THE EFFECT OF HYPOXIA ON CYTOTOXICITY, UPTAKE AND DNA BINDING OF CISPLATIN AND NOVEL BIS(PLATINUM) COMPLEXES IN CHINESE HAMSTER OVARY CELLS

by

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ABSTRACT

Disagreement exists in the literature as to whether the successful antitumour agent cisplatin exhibits greater toxicity in hypoxia than in air. In this study, Chinese hamster ovary cells were incubated in clinically relevant concentrations of cisplatin for periods of up to three hours in aerobic and hypoxic environments. For all incubation times studied, cells treated in hypoxia were more sensitive to cisplatin than those treated in air. Toxicity in hypoxic cells was up to two times greater than in aerobic cells. Detection of platinum using atomic absorption spectroscopy provided resolution fine enough to characterize whole cell uptake and DNA-binding of cisplatin at these concentrations. Cells treated in hypoxia showed consistently higher levels of platinum within the cells and bound to DNA than those treated in air. Furthermore, cells treated in hypoxia showed greater toxicity per platinum-DNA adduct and per atom of platinum bound per cell than those treated in air.

Two novel bis(platinum) compounds AN-38 and AN-36, of structural formula $\{[Pt(malonate)(NH_3)]_2H_2N(CH_2)_nNH_2\}$ (n=6 and 4, respectively), were also found to be preferentially toxic to hypoxic cells, up to four times as toxic as in air (for a four hour incubation), although there was little increase in platinum uptake levels.

To date, this study represents the most thorough investigation of cisplatin properties in air versus hypoxia, given that survival, whole cell uptake and DNA binding in the same cell population each showed enhanced effects in hypoxia. Suggestions of possible mechanisms explaining these data could form the basis for future studies.

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1. INTRODUCTION

1.1 Cisplatin

1.1.1 History

In a series of experiments in 1965, Barnett Rosenberg, investigating the effect of electric current on bacterial mobility, noticed that the cells under study formed a filamentous structure, one characteristic of undividing cells[1]. He found that the chemical causing this structure was *cis*-diamminedichloroplatinum (II), or cisplatin, a compound known since the 1860's as "Peyrone's chloride". Rosenberg went on to show that cisplatin had a potent antitumour effect in Sarcoma 180 and L1210 cells[2]. After similar activity having been shown in a wide variety of animal tumour systems and proven effective in a great many clinical trials[3], cisplatin was accepted for clinical use in 1979. Since then, cisplatin has become one of the most widely used anticancer drugs. Primarily used in testicular, ovarian, and bladder cancers, it is also used as an adjunct for treatment of many other tumour types.

But the importance of cisplatin goes much deeper. Since its discovery, well over 1000 platinum analogues have been tested for antitumour activity[4], relying on the example of cisplatin for their development. Furthermore there has been considerable interest in cisplatin as a radiosensitizer[5]. However, there are still many aspects of its properties and mechanism of action which remain, despite intense study, elusive.

1.1.2 Structure and Chemistry

The chemical structure of cisplatin and its isomer, *trans*-diamminedichloroplatinum (II) (abbreviated here as "trans-DDP") are shown in figure 1-1. The two chloride ligands

in trans and cisplatin are open to substitution. Commonly, substitution occurs for ligands which are in a large excess or for those which form more stable bonds with platinum than do the chlorides. The two ammine groups are relatively inert to substitution reactions. Therefore, both compounds may undergo substitution of two ligands, although it is important to note that there is a difference between the two isomers in the geometry of the ligands which will bind to them.

In aqueous solution, the Cl^- ions are slowly lost, replaced by H_2O or OH^- molecules[6], and an equilibrium between DDP, mono and diaquo species is established. However, at physiological pH, the equilibrium shifts and the vast majority of molecules present are in the diaquo form. It has been suggested that it is this form of cisplatin which is the active form[7]. There is also evidence to suggest that due to the high concentration of Cl^- ions outside cells, cisplatin remains in the dichloro form until after it passes through the cell membrane, where hydrolysis takes place[6].

1.1.3 **Interaction with the Cell**

The exact method by which cisplatin crosses the cell membrane remains in contention. The Michaelis-Menten constant, which describes the formation rate of a carrier-substrate intermediate, has been found to be infinite for cisplatin[8], indicating simple diffusion drives cisplatin into the cell. One study which presented indirect evidence of a carrier-dependent pathway was subsequently explained by cell cycle effects[9], and yet another study proposed that a part of the accumulation of cisplatin in cells can be attributed to energy dependent methods[10].

Nonetheless, cisplatin does interact with the extracellular membrane. Most significantly, it has been shown to inhibit the transport of neutral amino acids across the membrane[11]. Studies of cisplatin-sensitive and resistant L1210 and K562 cell lines have shown this to be a more pronounced effect in sensitive cells[12,13]. Furthermore, these studies showed the hydrated mono and diaquo species formed by cisplatin were the primary cause of its inhibitory effects on membrane nutrient transport. However, due to the low concentration of the hydrated species in the extracellular environment, it is likely that this plays only a minor role in the production of cisplatin toxicity.

Other interactions are also known to occur. Cisplatin binds to macromolecules within the cell, such as DNA, RNA, and proteins. Studies have shown that inhibition of protein and RNA synthesis by cisplatin is weak and most importantly, temporary, whereas DNA synthesis inhibition appears longer lasting[14,15]. It is further known that cisplatin is capable of causing mutagenesis and chromosome abnormalities[16], pointing to the nucleus, and specifically DNA, as the site for the principal cytotoxic damage caused by cisplatin.

1.1.4 **Interactions Between Cisplatin and DNA**

The mechanism by which cisplatin causes damage in cells is still not certain, but the prevailing theory suggests that the most important intracellular target of cisplatin is DNA[17]. Some of the evidence supporting this theory is listed below:

- the observed inhibition of cell division in E. coli while RNA and protein

synthesis continue[1]

- the prophage induction in lysogenic bacteria exposed to cisplatin[18]

- the observed inhibition of DNA synthesis in human tumour cells[15], while there is no inhibitory effect on DNA polymerase activity[19]

- base-substitution and frame-shift mutations in DNA caused by cisplatin interactions[20]

- increased toxicity of cisplatin in DNA repair deficient cell lines[21]

- many of these effects are seen with other DNA damaging agents, such as radiation[22]

The ways in which cisplatin binds to DNA have been studied thoroughly[23-29]. Cisplatin forms several types of adducts: interstrand crosslinks, intrastrand crosslinks, DNA-protein crosslinks, and monofunctional adducts (shown schematically in figure 1-2). The exact contribution of each of these to cisplatin toxicity has not yet been established. Monofunctional binding is unlikely to be the principal cause since *trans*-DDP is at least as effective in forming these adducts as cisplatin[24], yet it is much less toxic. There is a wealth of information available about interstrand and DNA-protein crosslinks, even though they represent only about 1% of the total platination of DNA, because the techniques for quantifying these adducts have been available for quite some time. Attempts to correlate either adduct to cisplatin toxicity have yielded conflicting results [25,26].

Yet there is a growing body of evidence suggesting that intrastrand crosslink formation contributes to the majority of damage inducing lesions. The majority of DNA-platinum adducts formed by cisplatin are intrastrand crosslinks[27], most often formed between (in order of descending frequency) adjacent guanosines, a guanosine-adenosine pair or two guanosines separated by one base[28]. The less toxic stereoisomer of cisplatin, trans-DDP, is incapable of forming this type of crosslink to DNA. Even when treatments result in binding of equal amounts of platinum to DNA, the cisplatin adducts are much more toxic than those of trans-DDP[29]. Studies of resistant cell lines have shown enhanced repair of DNA intrastrand crosslinks in these lines may be responsible for resistance to cisplatin[30], thus suggesting an important contribution to toxicity. Finally, intrastrand crosslinks have been found to inhibit both transcription of plasmid DNA in L1210 cells[31] and DNA synthesis in a defined DNA template[32]. It has been proposed that intrastrand crosslinks between two adjacent guanines cause a localized conformational change, or kink, in the DNA[33,34]. This kink, which would block DNA replication, is considered to be especially difficult for DNA repair systems to correct.

There is also one other type of interaction which has been shown to occur. Glutathione, or GSH, is a molecule produced by cells to aid in detoxification. It has recently been implicated in causing resistance to cisplatin in L1210 cells[35,36] and some human ovarian carcinoma cells [37,38]. The role of glutathione in cisplatin-resistance has not been identified, but there are several possible interactions which may

take place. Since Pt(II) is a soft lewis acid, sulfhydryls such as GSH react readily with cisplatin[35] and therefore may be actively involved in substitution of the cisplatin chloride ligands, preventing further cisplatin reactions with DNA. It has also been suggested that GSH may bind to monofunctional cisplatin-DNA adducts, preventing the more toxic bifunctional lesions from forming[39]. There has also been evidence to suggest that glutathione may modulate the cellular repair processes which correct cisplatin-damaged DNA templates[40].

1.1.5 **Repair of Cisplatin-DNA Adducts**

Once DNA is damaged, most cells can act to repair the lesions. Studies with Xeroderma pigmentosum cells and some Chinese hamster cell mutants, both deficient in the ability to repair UV damage, are highly sensitive to cisplatin treatment[41,42]. This suggests first, that it is only unrepaired or misrepaired lesions which are responsible for cisplatin's toxicity, and second, that the method of repair of cisplatin damage may be similar to that of UV-induced damage.

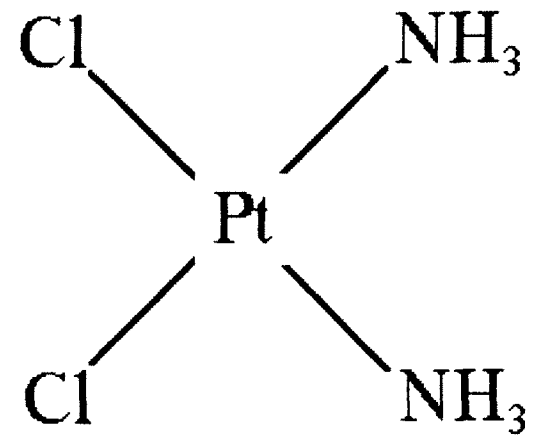
Bacteria repair UV-induced thymine dimers by a well characterized process known as "excision repair". A schematic diagram of the steps involved in this process is shown in figure 1-3. Indeed, this same repair mechanism has been shown to recognize and repair cisplatin adducts in mammalian cells[43]. There is also evidence that cisplatin damage may be repaired by a process known as "post-replication repair"[44,45]. In this process, DNA replication may still take place even though cisplatin has damaged the DNA template. Thus even unexcised platinum adducts may not cause lasting cell damage.

The same studies proposed that post-replication repair may be induced by cisplatin damage[45].

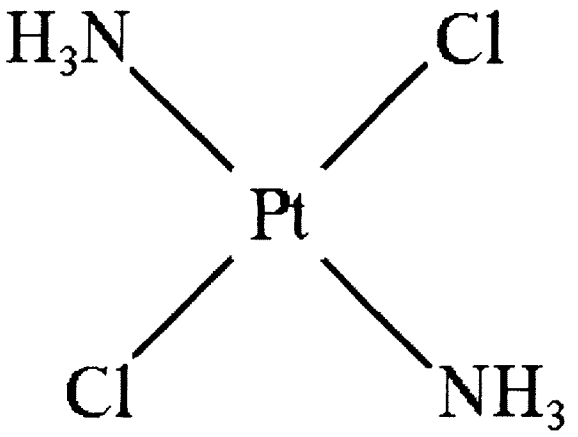
Other researchers have observed that cells are more sensitive to cisplatin in the G_1 phase of the cell cycle, a "gap" in the cell cycle before DNA synthesis(the S phase), than they are during S phase[46]. It is supposed that platinum lesions in the DNA will block DNA synthesis. But the S phase of the cell cycle is extended, allowing time for replication repair to ligate the DNA. Thus, synthesis of the full complement of DNA and mitosis, may proceed as normal. Large doses of cisplatin have been found to prevent cell division, possibly due to an overloading of the post-replication repair system. This may result in a variety of effects, including the appearance of chromosome abberations[47], as well as mutations, due to the error-prone nature of this repair system[48].

