were placed in flexible polyvinyl chloride containers, clear and amber glass flasks, and plastic syringes and stored at 22-25°C in the dark or exposed to measured amounts of light and assayed periodically for up to 96 days. The stability of cisplatin in the solutions was determined by measuring the concentrations of cisplatin, trichloroammineplatinate(II) (TCAP), and trans-dichlorodiammineplatinum(II) (transplatin) with high-performance liquid chromatography, and by measuring solution pH.

TCAP was identified as the predominant degradation product of cisplatin in all the solutions. The rate of degradation was dependent on pH: In the dark, about 0.04% and 0.21% of the cisplatin degraded to TCAP per week at pH 4.3 and 6.3, respectively. Degradation also occurred during exposure to short-wavelength (350-490 nm) visible light.

Cisplatin undergoes hydrolysis in water (figure 1-2). These aqua complexes are weak acids and the related pH

and pK_a have been investigated by many authors (Theophanides,1980). Reishus and Martin have investigated the acid hydrolysis of cisplatin at 25 and 35°C (Reishus,1961). For the first acid hydrolysis :

$$cis\text{-}[Pt(NH_3)_2Cl_2]+H_2O \rightarrow cis\text{-}[Pt(NH_3)_2(H_2O)(Cl)]^+ + Cl^-$$

The equilibrium constant, K_1, in their study was $3.3x10^{-3}$ mole/L. and the rate constant was determined as $2.5x10^{-5}$ $sec.^{-1}$. They did not find any significant direct exchange between the chloride ligands of cisplatin and Cl^-. For the second acid hydrolysis:

$$cis\text{-}[Pt(NH_3)_2(H_2O)(Cl)]^+ + H_2O \rightarrow cis\text{-}[Pt(NH_3)_2(H_2O)_2]^{++} + Cl^-$$

The equilibrium constant, K_2, was $4x10^{-5}$ mole/L., at 25°C. The exchange of chloride with mono-aquated cisplatin occurred at a rate which is chloride-independent and is characterized by a first order rate constant, $k_2=3.3x10^{-5}$ sec^{-1} at 25°C. It was suggested that this exchange also occurs by only an acid hydrolysis mechanism.

Miller's work resulted in a model of the complete Ph-rate profile for the hydrolysis of cisplatin. This model is summarized in figure 1-3(Miller,1989 and 1991).

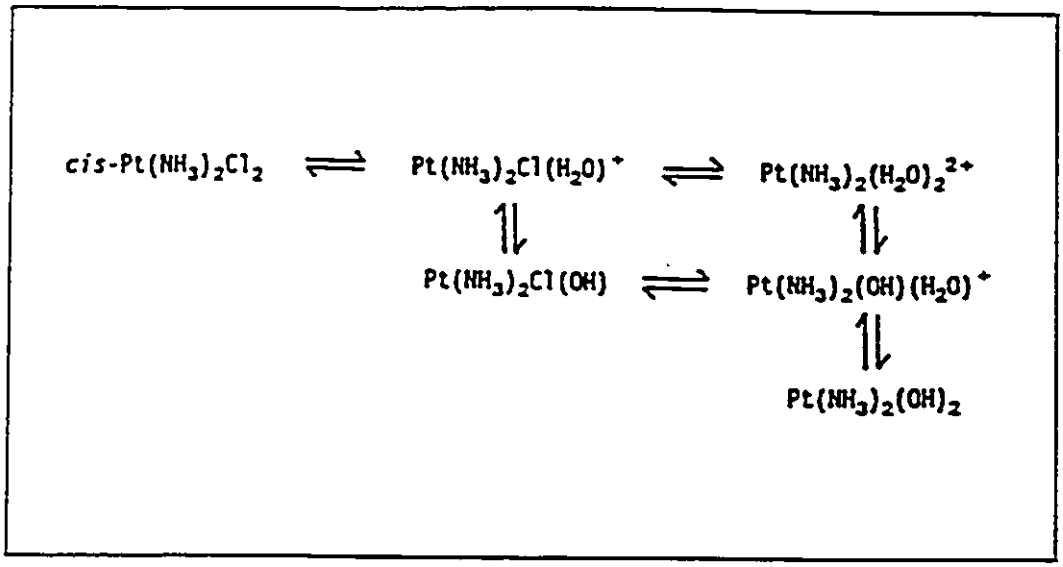

Figure 1-2: Hydrolysis of cisplatin.

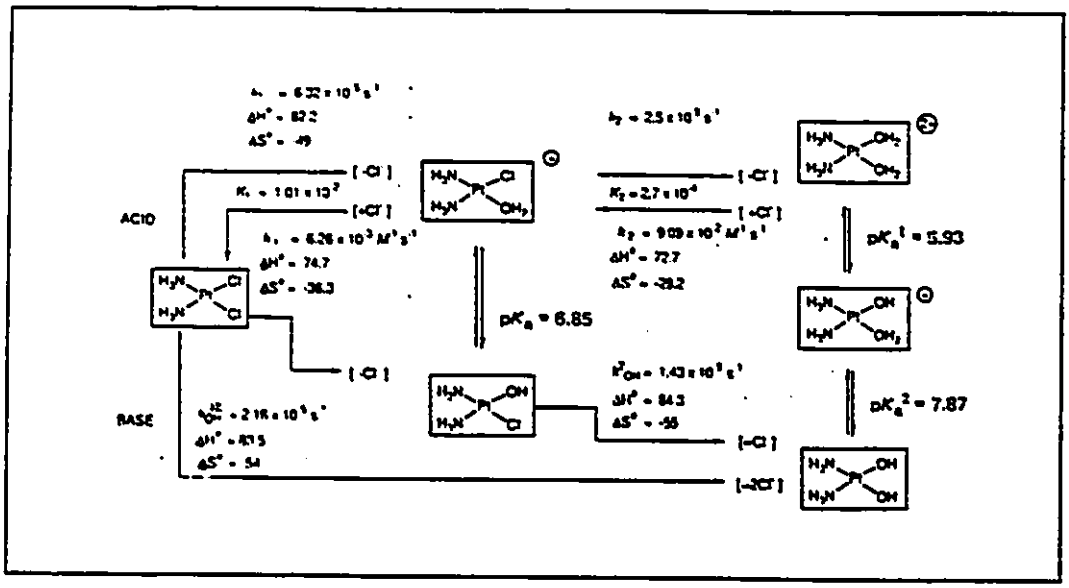

Figure 1-3 : Rate and equilibrium constants for cisplatin and its hydrolysis products in acidic and basic conditions at 25°C.

Cisplatin undergoes acid hydrolysis in aqueous solution. This reaction is pH dependent. Berners-Price and co-workers (1992) have determined the pK_a values of mono- and di- aquated cisplatin to be 6.41 and 5.37, respectively, via the use of ^{15}N-edited ^{1}H NMR spectroscopy. They found that the reactions of these species are complicated by further formation of hydroxo-bridged polymers (...Pt-O-Pt...) at different pHs. These hydroxylated species have the following general formula;

cis-$[PtCl(OH)(NH_3)_2]$

cis-$[Pt(OH)_2(NH_3)_2]$

cis-$[PtCl(OH)(H_2O)(NH_3)_2]^+$

Figure 1-4 presents the plots of ^{1}H NMR chemical shifts vs. pH for the above mentioned species. Their results will therefore allow more accurate preparation of different species and detailed investigations of the pathways of reactions of cisplatin under biologically-relevant conditions.

1.3.2 - Reactions with DNA :

We are still not in a position to pinpoint the exact products responsible for anti-tumour activity of cisplatin in a biological regime. However, a review of some work will help to predict the mechanism of action of cisplatin.

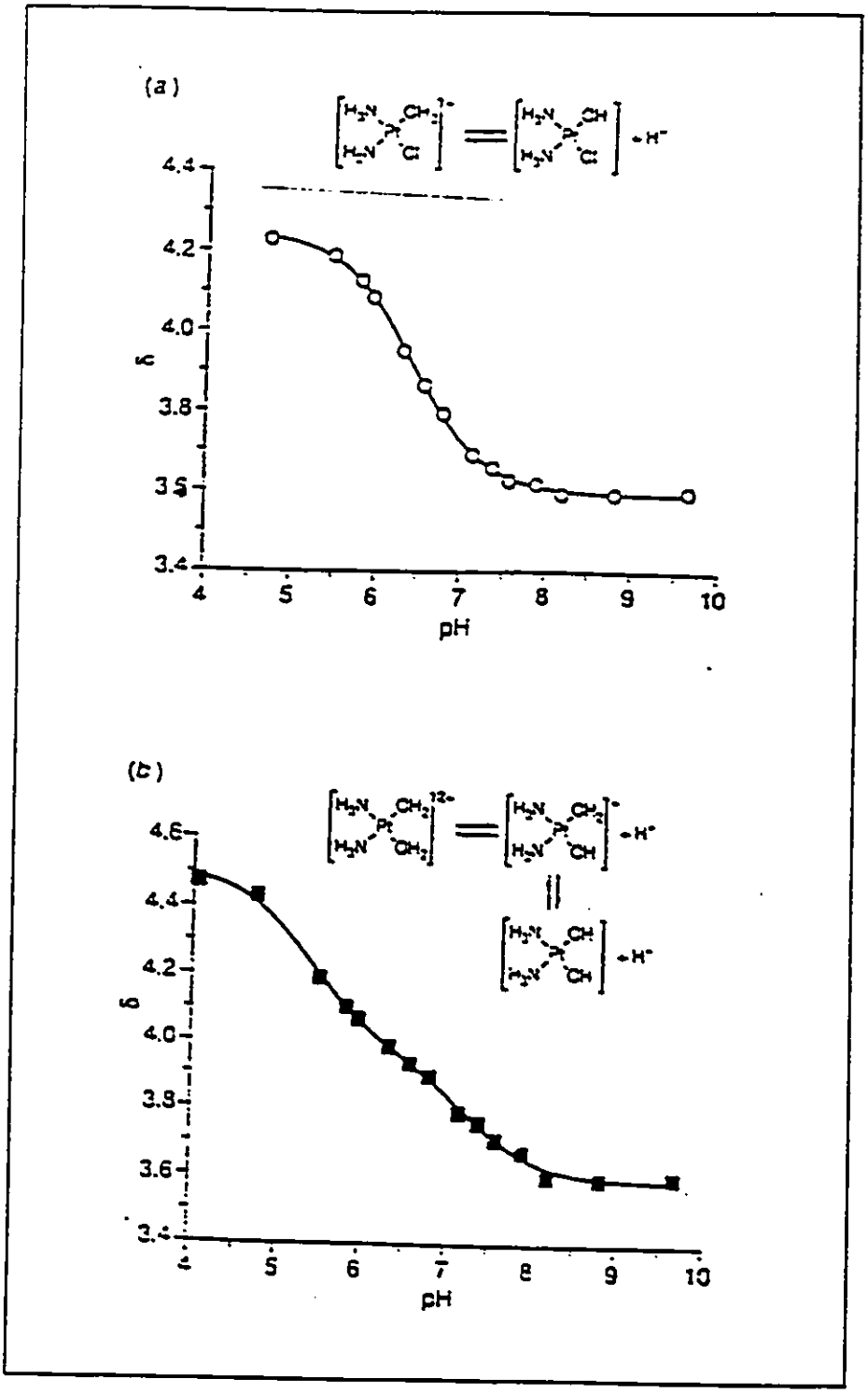

Figure 1-4 : Plots of ^{1}H NMR chemical shifts vs pH: a) *cis*-$[PtCl(H_2O)(NH_3)_2]^+$ & b) *cis*-$[Pt(H_2O)_2(NH_3)_2]^{2+}$. From Berners-Price et al. (1992).

Blood plasma has a pH of about 7.4 and under these conditions, base hydrolysis becomes a real possibility. The equilibrium end product composition would then be about 50% cis-$pt(OH)_2(NH_3)_2$ and 50% cis-$ptCl(OH)(NH_3)_2$ (Miller,1990) (See previous section).

The generally accepted mechanism for the interaction of cisplatin with DNA molecule is as follows (Rosenberg,1978): With clinical administration, cisplatin enters the blood stream and the neutral molecule passes through the cell wall. Once inside the cell, hydrolysis will occur, because of the low chloride concentration in the cell (about 4 mM); the positively charged (chloro)(aqua) product is the most likely labile complex for donor groups in the DNA target. On the other hand, if the base hydrolysis process were to operate at pH=7.4 (the pH of blood plasma), then an entirely different model could be proposed for the mechanism of cisplatin-DNA interaction. In this way, the final product would be a mixture of cis-$Pt(OH)(NH_3)_2(OH_2)^+$, cis-$PtCl(OH)(NH_3)_2$ and cis-$Pt(OH)_2(NH_3)_2$ (1989). The di(hydroxo) and (chloro)(hydroxo) forms are relatively inert to substitution, but capable of cell wall penetration in the neutral form, whereas the (hydroxo)(aqua) of (chloro)(aqua) ions would be the active reagent for attack by polar donor groups in the DNA target molecule. The

rapid protolytic equilibria between the (chloro)(hydroxo) and the (chloro)(aqua) species would allow facile transport of platinum species to suitable DNA sites. A speculative model is presented in figure 1-5.

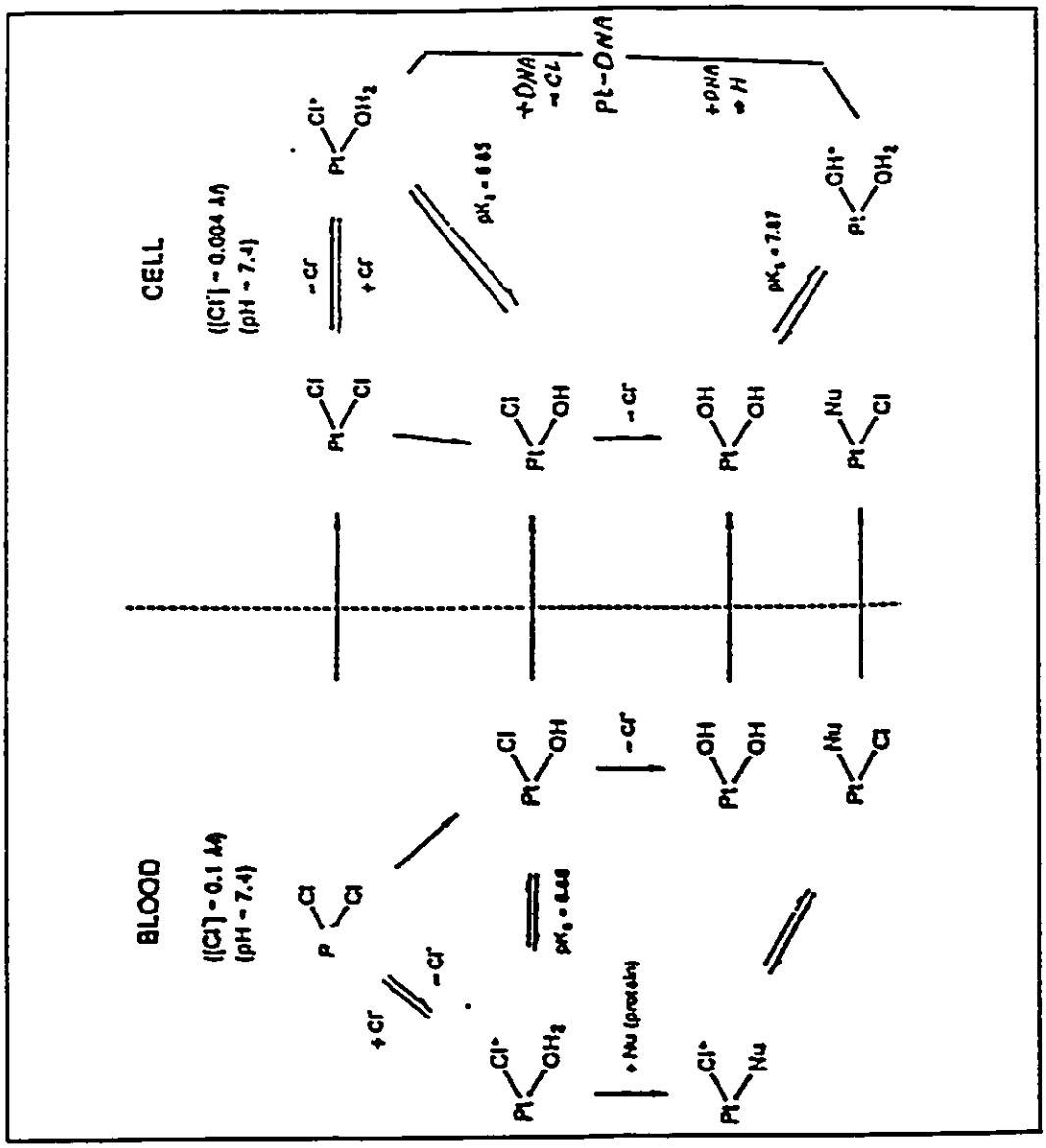

Figure 1-5 : A model of cisplatin modifications at the different pHs from the blood to the DNA. (NH_3 ligands have been omitted, Nu; Nucleophile)

The interaction of cisplatin and its analogues with DNA was reviewed by a number of investigators. The two chloride ligands can (usually after aquation) react with two guanine sites so as to produce crosslinks (Miller,1993). It has been established that platinum salts react preferentially with the N_7 site of guanine in nucleosides, nucleotides and DNA. Cisplatin reacts with guanine (G) first by substituting one of its two chlorine atoms with the N_7 atom of guanine, thus binding to DNA like a carcinogen (See figure 1.6- A).The second chloride reacts simultaneously or subsequently with a basic site nearby a distance of 2.9 to 3.4 Å. This second coordinating basic site may be another G molecule on the same strand of DNA just above or below the first G, again through the N_7 site and located at a distance of 3.4Å from the first N_7 forming an intrastrand cross-linked complex, (figures 1.6 and 1.7). The formation of an intrastrand cross-linked platinum complex involving N_7 sites are repairable. The repair mechanism of excision, re-synthesis of DNA by a DNA polymerase and the welding repair by ligase are shown in figure 1.6.C. However, formation of a cross-link with the other base on the other strand of DNA is cytotoxic (Gellert,1979).

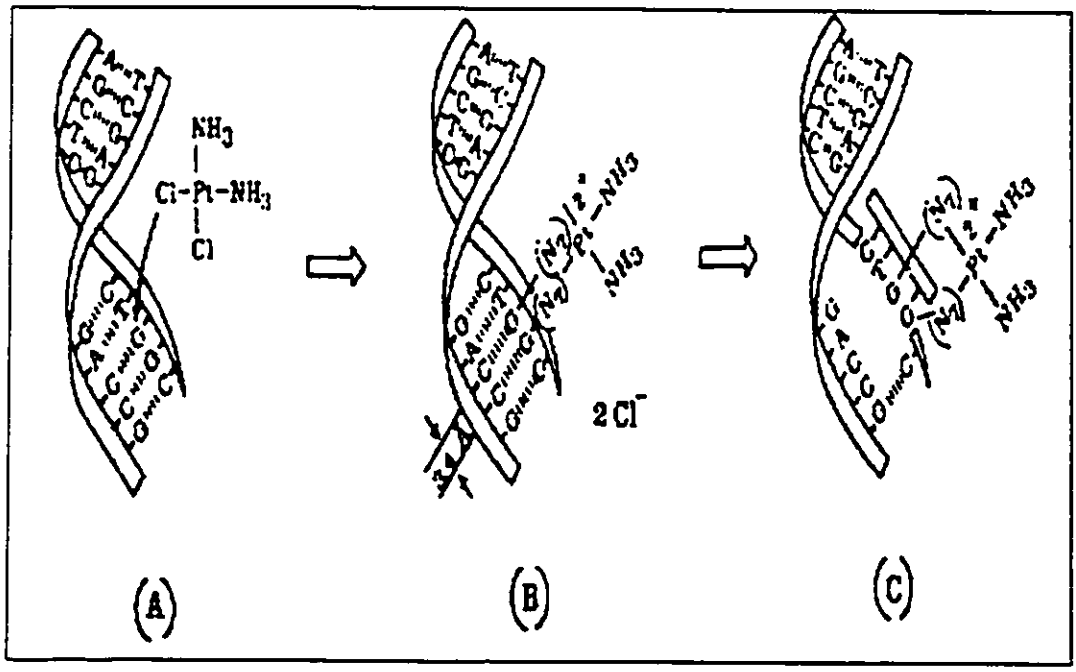

Figure 1.6 : Interaction of cisplatin with DNA.

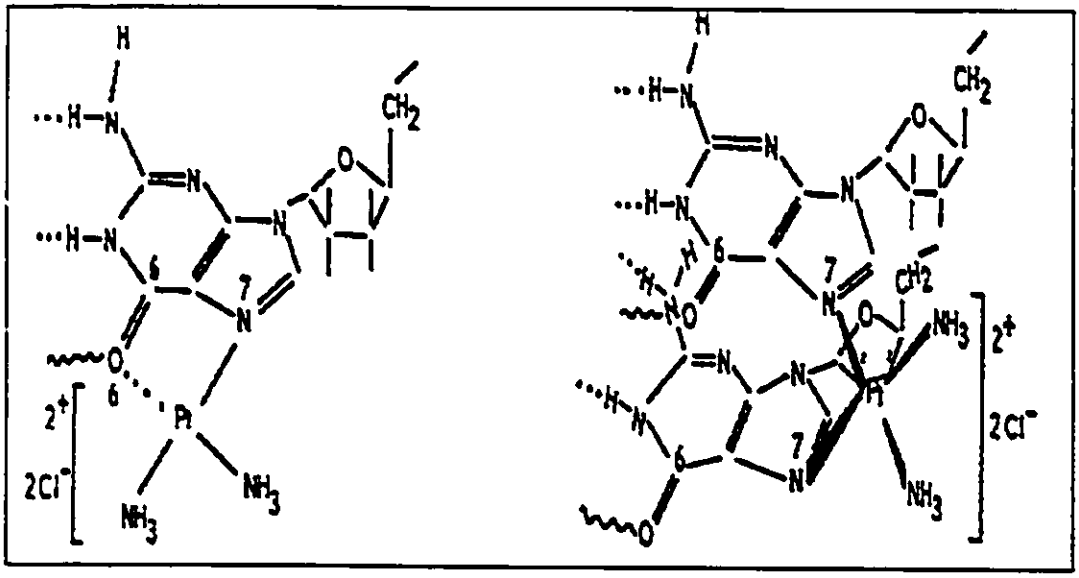

Figure 1.7 : Proposed model of binding of cisplatin to guanine bases.

1.4 - Pharmacology :

Chemistry and a part of the metabolism of cisplatin has been reviewed before. Some other important pharmacological aspects of this drug will be presented here.

1.4.1 - Clinical Applications :

Cisplatin is one of the most commonly used cancer chemotherapy drugs (Reed,1993). Combination cisplatin chemotherapy is curative for 85% of patients with advanced, testicular cancer. The drug is also potentially curative for advanced carcinoma of the ovary. Cisplatin causes significant responses in cancers of the bladder, head and neck (Douple,1990), small and non-small cell carcinoma of the lung; and some neoplasms of childhood. Interestingly, the drug also sensitizes cells to the cytotoxic effects of radiation therapy (Goodman and Gilman,1990). Its usual intravenous dosage is 50-125 mg/m^2 with a frequency of once every four weeks, when given as a single agent (Kalant,1989), 50-70 mg/m^2 as single dose every 3 weeks or 20 mg/m^2 for 5 days (Katzung,1992).

The usefulness of cisplatin as an anti-tumour drug is limited by drug toxicity and drug resistance. Some strategies that are being developed to overcome drug resistance include a) increase in dose-intensity, b) modulation of drug resistance, and c) the development of analogues of cisplatin. The latter strategy (c) is also being used to decrease drug toxicity (Freed,1993). We will be trying to further characterize the behaviour of cisplatin by studying the actions of the metabolites that are formed in plasma when it is administered to patients. This analysis may be of help in overcoming drug toxicity and drug resistance in the future.

1.4.2 - Pharmacokinetics :

1.4.2.1 - General parameters :

Cisplatin is not effective when administered orally. After rapid intravenous administration, the drug has a distribution half-life in plasma of 25 to 50 minutes; concentrations decline subsequently, with a elemination half-life of 58 to 73 hours. The half-life of the drug is longer in patients who receive high doses of cisplatin.

More than 90% of the platinum in the blood is bound to plasma proteins. High concentrations of cisplatin are found in the kidney, liver, intestine, and testes, but there is poor penetration into the CNS. Only a small portion of the drug is excreted by the kidney during the first 6 hours; after 5 days up to 43% of the administered dose is recovered in the urine. When given by infusion instead of rapid injection, the plasma half-life is shorter and the amount of drug excreted is greater. Biliary or intestinal excretion of cisplatin appears to be minimal. (Goodman and Gilman, 1990)

1.4.2.2 - Cell Membrane Transport :

It has been generally supposed that cisplatin enters the cell largely through passive diffusion (Gately,1993). However, there are several characteristics of intracellular cisplatin accumulation that seem incompatible with only simple passive uptake because it is: 1) energy dependent, Na^+ dependent and ouabain inhibitable (Andrews,1988) ; 2) stimulated by reduction in osmotic strength and pH (Smith,1989) 3) inversely related to membrane potential ; 4) stimulated by elevation of cAMP level (Andrews,1990); and 5) inhibited by aldehydes

(Dornish,1989-a). Despite this evidence, classical proof for a carrier system is lacking in that accumulation is not saturable up to 3 mM and can not be inhibited with structural analogs (Mann,1990). Some of these aspects will be described in detail below.

A double reciprocal plot of platinum accumulation vs drug concentration yields a straight line through the origin, indicating that the rate limiting factor for platinum uptake is the concentration of drug (Gale,1973 and Binks,1990). These observations are in agreement with a passive diffusion of cisplatin. A strong argument against the presence of active transport is that the accumulation is not inhibited by structural analogs of cisplatin (Andrews,1987-b). The only compound that inhibits cisplatin accumulation is cis-$PdCl_2(NH_3)$. However, even this analogue produces only 5% inhibition, which is attributed to non-specific damage from the highly reactive drug. Uptake of cisplatin is not saturable. This observation was confirmed by measuring the cellular uptake of cisplatin using either atomic absorption or radioactive cisplatin (Hormas,1987).

On the other hand, there are data suggesting that some components of cisplatin uptake must be mediated by a form of transport mechanism. Byfield postulated that cisplatin may be entering the cell via a carrier mediated

process (Byfield,1981). This hypothesis came from the observation that cisplatin showed differential toxicity in cycling and resting human T lymphocytes, a pattern shared by alkylating agents with known carriers such as melphalan and nitrogen mustard, but not in agents that enter the cell through passive diffusion, for example, mitomycin C.

The protein synthesis inhibitor benzaldehyde reduces the cytotoxicity of cisplatin, while another inhibitor of protein synthesis, cycloheximide, dose not have the same effect. Considering some control experiments, it has been postulated that the benzaldehyde reacts with membrane proteins to inhibit the uptake of cisplatin (Dornish,1986). It has been determined that a large number of aldehyde compounds inhibit the uptake of cisplatin, presumably by forming Schiff bases with membrane proteins (Dornish,1989-b). Cisplatin accumulation is potassium dependent, and therefore also appears to be dependent on the membrane potential. When the membrane is depolarized by incubation in high potassium media, the cells accumulate 5.4-fold more cisplatin than control cells (Andrews,1991). The uptake of cisplatin has also been shown to be inhibited by the overexpression of the c-Ha-ras oncogene by about 40% (Isonishi,1991). Cisplatin accumulation is also affected by a number of other intracellular signalling mechanisms, including protein

kinase C (PKC), protein kinase A (PKA), and the Ca^{++} calmodulin pathway. Calmodulin antagonists have been shown to decrease cisplatin accumulation (Kikuchi,1990). AMP-induced activation of PKA increases the cellular accumulation and toxicity of cisplatin in human 2008 cells (Mann,1991). Howell et al. showed that the nucleoside transport inhibitor dipyridamole increased cisplatin accumulation in 2008 cells in a dose-dependent manner (Howell,1987).

In conclusion, various studies present different mechanisms involved in the membrane transport of cisplatin, in favour of either active or passive uptake of the drug. Although there have been a number of studies of the uptake and accumulation of cisplatin, there has been relatively little work reported on the efflux of this drug. We will review some of these reports in Chapter 3.

1.4.3 - Mechanism of Action :

The cytotoxicity of cisplatin is believed to be mainly due to the formation of DNA adducts (Munchausen, 1975), which include DNA-protein cross-links, intermolecular cross-links, DNA monofunctional, and interstrand and intrastrand DNA cross-links (Figure 1.8).