1.1.6 **Toxicity in Mammalian Cells**

One of the most surprising elements in the study of cisplatin is the extreme variability in its toxicity in different cell lines, as illustrated in table 1-1. Many of these differences have been explained using arguments presented in the preceding sections. However, it is also of great importance to note the considerable variation in cisplatin toxicity within a given cell line as measured by different researchers. With relevance to the study presented here, wild-type Chinese hamster ovary(CHO) cells exhibit this effect quite markedly. The precise reasons for this are not known, and are difficult to explain in terms of experimental conditions (cell concentration, handling (eg. trypsinization), serum variations, etc.).



cis -DDP



trans -DDP

Fig. 1-1 : *Cis*- and *trans*-diamminedichloroplatinum(II) in their unsubstituted form.

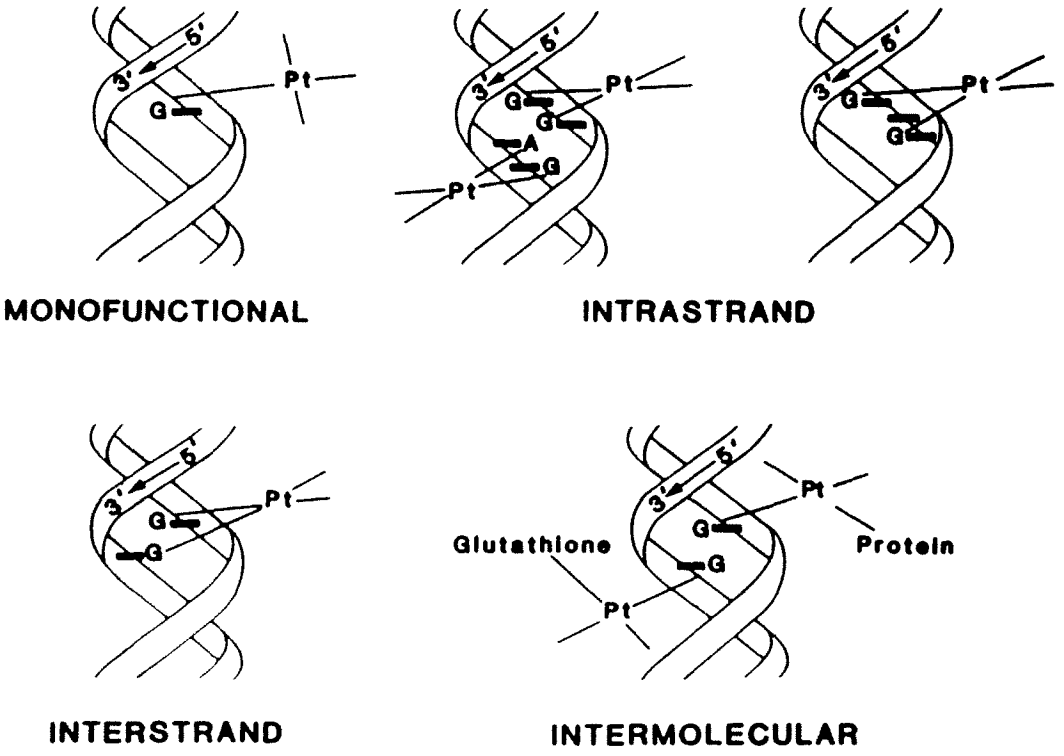


Fig. 1-2 : Various modes of binding of cisplatin to DNA. Note that its stereoisomer, trans-DDP, is sterically hindered from forming intrastrand crosslinks (from: Eastman, *Pharmac. Ther.* 34, 1987)

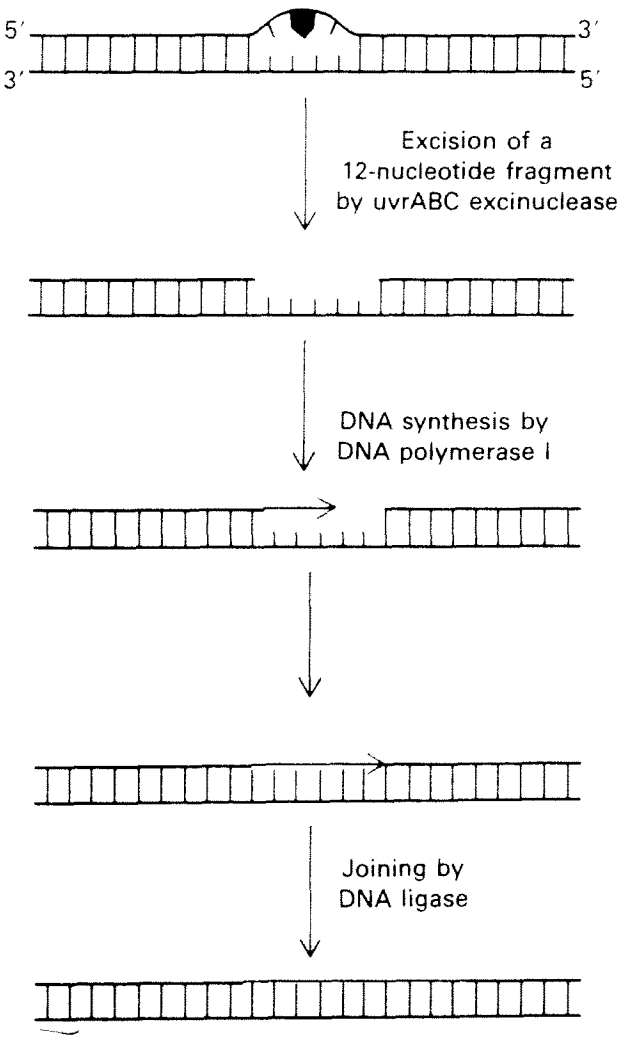


Fig. 1-3 : Sequence of action in excision repair of a damaged DNA template in *E. coli* [from: Biochemistry, L.Stryer, W.H. Freeman & Co., New York, 3rd ed. (1988)]

Cell line		Reference	Dose for SF = 0·3 (μM; 1 h exposure)
(1) UV-20	Hamster	Meyn *et al.* (1982)	0·3
(2) RIF1	Mouse	Begg *et al.*	2
(3) HA-1	Hamster	Evans *et al.* (1982)	2
(4) TC.SV-40	Hamster	Alvarez *et al.* (1978)	3
(5) WS	Human	Ducore *et al.* (1982)	3
(6) W-1	Human	Ducore *et al.* (1982)	3
(7) L1210	Mouse	Zwelling *et al.* (1978)	4
(8) CHO	Hamster	Herman (1983)	5
(9) L1210-K5	Mouse	Zwelling *et al.* (1981)	5
(10) HX109	Human	Jones *et al.* (1984)	5
(11) H4	Rat	Carde and Laval (1981)	6
(12) V79	Hamster	Fraval and Roberts (1978)	6
(13) HX61	Human	Jones *et al.* (1984)	7
(14) HeLa	Human	Pascoe and Roberts (1974)	8
(15) L1210-ZCRG	Mouse	Zwelling *et al.* (1981)	11
(16) HX110	Human	Jones *et al.* (1984)	11
(17) V79	Hamster	Douple and Richmond (1980)	12
(18) BHM	Human	Ducore *et al.* (1982)	13
(19) HX62	Human	Jones *et al.* (1984)	14
(20) V79	Hamster	Dritschillo *et al.* (1979)	16
(21) CHO	Hamster	Plooy *et al.* (1984)	16
(22) LoVo	Human	Bergerat *et al.* (1979)	19
(23) Lymphoma	Human	Drewinko *et al.* (1973)	20
(24) AA8	Hamster	Meyn *et al.* (1982)	21
(25) CHO	Hamster	Murthy *et al.* (1979)	34

Table 1-1 : Measurement of sensitivity to cisplatin varies markedly between cell lines, and also from laboratory to laboratory [from: Begg *et al, Int. J. Radiat. Biol.*, 50 (1986)]

1.2 Bis(platinum) Complexes

1.2.1 Rationale for Development

Cisplatin is indeed a useful anticancer agent, although it shows limited activity in many human cancers and is susceptible to acquired resistance[49]. There has thus been a considerable effort to develop new platinum-based compounds to overcome some of the shortcomings of cisplatin. A number of the new compounds are similar to cisplatin in that they have amines or complex diamines coordinated to platinum in a *cis* configuration (for a review of these compounds see [50]). However, many of these complexes have been found to bind to DNA in a similar fashion to cisplatin[51], and therefore offer little advantage against acquired resistance. The main lesion caused by cisplatin, the intrastrand crosslink, is believed to be effective due the conformational change it imparts in the DNA helix. The bis(platinum) complexes (U.S. Patent Appl. # SN 07/401919, issued to N.P. Farrell) were designed to impart a conformational change to the DNA different than that caused by cisplatin so that they might be active in cisplatin resistant cells.

There exists a broad series of bis(platinum) agents which are currently being investigated for antitumour activity. Some are clinical candidates. Following, is a summary of the current knowledge of the bis(platinum) series and their mechanisms of action.

1.2.2 Structure and Chemistry

The bis(platinum) complexes, in general, have two platinum moieties linked by a variable length alkyldiamine, with the simplest general formula given by

$[\{PtX_2(NH_3)\}_2H_2N(CH_2)_nNH_2]$. The anionic leaving groups, X, are non-specific and may be prepared in *cis* or *trans* configurations. However, bis(platinum) complexes with Cl^- leaving groups (Fig. 1-4a) are not very soluble in water. Derivatives with a dicarboxylate group, malonate (Fig. 1-4b), are much more soluble and are used in this study. The length of the diamine bridge is normally between 1 and 6. Details of their synthesis are available elsewhere[52,53].

1.2.3 **Interaction with DNA**

Restriction enzyme inhibition studies on plasmid DNA have been used to investigate the affinity of the dichloro bis(platinum) compounds for DNA. These studies found a 3-4 fold greater inhibition of enzyme activity of these compounds relative to cisplatin. Binding also appears to be chain length dependent, the n=4 compound forming the greatest number of adducts, with binding decreasing with a further increase in length. In a different assay, the malonate substituted n=4 compound was found to bind only 40-80% of cisplatin levels[54], likely due to the relative inertness of the leaving group[55].

But it is the modes of DNA binding of bis(platinum) compounds which sets them apart from cisplatin and its monomeric analogues. Specifically, they form unique interstrand crosslinks, binding a single platinum atom to each strand of DNA, as shown in figure 1-5. Intrastrand crosslinks are also formed, where the two platinum atoms are bound to the same strand of DNA. Interstrand crosslinks by the bis(platinum) complexes are up to 250 times more frequent than by cisplatin[54]. The n=4 compound, however,

has been found to distort the DNA conformation in a way quite similar to cisplatin[56], while n=5 and n=6 complexes seem to cause more conformational change. The formation of a large proportion of cisplatin-like intrastrand crosslinks by the n=4 complex has been suggested to account for this.

1.2.4 **Toxicity**

The most striking feature of the bis(platinum) compounds is their activities in cisplatin resistant cell lines[57], and lines resistant to other platinum analogues. In cell lines which are not resistant to cisplatin, bis(platinum) compounds with chloride ligands tend to show similar toxicity to cisplatin. Compounds with malonate groups tend to be somewhat less toxic in these lines, again, most likely as a result of different pharmacokinetics caused by the lesser reactivity of the leaving group.

In vitro toxicity of the compounds has also been found to be dependent upon the length of the diamine backbone[58]. The n=5 and n=6 compounds show a significant increase in activity over cisplatin in cisplatin-resistant lines, while the n=4 shows a lesser gain. Significantly, the bis(platinum) compounds also show activity in murine tumours, approximately equivalent to cisplatin, except that they display a more selective toxicity to tumour cells than cisplatin, and have much higher maximum tolerated doses[59].

Two of the bis(platinum) complexes were chosen for this study, the n=4 and n=6 malonate complexes. See figure 1-4 for details of their chemical structure.

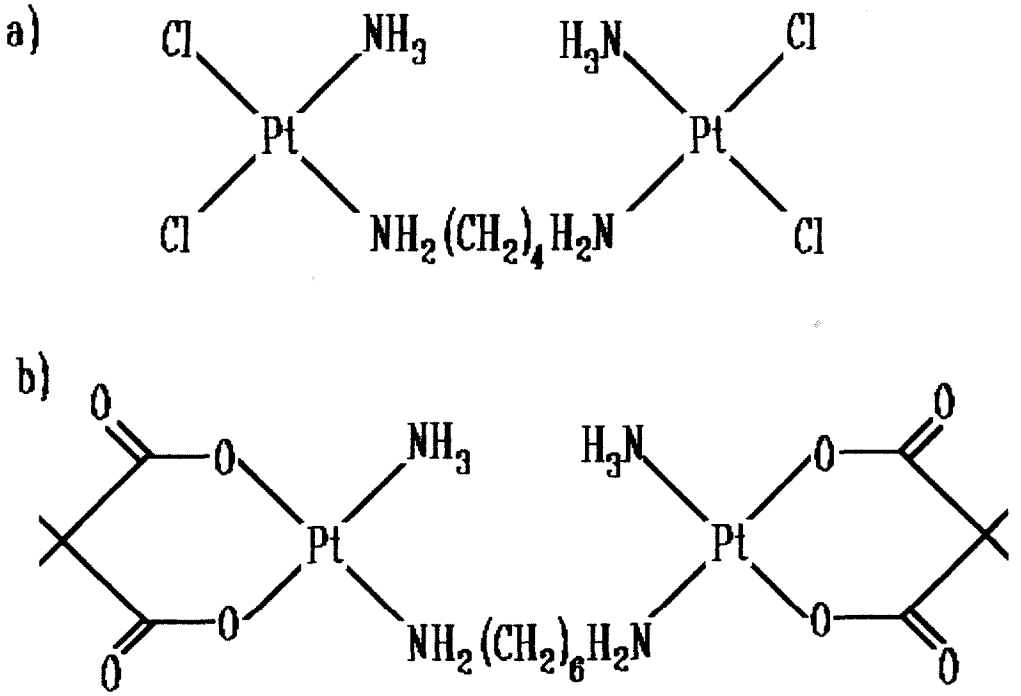


Fig. 1-4 : Chemical structures of two of the bisplatinum compounds: (a) the n=4 chloride $[\{Pt(X_2)(NH_3)\}_2H_2N(CH_2)_4NH_2]$ (b) the n=6 malonate $[\{Pt(mal)(NH_3)\}_2H_2N(CH_2)_6NH_2]$, where 'mal' = malonate = $(CH_2[COOH]_2)$. Compounds used in this study are the n=6 and n=4 malonate complexes. The n=4 malonate differs from structure (b) only in the length of the diamine backbone.

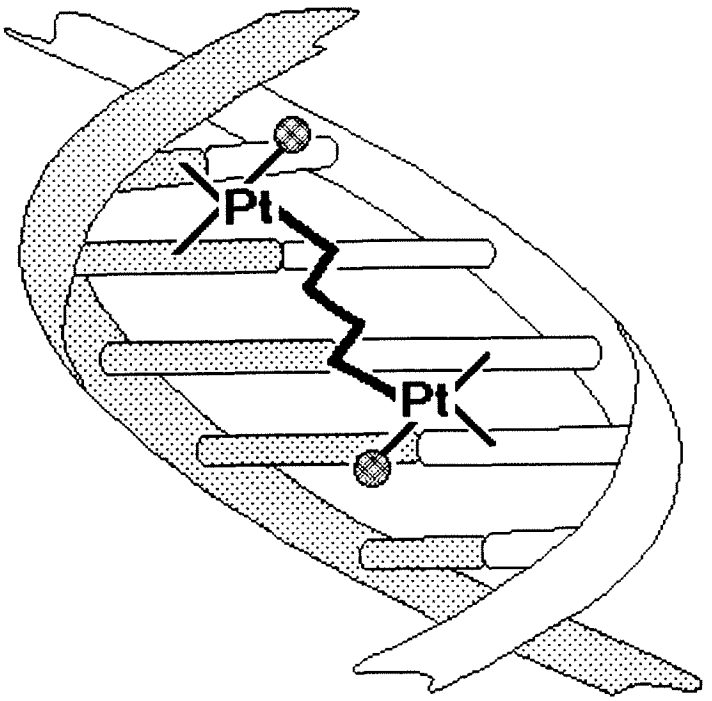


Fig. 1-5 : Schematic representation of the unique interstrand crosslinks formed by bisplatinum complexes (n=4 complex depicted here). Each platinum molecule is bound in a bidentate fashion to a single strand of the DNA [adapted from: J.D. Roberts *et al, Nucl. Acids Res.*, 17(23) (1989)]

1.3 Hypoxia

1.3.1 Hypoxia in Solid Tumours

It is now well established that nearly all solid tumours are heterogeneous with respect to oxygenation. That is, in many human tumours there exist populations of hypoxic cells, those which are poorly oxygenated[60]. Hypoxic cells may originate in one of two ways. Cells may become "chronically hypoxic" when they are situated in regions distant from blood vessels or in rapidly growing tumours with poorly developed vasculature[61,62]. As shown in figure 1-6, intervening cells utilize the oxygen from the blood vessels creating a decreasing oxygenation profile as distance from blood vessels increases. A second, less established case is known as "acute hypoxia"[63]. This mode of hypoxia occurs as a result of transient fluctuations in blood flow in tumour blood vessels.

1.3.2 The Effect of Hypoxia on the Cell

All cells can tolerate at least some period of hypoxia without sustaining irreversible damage. While acute hypoxia is thought to have little effect on cell viability, chronic hypoxia is believed to be more damaging[64]. In either case, there are documented effects of hypoxia on cell function[64].

One of the most profound effects of hypoxia is the inhibition of mitochondrial function, an effect which develops within a half hour of the onset of hypoxia[65]. Thus the availability of energy in the form of ATP is reduced. However, the mitochondria act to preserve their membrane pH gradients necessary for energy production, so that when

hypoxia is removed, ATP synthesis may proceed as normal[66]. Subsequently, hypoxia also acts to inhibit the Na^+,K^+-ATPase transport system of the cell, due to the decreased availability of ATP[67]. It has also been noted that hypoxia results in retention of Ca^{2+} ions within the mitochondria, impairing a cell's ability to maintain Ca^{2+} homeostasis. This can affect activities of Ca^{2+}ATPases in the plasma membrane.

Another important effect of hypoxia is a decreased ability to synthesize reduced glutathione. Normally, the rate of GSH synthesis is dependent upon the rate of depletion. It is resynthesized from its constitutive amino acids through an NADPH dependent process. NADPH supply has been found to be oxygen dependent, and the rate of GSH synthesis has been found to follow a similar oxygen dependency[68,69]. Thus it is clear that cells under hypoxic stress are more susceptible to injury due to their decreased ability for detoxification.

Because of the metabolic effects of hypoxia there is a rapid cessation of cell growth[70]. However, growth resumes when cells are reoxygenated, but at a slower rate depending on the duration of hypoxia[71]. Cells in hypoxia for less than 6 hours generally show no such growth lag. However, hypoxia tends to increase the length of time cells spend in the S phase of their cycle, presumably because DNA synthesis takes place at a lower rate[72]. This leads to a longer doubling time and lower rate of division[73].

1.3.3 **The Problem of Hypoxia in Cancer Therapy**

The importance of hypoxia to the treatment of cancer has been very well

established. It was known quite early in the study of radiobiology that the absence of oxygen greatly reduced the sensitivity of cells to radiation damage[74], a factor relevant to the success of radiotherapy. This phenomenon is known as the "oxygen effect". Figure 1-6 shows a typical survival curve for cells irradiated in the presence or absence of oxygen. Radiation may cause damage to a cell by one of two general interactions: direct or indirect. Direct action of is the process whereby ionizing radiation deposits its energy directly in the DNA of a cell. Conversely, in indirect action, the radiation interacts with an H_2O molecule to produce free radicals such as $OH\cdot$, $H\cdot$, e^-_{aq} and $HO^{\cdot}_2$. It is these highly reactive species, paricularly $OH\cdot$, which react with and damage DNA. The majority of biological damage produced by X-rays in air is by the latter process. The interaction of an $OH\cdot$ with DNA results in a free radical, $R\cdot$, being formed on the DNA. If no oxygen were available, the cell could act to repair the damage. But in the presence of oxygen this highly reactive species will form a peroxide, RO_2, a more permanent type of damage, a process known as "oxygen fixation". Thus hypoxic cells are said to be "radioresistant", that is, they show less response to radiation than do well oxygenated cells.

The implications of the oxygen effect on radiotherapy are obvious. To date, the most effective way of minimizing the oxygen effect is fractionation of the radiation dose. Treatment normally takes place in multiple, smaller radiation doses and because there is a tendency for tumours to reoxygenate following radiotherapy[75], this practice allows time for previously hypoxic cells to become oxygenated between successive irradiations.

However, this method is not always effective, as a result of varying patterns of reoxygenation between different irradiation conditions and tumour lines.

Furthermore, there is evidence that many commonly used chemotherapeutic agents, such as adriamycin and vincristine may be less effective in hypoxic cells[76]. This may be due to an inherent ineffectiveness to quiescent, non-cycling cells or may simply reflect the inability of the drugs to penetrate through cell layers to reach hypoxic cells because of pharmacodynamic considerations. In any case, hypoxia in tumours remains an obstacle in the effective treatment of cancer.

In one approach to overcoming the hypoxic problem, there has been a concentrated search for drugs which selectively enhance the effect of radiation in hypoxic cells. These agents are collectively known as "hypoxic cell radiosensitizers." Examples of this type of agent are the nitroimidazole class of compounds, but they have shown only limited clinical effectiveness[77]. There is also an effort being made to produce "hypoxic cytotoxins", drugs which alone act in hypoxic cells as well as, or better than in aerobic cells. An example of such an agent is SR 4233, which is up to 100 times more toxic to hypoxic cells than aerobic[78].

1.3.4 **Rationale for this Project**

Several groups have studied the effect of hypoxia on cisplatin toxicity *in vitro*. A summary of these results is given in table 1-2. A number of groups have found no difference in toxicity to aerobic and hypoxic cells[79,80,81,82,83]. Others have found a greater effect in hypoxia[84,85,86] and at least one group has

observed the opposite[87] in a human cell line and also noted that cisplatin uptake, determined by atomic absorption spectrometry, was the same in air and hypoxia. Some of the differences may be attributed to cell-line dependent effects, yet even within the same line, there are conflicting results as to the effects of hypoxia on cisplatin toxicity. Experimental conditions seem the most likely explanation for discrepancies (for example, different lengths of incubation in cisplatin), although to date the exact reasons have not been adequately demonstrated.

In vivo results are equally confusing. One group reports two-fold greater aerobic toxicity[88] and another shows little effect of oxygenation status[89].

Thus, the following work is aimed at providing a comprehensive study of cisplatin toxicity in air and hypoxia in Chinese Hamster Ovary (CHO) cells. To support the results, the whole cell uptake and DNA-binding of cisplatin is measured. Similar measurements are made for two of the new bis(platinum) compounds, the n=4 and n=6 malonates.

Platinum levels for DNA-binding and whole cell uptake measurements were made using Atomic Absorption Spectrometry(AAS). For the reader unfamiliar with this technique, a brief review of the principles of AAS is provided.