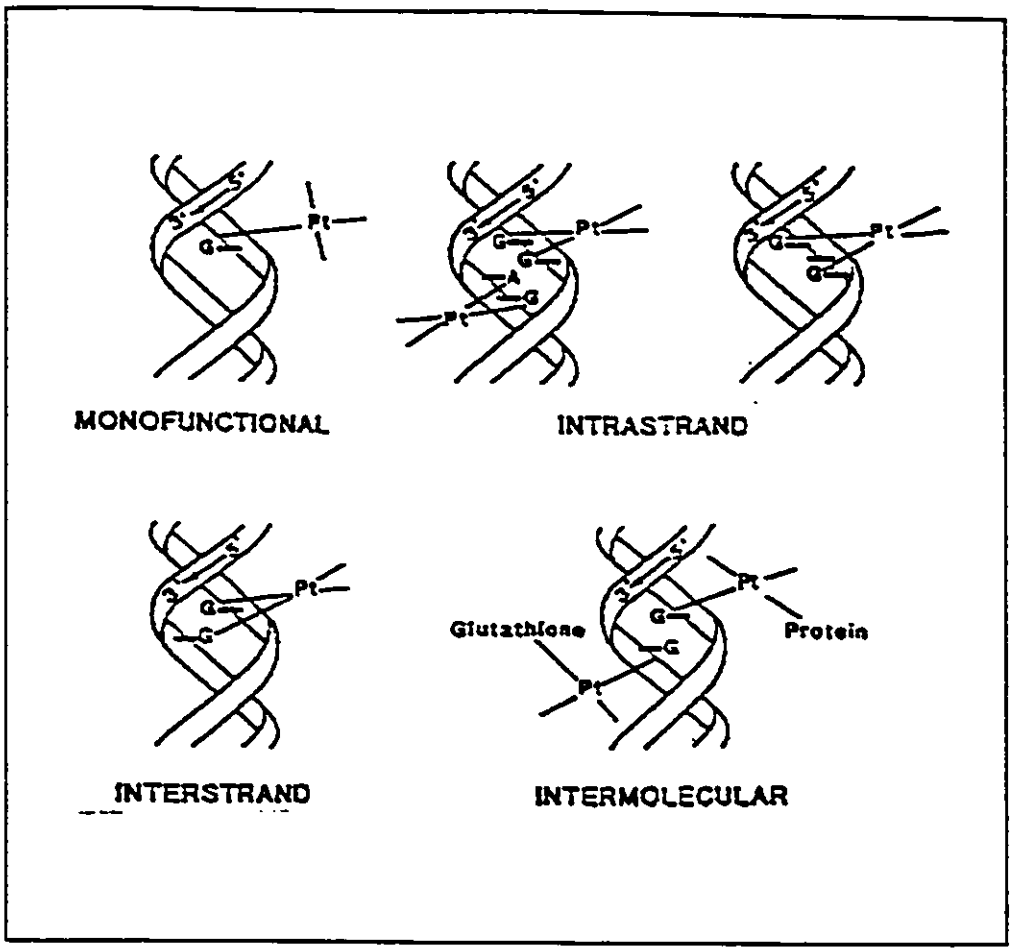

Figure 1.8 : Structures of various adducts produced in DNA by cisplatin.

The interactions of cisplatin with DNA have been defined in detail; however, the interactions of this drug with other components of the cell are less well understood.

1.4.3.1 - DNA Interactions :

Quantitative studies show that 1,2-intrastrand d(GpG}, and d(ApG) cross-links account for 65 and 25%,

respectively, of the cisplatin adducts formed in vitro (G: Guanine) (Eastman,1986). X-ray diffraction of the cross-linked dinucleotide cis-$Pt(NH_3)_2$(d(pGpG)) reveals that the two guanines are completely destacked. Thus, the intra-strand cisplatin cross-link produces a severe local distortion in the DNA double helix, leading to unwinding and kinking (Bellon,1991).

The extent of cisplatin-pGpG cross-link formation in leukocytes from different cancer patients may differ up to 4.5-fold (Vermorken,1986). Fichtinger-Schepman *et al.* also reported a positive correlation between the extent of in vitro and in vivo formation of cisplatin-pGpG cross-links in DNA of human leukocytes (Fichtinger-Schepman,1987). Interindividual variation in the level of cisplatin-DNA adducts has also been reported for rat tissues (Reed,1987). In vivo experiments showed that nuclear staining density measurements of buccal cells from ten cancer patients after infusion of ≥20 mg/m^2 cisplatin were always higher than pretreatment values; however, variation in DNA modification by cisplatin in pretreatment buccal cells from the same patients treated in vitro with 33 μM cisplatin (therapeutic concentration) for 1 hour was low (Terheggen,1988).

The binding of cisplatin to DNA is not in itself sufficient to cause cell death. Cisplatin is much more

toxic to dividing cells than to resting cells, suggesting that some cell-cycle associated event is required for its toxicity (Frava,1979). Although its action is not dependent on a specific phase of the cell cycle, cisplatin can be up to 10 times more toxic to cells that are about to enter into S phase (Dornish,1987). Moreover, its cytotoxicity is a function of the amount of DNA damage remaining at the time cells enter S phase (Pera,1981). These observations, plus the finding that DNA synthesis is inhibited in a variety of cells following platination have led to speculation that inhibition of DNA synthesis may be the critical step in toxicity (Harder,1970).

However, DNA repair-deficient Chinese hamster ovary cell that were highly sensitive to cisplatin were found to progress through S phase at a normal rate following exposure to the drug; the cells then arrested in G_2 and subsequently died. There was no apparent inhibition of DNA synthesis in these cells during S phase (Sorenson,1988). Thus cell death does not correlate with inhibition of DNA synthesis. In contrast, DNA repair-competent cells could survive incubation with higher concentrations of cisplatin that inhibited DNA synthesis and slowed passage through the S phase. These cells also arrested in G_2 following exposure to the drug. It should be emphasized that although G_2 arrest appears to be a prerequisite for cell

death (except at very high drug concentrations), all such arrested cells do not die. At minimally toxic concentrations of cisplatin, cells may eventually bypass the block and return to normal cycling. Hence, there are two fates for a G_2-arrested cell: survival or death (Eastman,1990). In any event, study of the relationship between cell killing and the binding of cisplatin to DNA in six different mammalian cell lines indicates that the initial level of cisplatin-DNA binding may be a reasonable predictor of sensitivity to this chemotherapeutic drug (Terheggen,1990, Chu,1994).

1.4.3.2 - Apoptosis :

In cells that eventually die with cisplatin, total transcription, polyadenylated RNA synthesis, and protein synthesis were found to be markedly inhibited only after 48 hours. Nicotinamide adenine dinucleotide (NAD) and adenosine triphosphate (ATP) levels decreased after 3 days. Cell membrane integrity was lost after 4 days. These results demonstrate that cells can be lethally damaged, yet continue to undergo apparently normal metabolic activities for several days. DNA double-strand breaks are detectable after 1 day, but breaks are visible as

fragmentation in the nucleosome spacer region of chromatin after two days. This type of damage is consistent with cell death occurring by the process of apoptosis (Sorenson,1990).

The analysis of cell death induced by cisplatin reveals DNA fragmentation into multimers of 180 base pairs, consistent with internucleosomal cleavage of chromatin by an endonuclease, followed by loss of membrane integrity and cell shrinkage. This process is inhibited by cycloheximide, suggesting that death requires new protein synthesis. These findings are consistent with the induction of programmed cell death or apoptosis (Barry,1990). Furthermore, studies with repair-deficient cells indicate that apoptosis is triggered by the presence of cisplatin- DNA adducts.

There is nothing unique about cisplatin that facilitates the activation of apoptosis. However, it is of particular interest that under conditions that kill 90% of the cells, the appearance of DNA degradation is almost always delayed for several days after drug treatment. With cisplatin, this delay is associated with arrest in G_2 phase of the cell cycle. In contrast, following heat treatment, DNA digestion occurs within 30 min. At high concentrations of cisplatin, DNA digestion can be observed within 6 hours. Hence, passage to G_2 is not essential for

cell death but may facilitate the process. Indeed, cell death induced by physiological rather than pharmacological agents often appears to occur during the G_1 phase of the cell cycle (Eastman,1990). It has been shown that 60 and 70% of the initial cell number of a human ovarian carcinoma cell line were described as showing apoptotic changes 72 hours after treatment with 10 or 25 μM cisplatin, respectively. However, this apoptotic cell death was not associated with degradation of DNA into nucleosomal-sized fragments (Ormerod,1994-a, 1994-b). Electron microscopy of the kidney tissue of Male BDF_1 mice, 4 days after i.p. injections of cisplatin showed many lesions, characteristic of apoptosis (Montpetit,1994).

1.4.3.3 - Interactions with Proteins :

Although DNA synthesis has been reported to be more sensitive to cisplatin than either RNA or protein synthesis (Roberts,1979), the role of cisplatin interactions with proteins have been considered recently. The combination of HPLC with a dual detection system of on-line UV spectroscopy and off-line flameless atomic absorption spectroscopy permits quantifiable separation of cisplatin, its reaction products with various peptide and

protein substrates, and the unreacted substrates (Riley,1982-b). Platinum compounds are irreversibly bound to plasma proteins (Repta,1980). In vitro binding to human plasma proteins is different for different platinum compounds, corresponding to the initial half-lives of those compounds in plasma and their decomposition half-lives of intact drug in plasma ultrafiltrate (Van der Vijigh,1986). There are reports that human malignant glioma cells may effectively repair DNA interstrand cross-links induced in their genome by cisplatin (Sriram,1990). Ali-Osman and coworkers have shown that the potentiating effect of novobiocin on cisplatin cytotoxicity in brain tumour cells is, at least in part, the result of decreased repair of DNA interstrand cross-links induced by cisplatin in the cells (Ali-Osman,1993).

Are there other proteins that bind to cisplatin damaged DNA? Cisplatin-cross-linked DNA(CCD) probes have been used in the gel mobility shift assay to identify such proteins in cell extracts (Chu,1988). A small mobility shift suggested involvement of a protein with a low molecular mass. A second factor, cisplatin-DNA damage recognition protein, was also specific for cisplatin adducts and had a molecular mass of 100 kDa (Toney,1989).

CCD probes were also used to screen a human cDNA expression library, identifying a cDNA encoding an 81-kDa

structure-specific recognition protein (SSRP1) that bound to cisplatin but not to its trans isomer (Bruhn,1992).

There are several possible mechanisms for how binding proteins might confer sensitivity to cisplatin. For example, the proteins may shield cisplatin adducts from repair or may signal apoptosis (Donahue,1990). Alternatively, the binding of proteins to the cisplatin adduct may lead to cell cycle arrest and trigger a program for cell death. The binding of proteins to cisplatin adducts may enhance blocks to replication of transcription, or may interfere with control systems that induce cell cycle arrest after DNA damage (Chu,1994). Clugston and coworkers have demonstrated the presence of at least two protein complexes in human cell extracts which bind selectively to DNA modified with cisplatin, Using monoclonal antibodies, they have shown that one complex, namely B1, contains human single-stranded DNA binding protein, a protein known to be involved in the in vitro repair synthesis assay of mammalian excision repair (Clugston,1992). Single-stranded DNA binding proteins have previously been suggested to have a role in the recognition of damaged DNA prior to DNA repair on the basis that the T4 gene 32 protein has a higher affinity for DNA modified by chemical adducts, including cisplatin, than for unmodified DNA (Toulme,1983). These mechanisms

may have important roles in the cellular resistance to the drug (see the following sections).

1.4.3.4 - In vivo Studies :

In vivo studies in rats illustrated that in addition to the platination of DNA and nuclei proteins, significant amounts of platinum are also incorporated into the chromosomal protein and cytosolic fractions. The localization of platinum in the cytosolic fractions was highest in the kidney, followed by the tumour, and lowest in the liver when determined as the fractional percentage of the total amount of injected cisplatin (Parti,1990). Cisplatin showed a significant high affinity for nuclei and microsomes in the kidney, but not in the liver. Renal mitochondria and plasma membranes were associated with low concentrations of platinum (Choie,1980). However, it has been suggested that the high localization of cellular platinum 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 cisplatin (Sharma,1983).

1.4.4 - Cellular Resistance to Cisplatin :

There are questions with respect to how cisplatin gets into cells, how it is transformed and inactivated,

how it and its biotransformation products efflux from the cell, how the DNA damage is repaired, and how all of these processes can be modulated for therapeutic gain.

Recent years have seen much progress toward defining the cellular pharmacology of cisplatin and, in the process, several mechanisms of acquired resistance to this drug have now been elucidated. A model that focuses on the biochemical systems that modulate the cellular pharmacology of cisplatin is outlined in figure 1-10. One might predict that a cell's acquired tolerance to a noxious xenobiotic like cisplatin would include changes in key aspects of its cellular pharmacology, such as membrane permeability, detoxification pathways and the ability to remove cytotoxic lesions from DNA. This review will focus on how specific alterations in cellular pharmacology can produce resistance to the cytotoxic effects of cisplatin.

1.4.4.1 - Accumulation :

Decreased cisplatin accumulation has been a consistent finding in many cisplatin-resistant cell lines from a variety of species. The decreased drug accumulation found in most of these resistant cells could thus be attributed to decreased influx, decreased intracellular binding or sequestration, or increased efflux (chapter 3).

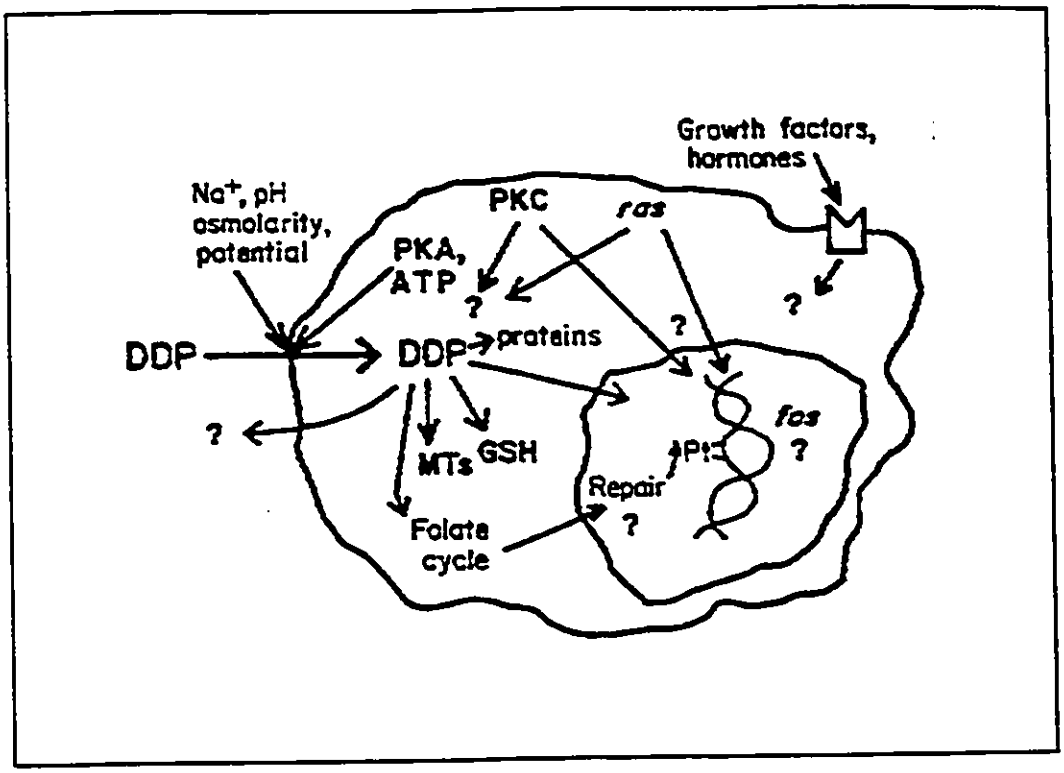

Figure 1-9 : Model depicting the possible elements involved in cellular pharmacology of cisplatin (DDP).
Abbreviations: PK:protein kinase. MTs:Metallothioneins. GSH:Glutathione

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The biochemical change underlying this decreased drug influx is presently unclear. It is assumed that a change in the passive permeability of the plasma membrane is responsible (Popovic,1993) because cisplatin- resistant 2008 cells do not have the same membrane fluidity as sensitive cells, and some changes in the cellular phospholipid composition were found (Popovic,1994).

However, if this is true, this should affect both influx and efflux. Timmer-Bosscha et al. also found changes in the lipid composition of cisplatin-sensitive versus cisplatin-resistant small cell lung carcinoma cells, although the latter cells do not have an accumulation defect (Timmer-Bosscha,1989). Polyunsaturated fatty acids can sensitize drug resistant cell lines to cytotoxic drugs. With respect to the synergist effects of cisplatin and γ-linolenic acid (polyunsaturated fatty acid), the isobologram method application to the interaction experiment between these two compounds showed clearly that the two agents demonstrate additive toxicities (Plumb,1993).

Accumulation could be affected by treatment of cells at higher temperatures, in cisplatin resistant as well as sensitive cells. Therefore hyperthermia may be a good modulator when it is possible to reliably increase tumour temperature to decrease resistance (Eichholtz-wirth,1990).

1.4.4.2 - Glutathione :

Cisplatin is an electrophile that is quite reactive toward sulphur-containing nucleophiles. One would thus predict that the principal intracellular thiol, glutathione (GSH), may participate in modulating cisplatin

cytotoxicity and nephrotoxicity, and that resistant cells may contain elevated levels of GSH. Cytotoxic effects of certain 1,2-diamino saturated cyclic platinum derivatives which have a high degree of activity against leukemias can be blocked by certain sulphur containing compounds such as methionine (Burchenal,1978). Evidence for a direct reaction of GSH with cisplatin comes from a report that GSH can bind to monofunctional adducts on DNA (Figure 1.8) (Eastman,1987). Mayer et. al. (1989) have reported that depletion of kidney GSH by an inhibitor of its synthesis decreased cisplatin induced nephrotoxicity in rats. Hospers et al. (1988) found that GSH was elevated 3.4-fold in a cisplatin-resistant human small cell lung carcinoma cell line, and Hamilton et al. (1985) reported that 14-fold cisplatin resistant human ovarian carcinoma cells (A2780) had 3.2-fold elevated GSH. However, 2 to 3-fold cisplatin-resistant 2008 cells did not have elevated levels of GSH (Andrews,1985).

GSH is also involved in DNA-related mechanisms of resistance (Hamers,1993). In a study by Lai et al., GSH reduction inhibited DNA repair, possibly by destabilizing the DNA repair enzymes or by reduction of the deoxyribonucleotide triphosphate pools (Lai,1989). Data suggest that this mechanism may also be involved in the ovarian cancer cell's resistance to cisplatin (Johnson,1993).

The exact role of GSH in mediating cisplatin resistance is unclear at present. There is no direct evidence that GSH inactivates enough intracellular cisplatin to affect cisplatin cytotoxicity. In combination with GSH, the enzyme glutathione S-transferase (GST) might play a role in cisplatin resistance, as it is responsible for the conjugation of chemicals to the thiol group. The activity of GST is also often found to be increased in cisplatin resistant cell lines (Saburi,1989).

1.4.4.3 - Metallothioneins :

Metallothioneins (MTs) comprise a family of proteins involved in Zn^{2+} homeostasis and in the detoxification of heavy metals. They account for a large percentage of the intracellular thiol content and likely are targets for electrophilic agents such as cisplatin (Farnworth,1989). In rat liver and kidneys, 25% of the total cytosolic platinum was reported to be bound to MT-like proteins following cisplatin treatment (Zelazowski,1984). Andrews and coworkers (1987-a) have shown that human ovarian carcinoma cells selected for cadmium resistance had elevated MTs and were cross-resistant to cisplatin, and that the elevated MTs bound 28% of the cytoplasmic

platinum after a three hour exposure to cisplatin. What is not clear is whether elevation of MTs is an important mechanism of resistance in cells subjected to clinically relevant selection procedures. All of the cisplatin-resistant cell lines associated with elevated levels of MTs have been exposed to high concentrations of cisplatin for long periods of time.

1.4.4.4 - DNA Repair :

A role for increased DNA repair capacity as a mechanism of cisplatin-resistance has been established (see also section 1.4.3.3). Eastman and coworkers provided direct evidence for increased DNA repair, showing that cisplatin-resistant cells remove the predominant lesion (the dGpdG intrastrand cross-link ; section 1.4.3.1) more rapidly than do sensitive cells. These resistant cells can also reactivate a cisplatin-damaged plasmid more readily than can sensitive parent cells (Sheibani,1989).

Kraker and Moore reported that DNA polymerase beta activity was elevated in cisplatin-resistant P388 leukaemia cells and that these cells also exhibited increased unscheduled DNA synthesis (Kraker,1988). A DNA-binding protein that recognizes a cisplatin-damaged DNA

fragment was described. It is not yet known whether this protein is involved in DNA repair or altered in cisplatin-resistant cells (Chu,1988).

1.4.4.5 - Other Mechanisms :

Many other mechanisms have been suggested to be responsible for the cellular resistance to cisplatin. For example, in some cell lines, protein kinase C (PKC) activation and in others, PKC inhibition seemed to potentiate cisplatin cytotoxicity (Timmer-Bosscha,1992). However, drug resistance in ovarian cancer is multifactorial (Ozols,1993), and it is likely that additional mechanisms are involved than those previously discussed in this review. For example Tumour Necrosis Factor-alpha (TNF-α) may have a role in the resistance to cisplatin, since a combination of cisplatin and TNF-α can overcome the cisplatin resistance of human ovarian tumour cells. Moreover, down regulation of TNF-α mRNA by cisplatin may play a role in the enhanced cytotoxicity seen with the combination of cisplatin and TNF-α (Mizutani,1993). Fluorescence microscopy of living cells stained with the mitochondria-specific dye revealed that ovarian carcinoma resistant cells have significant changes in their mitochondrial morphology (Andrews,1992). It was

concluded that an elevated plasma membrane potential and alterations in mitochondria is involved in cisplatin resistance of these cells. These mitochondrial changes have been found to be central to the expression of cisplatin resistance in the C13 cell line (a cisplatin resistant OV 2008) (Zinkewich-Peotti,1992).

1.5 - Toxicity :

Administration of cisplatin may be associated with a number of serious side effects, including nephrotoxicity, peripheral neuropathy, gastrointestinal side effects (nausea, vomiting, and diarrhea), myelosuppression, and occasional transient elevations in liver function tests. In addition, ototoxicity (tinnitus and hearing loss), anaphylactic reactions and hypomagnesemia with resulting tetany may also be encountered (Daniel,1979). The toxic potential of this drug necessitates careful clinical monitoring during treatment. We will review two of the major toxic effects of this drug below.

In early clinical trials with cisplatin, serious irreversible renal failure was encountered. Single courses of 2 mg/kg or 75 mg/m^2 cisplatin are associated with nephrotoxicity in one-third of the patients treated

(Kovach,1973). There is evidence that the therapeutic efficacy of cisplatin increases with increasing dose. Higher doses of cisplatin also increase the vulnerability of the kidney as the primary excretory organ for platinum (Daugaard,1989).

The cellular mechanism of cisplatin nephrotoxicity is unknown (Anand,1993) . Levi et al. have reported decrease in protein-bound sulfhydryl groups in the renal tissue in rats prior to the development of renal failure (Levi,1980). In vitro studies showed no direct interaction of platinum with sulfhydryl groups of cysteine or renal homogenates (Slater,1977). The decrease in sulfhydryl groups was similar in magnitude in the outer medulla and cortex, whereas platinum concentrations were higher in the medulla.

Peripheral neuropathy as a toxic effect of cisplatin was first reported Kedar et al (1978). The features of the neuropathy are uniform in most reports and are consistent with damage predominantly to large sensory fibres. Patients usually report numbness or tingling as the first symptom. Examination initially reveals decreased vibratory sensation and decreased or absent deep-tendon reflexes. Lhermitte's sign (a sensation during neck flexion resembling or electric shock) is often present. As the neuropathy continues, the sense of joint position is also

impaired (Mollman,1990). Severely affected patients become markedly disabled by sensory ataxia. The sensations of pain and temperature remain relatively well preserved. Symptoms may begin and often progress after cisplatin has been discontinued (Mollman,1988). Swelling of the optic disc and loss of tendon reflexes has been reported in a 3.5 year old girl after 11 courses of high dose cisplatin therapy for recurrent sacrococcygeal teratoma (Walsh,1982).

Neurotoxicity is dose-dependent (Hamers,1991). Pateints generally become symptomatic after they have received a total cumulative dose of 300 to 600 mg/m^2, although there are individual variations in susceptibility. The neuropathy is reversible, but recovery may take a year or more, depending on its severity (Roelfs,1984).

The cellular mechanism(s) of cisplatin toxic effects on the nervous system is poorly understood. Blisard et al. used a model system of cultured embryonic chick dorsal root ganglion cells to investigate cellular mechanisms of cisplatin toxicity (Blisard,1992). They found abnormalities in patterns of nuclear staining, but not in the neuronal cell membrane.

2

Background and Hypothesis for this Project :

Background :

a) Cisplatin metabolism:

After introduction into animals or biological fluids, cisplatin undergoes ligand exchange reactions leading to the production of a mixture containing high molecular weight (protein or amino acid bound) and soluble transformation products and unchanged cisplatin (parent drug) (Daley-Yates,1982). The biotransformation products of cisplatin have not been characterised owing to the lack of a suitable procedure for their separation.

A separation method which is potentially useful is high performance liquid chromatography (HPLC), but the application of this technique to the study of cisplatin pharmacokinetics is limited by the problem of species detection. A U.V. detection of metabolites after

derivatization of species has been described by Borch (1979); however, the stability of metabolites under derivatization is under question. Atomic absorption spectroscopy (AAS) of total platinum compounds of metabolites (Gromley,1979), either following their separation on an HPLC system or not (Goel,1990) has been used by some investigators as well. Monitoring of the radioactivity from ^{193}Pt-labelled cisplatin has been used to study the metabolites of this drug (DeConti,1973). X-Ray fluorescence has been used for the monitoring of cisplatin in urine too (Bannister,1977). However, although these techniques provide the necessary sensitivity for platinum level measurements, they suffer from matrix effects when applied to clinical samples for pharmacokinetic purposes (Riley,1982-a).

Metabolites of cisplatin are detectable in plasma within 15 min of cisplatin being injected into rats (Daley-Yates,1984). In the period following injection, cisplatin is removed from the plasma by a number of processes, including binding to plasma proteins, dissemination into tissues and removal by the kidney.

Mason and co-workers (1986-b) investigated intracellular metabolites of cisplatin using gel filtration and ion exchange chromatography. They demonstrated the presence of both neutral and positively

charged low-molecular weight species within 15 min of cisplatin administration. Mistry and co-workers (1989) used HPLC to separate the low-molecular-weight metabolites of cisplatin found in the cytosol of cells from the cortex and outer medulla of kidneys of rats up to 24 hours following cisplatin treatment. They compared the chromatographic behaviour of these metabolites with that of metabolites found in plasma, urine and cells from the liver, an organ on which cisplatin has no toxic side effects at therapeutic dose levels (Slater,1977).

The results for in vitro incubation in plasma show the appearance of a number of (bio)transformation products originating from cisplatin. Preliminary results for clinical samples indicate that, besides parent drug (cisplatin), other platinum species are present in plasma and plasma ultrafiltrate. After 24 hours incubation of cisplatin in human plasma ultrafitrate at 37°C, at least seven platinum-containing species can be described, in addition to cisplatin (De Waal,1987). The retention time of two species coincides with that of two aqua hydrolysis products of cisplatin (section 1.3.1):

cis-$[PtCl(NH_3)_2(H_2O)_2]^+$ and cis-$[Pt(NH_3)_2(H_2O)_2]^{++}$

This behaviour indicates that it is possible that the aqua complexes are formed in the plasma incubated in vitro.

Studies have shown that cisplatin reacts rapidly with nucleophiles containing divalent sulphur and that such reactions may be responsible for its biotransformation. Methionine has been identified as a possible in vivo substrate for cisplatin because of its high reactivity and significant concentration in plasma (Riley,1982-b, Norman,1992). Mouse plasma methionine levels dropped rapidly in the first hour to 62% of pretreatment levels, following 2 mg/Kg intraperitoneal injection of cisplatin. Methionine levels remained depressed for 5 hours and then returned to normal by 24 hours. Methionine levels were also determined in plasma samples from 6 courses in 4 patients receiving a 2 hour intravenous infusion of cisplatin 100 mg/m2. In 5 courses, plasma methionine levels declined to 35% of pretreatment levels within the first 6 hours (Lee,1988).

Attempts were made to synthesize the 2:1 complex of L-methionine with platinum by the reaction of cisplatin with excess L-methionine in aqueous solution (Christopher,1983). As the methionine to cisplatin ratio is increased in reaction, the product shifts from the monomethionine complex to a bismethionine complex in which one methionine is joined cyclically to Pt(II) through the sulphur atom and amino group; one methionine is joined only through the sulphur atom, and one amine group from

the parent cisplatin is retained (Andrews,1984). A preincubated mixture of cisplatin and methionine gives three peaks when separated using the HPLC technique: one peak is unchanged cisplatin and the other two are presumed to be mono-methionine and di-methionine substitution complexes of cisplatin, since this has been proved by thin layer chromatography in comparison to the peaks which arose from in vivo experiments (Daley-Yates,1983). Figure 2.1 presents the chemical structures of above mentioned metabolites.

Significance of cisplatin metabolism :

Cisplatin is one of the most widely used anticancer drugs. Cisplatin-based chemotherapy is highly effective against many tumours including testicular carcinoma, localized small cell lung carcinoma, head and neck carcinoma and bladder carcinoma (Chabner,1990).

The clinical administration of cisplatin at doses that are necessary to produce significant tumour cell kill is often associated with significant toxicity (see also section 1.5). Renal toxicity is often dose-limiting, but peripheral neuropathy and ototoxicity may also be severe. Although the mechanisms of cisplatin-induced nephrotoxicity are not well elucidated, it has been

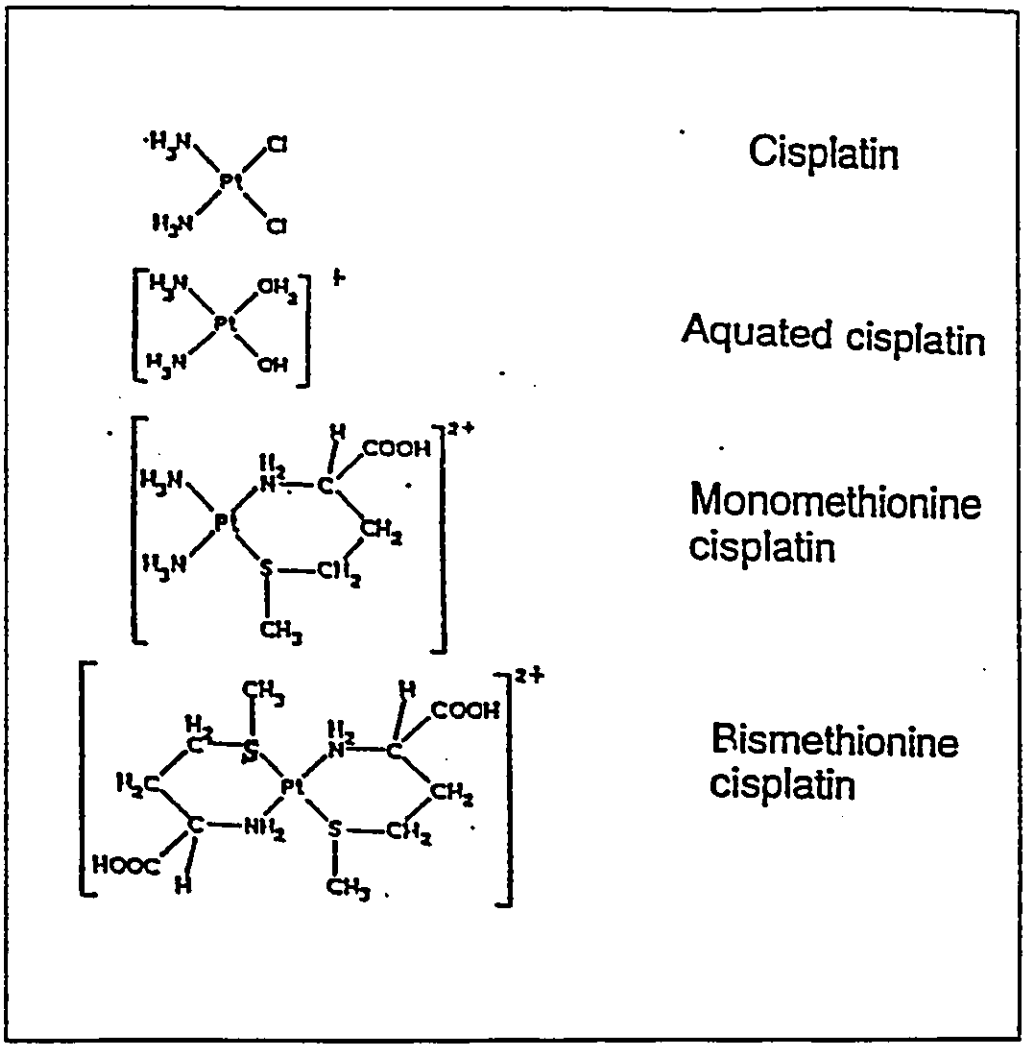


Figure 2.1 : Model platinum compounds originating from cisplatin as a result of hydrolysis and reaction with L-Methionine.

suggested that different cisplatin metabolites may be responsible for its nephrotoxic and antitumour effects (Daley-Yates,1984, Jones,1991).

Cisplatin forms metabolites in animal and human biological fluids, both in vivo and in vitro. These metabolites probably arise by simple ligand-exchange

reactions with substances present in the fluids. By HPLC analysis, there are at least 7 platinum-containing species in addition to cisplatin present in the plasma of rats dosed with cisplatin. The same species form when cisplatin is incubated in vitro in plasma ultrafiltrate.

When cisplatin is incubated in water or in a methionine solution in vitro, various aquated cisplatin species and methionine substitution products are formed (See also section 1.3.1). These species gave unique elution patterns by HPLC. The principal cisplatin metabolites in plasma ultrafiltrate show the same elution patterns as the aquated and methionine species of cisplatin, and are considered to be the putative aquated and methionine species of cisplatin.

Although no metabolites achieve a plasma concentration as high as that of cisplatin (section 1.4), the fact that cisplatin is rapidly removed from the plasma whilst metabolite species persist for longer times, means that although they are present in lower concentration, the metabolite species have more time to have an effect. The difference in the proportions of the metabolites can be explained as follows. In vitro, the relative proportions of the metabolites are not affected by different rates of excretion.

A dose of ultrafiltrate-cisplatin species, when injected intraperitoneally into rats, is more nephrotoxic than an equivalent dose of cisplatin (section 1.5). It is suggested that a low molecular weight platinum-binding fraction of protein may play a significant role in the accumulation and retention of platinum and may provide a site of detoxification during the second phase of influx of platinum into the kidneys (Mason,1986-a). Aquated cisplatin species are also more nephrotoxic than cisplatin, while the methionine-cisplatin species are less nephrotoxic than cisplatin. With respect to relative antitumour effects in mice carrying L1210 leukaemia, ultrafiltrate- and methionine-cisplatin species are much less effective than cisplatin itself (Daley-Yates and McBrien, 1984 - See also section 1.4).

In other experiments, methionine has variable effects on cisplatin-induced toxicity in vitro (Melvik,1987) and in vivo (Ormond,1988), and this may be related to the sequential order of administration of methionine and cisplatin.

Chromatograms performed on the plasma and urine of rats injected with cisplatin give peaks with elution patterns corresponding to all of the above-mentioned species. The nephrotoxicity of cisplatin is decreased if the rats are pretreated with saline. An associated

decrease in the concentration of the aquated species in plasma and urine is seen, suggesting that it was the nephrctoxic species. A decrease in the total and cytosolic kidney platinum concentrations is also seen with saline pretreatment, suggesting a decreased cellular platinum uptake in the nephron.

The evidence presented above (and in chapter 1), suggests that cisplatin and its metabolites in vivo may have specific effects.

The effects of identical doses of solutions containing cisplatin (parent drug), aquated cisplatin (formed by dissolving crystalline cisplatin in water), ultrafiltrate-cisplatin species (formed by dissolving cisplatin in human plasma ultrafiltrate), monomethionine-cisplatin complex (formed by adding equimolar amounts of methionine and cisplatin to a saline solution, and bismethionine-cisplatin complex (formed by adding a ten fold molar excess of methionine to a cisplatin solution), were each tested for relative cytotoxicity produced against several cell lines. The cells were grown as monolayer cultures on plastic dishes. The parent drug was generally found to be the most toxic metabolite in vitro, while the ultrafiltrate-cisplatin species were the least cytotoxic in the study.

Additional studies were then performed with mice injected with cisplatin dissolved in ultrafiltrate samples derived from plasma samples from different patients. The rationale is that the variable toxicity seen from patient-to-patient may result from differences in cisplatin species' profile in each patient that depends, in turn, on each patient's plasma characteristics. Results of these experiments confirmed that the propensity of developing cisplatin-induced side effect toxicities may depend upon the nature of cisplatin metabolites formed in plasma.

Hypothesis :

Cisplatin forms different metabolites in biological fluids, and each cisplatin metabolite has different effects against the human ovarian tumour cells.

Objectives :

The effects of different cisplatin metabolites formed in human plasma will be studied using the human ovarian carcinoma (OV 2008) cell line as a human tumour model. We will :

1. determine the relative cytotoxicities of cisplatin metabolites against OV 2008 cells.
2. determine the accumulation and efflux patterns of cisplatin metabolites in OV 2008 cells.
3. determine the biochemical changes induced by cisplatin metabolites in OV 2008 cells.

Significance :

One may eventually be able to predict a patient's response to cisplatin chemotherapy on the basis of the concentration of different cisplatin species in plasma and thus be able to optimize his/her dose schedule.

3

Cisplatin and its Metabolites Cytotoxicity Measurements :

A well established ovarian carcinoma cell line; OV 2008, was selected for this study. The following parts of this thesis are going to describe experiments and results on this subject. Generally speaking, two different (quantitative and qualitative) methods were selected to look at the different effects of cisplatin and three of its major metabolites (aquated cisplatin, monomethionine cisplatin and bismethionine cisplatin) and cisplatin dissolved in ultrafiltrate plasma on the OV 2008 cell line. Results are compared to cells exposed to saline (as a negative control) and/or each other by using appropriate statistical methods.

Qualitative measurement was done with infrared spectroscopy. Cells exposed to different species were examined with Fourier transform infrared spectroscopy and various effects of each metabolite on different chemical characteristics of the cells were recorded. Chapter four

will describe our results on the application of this technique to OV 2008 cells.

Various methods were applied to collect quantitative results on metabolites' effects on OV 2008 cells: clonogenic assay, IC_{50} calculations, and the time course of metabolites efflux. This chapter will discuss these results.

3.1 - Survival Curves :

Survival curves of OV 2008 cells were measured after one hour exposure to each of the cisplatin metabolites to investigate the various effects of these species on this cell line.

3.1.1 - Materials :

"Species", "Metabolites" and "platinum compounds under investigations" are terms used for all of following solutions interchangably: cisplatin, aquated cisplatin, monomethionine cisplatin, bismethionine cispaltin and plasma ultrafiltrate cisplatin.

Drugs and Chemicals;

Pure cisplatin powder was obtained from Sigma laboratories to make the aquated cisplatin using the

method of De Waal (1987). Cisplatin powder was dissolved in double distilled water in the proper concentrations to obtain different concentrations of aquated cisplatin. The pH of the final solutions was not adjusted, but was around 4.5, which would most probably yield the diaquated cisplatin derivative. This compound will then change to the monohydroxy- monoaquated derivative when dissolved in culture media which has a pH of about 7. Parent cisplatin was prepared from the clinical formulation of Horner Laboratories (Montreal, Canada). If it was necessary to dilute this formulation, which is shipped in 50 ml bottles of 1 mg/ml cisplatin in saline (0.9% NaCl in water), saline was always used.

Solutions of mono- and bis- methionine metabolites of cisplatin were prepared from this formulation based on De Waal's publication (1987). L-Methionine was purchased from Sigma laboratories. Monomethionine cisplatin was prepared with a mixture of a one to one molar ratio of cisplatin and methionine solutions. Bismethionine cisplatin was prepared with a 10:1 molar ratio of methionine to cisplatin in saline. Lower concentrations of these species were prepared by diluting the solutions with saline, but higher concentrations were prepared by directly dissolving the proper amounts of methionine powder in a saline solution of cisplatin calculated for

the proper molar ratio, to avoid further dilutions of final concentrations.

A mixture of all cisplatin metabolites in plasma was prepared by dissolving cisplatin in ultrafiltered plasma. Plasma was prepared from the Ottawa Civic Hospital Blood Bank supplies, and was ultrafiltered with Amicon membrane filters in a Beckman J2-21 centrifuge at 4500 RPM for 20 minutes at ambient temperature. Cisplatin powder was added to this preparation in the proper amounts to get the required final concentrations.

Cisplatin , its metabolite solutions and a negative control of saline were passed through a 0.45 μm sterilization filter (Acrodisc sterile filters) under sterile conditions and were incubated after preparation for one hour at 37°C before use.

Cell line :

The human ovarian cell line (OV 2008) was a gift from Dr. Stephen B. Howell, California, San Diego. The cells were cultured following the standard methodology of the American Type Culture Collection (ATCC, Rochville M.D.). These cells were grown on tissue culture dishes in a humidified incubator at 37 °C with a 5% CO_2 atmosphere. They were maintained in complete medium consisting of RPMI 1640 without glutamine, supplemented with 10% heat-

inactivated fetal calf serum. RPMI media, fetal calf serum and glutamine were purchased from Gibco BRL laboratories. Glutamine was added to the media in the amounts of 300 mg per litre before its usage.

3.1.2 - Method :

OV 2008 cells were cultured in cell culture plates up to the beginning of the plateau phase, which was indicated by confluency of the cells. Media was aspirated and cells were rinsed twice with saline. Cells were trypsinized and 5 ml of media containing 60 cells per millilitre were seeded in each tissue culture dish. Cells were incubated overnight to allow them to attach to the surface of the dishes.

Solutions of cisplatin, aquated cisplatin, monomethionine cisplatin, bismethionine cisplatin and plasma ultrafiltrate cisplatin were prepared fresh as mentioned above in the concentrations of 2500, 1665, 830, 667, 500, 333, 250, 166, 83, 42 and 8.3 μM of platinum content of metabolites. After addition of 50 μl of each of these solutions to each tissue culture dish, the following final concentrations of each species would be achieved; 25, 16.65, 8.3, 6.67, 5, 3.33, 2.5, 1.66, 0.83, 0.42 and 0.083 μM. Four tissue culture dishes were selected for

each concentration of each species, and the experiments were performed in triplicate. Cells were exposed to these different concentrations of different metabolites for one hour at 37°C. Media without fetal calf serum was used for this period of time (one hour exposure) to minimize protein binding of metabolites and to maintain the chemical structure and proper concentration of free metabolites as much as possible. Media was then aspirated and cells were rinsed with saline two times for each tissue culture dish. Five ml of fresh media were then added to each tissue culture dish and all dishes were incubated for 10 to 12 days until enough colonies containing more than 50 cells per colony were observed in control dishes, which had been exposed to saline.

After 10-12 days incubation, media were aspirated from dishes and cells were fixed and stained with crystal violet dye. Colonies containing more than 50 cells were counted under the microscope using a colony counter pen. Considering the average number of colonies in control dishes as 100%, the percentage of colonies in each dish was calculated, and the average, standard deviation and standard error of percentage of colonies for each concentration of each species were calculated.

A dose-response curve for each metabolite was plotted, and a corresponding IC_{50} calculated.

3.1.3 - Results :

Figures 3.1 to 3.5 present the survival curves of the OV 2008 cell line exposed to cisplatin and its metabolites for one hour. Figure 3.6 compares survival curves of different metabolites.

The Y axis represents the percentages of surviving colonies compared to control, and the X axis is the platinum content of the metabolite solutions in μM. A dotted line identifies the 50% survival of OV 2008 cells exposed to any metabolite. The relative concentrations on the X-axis of this line's intersection with the survival curve is the IC_{50} of that particular metabolite. Table 3.1 summarizes IC_{50}s of different metabolite solutions.

Metabolite Solution	IC_{50} (μM)
Aquated Cisplatin	1.25
Cisplatin	1.55
Plasma Ultrafiltrated Cisplatin	2.61
Monomethionine Cisplatin	3.92
Bismethionine Cisplatin	>25

Table 3.1 : IC_{50} of cisplatin and its metabolites for OV 2008 cell line, based on the platinum content of their solutions.

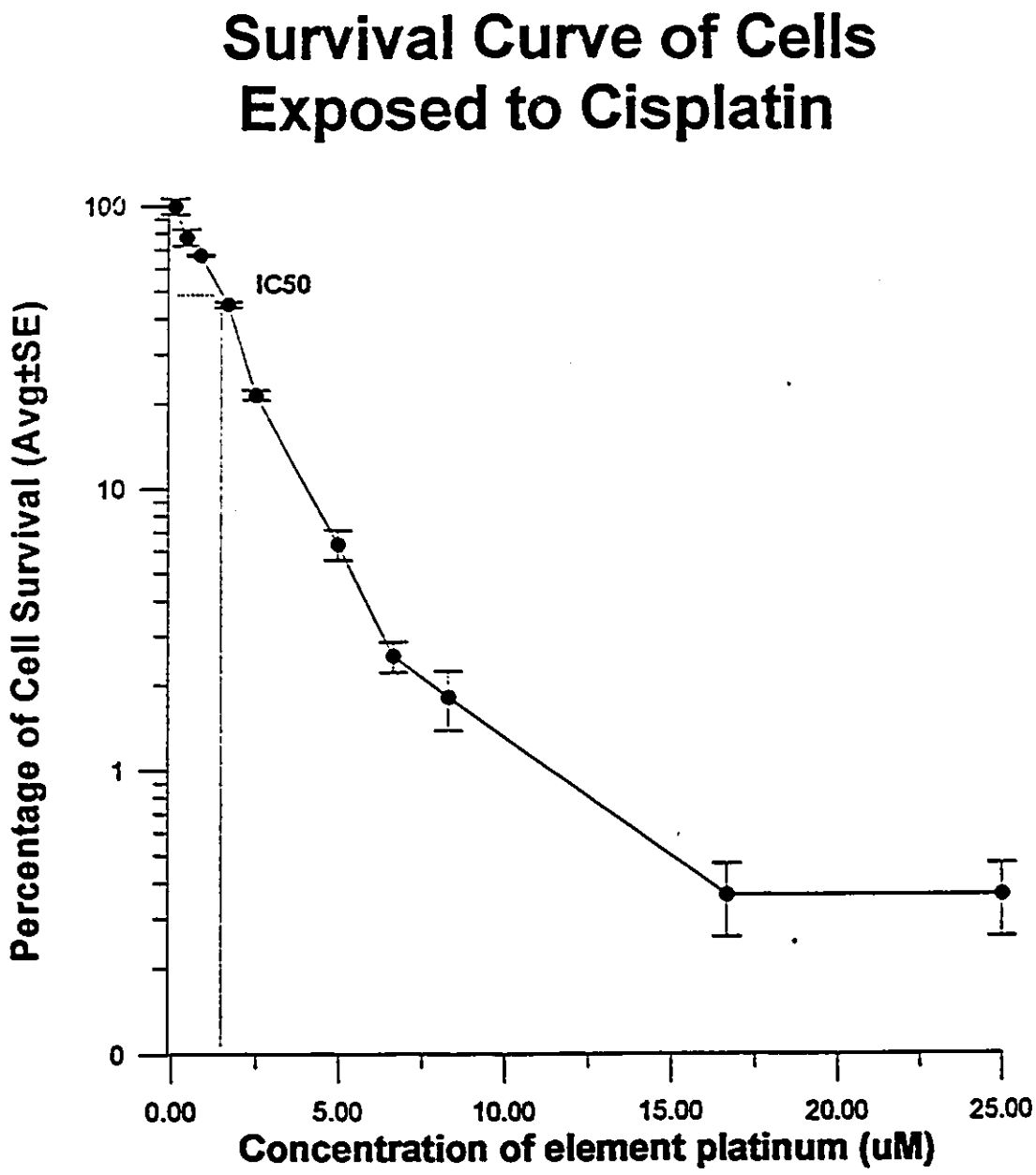


Figure 3.1 : Survival curve of OV 2008 cells following one hour posure to cisplatin in clinical formulation.(n=12, Mean±SE)

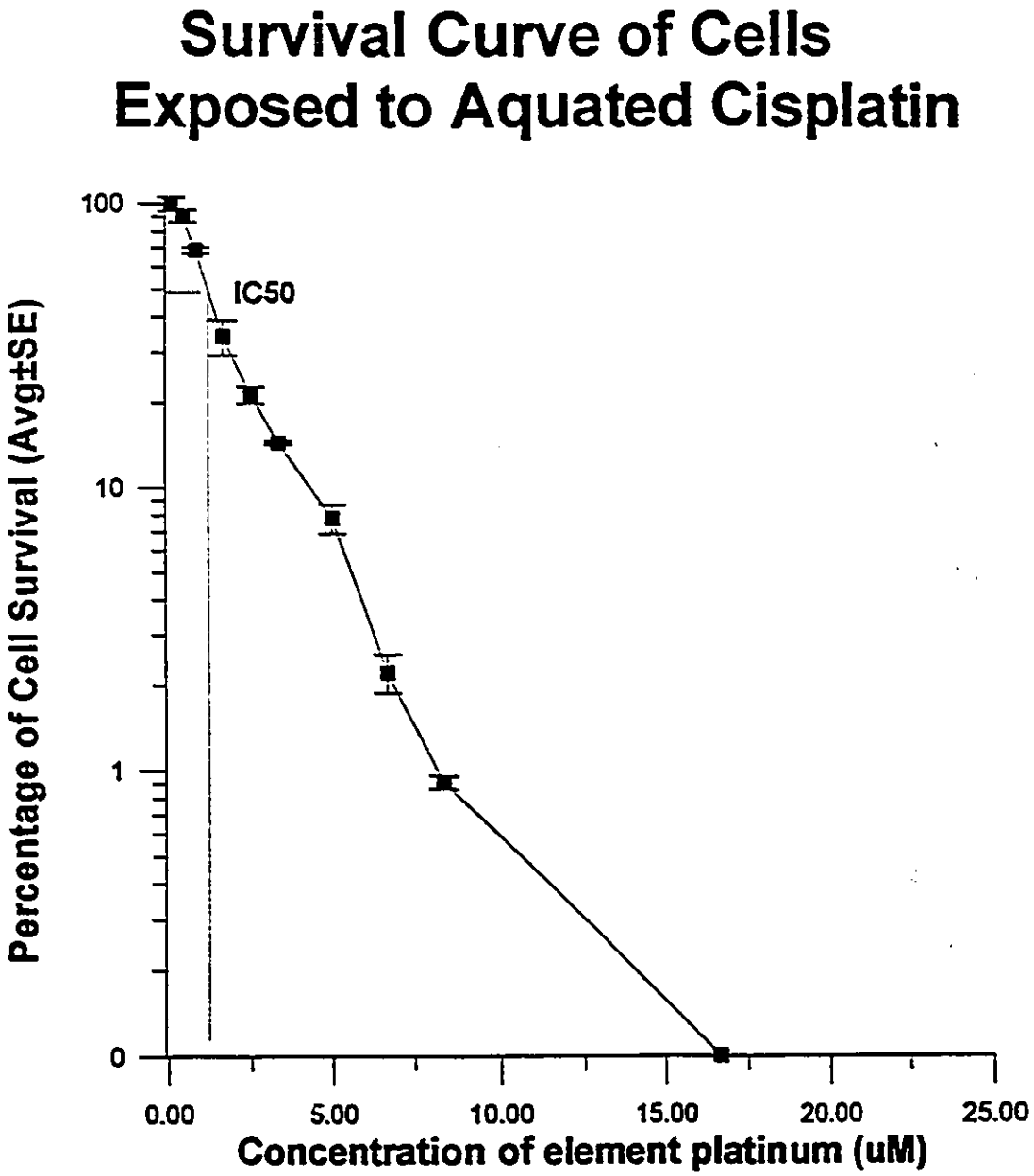


Figure 3.2 : Survival curve of OV 2008 cells following one hour exposure to aquated cisplatin solution. (n=12, Mean±SE)

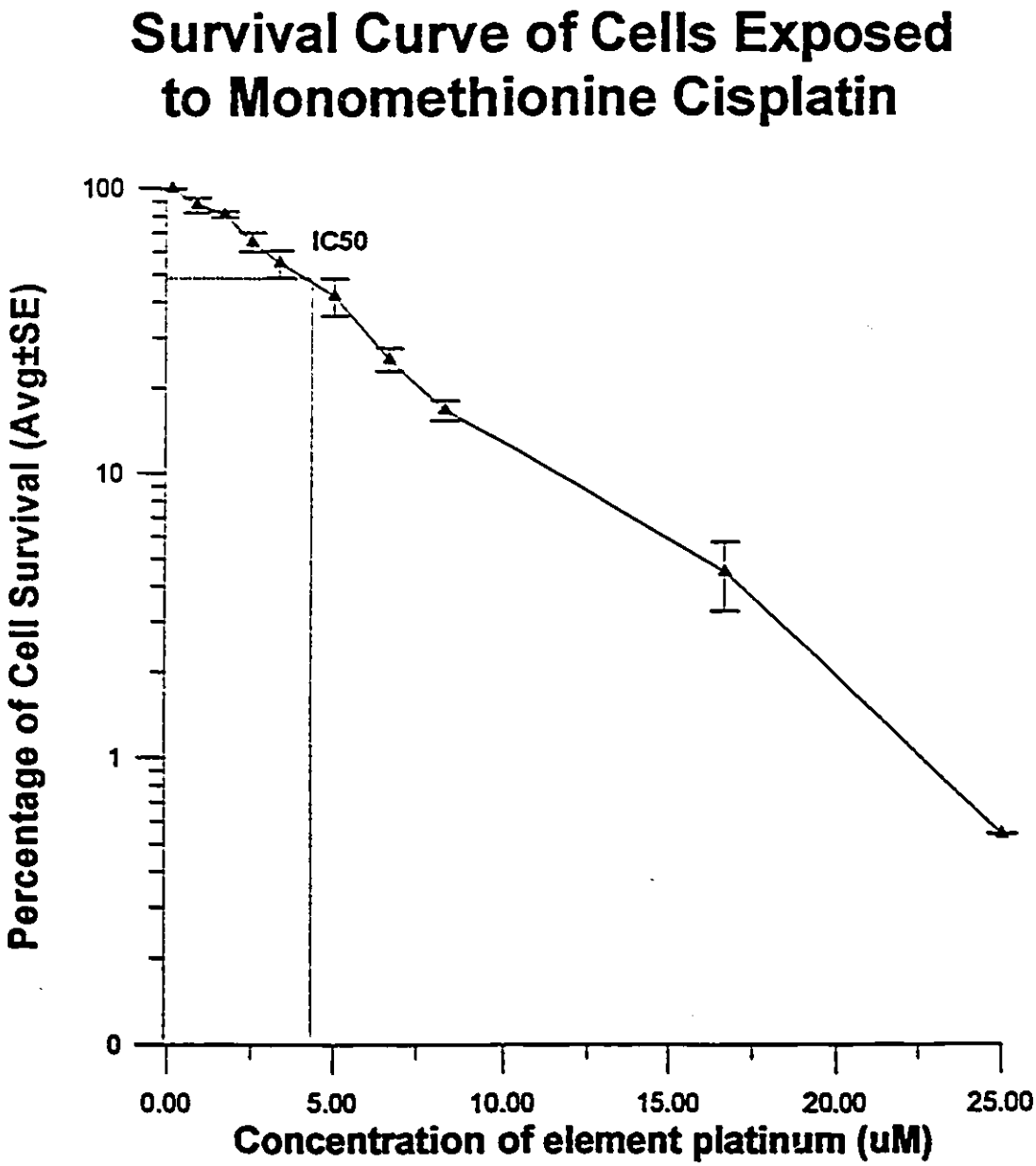


Figure 3.3 : Survival curve of OV 2008 cell following one hour exposure to monomethionine cisplatin solution. (n=12, Mean±SE)