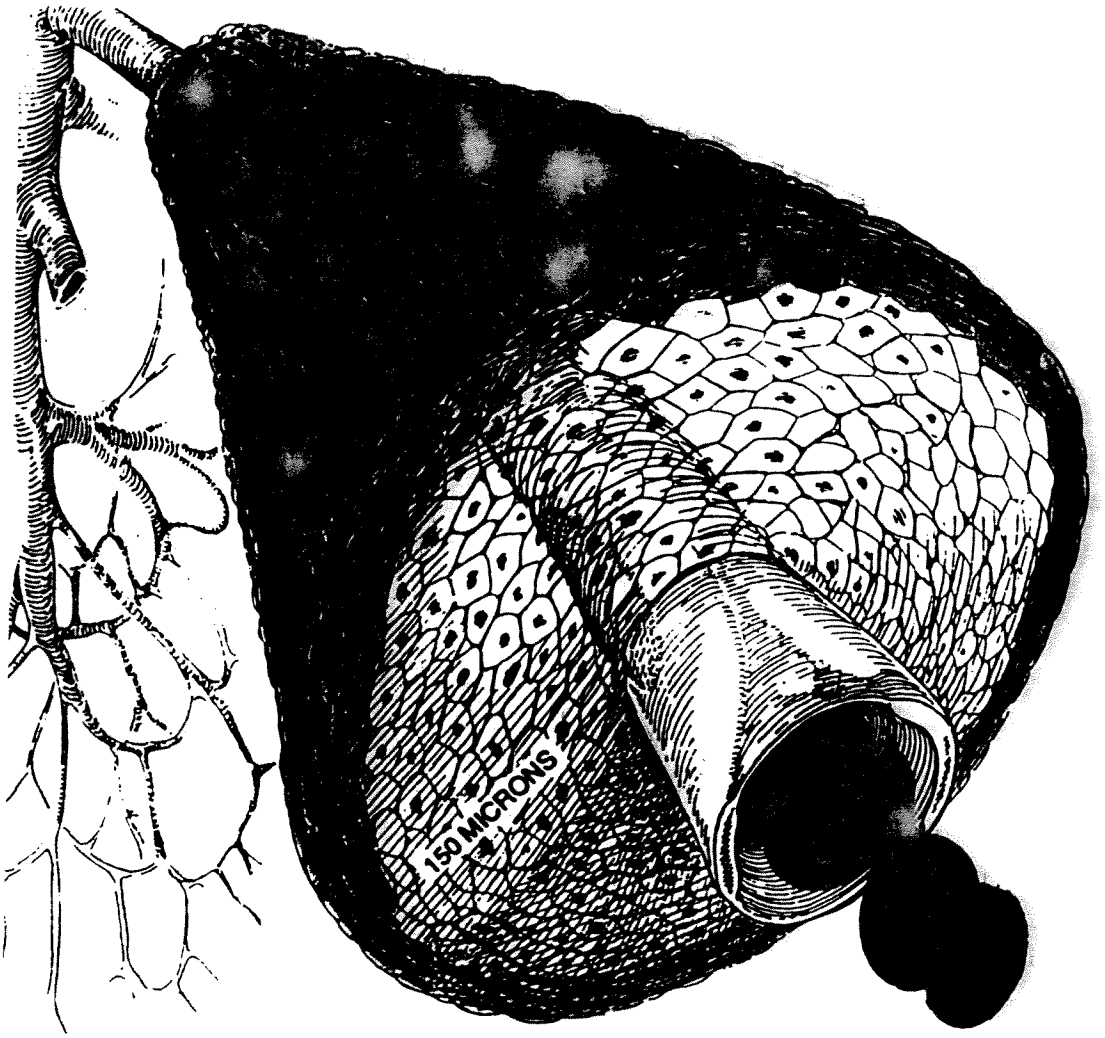


Fig. 1-6 : Oxygen utilization by intervening cells causes cells distant from blood vessels (shaded) to become hypoxic. In radiotherapy, the presence of hypoxic cells in tumours is thought to be a major cause of failure [Courtesy Dr. D. Chaplin]

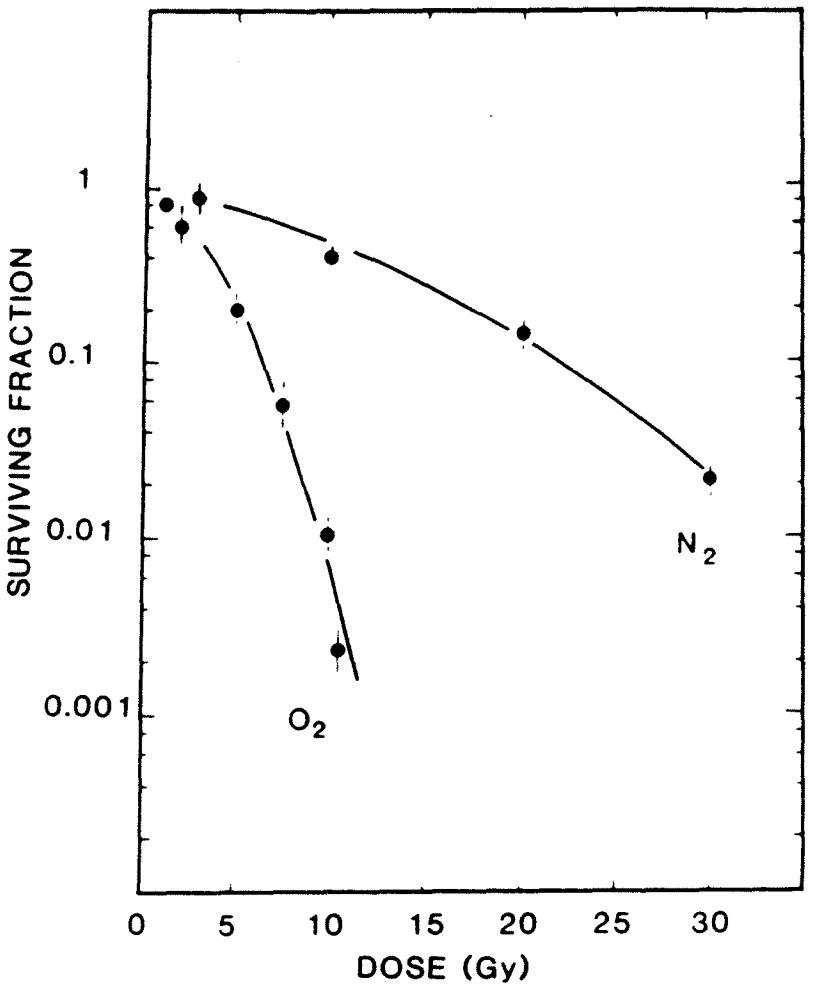


Fig. 1-7 : Cell survival curve illustrating the reduced sensitivity of hypoxic cells to treatment by X-irradiation [from Palcic and Skarsgard, Radiat. Res., 100, 328 (1984)]

Hypoxia and Cisplatin Toxicity in Mammalian Cells

Toxicity	Conc (μM)	Cells	Reference
$N_2 >> O_2$	10	V79 *spinner*	*Stratford et al. 1980*
>>	1-25	MTG-B	*Douple E. pers. comm.*
$N_2 > O_2$	1 - 20	V79 *suspension*	*MacPhail unpubl. 1989*
$N_2 = O_2$	10^{-5} - 1	EMT6 *monolayers*	*Teicher et al. 1981*
=	10	CHO	*Skov et al. 1987b*
=	1 - 20	CHO *suspension*	*Adomat; Harrison; Wurst; unpubl. 1980-88*
=	1 - 10	V79 *suspension*	*Korbelik & Skov 1989*
=	<1	V79 *spheroids*	*Durand R. pers. comm.*
$N_2 < O_2$	to 15	NHIK3025	*Melvik & Pettersen 1988*

Table 1-2 : The variability of cisplatin toxicity in hypoxic mammalian cells [from: K. Skov, in *Selective Activation of Drugs by Redox Processes*, G.E. Adams *et al*, Eds., NATO ASI Vol 198, p263 (1991)]

1.4 Atomic Absorption Spectroscopy

Atomic absorption spectroscopy(AAS) is a versatile and specific method for the measurement of trace quantities of metals. It is a useful tool for analyzing many types of samples in agricultural, geochemical and other sciences, including biology. Many examples exist of the application of AAS to the determination of metal quantities in biological samples such as human plasma and tissues[90]. More specifically AAS techniques have been used to measure the amount of platinum present in cells previously treated with cisplatin[91].

1.4.1 Energy Levels and Atomic Structure

In order to understand AAS, one must first have an understanding of the structure of the atom and its various energy states. Each type of atom has a unique number of protons and electrons, which, through electrostatic interactions cause the electrons to occupy a unique, discreet set of energy levels, or orbitals. Under normal circumstances, the electrons are arranged within the orbitals in the lowest overall energy state, known as the "ground state". It is possible, however, through the use of light or heat energy, to change the arrangement of the electrons within the orbitals. An electron may absorb just the right amount of energy and jump to a higher energy level. Under these circumstances, where the electronic arrangement differs from the ground state configuration, the atom is said to be excited. After a short interval, the electron will then return to its ground state level by emitting a photon equal in energy to the difference between the ground and excited state energies. It is important to note that because a photon's energy is propor-

tional to its wavelength, atoms will also only absorb and emit specific wavelengths of light, unique to each atom.

Resonance lines are those wavelengths of light produced or absorbed from transitions involving the ground state of the atom. Transitions from one excited state to another are known as non-resonance transitions. Resonance transitions are of particular importance to atomic absorption spectroscopy.

1.4.2 **Absorbance Measurement and Calibration**

In AAS, a monochromatic beam of light is passed through the "atomized" sample, one that has been prepared in such a way as to produce ground state atoms confined in the optical path. The wavelength of the incident light is chosen to be identical to one of the resonance lines of the metal being analyzed (known as the "analyte"), more specifically, one that is unique to that metal. For platinum, this characteristic wavelength is usually 265.9 nm. As the incident light passes through the sample, the ground state atoms absorb the light and become excited. What is not absorbed by the analyte atoms is measured and used to calculate the absorbance.

The absorbance is calculated by the formula:

$$A = \log \frac{I_0}{I},$$

where I_0 is the incident light intensity and I is the transmitted light intensity. According to Beer's law, the absorbance in a sample is directly proportional to the concentration of the analyte in the sample:

$$A = abC$$

where **C** is the analyte concentration, **a** is the extinction coefficient, a measure of the amount of absorption to be expected per mole of analyte and **b** is the optical path length. In practice, however, deviations from the straight line relationship predicted by Beer's law are almost always found at higher concentrations, presumably due to quenching effects. For this reason, AAS is a comparative technique, where a set of standards with known analyte concentrations are tested for absorbance, generating a calibration curve to which unknown samples may be compared to determine their analyte concentration. A sample calibration curve is shown in figure 1-8.

1.4.3 The Atomization Process

In order to measure the absorbance of a sample, a population of ground state atoms must be produced within the chamber, by a process known as atomization. This process also serves to eliminate unwanted species from the solvent or matrix material before the absorbance measurement is taken. The atomization process used in the following study utilized a graphite furnace as shown in figure 1-9. Similar techniques such as flame atomization are common in other disciplines, but in general, lack the sensitivity necessary for biological assays and are more susceptible to background interferences.

Once an aliquot of the sample (typically 10-100 μL) has been injected into the partition tube, three steps are required to reach atomization. The first is the **drying stage**, where the solvent material is burned off by raising the furnace temperature to a level just

below the boiling point of the solvent, typically 90 to 120°C. The second step is the **ashing stage**, where the temperature is increased to remove as much of the organic or inorganic matrix material as possible without losing any of the analyte. Temperatures for this stage range from 350 to 1200°C, depending on the analyte under consideration. During each of the preceeding steps the chamber is filled entirely with an inert gas, commonly argon, to prevent premature oxidation of the analyte. The third and final step is the **atomization stage**. The temperature for atomization is raised, typically, in excess of 1800 C, and again is dependent upon the analyte being studied. It is at this stage that ground state atoms are produced in the path of the light beam and the absorbance is measured.

The temperature profile for atomization of a given analyte and its associated matrix is by no means fixed. There are a variety of interference effects which can occur that must be minimized. The usual technique for determining the best profile is by trial and error: changing both the temperature and times in a systematic way to find the profile which leads to the highest, or most consistent absorbance reading for a given concentration of analyte.

1.4.4 Interference and Zeeman Atomic Absorption Spectroscopy

Several types of interference may be encountered in AAS which may affect the absorbance values.

Spectral interference is the overlapping of the resonance line of the analyte by an absorption line of a matrix component, causing an increased absorbance signal at the

studied wavelength. This type of interference is rare in graphite furnace AAS.

In the case of some combinations of analyte and matrix, the analyte may form a more volatile compound which is lost at lower temperatures, during drying or ashing stages. Alternatively, the analyte may form a more stable compound with a matrix component. This could cause the appearance of more than one peak, separated in time, during atomization. In either case, addition of a chemical modifier to the matrix, thereby changing the analyte or matrix atomization characteristics can overcome this type of interference.

Sample viscosity and surface tension are important considerations affecting the reproducibility of AAS measurements. It is important that the deposited sample spreads to uniform thickness within the tube to allow even heating and atomization. It can also affect the pipeting of the sample into the tube, as very viscous samples can leave residues in the pipet, decreasing the sample volume within the tube. Assuring low sample viscosity will minimize these effects.

The most important type of interference in graphite furnace AAS is due to attenuation or absorption of the incident wavelength by species other than the analyte, referred to as background. Background may be caused by scattering of the incident light by matrix particles ("smoke") present in the path during atomization, which can change the wavelength of the light such that it is outside of the spectral bandwidth of the detector. This may be corrected by optimizing the furnace temperature profile, particularly by including sufficient drying and ashing times.

Background may also occur by non-atomic or molecular absorption processes, where matrix molecules formed during the atomization process absorb light of the incident wavelength thereby increasing the observed absorbance reading. To correct for this type of interference, a special technique called Zeeman Background Correction is employed.

When atomic absorption lines are exposed to a strong magnetic field, they split into several different lines about the original wavelength. This effect is due to the interaction of the angular momentum of an atom's valence shell electrons with the magnetic field orientation, and is known as the Zeeman Effect. In Zeeman AAS, an absorbance measurement is first taken in the absence of a magnetic field, and thus has contributions from both the analyte and molecular species. Then with the magnetic field turned on, another measurement is taken: the absorbance line of the analyte atoms shifts to a wavelength outside of the spectral bandwidth of the detector due to the action of the Zeeman Effect. Thus no absorbance due to the analyte is measured. The resulting background absorbance is due entirely to absorption by matrix molecules, which are insensitive to the magnetic field, and continue to absorb at the analytical wavelength. Thus a background corrected absorbance may be determined by the difference in measurements with the magnetic field on and off, which represents the true atomic absorbance of the analyte in the sample. It is this corrected absorbance which is used for both calibration and sample measurements.

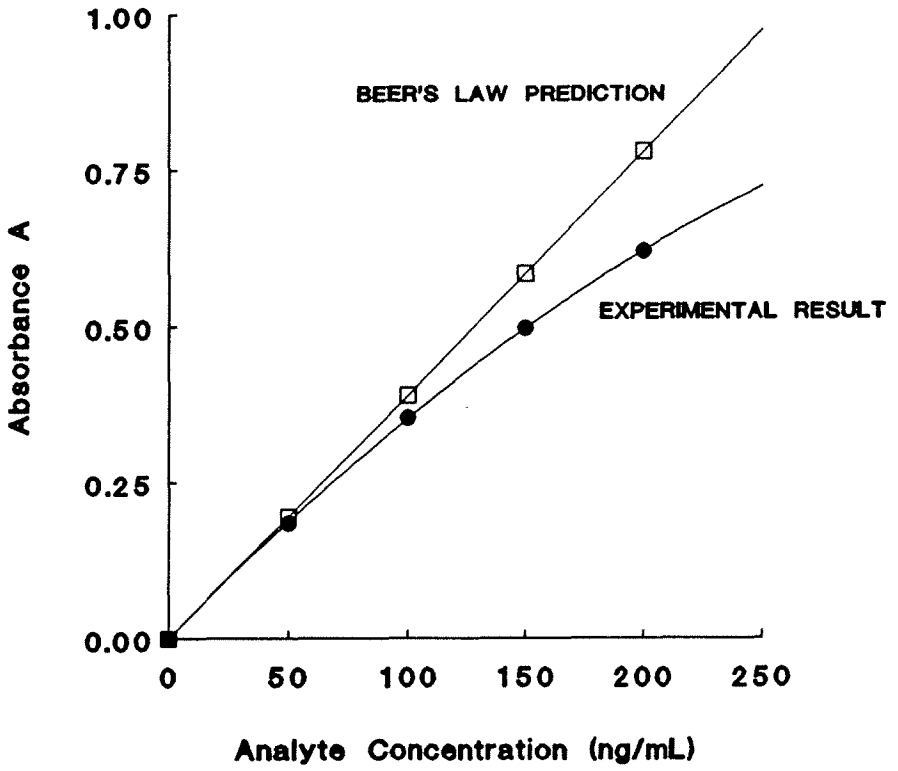


Fig. 1-8 : Sample atomic absorption calibration result. Deviations from the Beer's Law prediction occur due to quenching of the AAS signal.

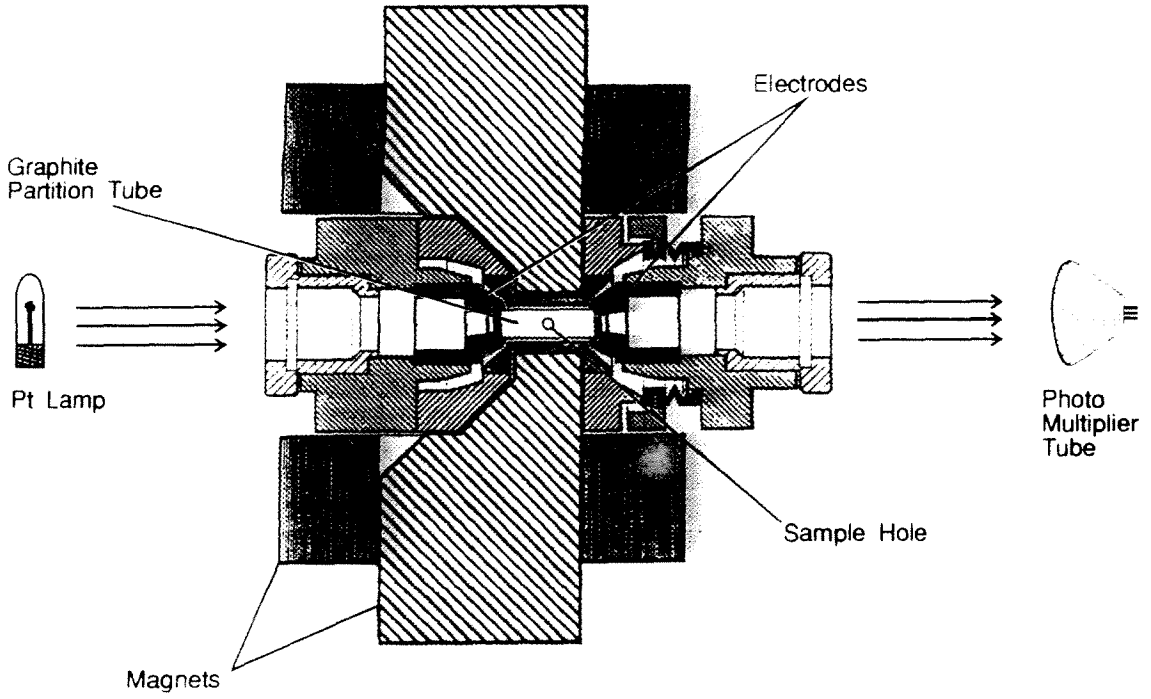


Fig. 1-9 : Schematic representation of the graphite tube atomic absorption spectrometer used in the studies to follow.

2. MATERIALS AND METHODS

2.1 Special Solutions

2.1.1 Media

All experiments used an alpha-modification of Eagle's minimum essential media (GIBCO, Burlington, Ontario) in three different forms: α-/-; α+/-, with 10% foetal bovine serum (FBS, Gibco); and α+/+, with 10% FBS and 2.0 g/l $NaHCO_3$.

α+/+ and α-/- were prepared by dissolving α-media powder in 10 l of double-distilled H_2O (ddH_2O) , supplemented with 10,000 units of Penstrep antibiotic (Gibco), and stirred for two hours at room temperature. FBS and bicarbonate were added to the α+/+ preparation. The pH was then adjusted to 7.3 with 4M NaOH.

Media were sterilized by filtration (0.22μM pore size) under pressure, asceptically dispensed into 500 ml bottles and stored at 4°C until use. Each batch was tested for contamination and proper cell growth.

When needed, α+/- was made by adding 50 ml FBS to an α-/- bottle.

2.1.2 EDTA

Stock solutions of 250 mM were prepared using EDTA disodium salt (Sigma) dissolved in approximately 0.5 l ddH_2O. pH was adjusted to 8.0 with 10 N NaOH to aid in dissolution.

2.1.3 Trizma Buffers

Tris stock solutions were prepared in 1 l batches with Trizma-HCl(Sigma) to a concentration of 0.5M. 4N NaOH was used to adjust the pH to 7.5 or 8.0 depending on the use.

2.1.4 Tris-EDTA (TE)

A 1 l stock solution of 50x TE with final concentration of 0.5M Tris-HCl and 50 mM EDTA was prepared and the pH adjusted to 8.0 with 4M NaOH. 500 ml TE solutions were then prepared by a 1:49 dilution with ddH_2O prior to use.

2.1.5 TNE

TNE was prepared from stock solutions, with a final composition of 10mM Tris, 150mM NaCl(Sigma) and 10mM EDTA. Final pH of the solution was 8.

2.1.6 TNE Equilibrated Phenol

99%+ pure phenol(Aldrich) was placed in a water bath and melted at 60°C for 30 minutes. The liquified phenol was saturated with an equal volume of 0.5M Tris pH 8.0 and the two phases allowed to separate. The upper layer was removed and the process repeated. Similar washes with equal volumes of TNE were repeated until the pH of the upper phase reached 7.

The equilibrated phenol was then aliquotted into 50 ml screw-capped polypropylene tubes (Falcon), wrapped in foil and stored at -20°C until used.

2.2 Drug Preparation

Cisplatin (a gift from Bull Laboratories, Mulgrave, Australia) and bis(platinum) complexes (courtesy of N. Farrell, University of Vermont) were stored at 0°C until use. Drugs were dissolved in α+/- medium to a concentration of 200μM by stirring at 37°C for 1 hour. All drug solutions were passed through Millex-GV 0.22μM sterilizing filter units immediately before use.

Flasks containing dilute cisplatin and bis(platinum) solutions were placed in a shaker bath at 37°C. Hypoxic conditions were achieved by a continuous flow (1 cm^3/s) of humidified, oxygen-free nitrogen gas (Medigas) through the flasks (see fig. 2-1). One hour was adequate to attain radiobiological hypoxia[92], before the addition of cells. Aerobic conditions were similarly maintained using a water saturated air flow through the flasks.

2.3 Cell Maintenance

CHO cells were carried in spinner culture in α+/+ medium at 37°C in a 95% air/5% CO_2 atmosphere. Daily dilution to approximately $1x10^5$ cells/ml in 250 ml maintained the cells in exponential growth phase, with a doubling time of approximately 13-14 hours. Spinner flasks were changed twice weekly.

Once a week, 10^5 cells were dispensed into T-75 flasks (Falcon) for incubation, as a reserve in case of contamination of the spinner culture.

2.4 Cell Preparation

Immediately prior to use, cells were centrifuged at 93g and 4°C for 7 minutes, and resuspended in an appropriate volume of chilled α+/- to give a concentration of $2\text{-}4x10^6$ cells/ml.

2.5 Clonogenic(Survival) Assay

Cells were added to drug solutions to yield a concentration of $2x10^5$ cells/ml. One ml cell samples were taken at various time intervals and diluted into 9 ml of chilled α-/-. Samples were immediately centrifuged at 93g and 4°C for 7 minutes, the supernatant was

poured off and the cell pellet was resuspended in 10 ml cold, fresh α-/-. Appropriate numbers of cells needed to produce approximately 100 surviving colonies were then seeded, in duplicate, into 115x25 mm disposable petri dishes (Falcon) containing 5 ml of pre-gassed α+/+. Suspensions were incubated at 37°C in a 95% air/5% CO_2 atmosphere.

After 7 days the excess medium was poured off and the colonies were stained for 10 minutes with 5 ml of a 2.0 g/l methylene blue solution (Fisher). The colony counting criterion was chosen as 50 or more cells. Plating efficiencies were determined as the number of colonies formed divided by the number of viable cells plated.

2.6 Uptake and DNA Binding Studies

2.6.1 Removal of Extracellular Platinum

Cells were added to pre-gassed drug solutions to give a concentration of 2-4x10^5 cells/ml. At various time intervals, 20 ml cell samples were taken and immediately centrifuged under the usual conditions. After removing the supernatant, two washes with 10 ml ice-cold phosphate buffered saline (PBS) were performed. The pellet was resuspended in 4.5 ml ice-cold PBS and the cell concentration was determined. Four ml of cell suspension was then accurately transferred to a 5 ml capacity polypropylene tube (Falcon) and centrifuged once again. The supernatant was discarded and the inside of the tube was wiped dry with a tissue.

2.6.2 Whole Cell Uptake

Pelleted cell samples were prepared for atomic absorption analysis by digestion in 100 µl concentrated HNO_3 at 37°C overnight. The samples were then diluted to 600

μl with 490 μl ddH_2O, allowing 10 μl for the volume of the cells.

2.6.3 DNA Binding

Pelleted cell samples were digested in a mixture of 960 μl TNE, 10 μl of 10 mg/ml proteinase K(Sigma) and 10 μl of 10% SDS(Aldrich) at 37°C, overnight. DNA extraction was accomplished via two separate washes in an equivalent volume of TNE-equilibrated phenol followed by two washes in 24:1 chloroform/iso-amyl alcohol. Platinum-DNA products have been found to remain stable during this extraction procedure [93]. The DNA was precipitated by adding 2x the sample volume of 99.5% ethanol and placing the tubes at -20°C overnight. Precipitated samples were centrifuged at 9600g for 3 hours, the supernatants were discarded and the samples thoroughly dried by a gentle flow of warm air through the tubes. The DNA was then re-hydrolysed in 200 μl TE. The DNA concentration was determined by measurement of the optical density(OD) of a one-hundred-fold dilution of a 20 μl sample of each tube. OD measurements were made at 260 and 280 nm on an Aminco DW-2 UV/vis spectrophotometer. An OD_{260} of 1.0 is equivalent to a DNA concentration of 50 μg/ml, which, when used to calculate the DNA concentration of a typical sample, gave between 0.8 and 1.2 mg/ml. The ratio of the OD_{260} to OD_{280} values was typically 1.8 to 1.9, indicating a very pure sample of DNA, with little RNA present.