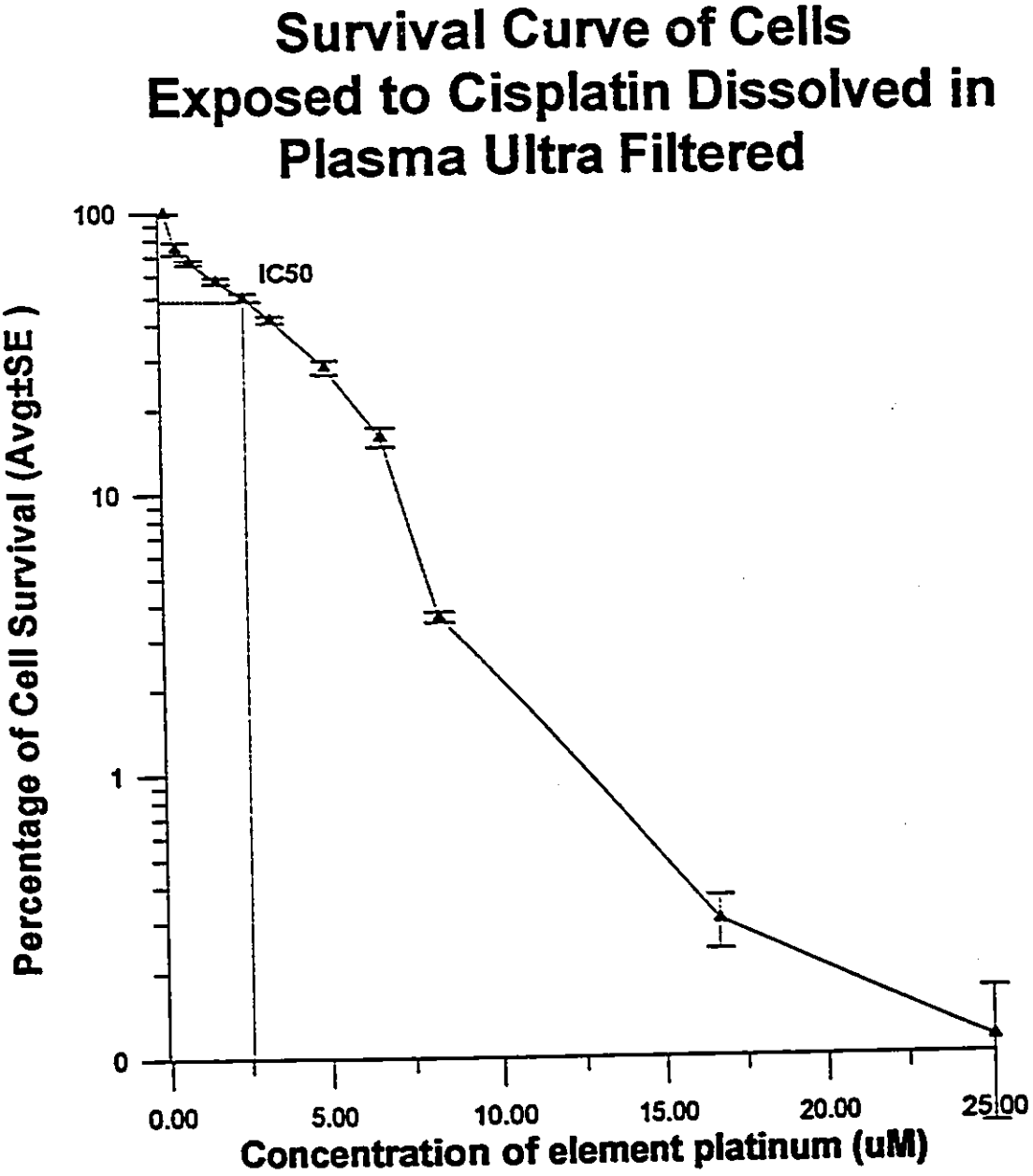


Figure 3.4 : Survival curve of OV 2008 cells following one hour exposure to cisplaitn in plasma ultrafiltrate solution. (n=12, Mean±SE)

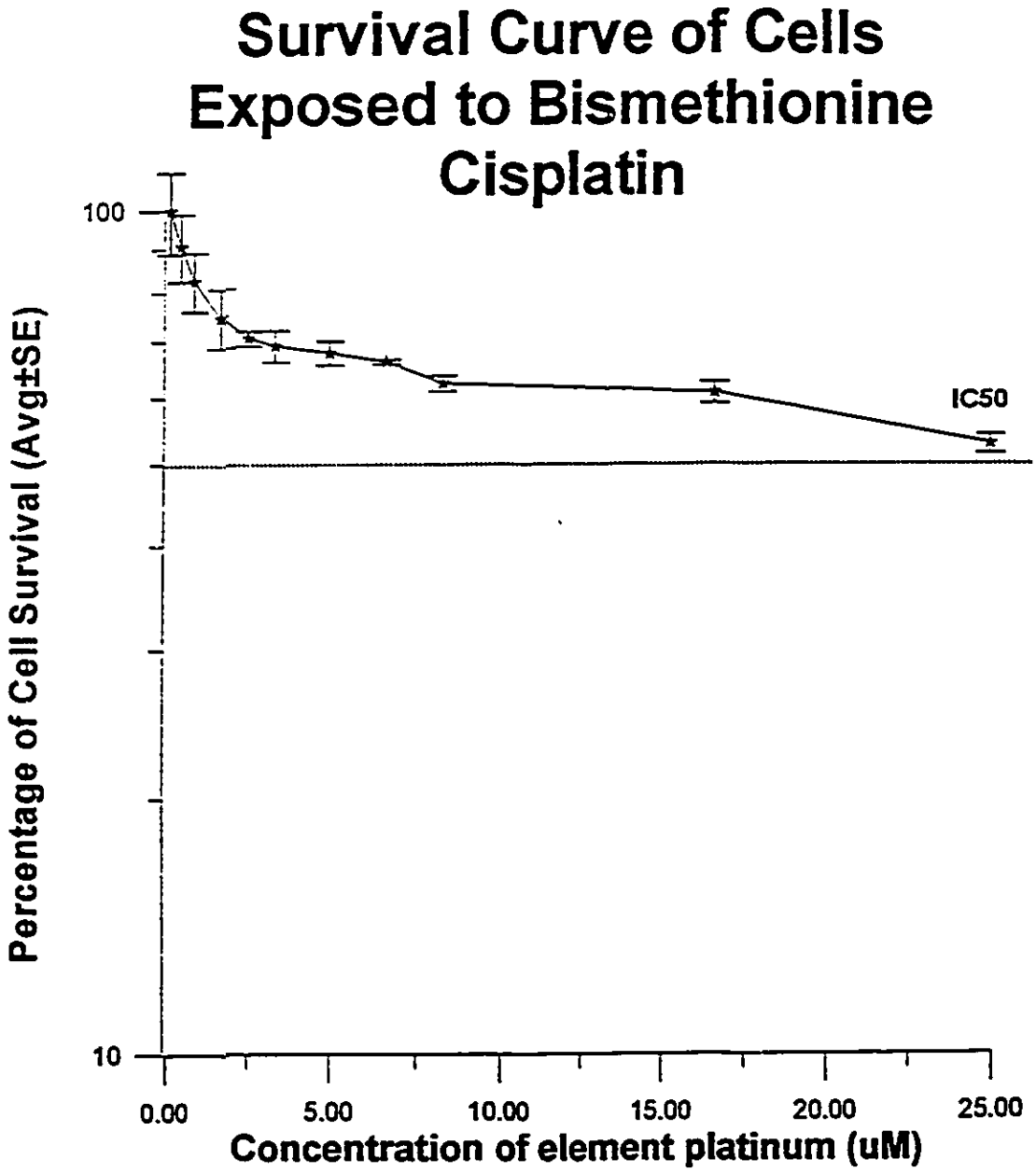


Figure 3.5 : Survival curve `V 2008 cells following one hour **exposure to bismethionine** splatin solution. (n=12, Mean±SE)**

Survival Curves of Cells Exposed to Solutions of Different Cisplatin Metabolites

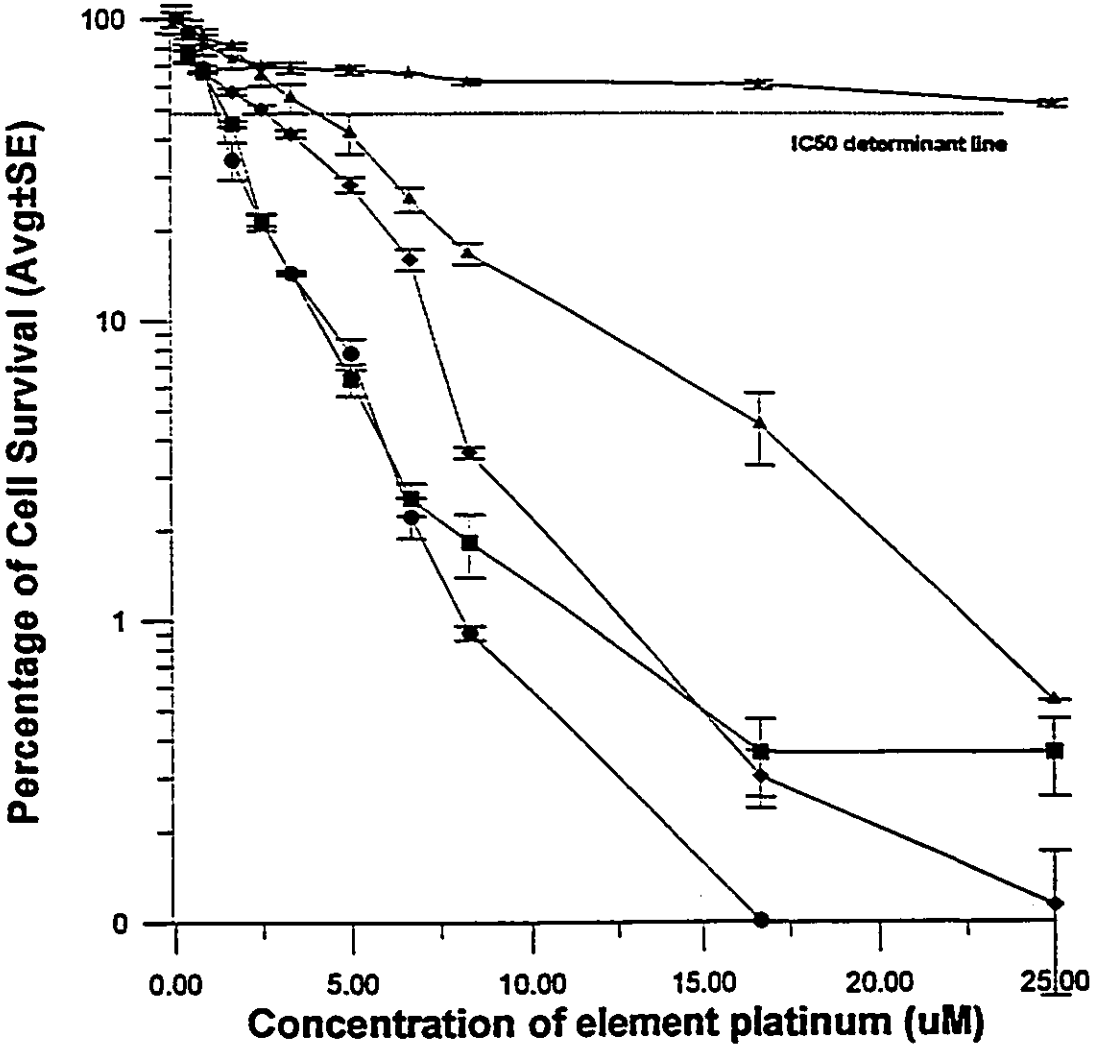


Figure 3.6 : Survival curves of OV 2008 cells following one hour exposure to the different solutions of platinum compounds

As is shown in table 3.1, aquated cisplatin has the greatest toxicity and bismethionine cisplatin is the least toxic metabolite for the OV 2008 cell line. The highest concentration of bismethionine cisplatin in the selected range of concentrations barely resulted in 50% killing of OV 2008 cells, while 20 fold lower concentrations of aquated cisplatin resulted in 50% colony survival.

The rank order of cytotoxic potencies of compounds under investigation for the OV 2008 cell line are therefore as follows:

Aquated cisplatin > Cisplatin > Plasma ultrafiltrate cisplatin > Monomethionine cisplatin > Bismethionine cisplatin.

3.2 - Clonogenic assay of cytotoxicity :

Relative antitumour activities of cisplatin and its metabolites on ovarian carcinoma cells were compared to each other at the same concentration for a one hour exposure. This is to confirm our previous results described in section 3.1.

3.2.1 - Materials :

Materials used were described in section 3.1.1.

3.2.2 - Method :

The one hour exposure of metabolites to OV 2008 cells was done in the same way as was described in section 3.1.2. The difference is that in this experiment, solutions of 1 µg/ml of each species, based on the amount of cisplatin, were added to the cells.

Colonies were counted in different dishes after about 12 days, when the control plates (exposed to saline instead of metabolites) contained at least 50 cells per colony. The number of colonies in each dish was counted and expressed as a percentage of colonies that grew in control plates. Numbers of colonies for each sample (Average±S.E.) were calculated and the survival due to each metabolite was compared to each other and controls using the ANOVA test and Tukey-Kramer multiple comparisons post test.

3.2.3 - Results :

Table 3.2 presents a summary of resultant colony percentages from different dishes containing the same

concentration of the different metabolites in comparison to the control cells. ANOVA p values are mentioned in the third column, for the comparison of each metabolite to the control cells. Table 3.3 lists the ANOVA p value for comparison of different metabolites with each other, after carrying out Tukey-Kramer multiple comparisons post test. Figure 3.7 represents a bar line graph of these results.

As is presented in table 3.2, different metabolites showed significant differences with respect to the control, and each other. The only exception is between plasma ultrafiltrate cisplatin and two of the species; cisplatin and monomethionine cisplatin. The clonogenic assay of OV 2008 cells exposed to plasma ultrafiltrate cisplatin did not show any significant difference with either of these two species, even though results with cisplatin and monomethionine cisplatin have significant differences with each other.

The relative cytotoxicity rank order of cisplatin and its metabolites for OV 2008 cell line will therefore be as follows:

Aquated cisplatin > Cisplatin > Plasma ultrafiltrate cisplatin > Monomethionine cisplatin > Bismethionine cisplatin.

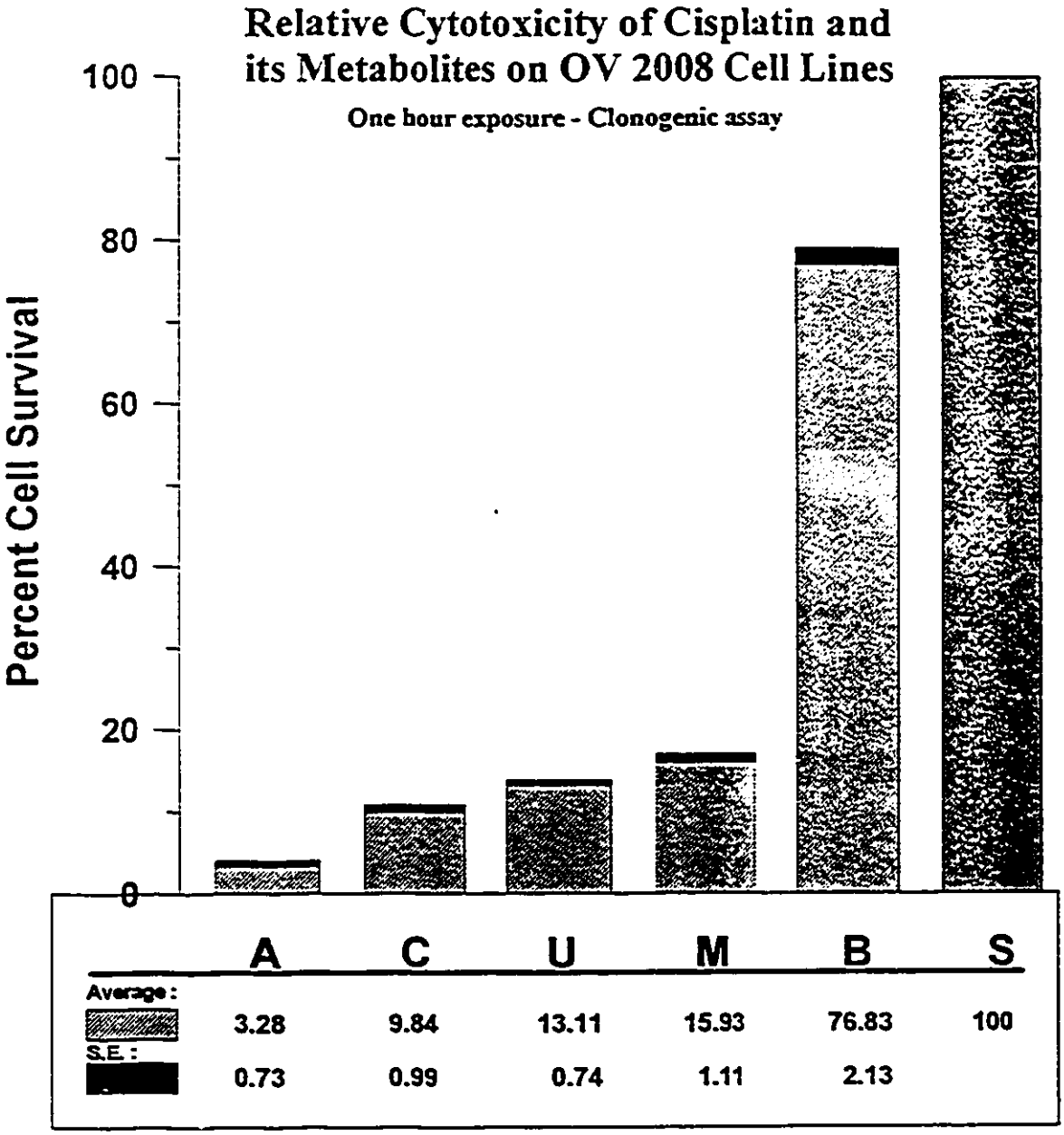


Figure 3.7 : clonogenic assay on OV 2008 cells following one hour exposure to : aquated cisplatin solution (A), cisplatin clinical formula (C), monomethionine cisplatin solution (M), bismethionine cisplatin solution (B) and cisplatin in plasma ultrafiltrate (U), compared to saline (S). (n=12 for each solution)

Species	Mean	S.E.	p<
Saline	100	0.02	-
Aquated Cisplatin	3.28	0.73	0.001
Cisplatin	9.84	0.99	0.001
Plasma Ultrafiltrate Cisplatin	13.11	0.74	0.001
Monomethionine Cisplatin	15.93	1.11	0.001
Bismethionine Cisplatin	76.83	2.13	0.001

Table 3.2 : Relative Cytotoxicity of cisplatin and its metabolites solutions to OV 2008 cell lines in comparison to cells exposed to saline. ANOVA $p<0.001$

(S.E.: Standard Error, P<:Tukey-Kramer, n=12)

Comparison	p value
Aquated C*. vs Cisplatin	p<0.01
Aquated C. vs PUF*	p<0.001
Aquated C. vs Monomethionine C.	p<0.001
Aquated C. vs Bismetnionine C.	p<0.001
Cisplatin vs PUF	p>0.05
Cisplatin vs Monomethionine C.	p<0.01
Cisplatin vs Bismethionine C.	p<0.001
PUF vs Monomethionine C.	p>0.05
PUF vs Bismethionine C.	p<0.001
Monomethionine C. vs Bismethionine C.	p<0.001

Table 3.3 : Relative cytotoxicity of cisplatin and its metabolites solutions versus each other to OV 2008 cell lines. ANOVA post test (Tukey-Kramer multiple comparison test) comparison of clonogenic assay results are presented in the right column.

*: (C.; Cisplatin, PUF; Plasma Ultrafiltrate Cisplatin)

3.3 - Cellular Accumulation of Species :

Atomic absorption spectroscopy has been used to measure cellular platinum content of OV 2008 cells after one hour exposure to each metabolite. Three different concentrations of each species have been used for this experiment (a pharmacological concentration, and higher concentrations). Cellular platinum accumulation of species resulting from exposure of cells to different concentrations were then correlated to each other to calculate the linearity of platinum compound accumulation in the OV 2008 cell line.

3.3.1 - Atomic Absorption Spectroscopy :

The atom is made up of a nucleus surrounded by electrons. Every element has a specific number of electrons which are associated with the atomic nucleus in an orbital structure which is unique to each element.

The quantity of interest in atomic absorption measurements is the amount of light at the resonant wavelength which is absorbed as the light passes through a cloud of atoms. The atom cloud required for atomic absorption measurements is produced by supplying enough

thermal energy to the sample to dissociate the chemical compounds into free atoms.

By far the most advanced and widely used sensitive sampling technique for atomic absorption is the graphite furnace. In this technique, a tube of graphite is located in the sample compartment of the atomic absorption spectrometer, with the light path passing through it. A small volume of sample solution is placed into the tube. The tube is heated through a programmed temperature sequence until finally the analyte present in the sample is dissociated into atoms and absorption occurs.

As the atoms are created and diffuse out of the tube, the absorbance rises and falls in a peak-shaped signal. The peak height or integrated peak area is used as the analytical signal for quantitation (Beaty,1988).

Cellular platinum measurement with atomic absorption spectroscopy:

Platinum element atomic absorption detection limit is about 60 µg/L for flame and about 5 µg/L for furnace atomic absorption spectroscopy with our instrument.

Figure 3.8 represents a diagram of our temperature program for the determination of platinum in a cell suspension sample (Priesned,1981).

As is shown in table 3.4, five different steps are presented in this program. 1 to 3 are drying steps, 4 and 5 are ashing with a temperature of 1200°C, atomization is after step 6 (preparation for atomization), at steps 7 and 8 with a temperature of as high as 2700°C. Steps 9 to 11 are for cleaning and re-setup purposes.

3.3.2 - Materials :

The same materials have been used as were described in section 3.1.1.

Equipment :

Varian techtrom AA-1475 spectrophotometer with a GTA-95 pyrolitically coated graphite tube was used for atomic absorption spectroscopy.

The spectrophotometer was operated at an excitation wavelength of 265.9 nm, at a current which provided a photomultiplier tube potential of 350 to 360 V producing a peak noise level of $\pm 1 \times 10^{-3}$ absorbance units. Hallow cathode lamp current was adjusted on 10 ma, and it was observed to be 7 to 10 ma depending on the age and condition of the lamp.

Step	Temperature (°C)	Time (S)
1	95	15
2	100	25
3	120	20
4	1200	25
5	1200	20
6	1200	2.0
7	2700	1.0
8	2700	2.0
9	2700	1.0
10	40	12
11	40	3.0

Table 3.4 : Furnace operating parameters for the measurements of platinum in a cell suspension sample.

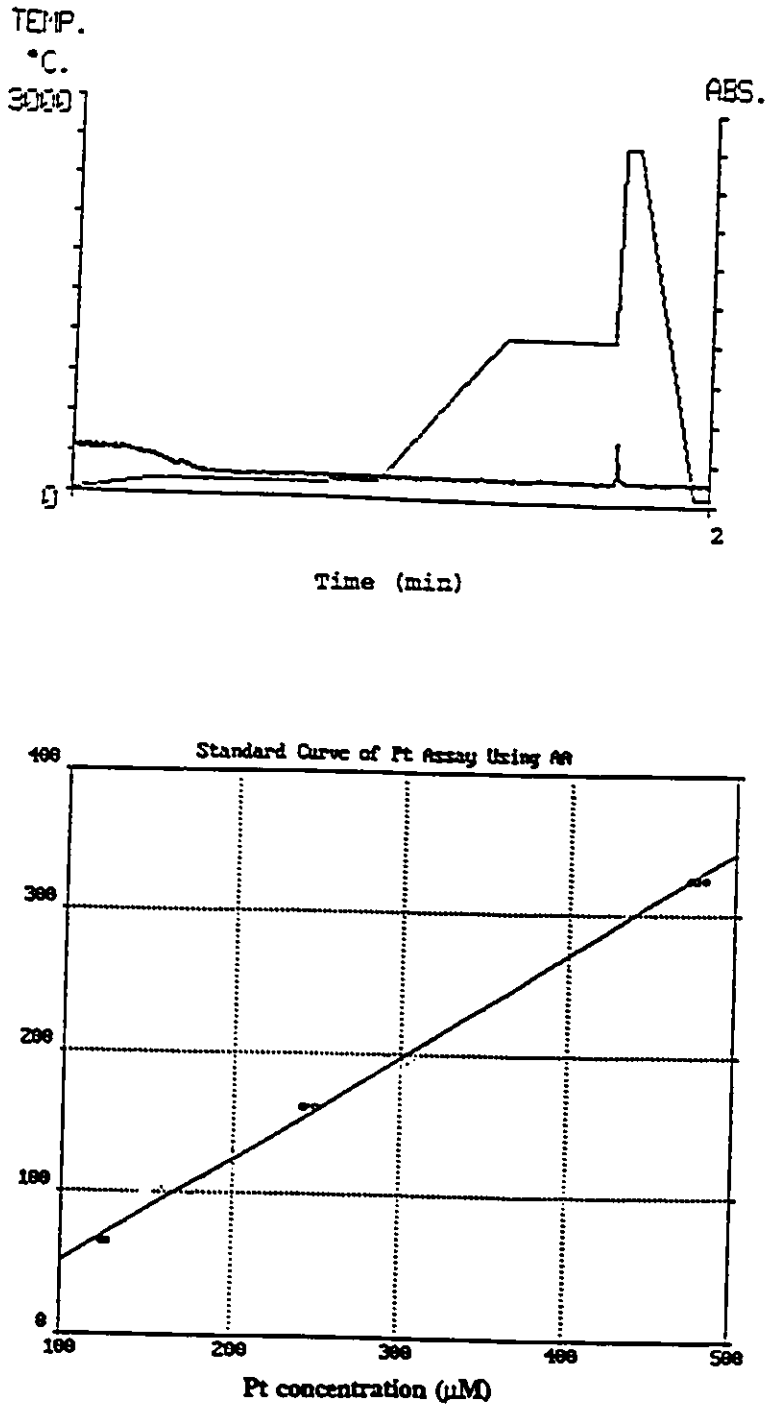


Figure 3.8 : Diagram of temperature program on atomic absorption spectroscope, and the resulted standard curve.

An ultrasonic homogenator of Vibro-Cell model V.C.375 was supplied from Fisher Scientific Canada, and was used with 90% output of 5 pulses per second.

An Elzone 80 cell counter was supplied by Particle Data Inc., USA. A 500 µl volume of cell suspension was used in this instrument with approximately a 14 second counting time. A 95 µm orifice size was used with 160 mm mercury to balance the volume of the cell suspension for counting. The threshold diameter was adjusted on 10 with a current of 6.0 and gain of 1 for the measurement of OV 2008 cell numbers in suspension.

3.3.3 - Method :

OV 2008 cells were plated in tissue culture dishes as was described in section 3.1.2, until the first stage of confluence. Different metabolites were prepared in the concentrations of 0.33, 1.6 and 2.5 mM, as was described in section 3.1.2. Cells were exposed to different concentrations of metabolites for one hour in serum free media. After a one hour exposure time, medium was aspirated and dishes were washed three times with saline as follows: three beakers, each of them containing one litre of saline, were prepared, and tissue culture dishes were floated two times in the saline of each beaker.

Five hundred microlitter of trypsin was then added to each dish, and dishes were then incubated for 3 minutes. A cell suspension was then produced by pipetting the contents of each dish up and down in 100 µl serum- and glutamine-free media to inhibit further digestion by trypsin. Cell suspensions resulting from each tissue culture dish were collected in separate micro cone tubes and 300 µl of each tube was used for cell counting after further resuspension by vortexing. The rest of the cell suspension was collected for atomic absorption measurement of cellular platinum content, as a measurement of cellular uptake of metabolites.

A standard curve of platinum measurement on atomic absorption spectroscopy was obtained by injecting 20 µl of 65.0, 162.5 and 325 µg/l of a clinical formulation of cisplatin. Temperature programming of the atomic absorption spectroscope was designed as described in section 3.3.1. Cell suspensions were sonicated with the ultrasonic homogenator until homogeneous suspensions were obtained. The suspensions were then vigorously vortexed for 30 seconds. An aliquot (20 µl) of homogenate was injected directly into the graphite atomizer using an appropriately-sized pipet. If the platinum signal was not in the linear range of the calculated standard curve, the aliquot would be further diluted with distilled water.

Platinum concentrations of different tissue culture dishes were calculated in µg of platinum per million cells, using the concentration of platinum measured by atomic absorption spectroscopy and the number of cells counted with the cell counter.

3.3.4 - Results :

Figure 3.9 represents the accumulation of cisplatin and its metabolites in OV 2008 vs concentrations. Linear curve fits' r values are presented in front of the corresponding legends. Figure 3.10 is the duplicate of the same graph, except that the accumulation of aquated cisplatin is also plotted so that it can be compared with the accumulation of the other four cisplatin species.

Aquated cisplatin accumulated to a much greater extent than any other species in OV 2008 cells. There is a close correlation between the cytotoxicity and accumulation of the cisplatin metabolites. The r squared value of a regression line weighted at all points for each metabolite is very close to 1. Cellular accumulation percentages of exposed drug are different for various metabolites.

The rank order of species accumulations in OV 2008 cell line is as follows:

Aquated cisplatin > Cisplatin > Plasma ultrafiltrate cisplatin > Monomethionine cisplatin > Bismethionine cisplatin.

Figure 3.11, demonstrates an inverse relationship between cell survival and cellular accumulation of metabolites. At pharmacological concentration (0.33 mM), a regression line between points indicating species uptake versus cell survival has an r squared value of 0.997. This good correlation accumulation and growth inhibition, may suggest that the amount of cellular platinum is a critical factor in platinum compounds' cytotoxicities.

3.4 - Species efflux from cells :

Intracellular cisplatin metabolites accumulation after one hour is clearly related to the cytotoxicity of the different metabolites. The question then arises whether the differences between metabolites cellular uptake and cytotoxicity is due to differences in influx or to differences in efflux. Hence we next studied efflux of the different solutions of cisplatin metabolites.

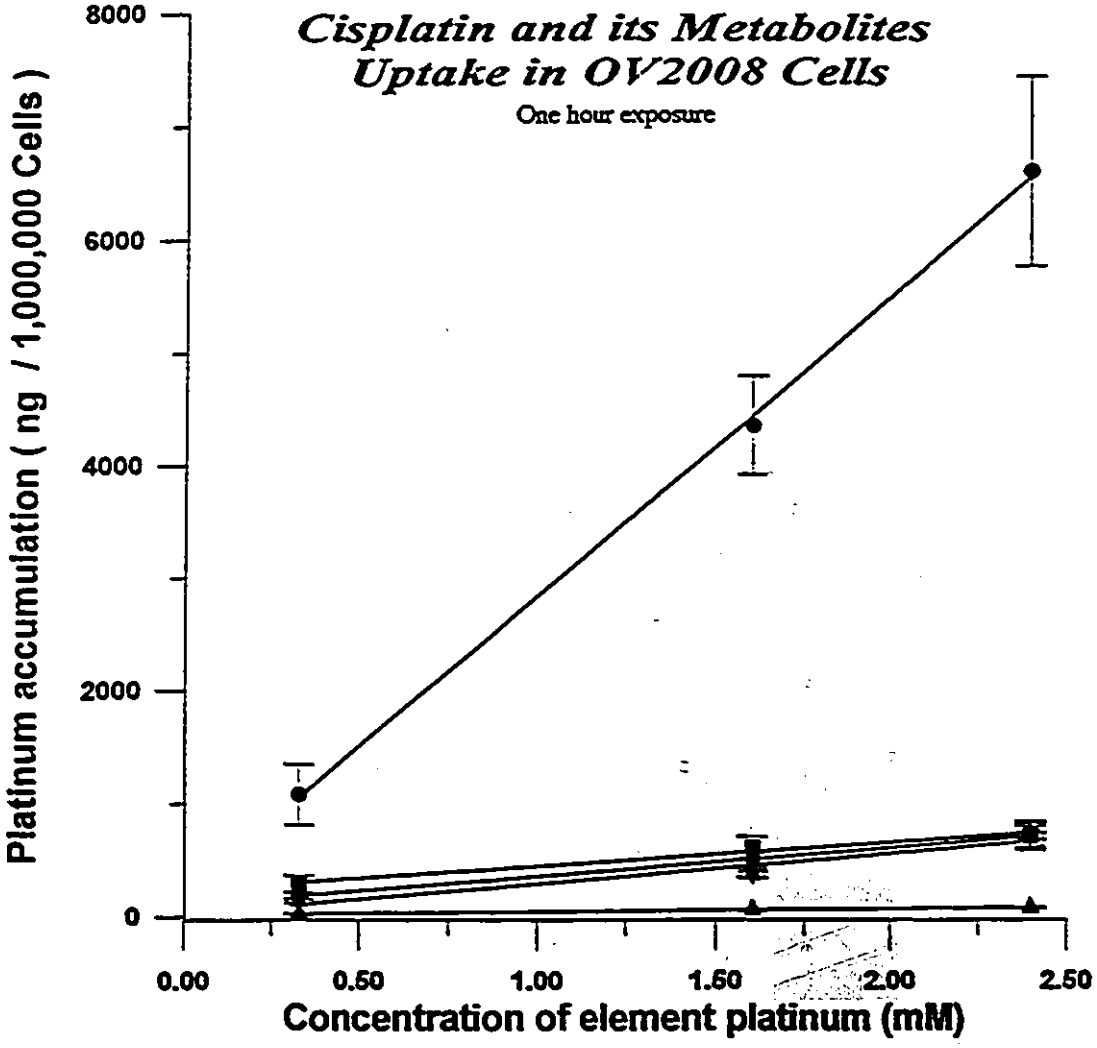


Figure 3.9 : Cellular platinum accumulation in OV 2008 cells (Avg±SE) following one hour exposure to the following solutions:

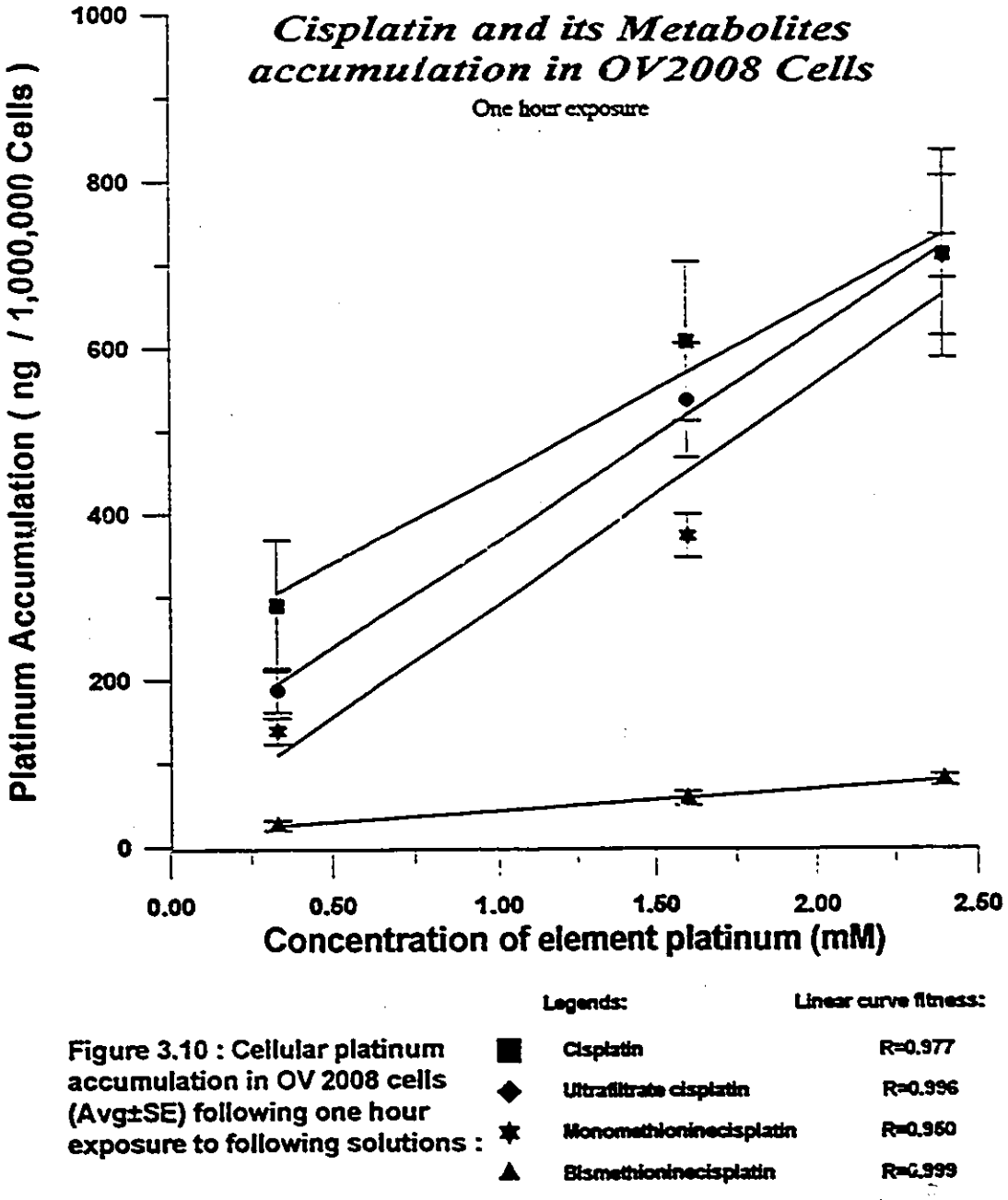


Figure 3.10 : Cellular platinum accumulation in OV 2008 cells (Avg±SE) following one hour exposure to following solutions :

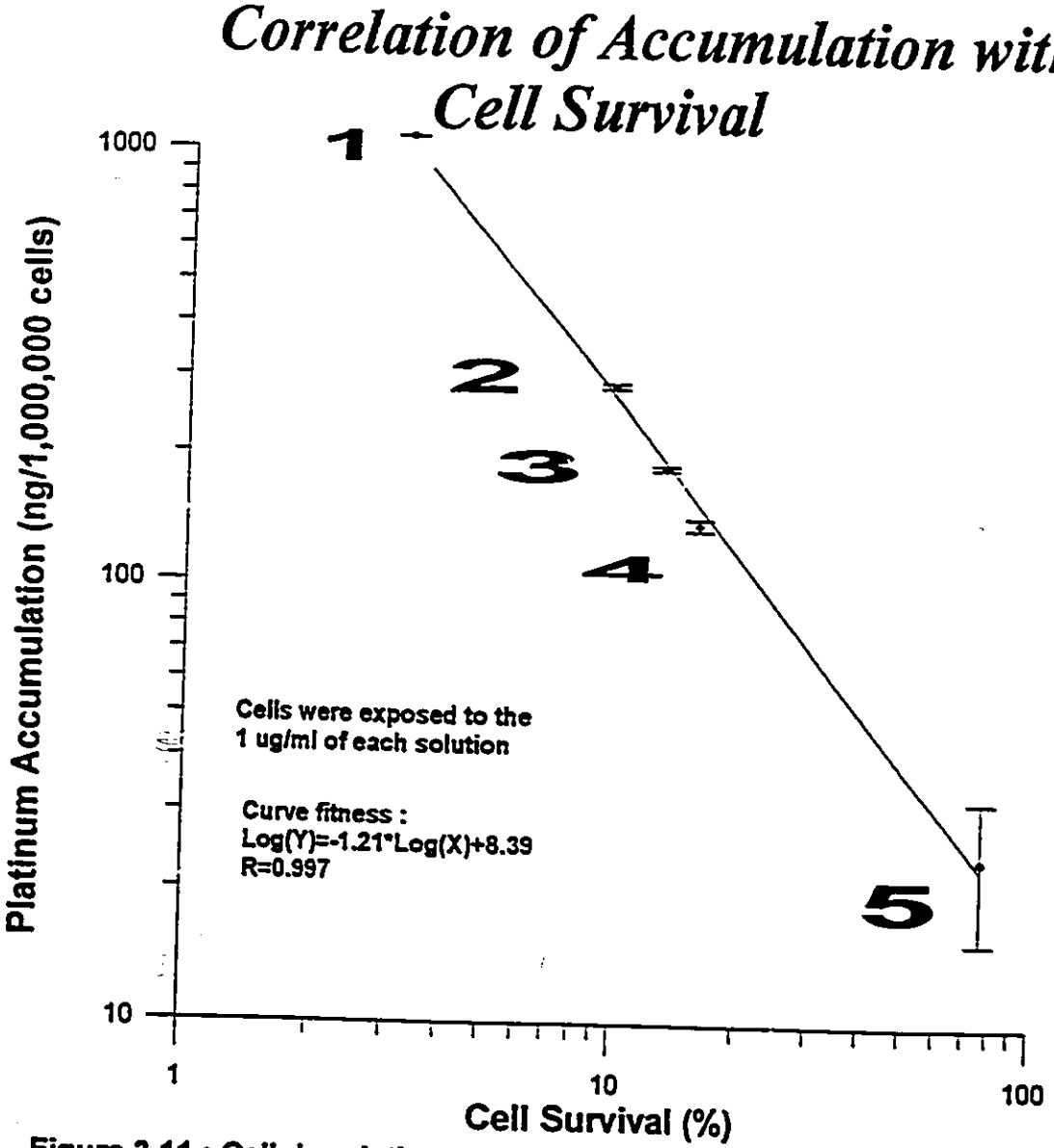


Figure 3.11 : Cellular platinum accumulation (Avg±SE) vs the precentage of survival of OV 2008 cells following one hour exposure to solutions 1 to 5 :

1 : Aquated cisplatin
2 : Cisplatin
3 : Ultra filtrate cisplatin
4 : Monomethionine cisplatin
5 : Bismethionine cisplatin

3.4.1 - Materials :

The same materials have been used as were described in section 3.3.2.

3.4.2 - Method :

One hour exposure of OV 2008 to 0.4 mM of different metabolites carried out exactly as described in section 3.3.3, but this time cells were not trypsinized after one hour exposure and aspiration of the medium.

After one hour exposure to materials, cells were washed three times in three beakers containing one litre of sterile saline, as was described in section 3.3.3. Five millilitres of media were then added to each tissue culture dish, and all tissue culture dishes were again transferred to the incubator. At different specified intervals, tissue culture dishes were taken from the incubator, washed rapidly and assayed for platinum accumulation as described in section 3.3.3.

The following time table was used for the sampling of tissue culture dishes: 0.5, 1, 1.5, 2, 2.5, 3, 4, 5, 7.5, 10, 15, 20, 25, 30, 45, 60, 75, 90, 120, 150, 180,

210, 240, 270 and 300 min after the end of a one hour exposure of cells to metabolites.

Platinum concentration per million cells was plotted versus time (min), and kinetic parameters were calculated using Grapher for Windows (version 4.1) software.

3.4.3 - Results :

Figure 3.12 represents curves fitted to platinum concentration versus time. As is shown in this figure, different metabolites have different efflux rates from OV 2008 cells, however, the general pattern is similar among all of them. Efflux is biphasic: there is a rapid initial decrease in cellular concentration of metabolites in the first 2 hours, followed by a slower efflux rate and eventually a plateau phase about 2.5 to 3 hours after the end of exposure.

Although different species both accumulate and efflux differently from cells, the efflux pattern for different species dose not seem to have an effect on the final cellular concentration of different metabolites, since the rank order of cellular platinum concentrations for different metabolites is still the same at the plateau phase of efflux, and there is not significant difference between the relative percentages of remaining platinum

concentration in the cells exposed to any species in each phase of efflux.

Figures 3.13 to 3.17 represent percentage of total platinum retained in cells (in ng per million cells) on a logarithmic scale, versus time up to 120 min. These curves have been used for the calculation of efflux constants. Tables 3.5-a and 3.5-b summarize the related kinetic calculations on the initial rapid portion and the second slow portion of species efflux.

The results were fit to first-order decay curves, yielding correlation coefficients of -0.98 to -0.99 for different metabolites. The calculated first-order efflux-rate constants are presented in tables 3.5-a and -b. Statistical calculations show significant differences in the efflux-rate constants of different species with respect to each other in most cases, in the initial rapid phase, but not the second phase of efflux. However, these differences were not important enough to effect the final relative platinum concentrations in the different groups.

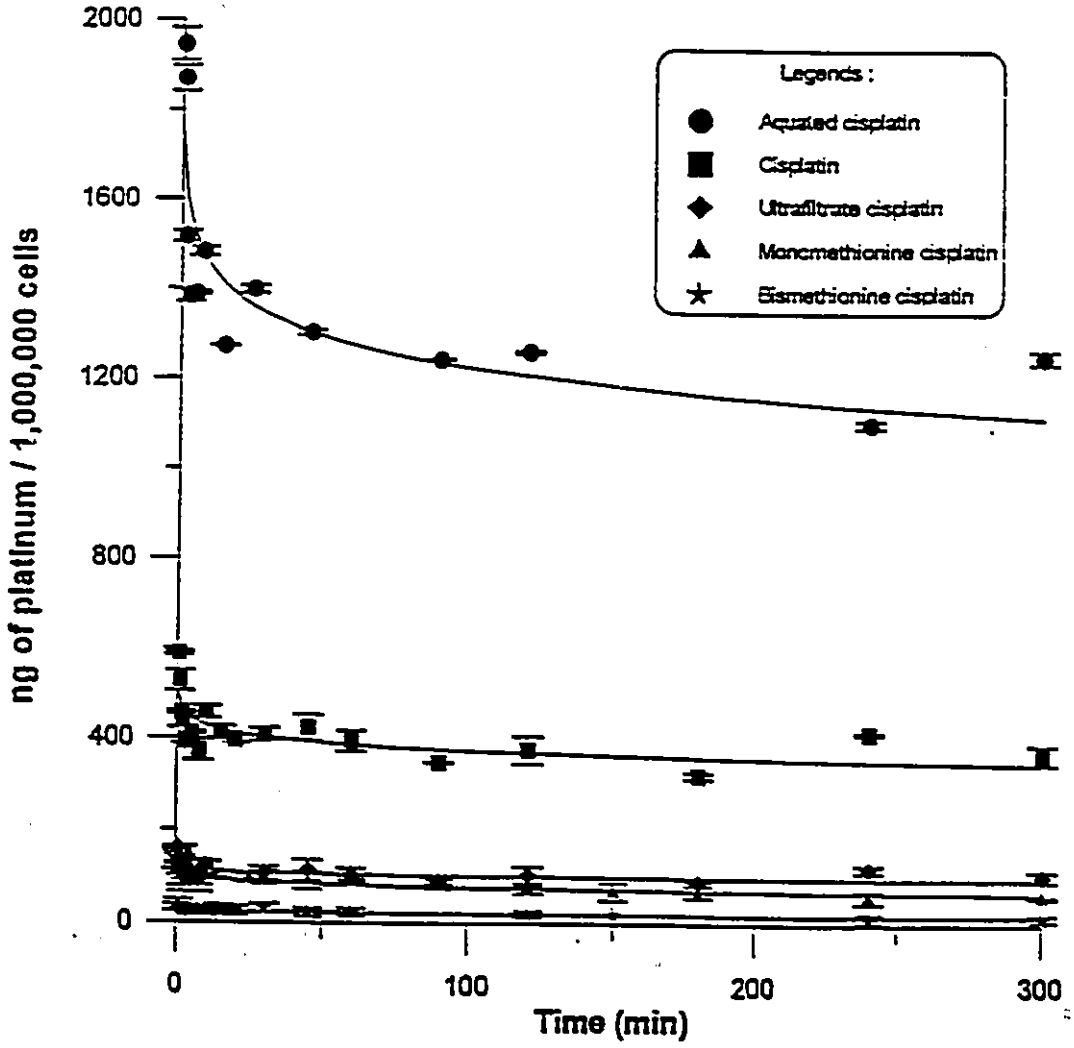


Figure 3.12 : Cisplatin and its metabolites concentrations in OV 2008 cells after one hour exposure. (Avg $\pm$ SE, n = 6)

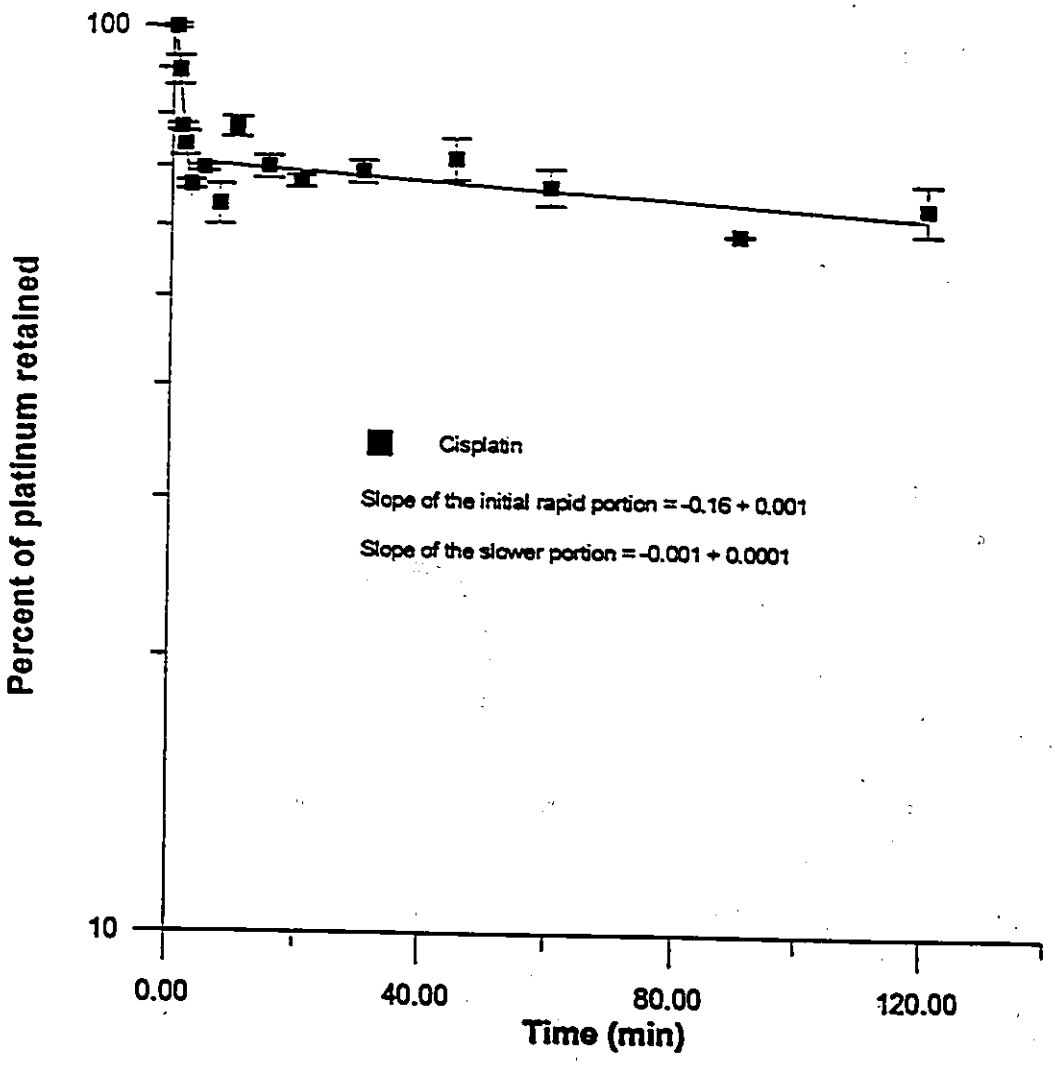


Figure 3.13 : Retained percentage of total platinum per million cells following from a one hour exposure of OV 2008 cells to 0.4 μM cisplatin, up to 2 hours after exposure. (Avg ± SE, n=6)

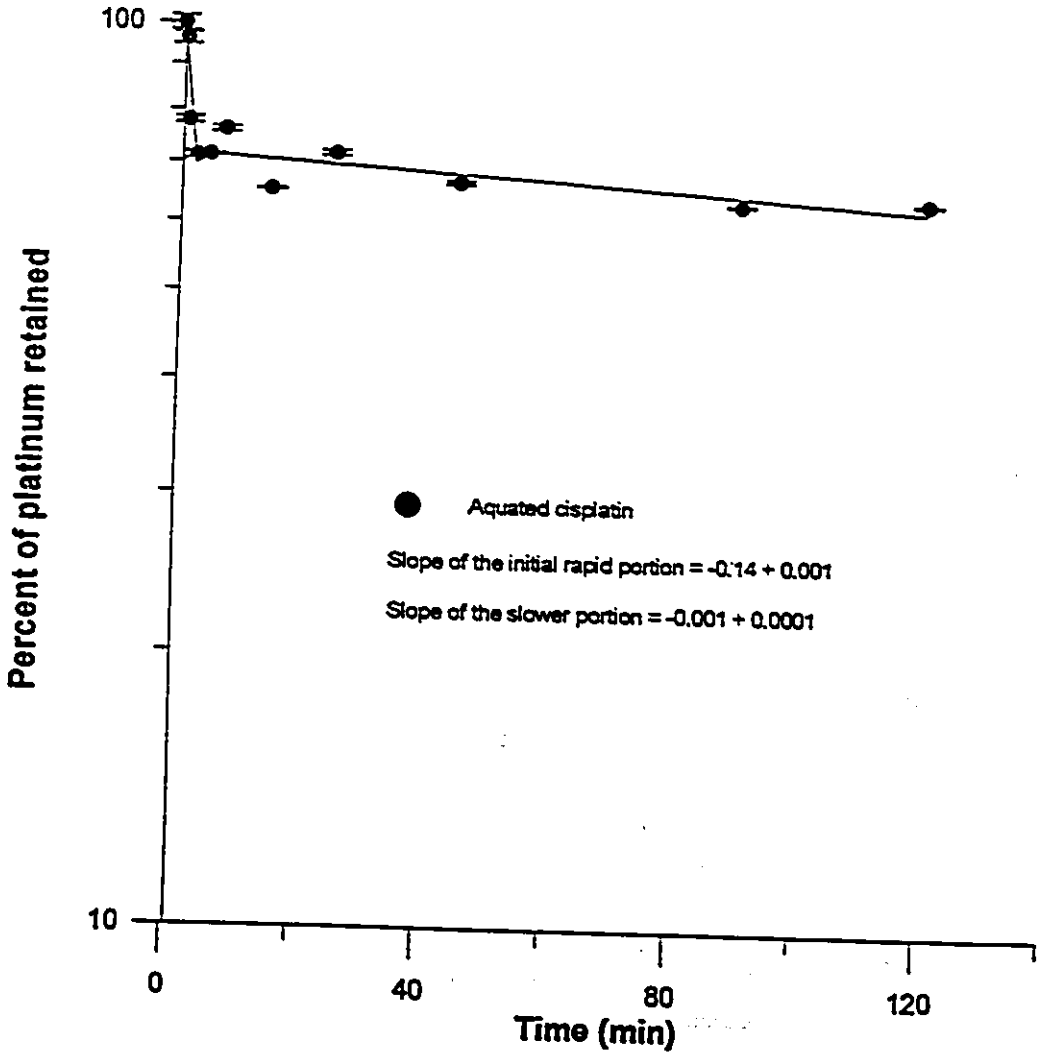


Figure 3.14 : Retained percentage of total platinum per million cells following from a one hour exposure of OV 2008 cells to 0.4 μM aquated cisplatin, up to 2 hours after exposure. (Avg ± SE, n=6)

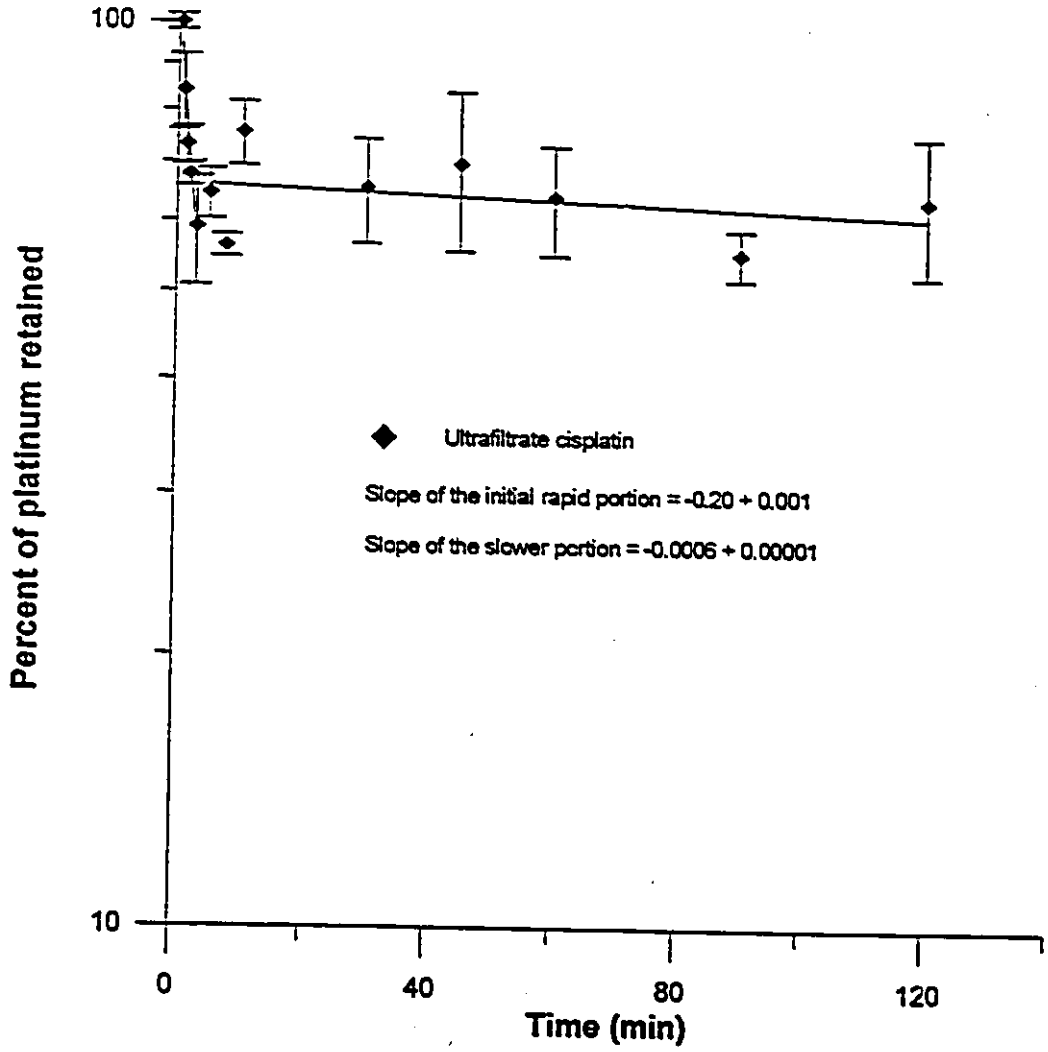


Figure 3.15 : Retained percentage of total platinum per million cells following from a one hour exposure of OV 2008 cells to 0.4 μM plasma ultrafiltrate cisplatin, up to 2 hours after exposure. (Avg ± SE, n=6)