2.6.3 Atomic Absorption Analysis

All atomic absorption measurements were made using a Varian SpectrAA 300 atomic absorption spectrometer with a graphite tube atomizer, and Zeeman background

correction. A Compaq Deskpro 386s computer with Varian atomic absorption software was used to control the instrument.

For each measurement, twenty-microlitre aliquots of sample were placed in the graphite tube, and then dried and ashed according to the temperature profile shown in figure 2-2. For DNA binding measurements, three successive aliquots were placed in the tube and then dried and ashed before the atomization stage was reached. Two 20 μl aliquots were used for whole cell measurements. Peak absorbances, measured with a platinum lamp at 265.9 nm wavelength, were used to find the platinum concentration by comparison with a calibration curve run immediately prior to the samples. The calibration measurements were performed at four concentrations (100, 200, 300, and 400 ng Pt/ml) by standard additions of the 400 ng/ml standard solution. Two replicates of each sample were performed and the average was determined.

Every five samples, a blank sample was analyzed followed immediately by a "reslope standard". The blank measurement was taken to ensure that no "memory effects" were present, caused by an incomplete atomization of the previous sample. Reslope measurements were performed with the 200 μg/ml standard. The calibration curve was then rescaled according to the new result. This accounted for any tube deterioration which may have occurred during the experiment.

Sample concentrations, in ng/ml were determined by interpolation from the calibration curve. Whole cell platinum concentrations were then normalized by division by the number of cells present in the digested sample, and converted to obtain a

measurement of atoms of platinum per cell. DNA bound platinum concentrations were divided through by the sample DNA concentration and then converted to give a measurement of the number of platinum atoms bound per 10^5 base pairs of DNA (assuming an equal representation of G, C, T and A bases in the DNA).

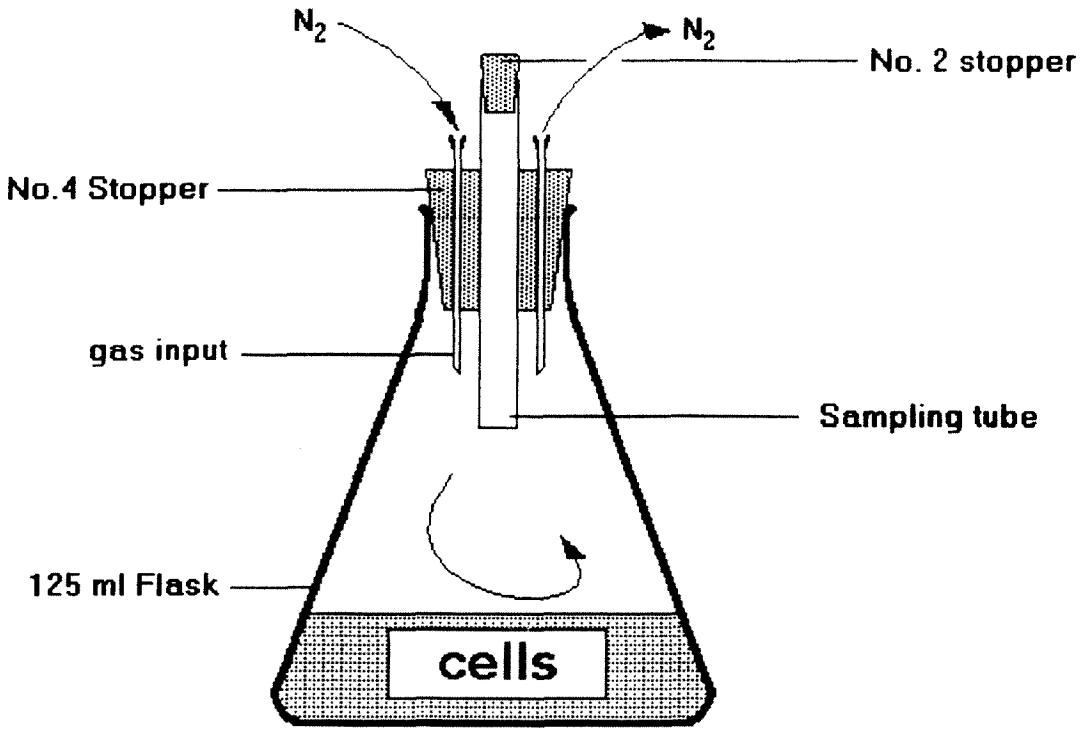


Fig. 2-1 : A schematic representation of the vessels used for cytotoxicity, whole cell uptake and DNA-binding experiments. Cell concentration was approximately $2x10^5$ cells/mL and a flow of 1 cm^3/s of N_2 (for hypoxic conditions) or air (for aerobic conditions) was maintained through the flasks for the duration of the experiments.

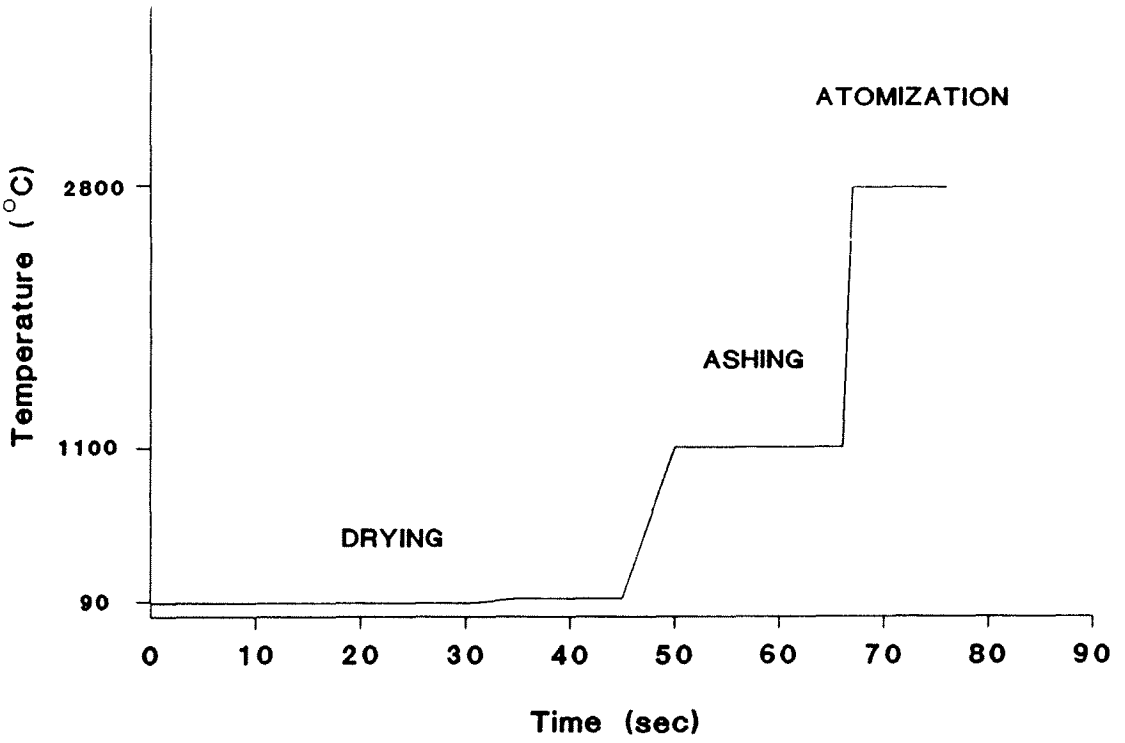


Fig. 2-2 : Temperature profile for atomic absorption measurements. Each aliquot of sample was dried for 30 seconds at 90°C followed by 15 seconds at 120°C, then ashed for 15 seconds at 1100°C. After the final aliquot was added, the entire sample was ashed for a further 7 seconds before absorption measurements were taken at an atomization temperature of 2700°C.

3. RESULTS

3.1 Studies with Cisplatin in Air and Hypoxia

3.1.1 Toxicity

CHO cells were incubated in 0, 5, 10 and 15 μM cisplatin solutions for up to 3 hours, under both aerobic and hypoxic conditions. Results are shown in figures 3-1 through 3-4 for 1, 1.5, 2 and 3 hour incubations.

All points represent the averages of 4 separate experiments except for the 1.5 hour data, averaged from 3 experiments. In most cases, the survival data were fitted to an exponential function of the form:

$$PE = Ae^{-\tau D}$$

by a weighted least squares fit to the linearized function. In the above equation, PE is the plating efficiency, D is the concentration of drug in μM, and A and τ are constants. The constant A is simply the control plating efficiency and τ is a measure of the toxicity of the drug, in μM^{-1}. In cases where survival data did not conform to the above relationship, data were fitted to an arbitrary function and were treated as noted below.

In all of the resulting data, cisplatin appeared preferentially cytotoxic to hypoxic, rather than aerated cells. As a measure of the degree of enhanced toxicity, the "hypoxic cytotoxicity ratio" (HCR) was calculated. The HCR is defined as the concentration of cisplatin required in air to produce a given level of toxicity relative to the dose required to produce the same effect in hypoxia[94]. For toxicity data where the log of both hypoxic and aerobic plating efficiencies are linear, the HCR is simply the ratio of the two τ's. This also has the added benefit of making conversions of plating efficiency to

surviving fraction, to account for the differential effects of air and hypoxia on control populations, unnecessary. There is, however, one exception. The result for a one hour aerobic incubation of cisplatin does not follow a simple exponential, rather it resembles the radiation survival curve, with an initial shoulder region at low concentrations, leading to a straight region at higher concentrations (see figure 3-1). For this case, a simple exponential was fitted to the linear portion of the curve. The HCR for one hour incubations in cisplatin was then taken to be the ratio of concentrations needed to produce a surviving fraction of 0.01 in air versus hypoxia. It should also be noted that survival curves for hypoxic incubations of greater than one hour also appear as if there may be a more complex relationship. For simplicity, these were analyzed using equation 1. Possible deviations from this fit will be investigated in the discussion.

Table 3-1 shows the τ values for each case, and the calculated HCR's for cisplatin. Student's t-test analysis (results also shown in table 3-1) show that the observed differences in toxicity between air and hypoxia are statistically significant. Figure 3-5 shows graphically that as incubation time increases, the difference between aerobic and hypoxic τ's, and hence the HCRs, increases.

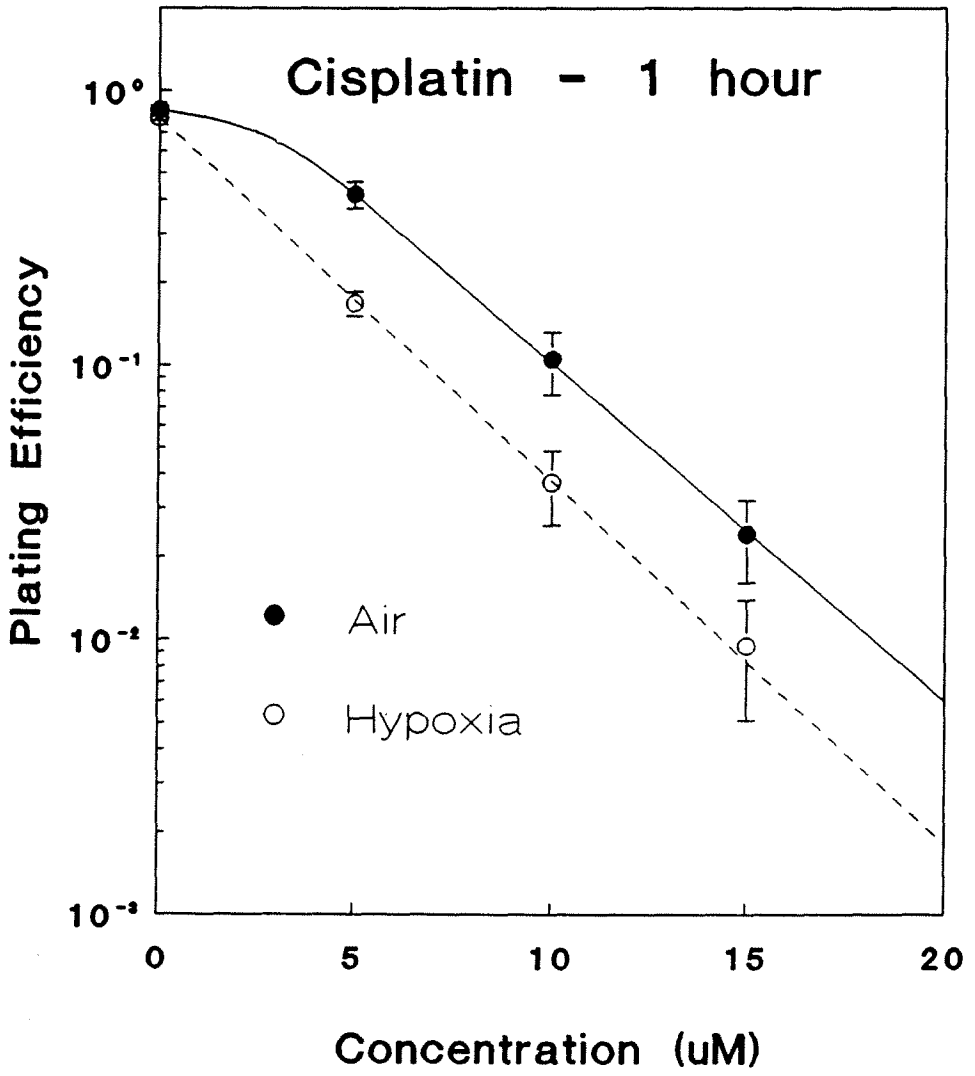


Fig. 3-1 : Comparison of hypoxic and aerobic cytotoxicity of cisplatin in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 1 hour. Error bars represent standard error from four separate experiments. The hypoxic cytotoxicity ratio, calculated as the ratio of doses necessary to produce a survival of 0.01 in air versus hypoxia, is 1.26±0.49 (in air, $D_{.01}$=18.2±6.4 μM; in hypoxia, $D_{.01}$=14.4±2.6 μM). Note that aerobic and hypoxic lines are effectively parallel for concentrations of greater than 5 μM.