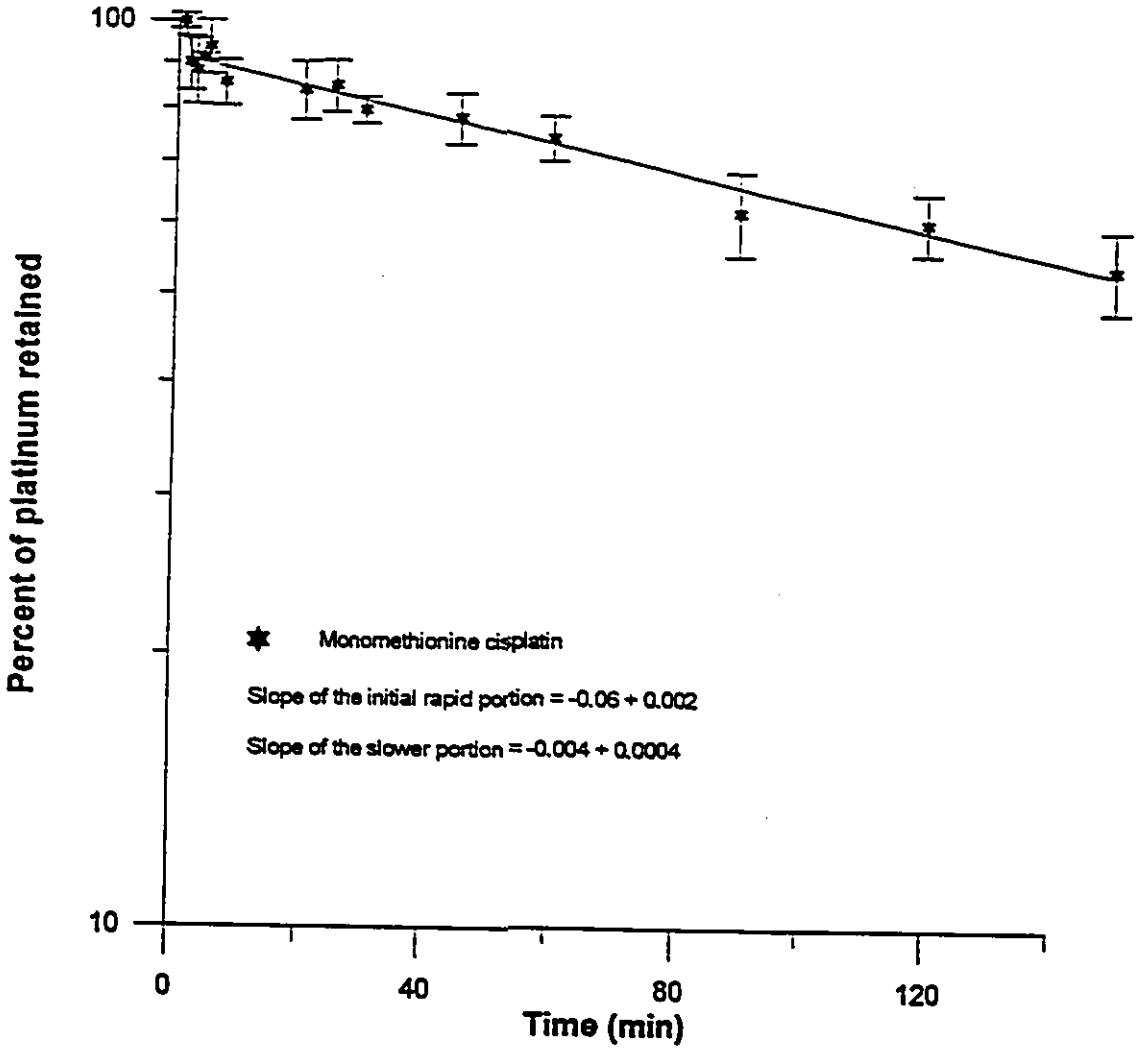


Figure 3.16 : Retained percentage of total platinum per million cells following from a one hour exposure of OV 2008 cells to 0.4 μM monomethionine cisplatin, up to 2 hours after exposure. (Avg ± SE, n=6)

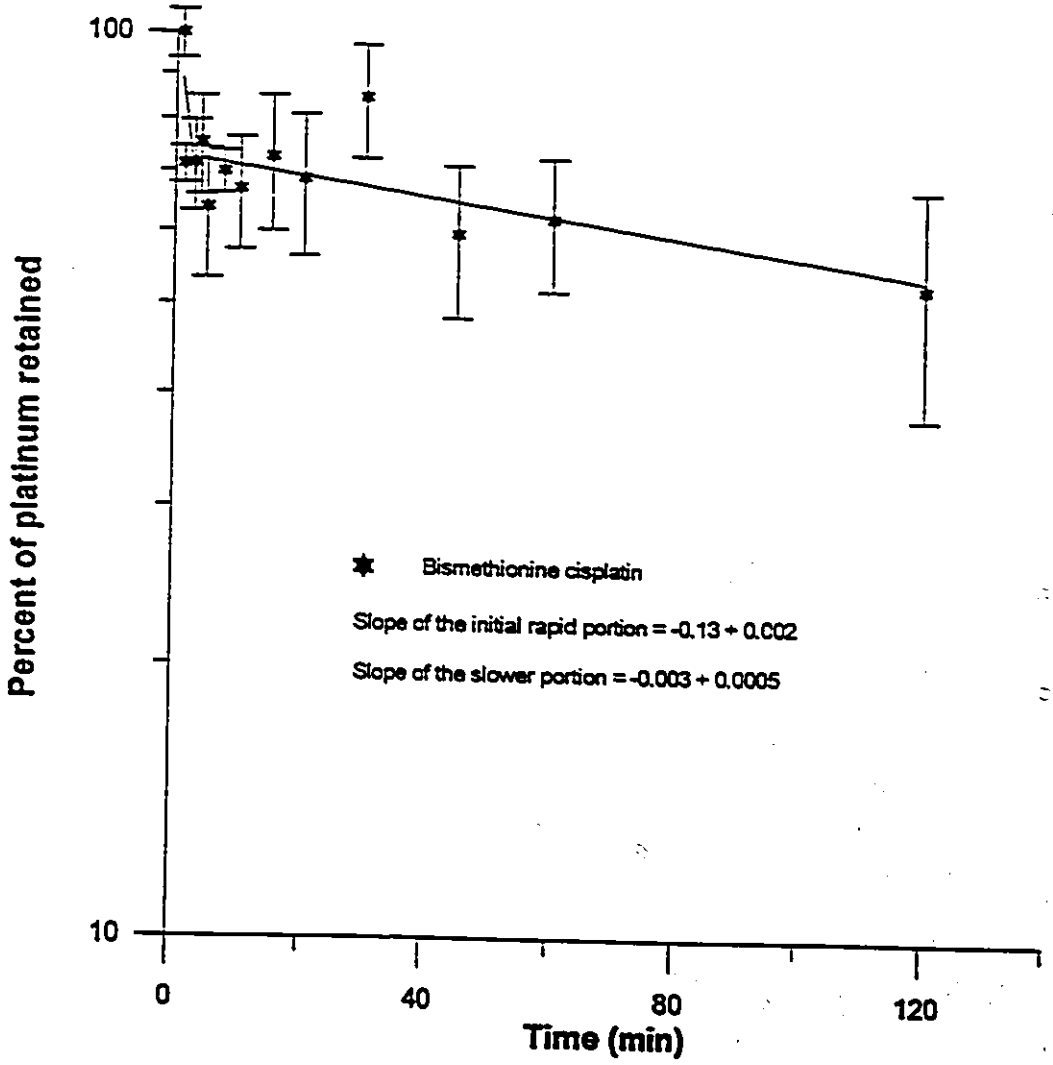


Figure 3.17 : Retained percentage of total platinum per million cells following from a one hour exposure of OV 2008 cells to 0.4 μM bismethionine cisplatin, up to 2 hours after exposure. (Avg ± SE, n=6)

Efflux Results

(Initial Rapid Portion)

Species	Slope	K (S^{-1})*
Cisplatin (C)	-0.16±0.001	0.37±0.001
Aquated C (A)	-0.14±0.001	0.32±0.001
Plasma ultrafiltrate C	-0.20±0.001	0.46±0.001
Bismethionine C (B)	-0.13±0.002	0.30±0.01
Monomethionine C (M)	-0.06±0.002	0.14±0.02

* Efflux-rate constant (K = slope x (-2.303))

Tukey-Kramer multiple comparison ANOVA post test

A vs C	p<0.05	C vs M	p<0.001
A vs M	p<0.001	C vs B	p<0.01
A vs B	N.S.	M vs B	p<0.001
A vs U	p<0.001	B vs U	p<0.001
C vs U	p<0.001	M vs U	p<0.001

Table 3.5-a : Kinetic and Statistical analysis of efflux results (initial phase).

Efflux Results

(Second Slow Portion)

Species	Slope	K (S^{-1})*
Cisplatin (C)	-0.001±0.0001	0.002±0.0001
Aquated C (A)	-0.001±0.0001	0.002±0.0001
Plasma ultrafiltrate C	-0.0006±0.0	0.001±0.0
Bismethionine C (B)	-0.003±0.0003	0.007±0.0003
Monomethionine C (M)	-0.004±0.0005	0.009±0.0005

* Efflux-rate constant (K = slope x (-2.303))

ANOVA Test

ANOVA showed that there were not any statistically significant differences between the various rate constants of efflux of second phase

F=0.968 p=0.4665

Table 3.5-b : Kinetic and Statistical analysis of efflux results (second phase).

4

Infrared changes of cells exposed to cisplatin and its metabolites:

4.1 - Introduction :

Spectroscopy techniques may be applied over a very wide range of scientific disciplines, from the endless investigations in space to the fine procedures under the microscopes. An introduction to atomic spectroscopy was presented in section 3.3.1. What is described here is a review of those aspects of this science and some basic spectroscopic terminology to provide a better understanding of how I have made use of molecular spectroscopy in my project.

4.1.1 - Introduction to spectroscopy:

Molecular spectroscopy may be defined as the study of the interaction of electromagnetic waves and matter. Electromagnetic radiation, of which infrared is a part,

may be considered as a simple harmonic wave propagated from a source and travelling in straight lines, as is shown in figure 4.1 (Banwell,1972). Wavelength (λ) is the distance which the wave travels during a complete cycle (Figure 4.1), with a variety of units chosen for any particular range for example, meters (m), centimetres (cm), micrometers (μm) or angstroms (Å). The reciprocal of the wavelength, expressed in units of cm^{-1}, is called wavenumber. These parameters are used to characterize electromagnetic radiation. Figure 4.2 illustrates in pictorial fashion the various regions into which electromagnetic radiation has been divided. The boundaries between the regions are by no means precise, although the molecular processes associated with each region are quite distinct.

When the velocity of radiation is equal to *c* (speed of light) meters per second, v, the frequency of the wave, may be defined as c/λ, with the SI unit of hertz (Hz) and the dimension of reciprocal seconds (s^{-1}). This parameter may be used to correlate the definition of waves with energy (E) with the help of universal constant of Planck (*h*).

If a light beam with a specific frequency range (for example, in the infrared region) passes through an empty sample container and then into a detector equipped with a

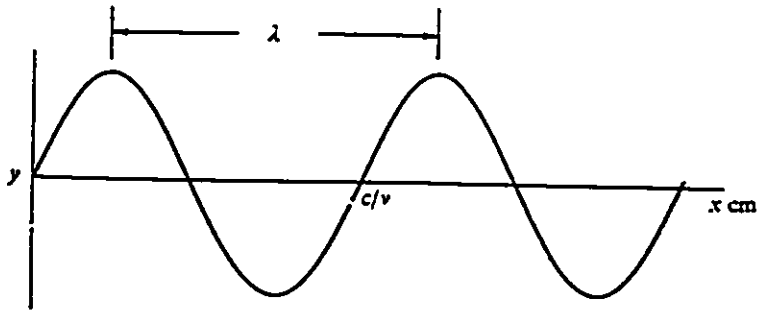


Figure 4.1 : A simple harmonic wave.

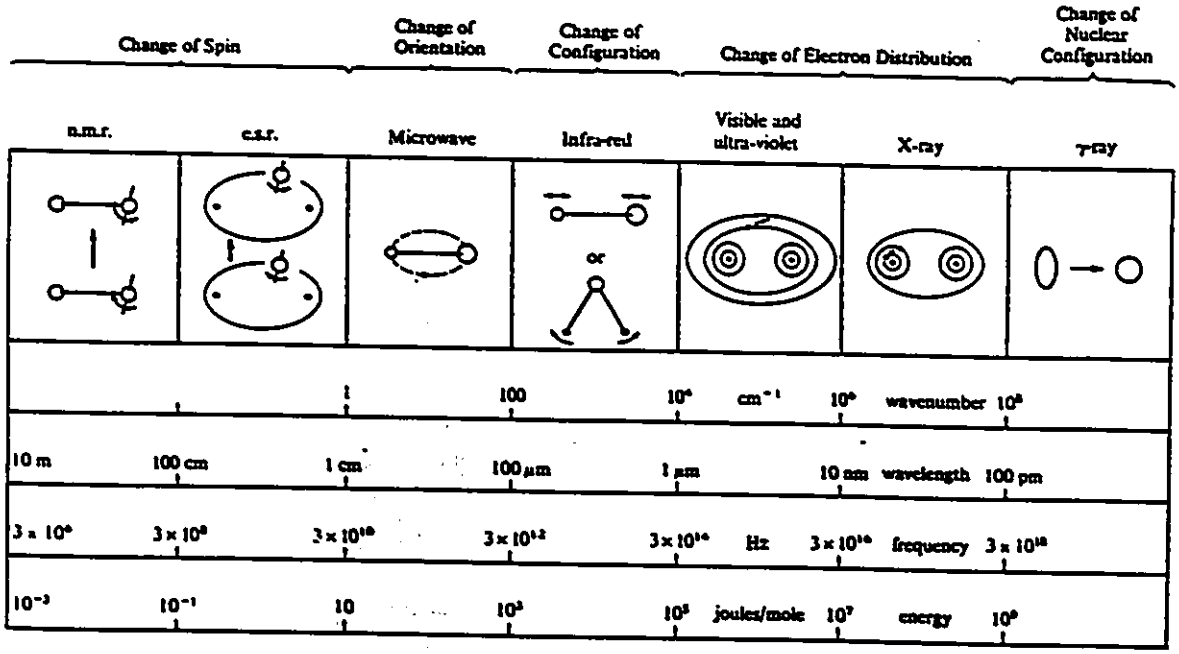


Figure 4.2 : Various regions of electromagnetic radiation.

photomultiplier tube, consisting of a light-sensitive surface which emits electrons when light falls upon it, the connected recorder to this photocell should draw a straight line (base line), since the frequency of the beam and therefore its energy level will remain the same. If one imagines the sample space to be filled with a substance having only two possible energy levels, E_1 and E_2, the recorder will show a base line at all points except at the frequency $\nu=\Delta E/h$, since energy at this frequency will have been absorbed by the sample in raising each molecule from state 1 to state 2. Further, if, as is almost always the case, there are many possible energy levels, $E_1, E_2, E_3, \ldots$ available to the sample, a series of absorption lines will appear on the recorder at frequencies defined with the proper energy state transitions. The absorbance of light passing through the sample may vary from zero, when there is no energy absorption matched with the resultant energy level of that particular frequency, to 100 percent, when all of the energy at the particular frequency is absorbed with the proper chemical group.

The derivative of a spectrum is another form of presentation which is often adopted (O'Haver,1976). The derivative of a curve is simply its slope at a given point. In calculus notation, the derivative of a spectrum

is dA/dv, where dA is the difference in absorbance and dv is the frequency distance in which the absorbance is occurred. The derivative record is thus a plot of the slope dA/dv against v. The derivative trace has advantages over the direct record in some circumstances. One advantage is that it indicates the centre of each absorbance peak in a rather more precise manner, since at the centre of a peak, the curve is horizontal. Hence, dA/dv is equal to zero, so the centres are marked by the intersection of the derivative curve with the axis. This technique is often used to determine the exact frequency of the maximum absorbance (peak), especially when comparison of peaks are proposed (Cameron,1987).

Deconvolution of a spectrum is another way of looking at a graph to get a more precise idea of intensities i.e. the percentage of absorbance at any particular frequency. This method is very useful to increase signal to noise ratio of the spectrum. Considering any particular absorbance peak which is real and/or of spectroscopist's interest as signal, noise may be defined as minor absorbances on the base line which come from impurities or are not of interest for a particular sample. Two computational techniques have been used in spectroscopy to deconvolute spectra. An iterative method based on Van Cittert's method (1931) has been developed and applied to

the study of gas phase spectra recorded on laser spectrometers (Pliva,1980). The second technique is based on the use of Fourier transforms which requires a particular form of apodization of the interferogram corresponding to the deconvoluted spectrum (Beer,1992). The feasibility of this technique has been pointed out (Betty,1976) and recently two specific applications to NMR (Ferrige,1978) and Raman (King,1980) spectra have been reported.

The application of this technique to infrared spectroscopy has been developed at the National Research Council in Ottawa (Kauppinen,1981).

4.1.2 - Biological applications:

There is some disagreement in the interpretation of IR spectra from whole cells or tissue samples among spectroscopy researchers (Wong,P.T.T., personal communication). However, infrared spectroscopy is no longer limited to structural studies of small molecules but is useful for the measurements of biological macromolecules and of suitably selected group vibrations in tissues.

Identification of infrared absorption bands led to qualitative and quantitative measurements of base stacking and hydrogen bonding in nucleic acids (Thomas,1969),

tertiary structures of ribonucleic acids, t-RNA (Schernau,1977), 5sRNA (Burkey,1983) and proteins (Susi,1969). These approaches require highly stable instrumentation and excellent signal-to-noise ratios to yield sufficiently reproducible spectral data. Infrared spectroscopy in the study of membrane structures has been reviewed by Fringeli and Gunthard (1981). Cameron and co-workers (1980) have studied C-H stretching and bending modes of fatty acid groups as a functions of temperature. Membrane-protein interactions have also been examined in studies of the polypeptide melittin dissolved in aqueous or organic solvents or incorporated into phospholipid multilayers by Lavialle *et al.* in 1982.

Theophanides and co-workers (1984) considered the interactions of metal ions with nucleic acids. They made comparisons of spectra from a number of series of nucleic acid derivatives and their metal complexes. They examined the in-plane and out-of-plane bending modes of CH, CO and NH of the pyrimidine or purine ring vibrations around 1300-1650 cm^{-1}, the fingerprint region, the CH, NH and OH stretching vibrations and finally the range of 400-1300 cm^{-1} which contains C-C and C-N single bonds and the exocyclic and the sugar-phosphate groups. With the help of the spectral changes of the nucleic acids, they determined the site of attachment to the metal ions.

Application of infrared spectroscopy in biological samples containing polypeptides, proteins, enzymes, prophyrins, hemeproteins, metaloproteins, carbohydrates, nucleic acids and related compounds, viruses, nucleoproteins, model membranes, biomembranes, lipid containing systems, carotenoids, polyene aggregates and steroids has been reviewed by Parker (1983).

4.2 - Material and Methods:

4.2.1 - Materials :

Instruments :

A Bomen-Michelson series 110 Fourier Transform spectrometer was used with a liquid nitrogen-cooled mercury-cadmium-telluride detector. The infrared beam was condensed onto the AgCl sample cell by a sodium chloride lens system. The system was purged for one minute with a nitrogen stream to remove any water vapour and carbon dioxide before any spectra were taken.

For each spectrum, 512 interferograms were averaged at a spectral resolution of 4 cm^{-1}.

The source of the light intensity infrared radiation was a temperature-stabilized glowing ceramic source. Wavenumber precision was controlled by an internal He-Ne laser.

The detector was a mercury-cadmium-telluride (MCT) detector cooled by liquid nitrogen.

Cell line :

OV 2008 cell line was used for the experiments with the specifications have been described before (section 3.1.1). Cisplatin and its metabolites were prepared as previously described (section 3.1.1).

4.2.2 - Method :

OV 2008 cells in plateau phase were exposed to each of the cisplatin metabolites at 0.33 mM, 1.6 mM and 2.5 mM for one hour (see description in section 3.1.2). One group of cells which were not exposed to drug, were used as negative controls.

After one hour incubation the media was aspirated and cells were washed for four times with saline. Cells were harvested from the plates by gentle scraping off the tissue culture plates in 10 ml saline. Preliminary experiments indicated that infrared spectra of these cells were not affected by either gentle scraping or trypsinization. Cells were collected in sterile plastic vials, washed two times with saline and pelleted by centrifugation at room temperature. The pellet cells were stored at -80°C until analyzed by infrared spectroscopy.

The whole procedure was repeated three times for each metabolite solution at each concentration.

Infrared spectra were obtained by placing small amounts (about 0.01 mg) of cell samples at room temperature between two AgCl windows of the infrared adsorption spectra sample holder. Fourier transform infrared spectra at the region of 400-4000 cm^{-1} frequency were measured with a Bowman spectrometer as was described in section 4.2.1. For each spectra 512 scans were coadded, at a spectral resolution of 4 cm^{-1}. Infrared spectroscopy was conducted on each sample between three to seven times.

The infrared spectra resulting from exposure of the cells to each cisplatin metabolite was analyzed and six selected regions (to be described below) were focused (after either derivatization or deconvolution). The frequencies (mean ± S.E.) of the spectral peaks were determined. The reproducibility of the infrared spectra was confirmed with the non-significance differences between repeated measurements on any sample, came from analysis of variance (ANOVA) test on the peak frequencies of any of six focused region.

Averages and standard errors of peaks in any of six well known regions were calculated for each sample (any species in any concentration and negative control). The significance of the peak frequency shift for any sample in

comparison to either saline (negative control) or the other samples (either different concentrations of the same species or different species) were measured using two-way ANOVA analysis and Tukey-Kramer multiple comparisons post ANOVA tests.

4.3 - Results :

Figure 4.3 shows the IR spectra of the OV 2008 cell line. Generally, the peak at 970 cm^{-1} is due to the symmetric stretching of dianionic phosphate monoester of phosphorylated proteins and cellular nucleic acids (Wong,1991-c). The band at 1086 cm^{-1} is due to the symmetric stretching of the phosphate group of nucleic acids while the antisymmetric stretching of phosphate has two overlapping bands (a hydrogen bonded band at 1220 cm^{-1} and a non-hydrogen bonded band at 1240 cm^{-1}. The band in the range 1155-1170 cm^{-1} is mainly due to C-O stretching of the C-OH groups of serine, threonine and tyrosine in proteins. The peak corresponding to the bending mode of CH2 of the fatty acid chains of lipids is in the range 1460 to 1480 cm^{-1}. The C=O stretching vibration of membrane lipids is expressed in the 1650 to 1750 cm^{-1} spectral region (Wong,1991-d).

The spectral region of 1600 to 1700 cm^{-1} is due to the in-plane C=O stretching vibration weakly coupled with C-N

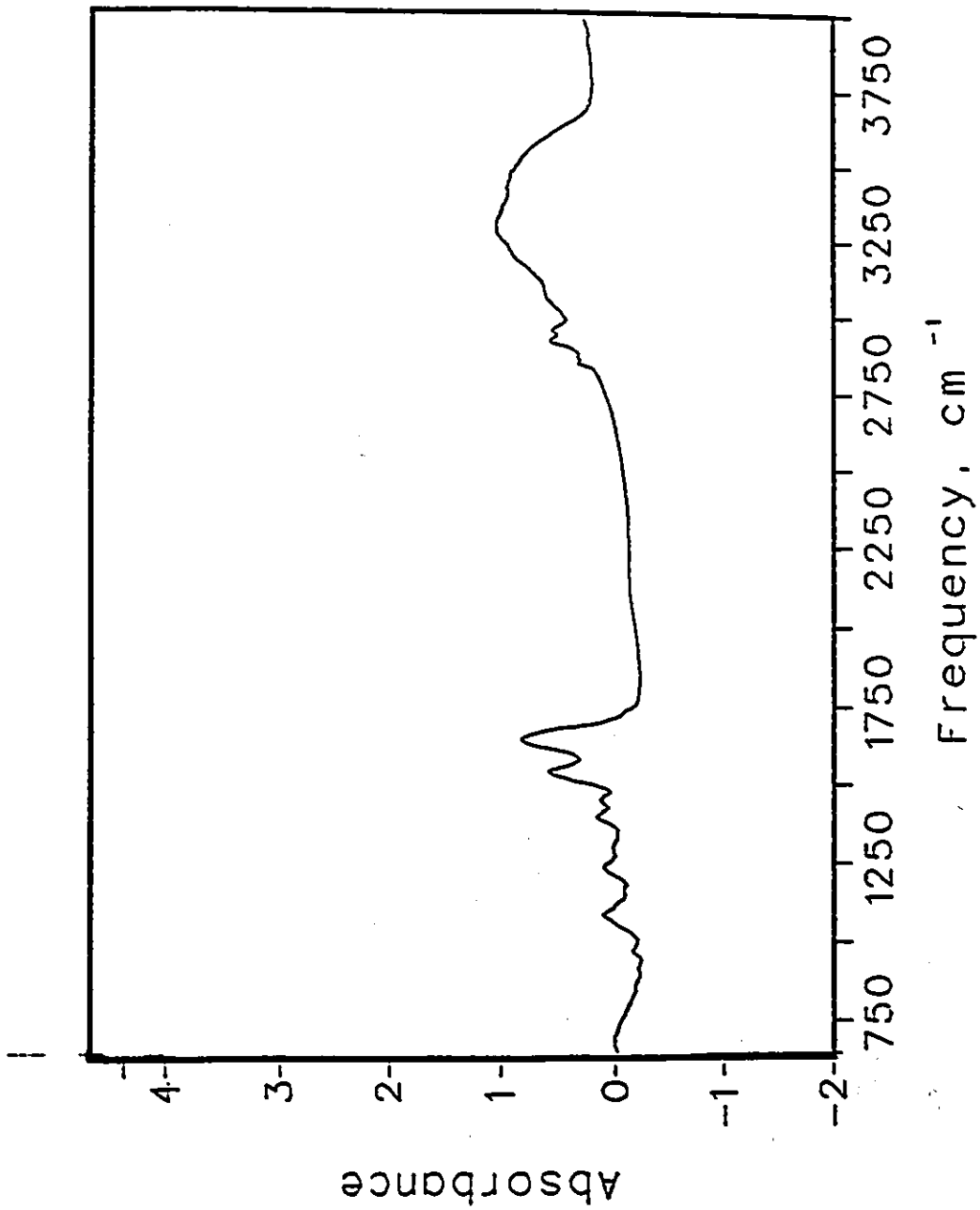


Figure 4.3 : The IR spectra of OV 2008 cells.

stretching and in-plane N-H bending of the amide groups in proteins. The region 2820-2910 cm^{-1}, is due to the stretching mode of methylene chains in membrane lipids.

The spectra of cells exposed to the different metabolites were compared to each other and to those of a negative control (cells exposed to saline) after derivatization (Cameron,1981) or deconvolution (Kauppinen,1981). Wavelengths are presented as average$\pm$ standard error where applicable. Table 4.1 summarizes the defined peaks of six spectral regions exposed to cisplatin species at 0.33 mM.