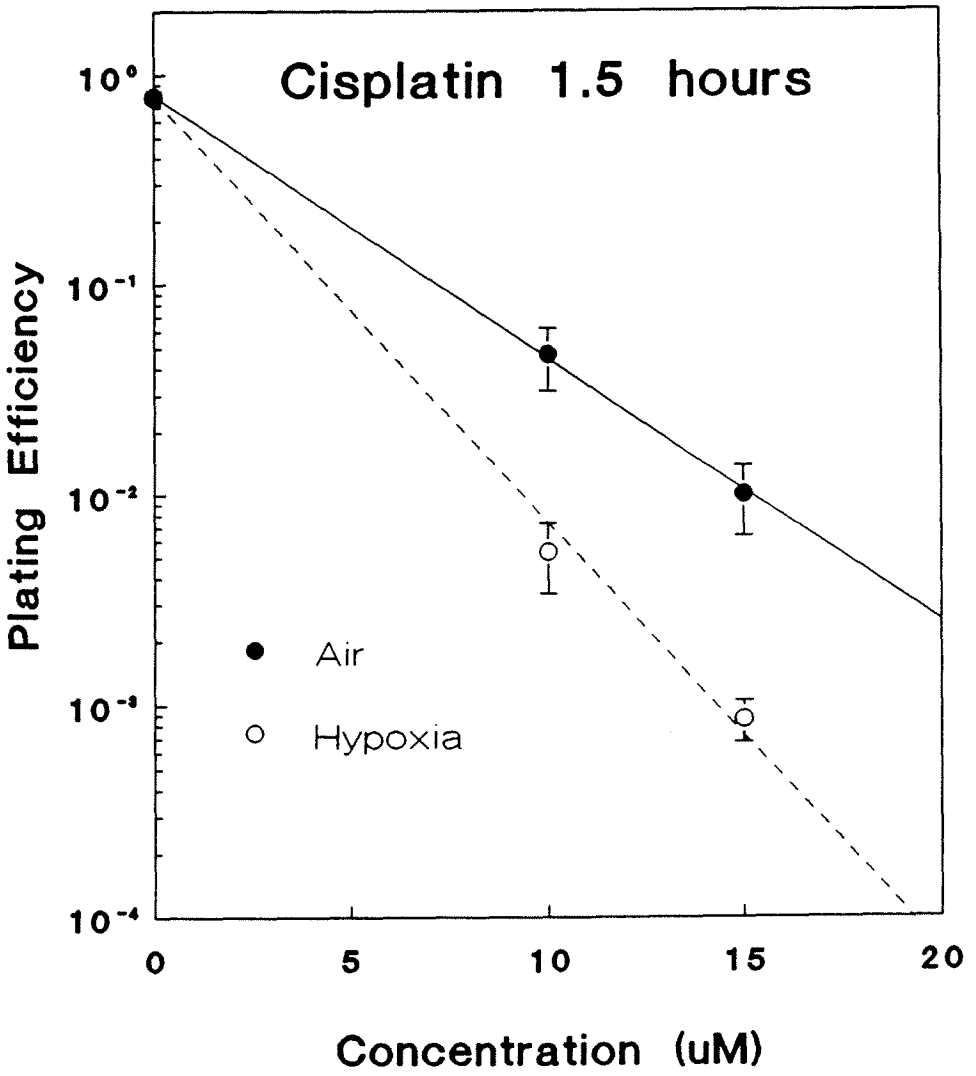


Fig. 3-2 : Comparison of hypoxic and aerobic cytotoxicity of cisplatin in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 1.5 hours. Error bars represent standard error from three separate experiments. The hypoxic cytotoxicity ratio (HCR), calculated using the ratio of τ values (as defined in equation 1) is 1.60±0.45.

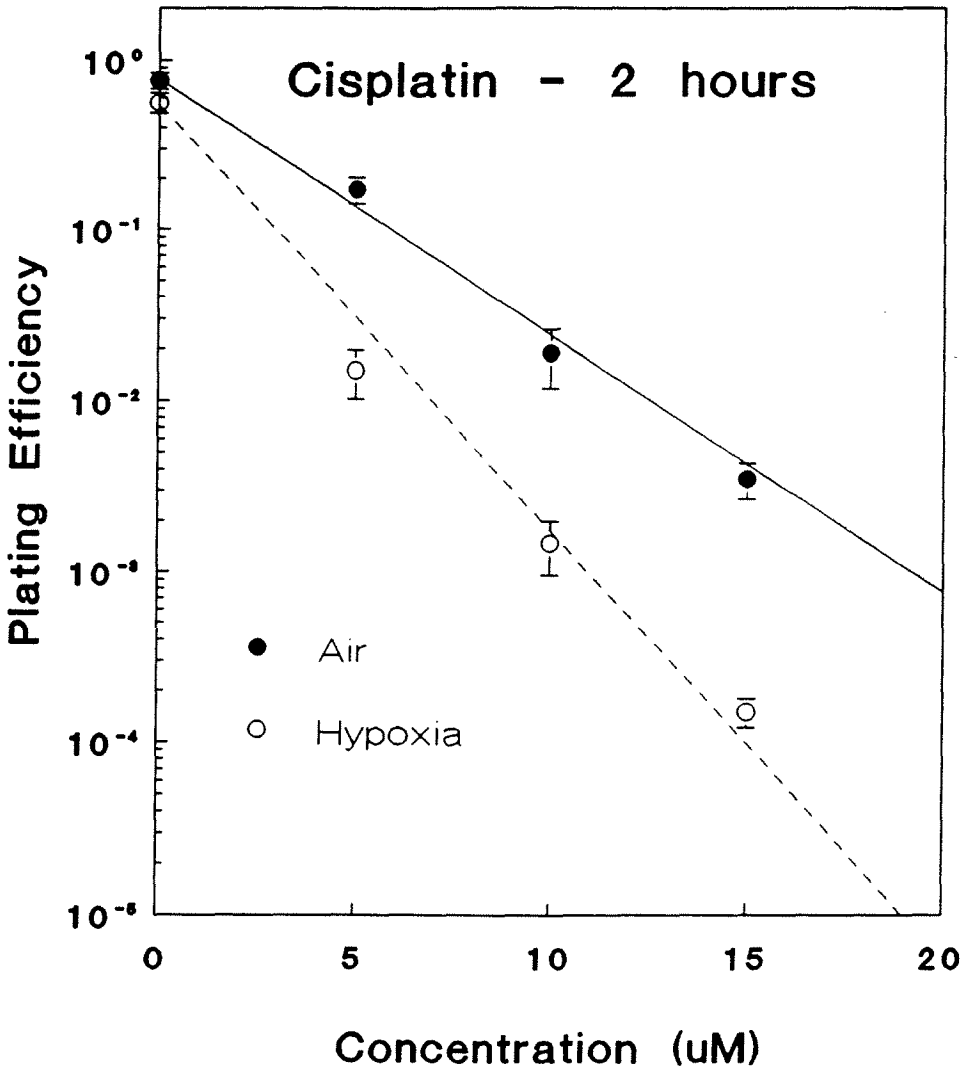


Fig. 3-3 : Hypoxic and aerobic cytotoxicity of cisplatin in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 2 hours. The hypoxic cytotoxicity ratio is 1.66±0.36.

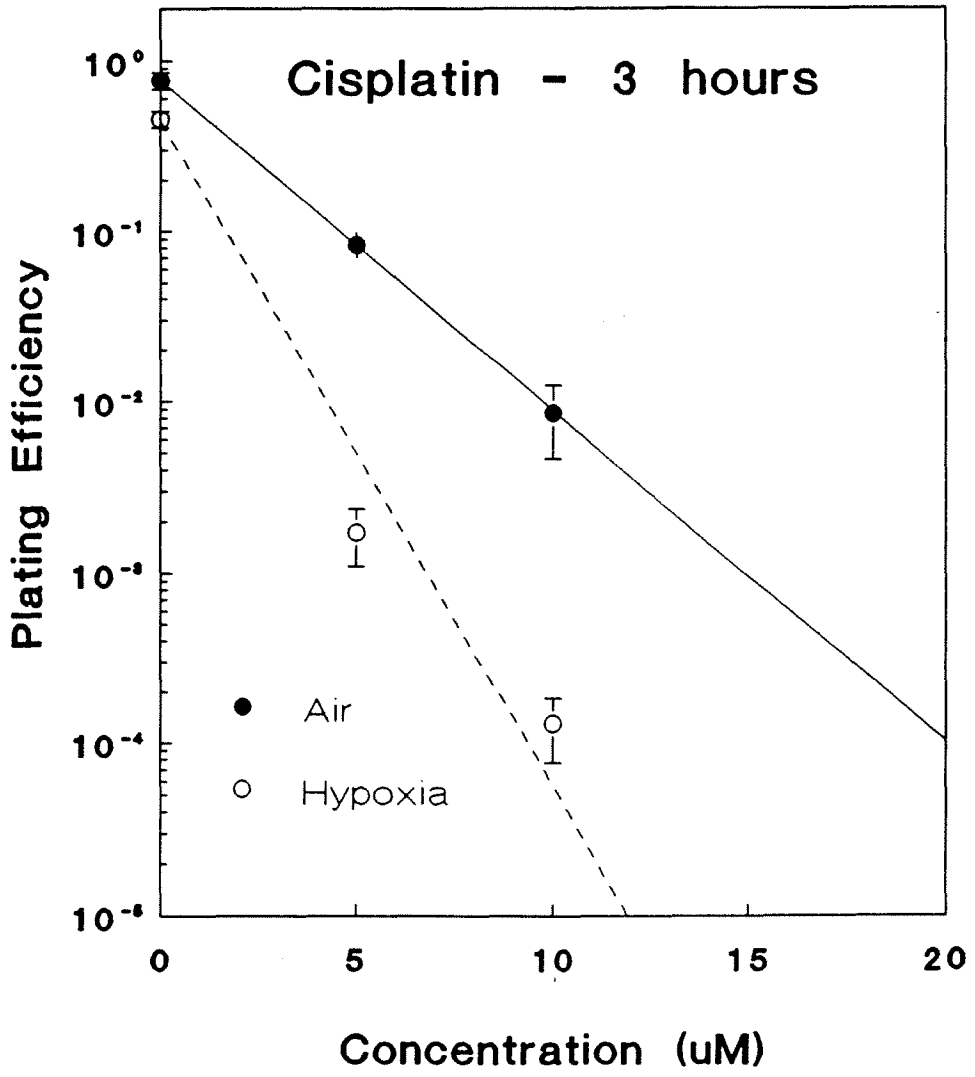


Fig. 3-4 : Comparison of hypoxic and aerobic cytotoxicity of cisplatin in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 3 hours. Error bars represent standard error from four separate experiments. The hypoxic cytotoxicity ratio is 2.01±0.50.

time (hr)	τ (μM^{-1})		HCR	p
	Air	Hypoxia		
1.0	N/A	0.304±.055	1.26±.49	.014
1.5	0.288±.066	0.465±.072	1.61±.45	<.01
2.0	0.346±.059	0.574±.076	1.66±.36	<.001
3.0	0.446±.072	0.896±.166	2.01±.50	<.001

Table 3-1 : Hypoxic cytotoxicity ratios and τ values for figures 3-1 to 3-4. Where applicable, toxicity data was fitted by least squares to the equation $PE = Ae^{-\tau D}$. N/A means data followed another relationship, and the HCR was calculated from the ratio of drug concentrations required for 0.01 survival. For one hour incubations this concentration, the $D_{0.01}$ was 18.2±6.4 μM in air and 14.4±2.6 μM in hypoxia.