4.3.1- Region 950-1020 cm^{-1}:

As shown in figure 4.4, there are two bands in this region of the IR spectra of the OV 2008 cell line, one at 971.01$\pm$0.08 cm^{-1} and the other at 1012$\pm$0.91 cm^{-1}. This figure originates from the third power derivative of the original spectrum with a breakpoint of 0.15. The band at 971 cm^{-1} is due to three separate biochemical groups give rise to the band at this region. These are nucleic acids (Wong,1991-b) and the symmetrical stretching mode of dianionic phosphate monoesters of phosphorylated

Table 4.1 : Important infrared peaks of different regions of OV 2008 cells exposed to 0.33 mM of different species. (N=4)

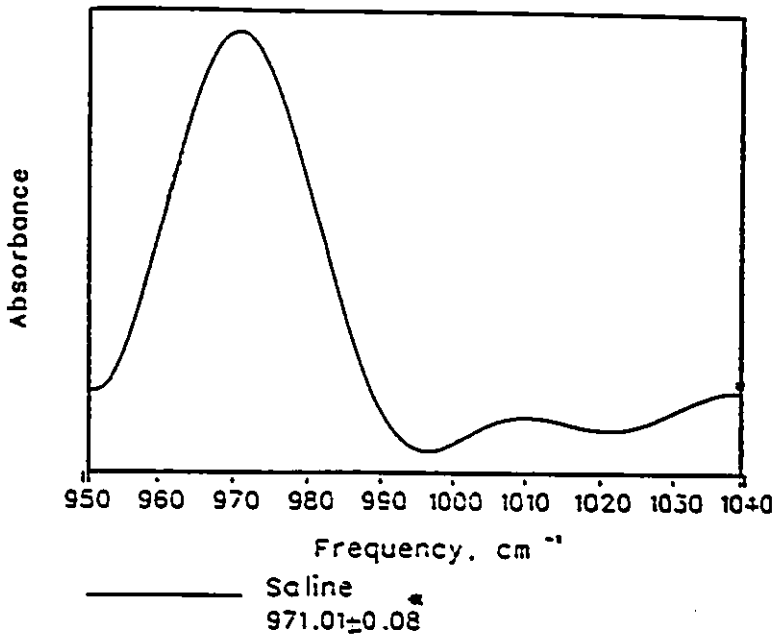
(CDDP: Cisplatin B: Bismethionine M: Monomethionine PUF: Plasma ultrafiltrate cisplatin)

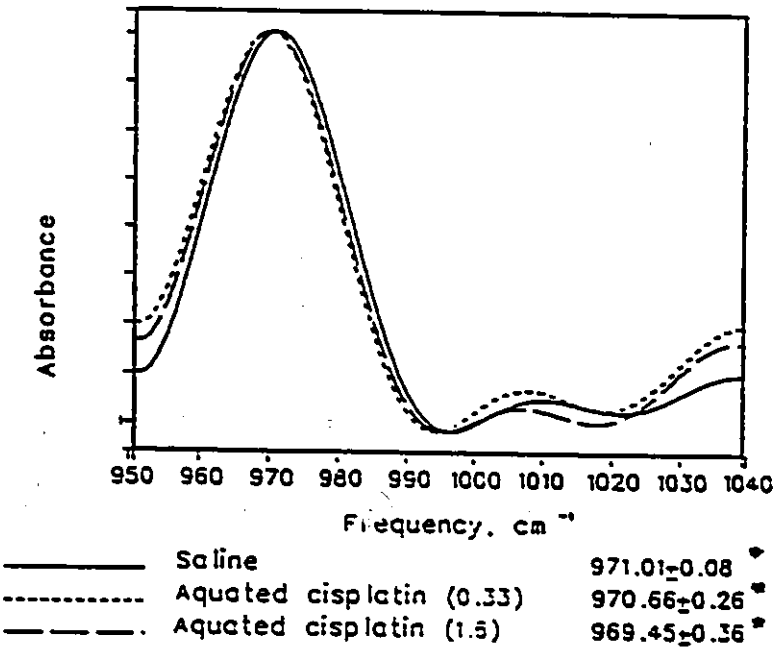
Saline	Aquated CDDP	B CDDP	CDDP	M CDDP	PUF
971.01±0.08	970.66±0.26	971.27±0.14	969.5±0.17	969.74±0.32	969.14±0.0
*:	N.S.	N.S.	p<0.001	p<0.001	p<0.001
1059.70±0.02	1059.38±0.12	1059.56±0.07	1060.11±0.05	1059.48±0.13	1060.6±0.08
*:	N.S.	N.S.	p<0.01	N.S.	p<0.01
1086.59±0.10	1086.67±0.14	1086.95±0.02	1086.31±0.11	1085.72±0.13	1087.20±0.42
*:	N.S.	N.S.	N.S.	p<0.001	p<0.001
1172.7±0.08	1171.85±0.18	1171.93±0.06	1171.44±0.03	1171.24±0.02	1172.30±0.15
*:	p<0.001	p<0.001	p<0.001	p<0.001	N.S.

Saline	Aquated CDDP	B CDDP	CDDP	M CDDP	PUF
1240.37±0.13	1239.69±0.51	1240.58±0.09	1239.65±0.13	1239.67±0.23	1238.13±0.66
*:	p<0.05	N.S.	p<0.05	p<0.05	p<0.01
1650.15±0.26	1653.31±0.18	1652.35±0.28	1653.73±0.4	1652.95±0.25	1655.17±0.14
*:	p<0.001	p<0.001	p<0.001	p<0.001	p<0.001
2852.44±0.02	2852.41±0.05	2852.33±0.01	2852.73±0.02	2852.24±0.01	2852.34±0.25
*:	N.S.	N.S.	p<0.01	p<0.05	p<0.05
2874.62±0.04	2874.60±0.10	2874.8±0.01	2874.91±0.01	2874.45±0.03	2874.32±0.0
*:	N.S.	N.S.	p<0.001	N.S.	p<0.05
2892.90±0.09	2892.77±0.23	2893.14±0.64	2893.27±0.20	2892.9±0.09	2893.82±0.49
*:	N.S.	N.S.	N.S	N.S.	N.S.

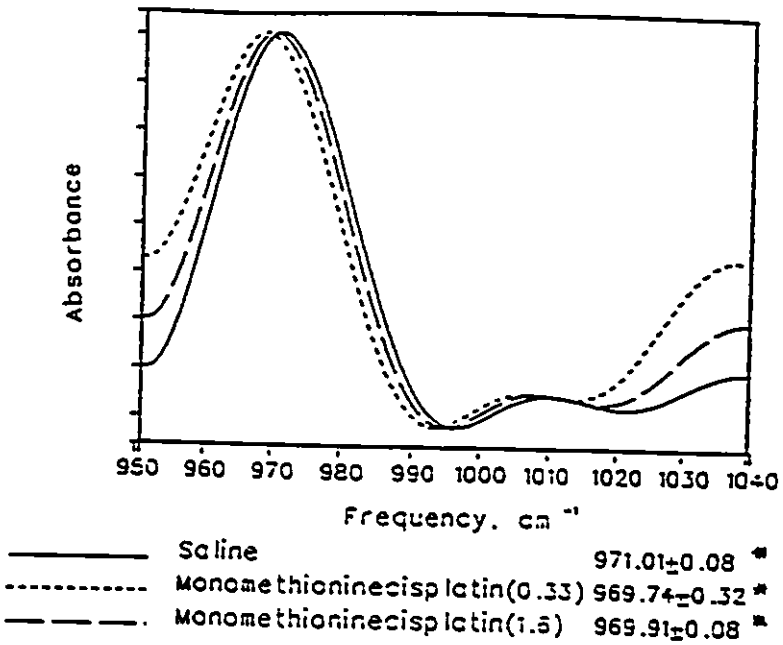
Table 4.1 : continue

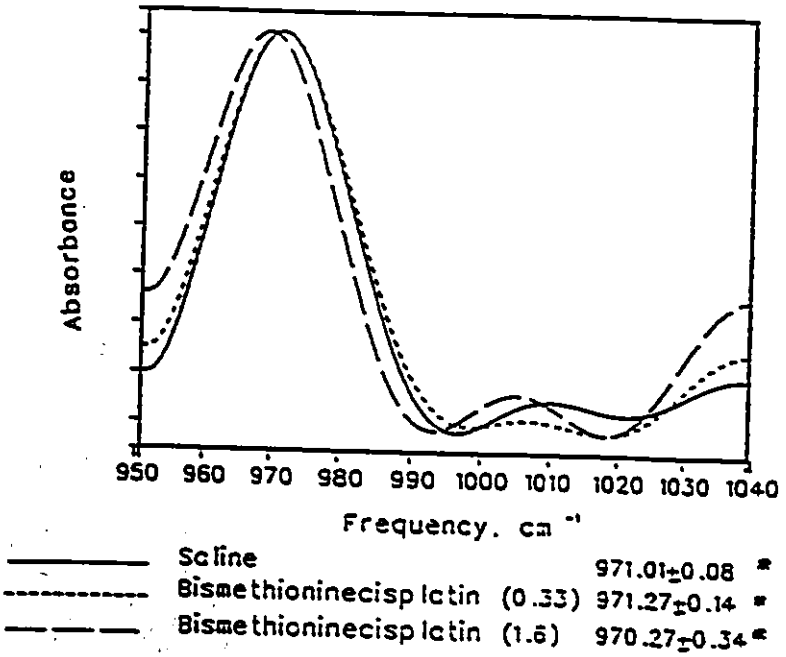
* : value of Tukey-Kramer multiple comparison ANOVA post test between cisplatin metabolites and saline control. (ANOVA for all 9 regions show a significant p value)



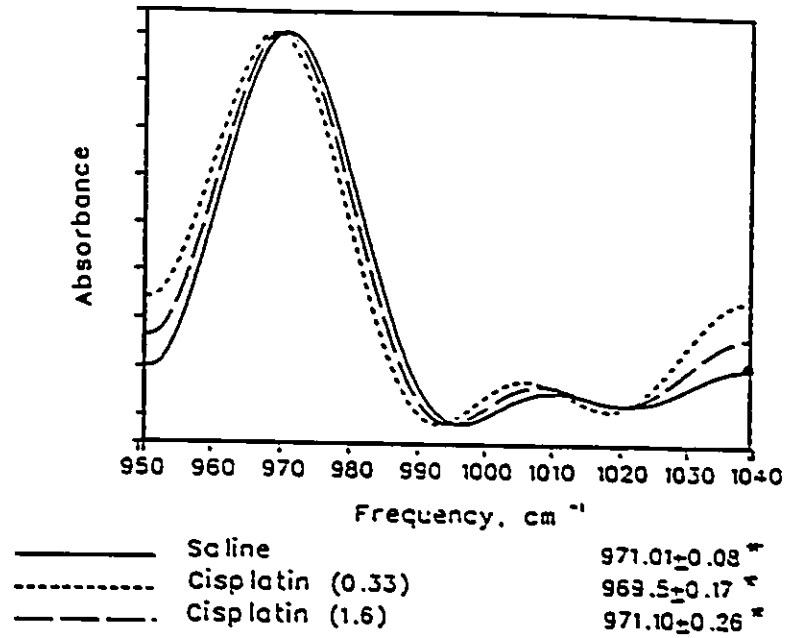


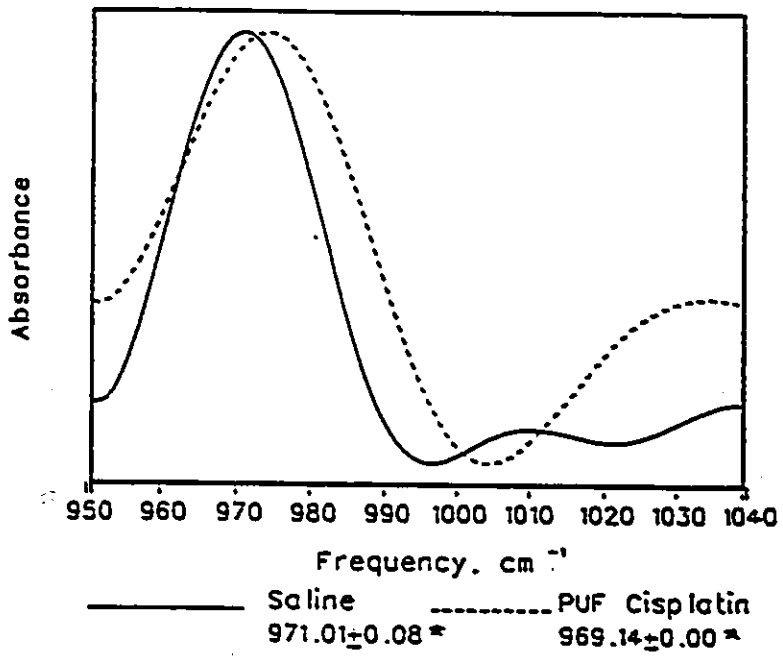
Figures 4.4 and 4.5 : IR spectra of OV 2008 cells (region 950-1020 cm^{-1}) - * peak frequency





Figures 4.6 and 4.7 : IR spectra of OV 2008 cells (region 950-1020 cm^{-1}) - * peak frequency



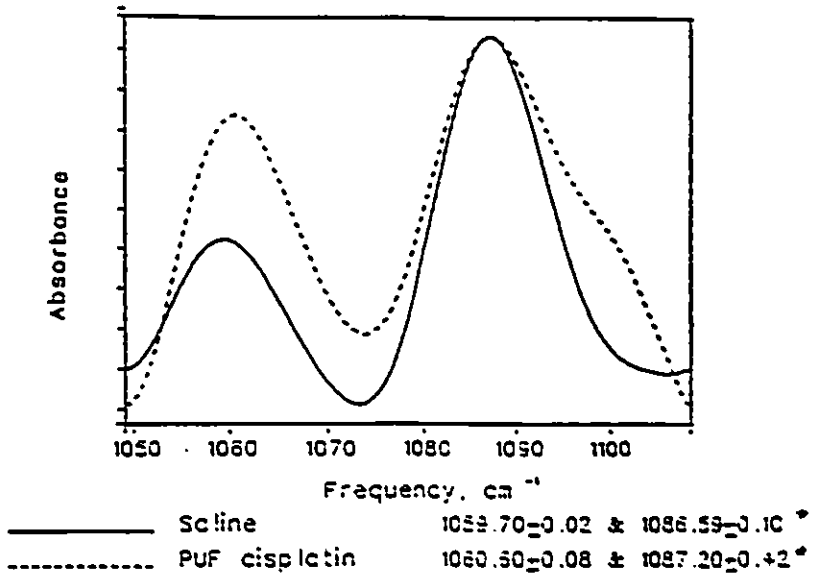


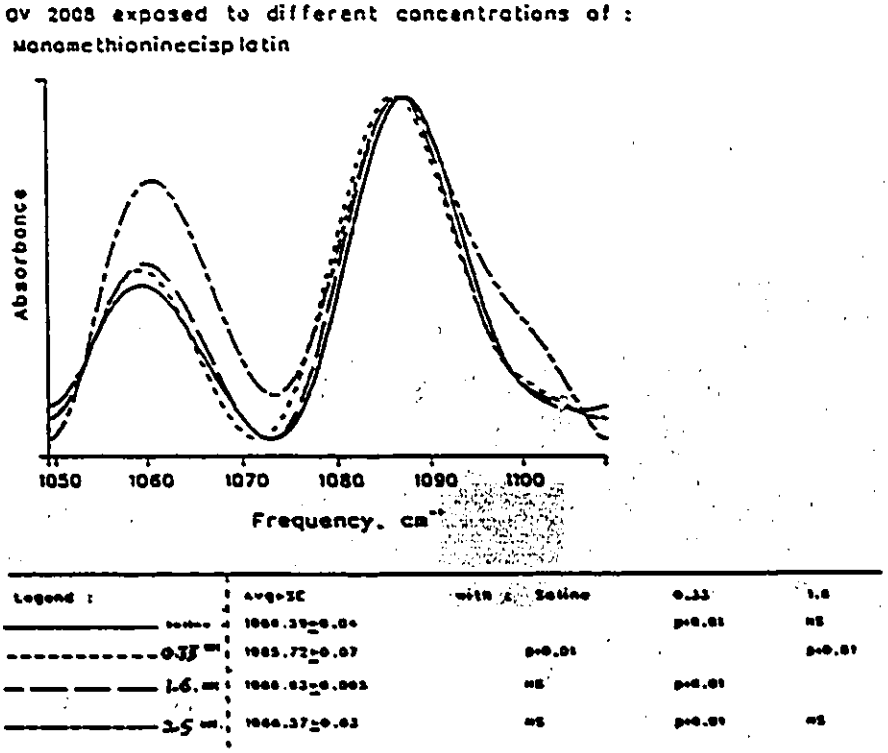
Figures 4.8 and 4.9 : IR spectra of OV 2008 cells (region 950-1020 cm^{-1}) - * peak frequency

proteins (Sanchez-Ruiz,1988), whereas the next band has not been exactly assigned yet. Sometimes the C-O group of carbohydrates may contribute to the spectra at this region as well. The bands are shifted to the lower frequencies as a result of exposure of cells to 0.33 mM cisplatin dissolved in ultrafiltrated plasma (the term frequency is used in this chapter for "wave number" as is usual in spectroscopy - see section 4.1). Figures 4.5 to 4.9 show the shift of these two bands as a result of cell exposure to 0.33 mM and 1.6 mM of different platinum compounds. Monomethionine cisplatin caused the most shift to the left of spectra in this region (969.74$\pm$0.32 cm^{-1} and 1009.36$\pm$1.14 cm^{-1} respectively, ANOVA $p<0.001$). The amount of the shift is greater with higher concentrations of species added to the cell media for example, the picked frequencies show significant differences from that of the control with the $p<0.05$ for 1.6 mM and $p<0.001$ for the 2.5 mM concentration of bismethionine cisplatin exposure to the cell in relative to the control.

4.3.2- Region 1050-1110 cm^{-1}:

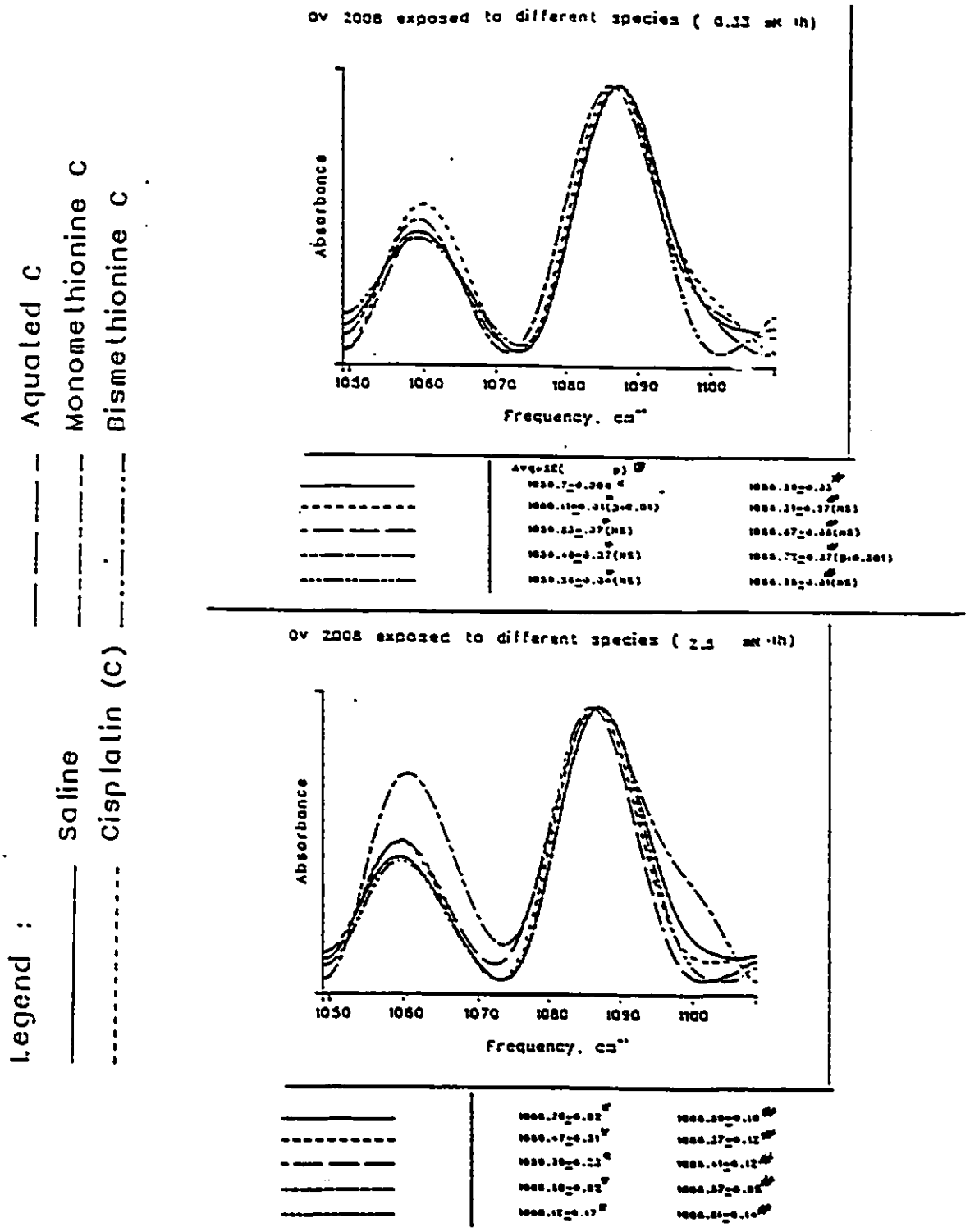
Figure 4.10 shows two bands in this region, after third power derivatization of the original spectra with





Figures 4.10 and 4.11 : IR spectra of OV 2008 cells (region 1050-1110 cm^{-1}) - * peak frequency

the breakpoint of 0.3. In control OV 2008 cells, these two bands are at 1059.70±0.02 cm^{-1} and 1086.59±0.10 cm^{-1}. The band at 1086 cm^{-1} is mostly related to DNA packing condition (Wong,1991-a). It is due mainly to the symmetric phosphate (PO_2-) stretching mode (Parker,1971). The band at this frequency may also represent glycogen (Wong,P.T.T. et al. 1991). Changes in the tertiary structure of DNA double strands will be reflected in this region by the shifting of bands to lower or higher frequencies. More unpacked nucleic acids will result in more of a shift to a lower frequency of this band. After one hour exposure to different platinum compounds, monomethionine cisplatin and the parent drug caused more changes in the pattern of spectra than did the other species. Post ANOVA test results showed significant differences from controls for the first peak with cisplatin ($p<0.01$) and for the second peak with monomethionine cisplatin ($p<0.001$), respectively. Figure 4.11 shows the same region with the same derivatization parameters of OV 2008 cell lines exposed to concentrations of 0.33 mM, 1.6 mM and 2.5 mM of monomethionine cisplatin for one hour. As is shown in this figure, there are dose-related changes in the intensities and frequencies of bands in this region with the increase in the drug concentration. Figures 4.12 and 4.13 show spectra for OV 2008 cells at the same region

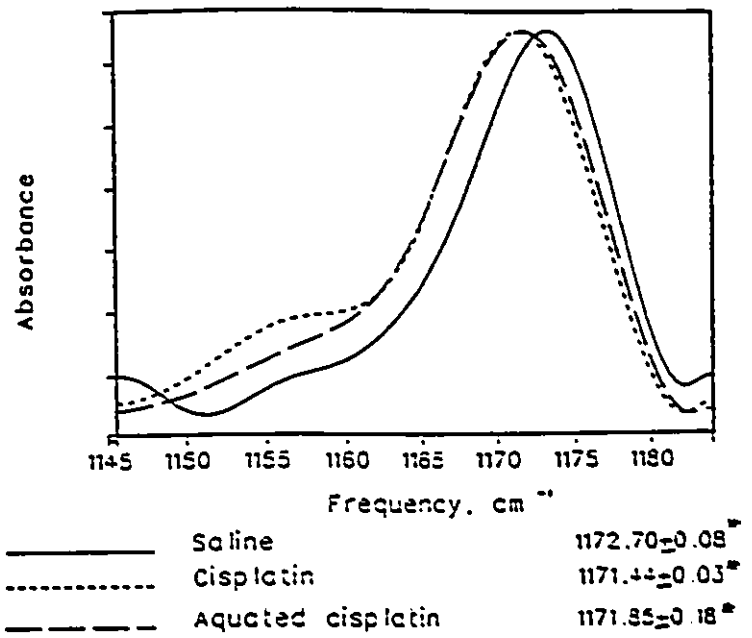


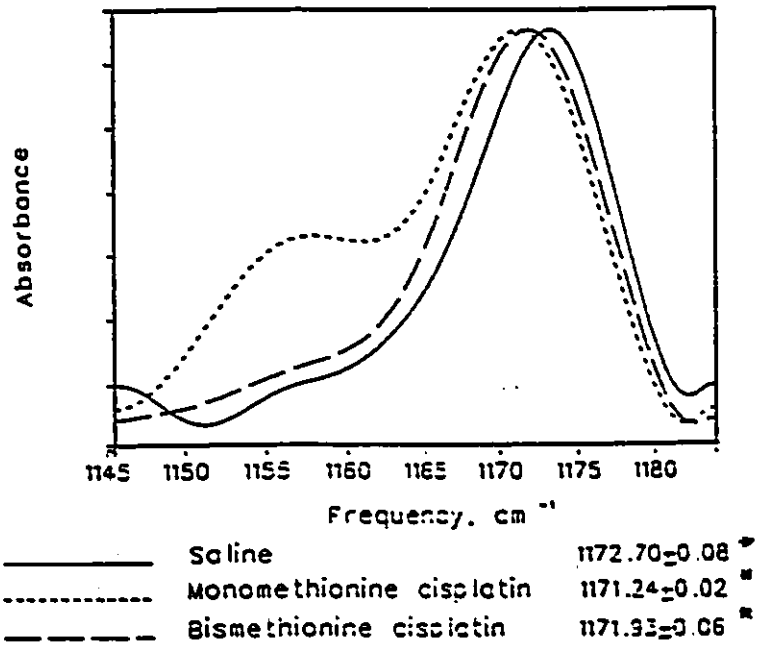
Figures 4.12 and 4.13 : IR spectra of OV 2008 cells (region 1050-1110 cm^{-1}) - * mean±SE frequencies for the first peak - # mean±SE frequencies for the second peak - ⊗ p value is for comparison to the saline control

after exposure to 0.33 mM and 2.5 mM of different platinum compounds.

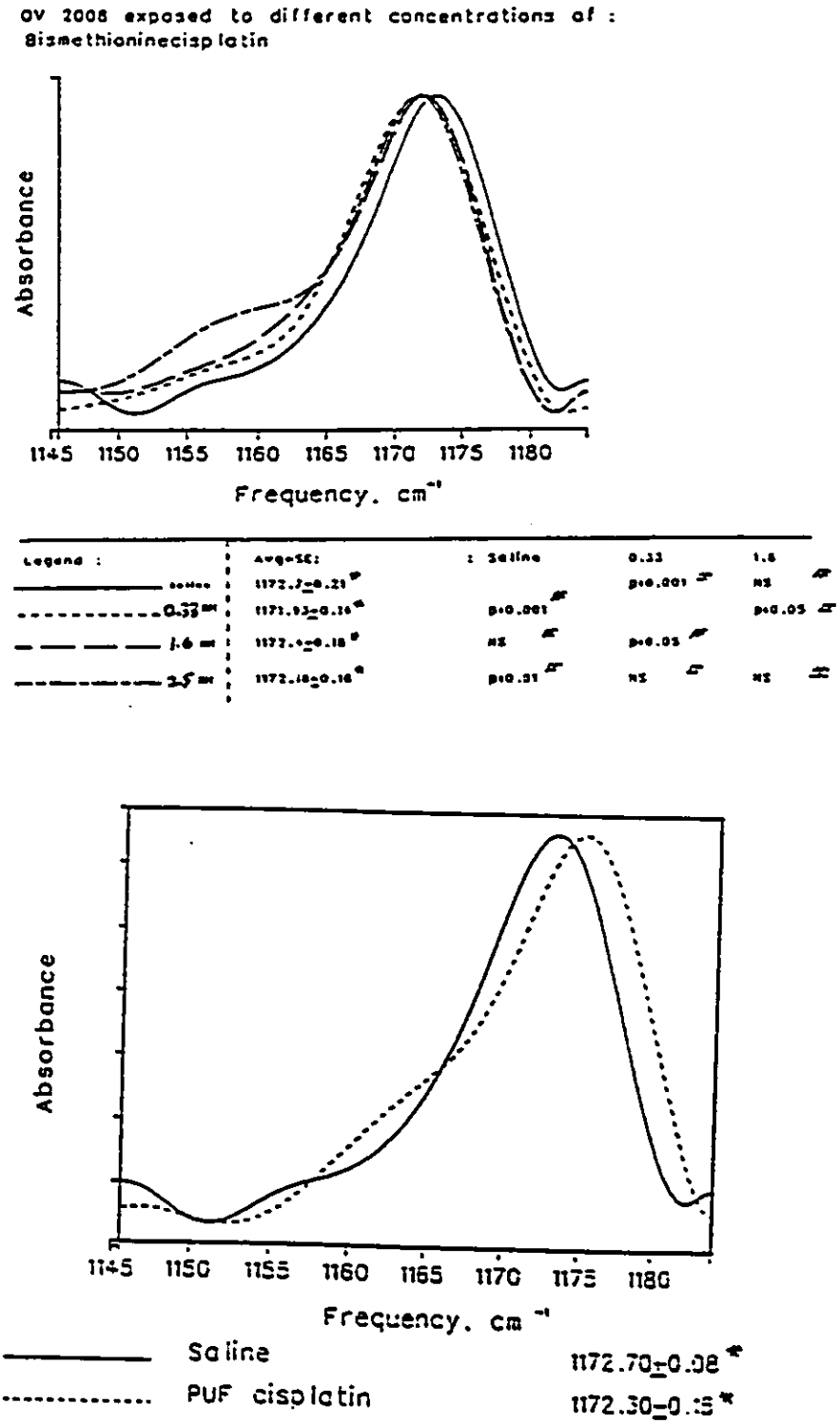
4.3.3- Region 1145-1180 cm^{-1}:

The band at 1172.7±0.08 cm^{-1} is due to the stretching mode of C-O groups of cell proteins. This band occurs at 1155 cm^{-1} in normal cells, but at about 1170 cm^{-1} in cancerous cells. This shift indicates the decrease in the vibration of the C-OH group due to bond with another group. The C-O band in this region originates mainly from the C-OH group of serine, threonine and tyrosine in cell proteins (Rigas,1990). Figures 4.14 and 4.15 show the shift of this band to a lower frequency in the cells affected with any of the studied compounds. Multiple comparison post ANOVA tests of peaks resulting from cells exposed to each of these compounds show significant difference from cells exposed to saline ($p<0.001$). Figure 4.16 compares the spectra of the cells exposed to increasing concentrations of bismethionine cisplatin and figure 4.17 compared the negative control and plasma ultrafiltrate dissolved cisplatin spectra at this region. The band near 1157 cm^{-1} is due to carbohydrates (Parker,1983). Variations occurring in the intensity of this band among cell





Figures 4.14 and 4.15 : IR spectra of OV 2008 cells (region 1145-1180 cm^{-1}) - * peak frequency



Figures 4.16 and 4.17 : IR spectra of OV 2008 cells (region 1145-1180 cm^{-1}) - * peak frequency - # values are being compared to saline and each other.

samples exposed to different species, indicate that different amounts of carbohydrates are present in each one of them, or that the chemical structure of carbohydrates has been changed to a different degrees in the cells exposed to the different cisplatin metabolites (Rigas,1992). As is presented in this figure, the degree of changes caused by cisplatin in plasma ultrafiltrate is more complicated than those caused by other metabolites, especially in the band related to carbohydrates.

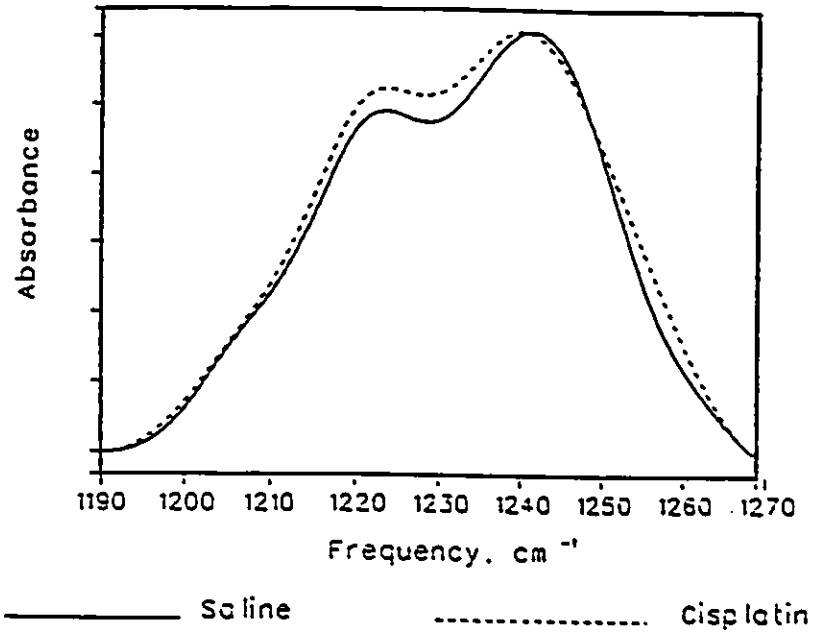
4.3.4 - Region 1190-1270 cm^{-1}:

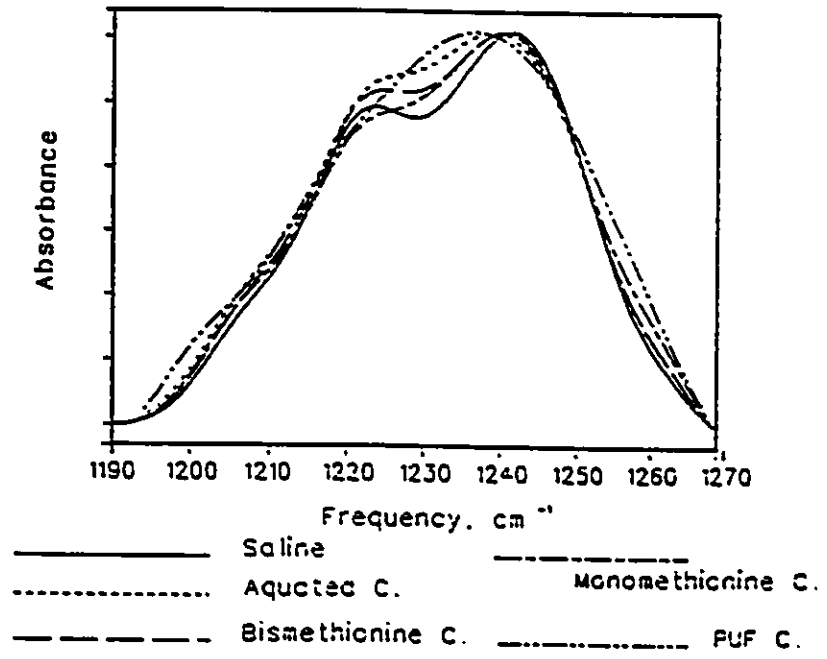
Figures 4.18 and 4.19 show the superimposed spectra of the asymmetrical PO_2^- stretching band of OV 2008 cells exposed to cisplatin metabolites. All of these graphs tolerate Fourier self-deconvolution with an enhancement factor of 1.7 and band width of 20 cm^{-1}. This region is broad and consists of two overlapping bands with the maxima near 1224.13$\pm$0.15 and 1240.37$\pm$0.13 cm^{-1}. The component band at 1240 cm^{-1} is mainly due to nonhydrogen-bonded phosphodiester groups and that at 1220 cm^{-1} is due to hydrogen-bonded phosphodiester groups (Wong,1991). The amide III band of proteins may also contribute to the intensity to the peak of the PO_2^- band at 1240 cm^{-1}.

However, the frequency of the amide III band varies according to the secondary structure of proteins, which can be determined by the peak maximum of the amide I band (Susi,1969). Since the phosphate stretching band originates from the vibrational modes in the phosphodiester group of nucleic acids(DNA and RNA) (Kauppinen,1981), these graphs indicate that the hydrogen bonding of the phosphodiester groups of nucleic acids increases with the concentration of the species to which the cells are exposed. A 1.35 ± 0.05 cm^{-1} shift to the left of the spectra of cells exposed to cisplatin metabolites is also apparent in the nonhydrogen-bonded phosphodiester related band relative to the control cells. This shift indicates that the nonhydrogen-bonded phosphodiester groups of nucleic acids are bonded to a heavy group as a result of exposure to any of these species.

4.3.5 - Region 1600-1700 cm^{-1}:

Figure 4.20 shows the deconvoluted amide I band spectra of OV 2008 cells (solid line) and the cells exposed to cisplatin in plasma ultrafiltrate. The amide I band in this region is due to the in-plane C=O stretching vibration weakly coupled with C-N stretching and in-plane

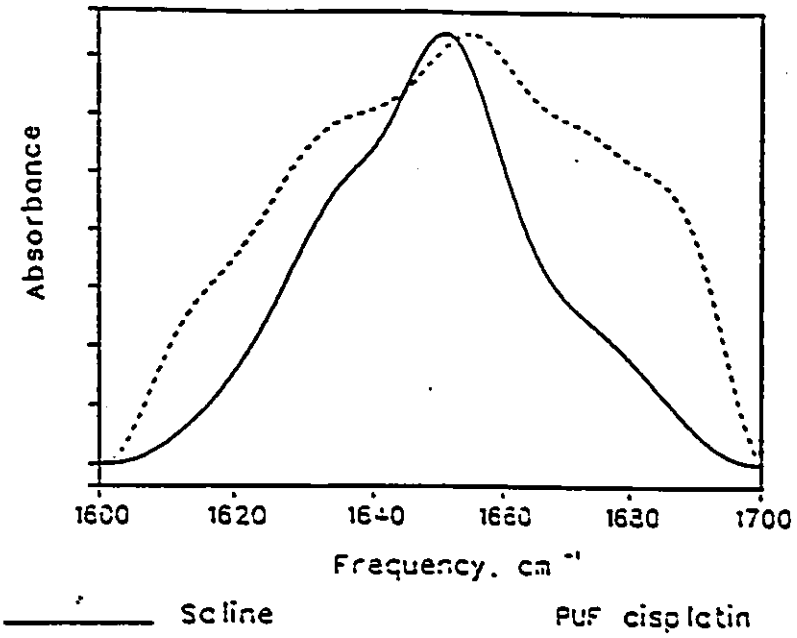


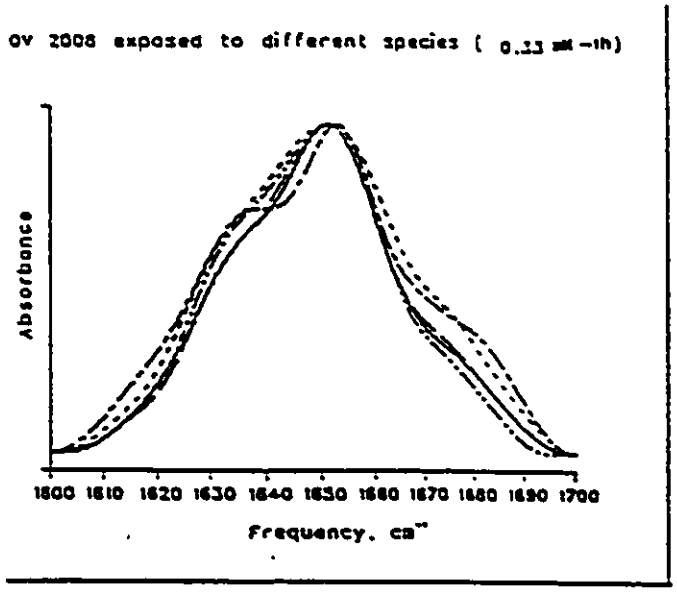


Figures 4.18 and 4.19 : IR spectra of OV 2008 cells (region 1190-1270 cm^{-1})

N-H bending of the amide groups in proteins, and the peak maximum absorbance of the group in this region is sensitive to the secondary structure of cellular proteins (Wong,1988). The α-helical structure peak maximum absorbance comes near 1650.15±0.26 cm^{-1}, the parallel ß-sheet near 1635 cm^{-1}, and that of antiparallel ß-sheet near 1680 cm^{-1}. Since each molecule of a globular protein contains segments with different substructures, the amide I band appears as a broad band with several maxima.

Figures 4.21 to 4.23 show the spectra of the same region of cells, exposed to different concentrations of different species. These graphs show that the cellular proteins are to a large extent α-helices in OV 2008 cells. The relative amount of ß-sheet segment in comparison to that of the α-helical segments is larger in all cells exposed to different platinum species. On the other hand the amide I shifts slightly towards higher frequencies, indicating that hydrogen bonds in the α-helical segments of the proteins are weaker as a result of exposure to the different platinum compounds (Wong,1989). Wideness of the whole complex peak is also an indicator of some degree of denaturation in the whole proteins of cells exposed to different platinum compounds. The degree of these effects vary among the different platinum metabolites.





Legend :

Saline

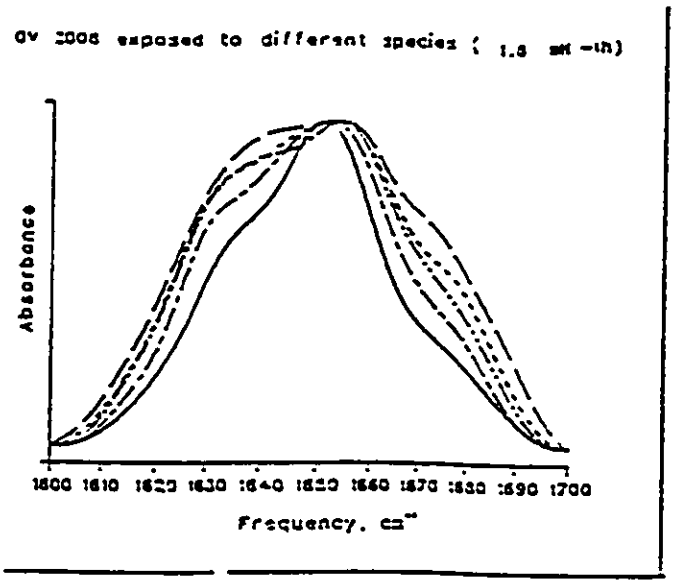
Cisplatin (C)

Aquated C

Monomethionine C

Bismethionine C

Figures 4.20 and 4.21 : IR spectra of OV 2008 cells (region 1600-1700 cm^{-1})



Legend :

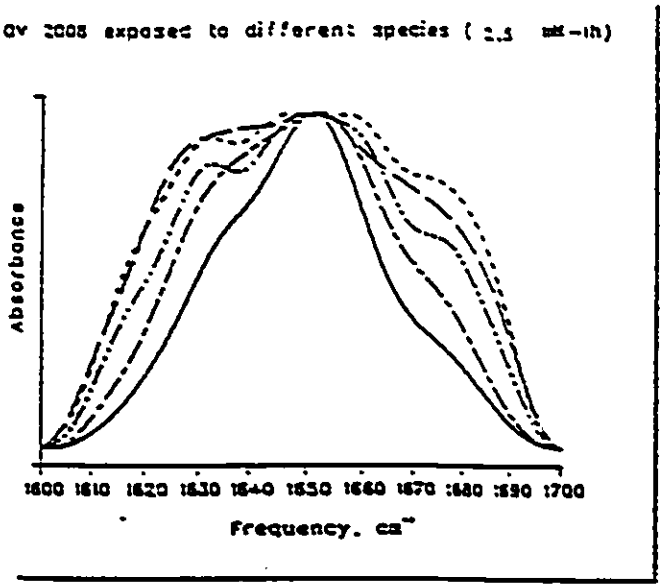
Saline

Cisplatin (C)

Aquated C

Monomethionine C

Bismethionine C



Legend :

Saline

Cisplatin (C)

Aquated C

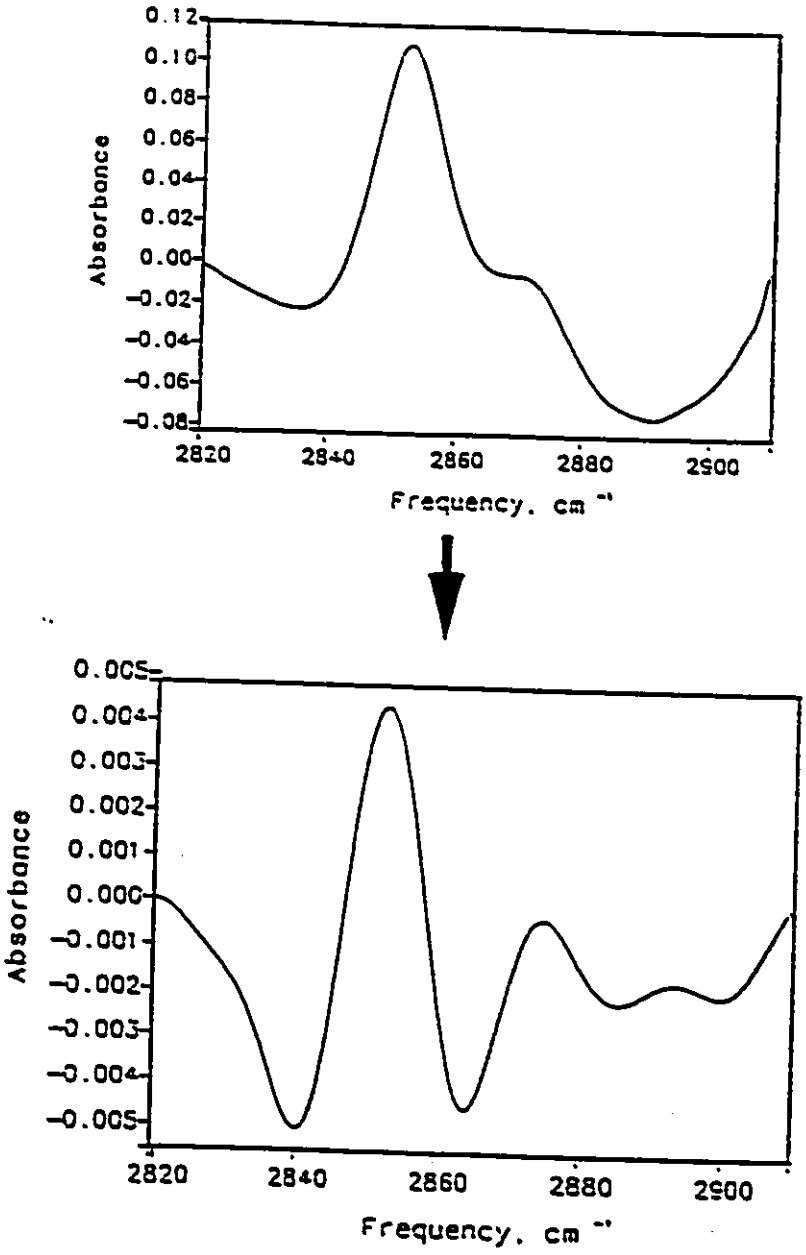
Monomethionine C

Bismethionine C

Figures 4.22 and 4.23 : IR spectra of OV 2008 cells (region 1600-1700 cm^{-1})

4.3.6 - Region 2820-2910 cm^{-1}:

Figure 4.24 represents the region of 2820-2910 cm^{-1} for OV 2008 cells, and figure 4.25 shows the same region with the third power derivatization of the original spectra and the breakpoint of 0.3. Figure 4.26 represents the same region after exposure to 0.33 mM of any of the investigated species, or the saline control (curves are indistinguishable due to significant overlap). Figure 4.27 shows the same region, this time after exposure to 2.5 mM of the different species and saline. This region is mainly related to the symmetric CH_2 stretching mode of methylene chains in membrane lipids (Fringeli,1981). The dominant infrared band at 2852.44±0.02 cm^{-1} arises from the CH_2 symmetric of the acyl chain methylene groups, while the weaker band at 2874.62±0.04 cm^{-1} is due to the symmetric stretching mode of CH_3. As is shown in these figures, there are no significant changes in the spectra of this region resulting from the exposure of cells to cisplatin metabolites compared to control, indicating almost no effect of platinum compounds on the methylene chains of the membrane lipids. A slight change on the band at 2874 cm^{-1} has been found in the case of cisplatin and aquated cisplatin after the exposure of the cells to a

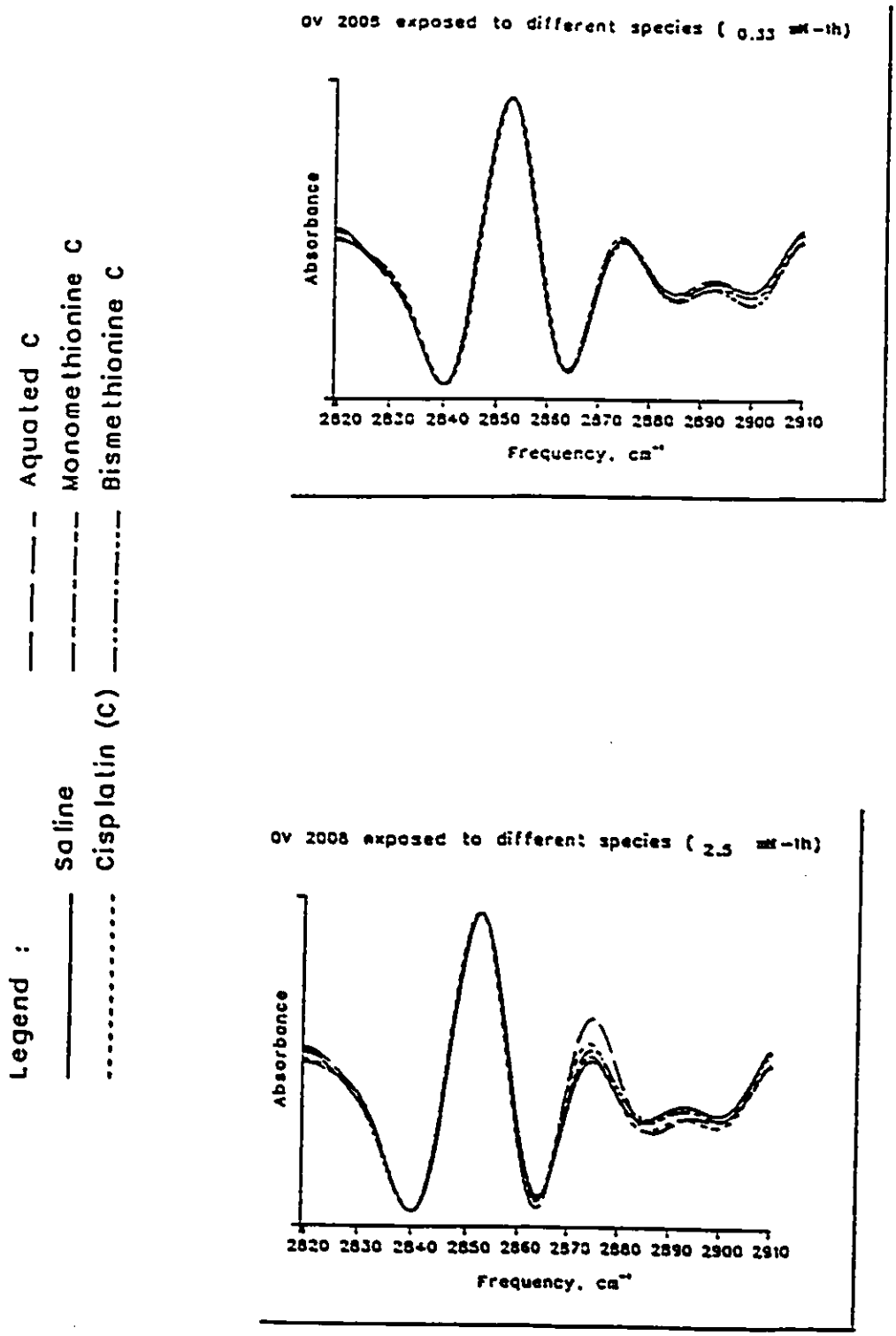


Figures 4.24 and 4.25 : IR spectra of OV 2008 cells (region 2820-2910 cm^{-1})

very high concentration of drug (2.5 mM). This region will not reflect the possible effects of these compounds on the other segments of the cell membrane (e.g. hydrophile groups), except for the CH_2 stretching mode of cell membrane lipids.

4.3.7. In summary :

Infrared spectroscopy studies showed that there was an interaction of cisplatin and its metabolites with the phosphate backbone of DNA, with the carbonyl groups of the three amino acids serine, threonine and tyrosine, of proteins, with amide I of proteins, and to some extent with carbohydrates. However, there was no significant interaction between cisplatin or its metabolites and cell membrane lipid bilayer and cellular carbohydrate.



Figures 4.26 and 4.27 : IR spectra of OV 2008 cells (region 2820-2910 cm^{-1})

5

Discussion :

The past two chapters have reviewed our findings in detail. In this chapter the data will be summarized and conclusions based on both the quantitative and qualitative measurements will be presented.

Tissue culture studies on tumour cell lines have greatly facilitated the understanding of the behaviour of chemotherapeutic agents. The ability to control the study environment may be assumed to be an advantage with in-vitro experiments, because one can mimic what is happening in-vivo with less interactive variables. These results may then have some clinical relevance.

The degree of accumulation of intracellular platinum resulting from the exposure to cisplatin metabolites is a strong determinant of the varying cytotoxicities; the rank orders of the cisplatin metabolites' relative cytotoxicities and relative intracellular accumulations were found to be identical. Cellular efflux of the cisplatin metabolites did not seem to be an important

factor in their relative cytotoxicities or drug accumulations; there was not relationship between the rank order of efflux and the other parameters.

The mechanism(s) of the intracellular uptake of cisplatin has been studied extensively, but it is still not well understood (Gately,1993). There are data that favour both passive and active membrane transport of cisplatin (Andrews,1988, Smith,1989 and Andrews,1990). However, our results are not sufficient to address the mechanism of cellular uptake of cisplatin and its metabolites. Aquated cisplatin, which is the most cytotoxic of the cisplatin metabolites that were studied, is associated with the greatest amount of intracellular accumulation of platinum. The increase in cellular accumulation of different species is directly proportional to the increase in metabolite concentration in the media. This is consistent with both passive and active mechanisms of cell transport of cisplatin and its metabolites.

Not all of these species have the required physio-chemical characteristics for passive diffusion through a cell membrane; passive diffusion occurs best with lipophilic compounds (Loomis,1978). Methionine derivatives of cisplatin meet the required specifications for a passive transport mechanism of a chemical, but aquated cisplatin does not. Cisplatin and its metabolites may have

different transporter systems to explain their different cellular accumulation patterns.

The charged species (e.g. aquated cisplatin) may not have the capacity to cross cell membranes easily, because only neutral molecules can diffuse passively into the cell (Harper,1979). The measured concentrations of cellular aquated cisplatin may not represent intracellular accumulation of platinum compound. This measurement may be determining the total amount of those species which are associated with the outer layer of the cell membrane, in addition to the species that are present intracellularly.

Previous investigators have showen many differences in the chemical nature of cell membranes in cancer cells. Amino acids, ceramide groups, sialic acids and surface DNA structures have been found in greater amounts on the outer surface of cancerous cells in comparison to their normal counterparts (Francina,1989 and Dobrossy,1981). These groups are all hydrophilic in nature and may be good binding sites for highly charged molecular species such as aquated cisplatin. It is possible that there is significant interaction of aquated cisplatin with these chemical groups on cell membranes of OV 2008 cells.

The efflux of cisplatin and its metabolites from the OV 2008 cells is biphasic. These findings are consistent with other investigators' data (Mann,1990). The cisplatin

species' efflux constants are significantly different from each other in the first rapid portion, but are not significantly in the second phase of slower efflux. It is possible that after the different cisplatin metabolites have entered the cell, they may either remain free or may bind to biomolecules. The cisplatin species that are free are rapidly effluxable, thereby contributing to different efflux rates in the first phase. The slowly effluxable component of the cisplatin species may represent those molecules that are present after dissociation from the intracellular biomolecules. It is therefore possible that the dissociated species resulting from each cisplatin species are identical. This could explain why the second phase of efflux is similar for all cisplatin species.

Infrared spectroscopy studies showed that there was an interaction of cisplatin and its metabolites with the phosphate backbone of DNA, with the carbonyl groups of the three amino acids serine, threonine and tyrosine, of proteins, with amide I of proteins, and to some extent with carbohydrates.

These interactions would respectively cause an unpacking of DNA, denaturation of proteins, and a relative increase in ß configuration of proteins. There was no significant interaction between cisplatin and its

metabolites with the lipid bilayer matrix of cell membrane.

While IR spectral changes caused by all cisplatin metabolites were qualitatively similar, these changes were quantitatively different. However, the design of the IR experiment dose not allow for the relative comparison of the band shifts due to each cisplatin metabolite.

The rank order of cytotoxicities resulting from each cisplatin metabolite match that of intracellular platinum accumulation. It would seem that intracellular accumulation of platinum is a strong determinant of the cytotoxicity to OV 2008 cells produced by each cisplatin metabolite.

Cisplatin dissolved in plasma forms different metabolites (Borch,1979, Gromley,1979 and Goel,1990). At least some of these metabolites correspond to the aquated and methionine complexes of cisplatin (DeConti,1973). Cisplatin metabolites cause different magnitudes of cytotoxicity, transmembrane platinum transport, and biochemical changes against OV 2008 cells. We hypothesize that the effects of cisplatin in patients' plasma against ovarian cancer (and other tumours) is a composite of the specific effects caused by each cisplatin metabolite.

Future studies are needed to determine the metabolite profile that is formed in plasma of patients

that receive cisplatin. From this research, one may be able to eventually predict the degree of antitumour response in a given patient based on the cisplatin metabolite pattern of that individual. One may eventually be able to increase the therapeutic index of platinum-based chemotherapy by isolating and administering a cisplatin metabolite that has a greater selectivity than cisplatin against tumour cells compared to normal cells.

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