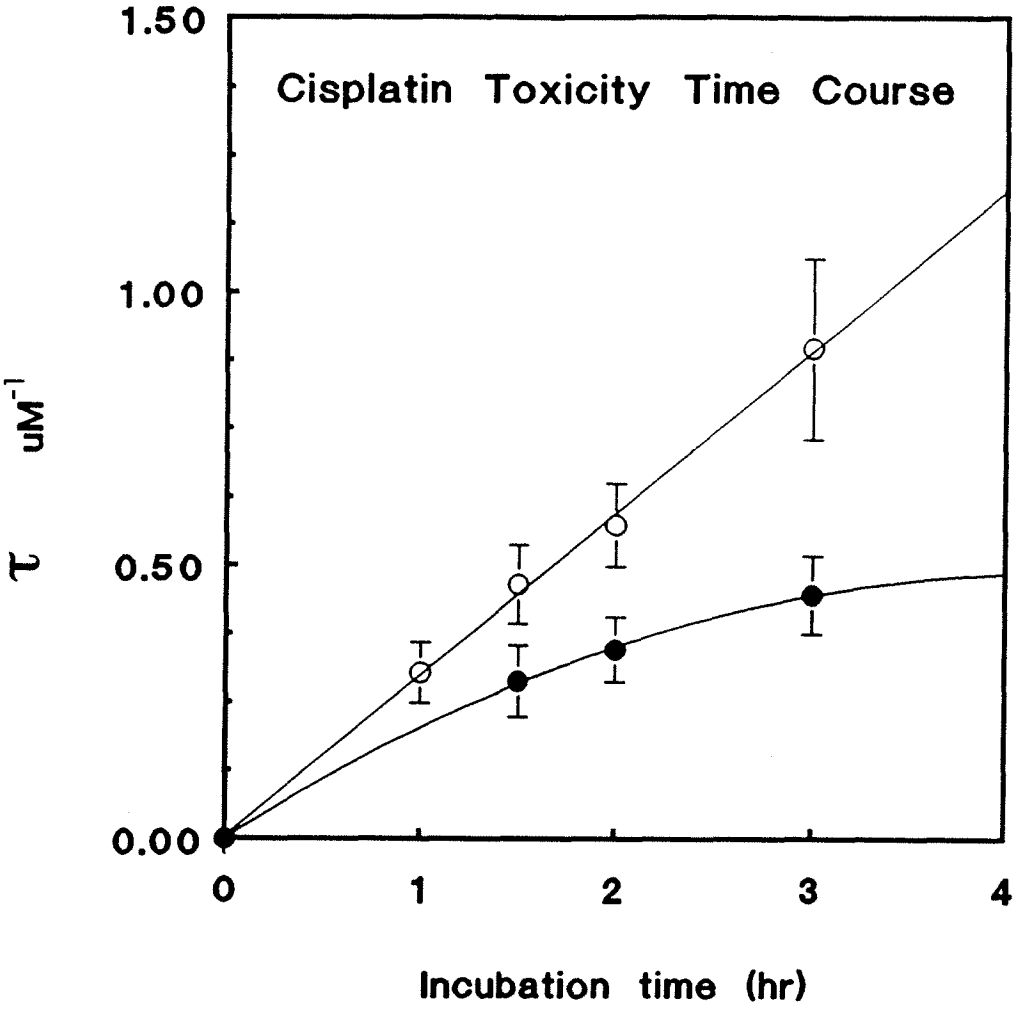


Fig. 3-5 : The variation of τ with the length of incubation in cisplatin. As the incubation time increases, so does the difference between aerobic(•) and hypoxic toxicity(o).

3.1.2 **Whole Cell Uptake**

Uptake measurements were performed at the same dose and time intervals as toxicity experiments. Atomic absorption was used to measure the total platinum, as described in section 2.6.4. One experiment was performed simultaneously with toxicity at each concentration, from the same vessels. In the three remaining experiments, toxicity was measured for the 10 μM vessel only, to ensure agreement between experiments.

Whole cell uptake is plotted against the concentration of cisplatin for each time interval in figures 3-6 through 3-9. Uptake for any given incubation period varies linearly with drug concentration under both aerobic and hypoxic conditions. Furthermore, cells incubated in hypoxia consistently bound more cisplatin than those in air. The slope, in each case, was calculated by the method of least squares, each point weighted by its associated uncertainty.The slope then represents the number of platinum atoms bound per cell for each μM of cisplatin concentration during incubation. The ratio of slopes of hypoxic and aerobic lines (calculated by the method of least squares and weighted by the associated uncertainties) was defined as the "Hypoxic Uptake Ratio", or "HUR". Table 3-2 lists the HUR for each incubation period (shown graphically in fig. 3-10), along with student's t-test results showing that differences between air and hypoxia are significant in at least three of the four cases.

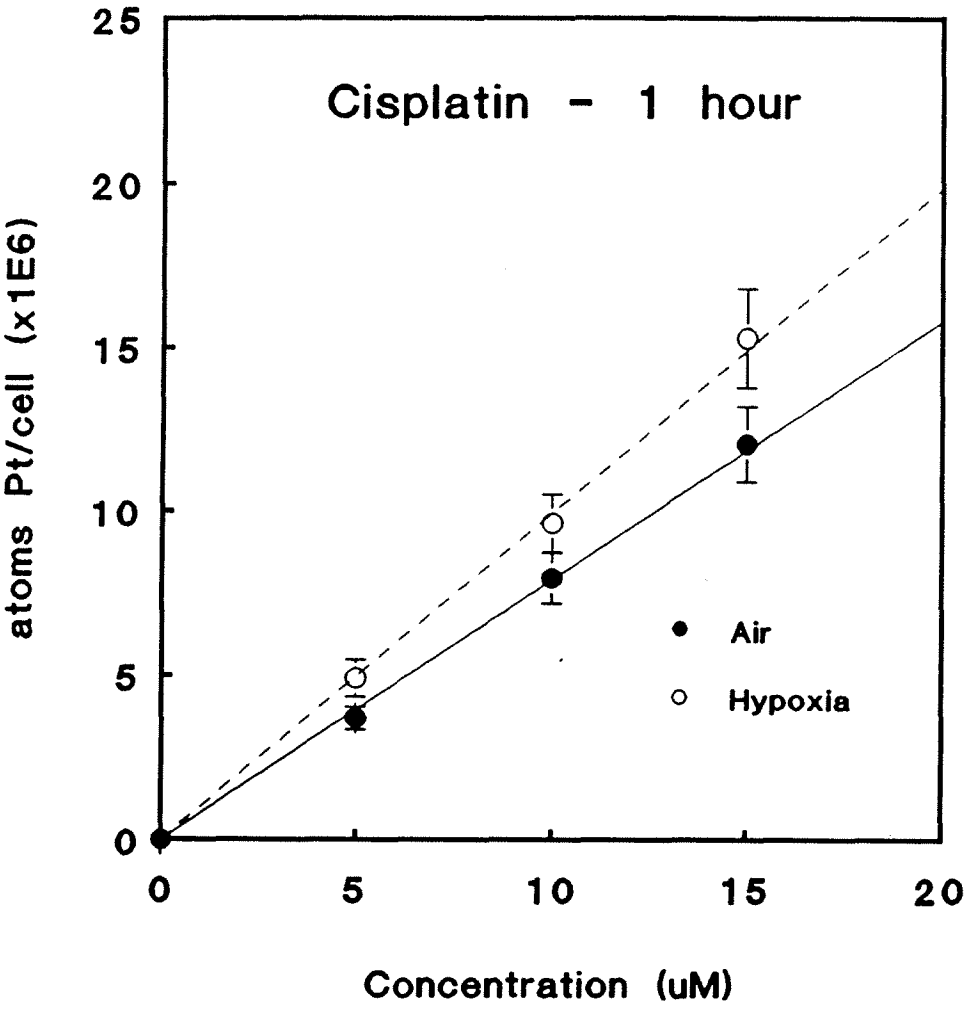


Fig. 3-6 : Comparison of whole cell uptake of cisplatin in exponentially growing CHO cells incubated in air and hypoxia at 5, 10 and 15 μM for 1 hour. Error bars represent standard error from four separate experiments. The hypoxic uptake ratio, calculated from the ratio of hypoxic versus aerobic slopes, is 1.19±.11 .

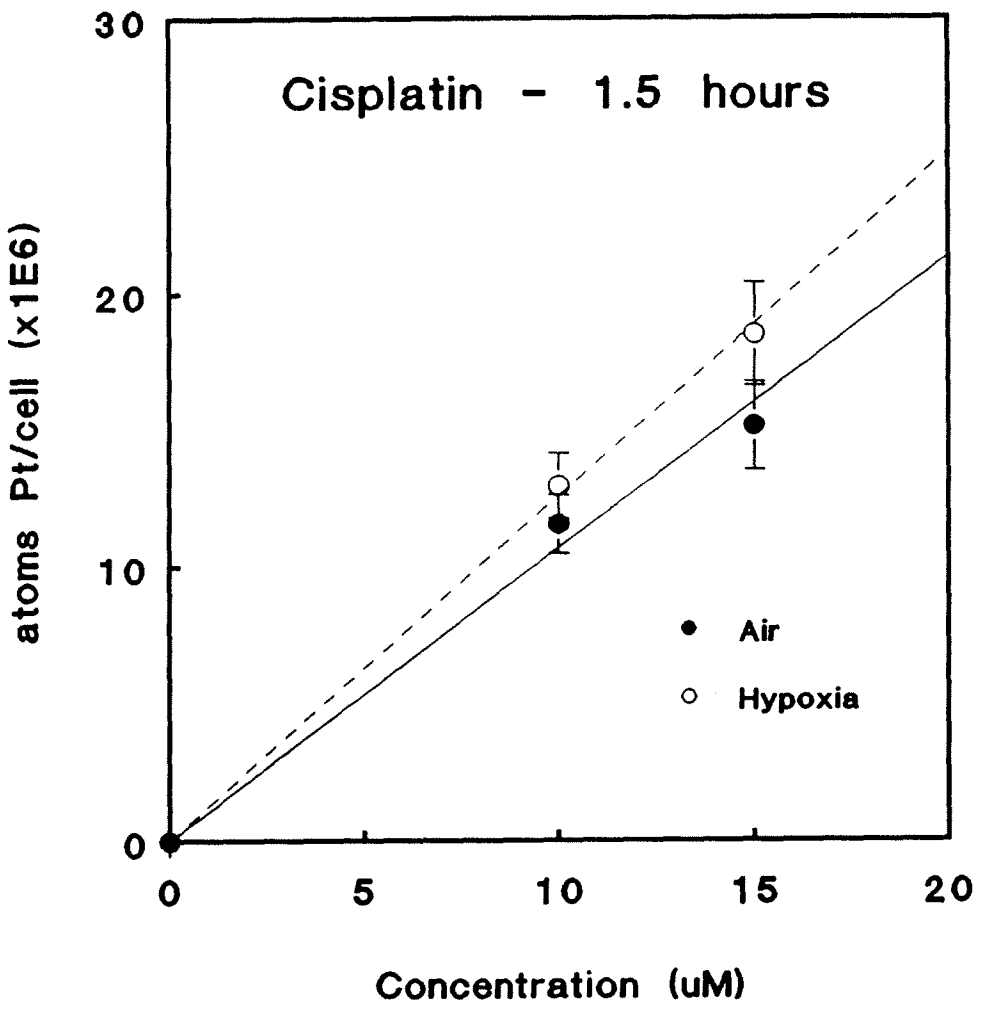


Fig. 3-7 : Comparison of whole cell uptake of cisplatin in exponentially growing CHO cells incubated in air and hypoxia at 10 and 15 μM for 1.5 hours. Error bars represent standard error from three separate experiments. The hypoxic uptake ratio is 1.18±0.09 .

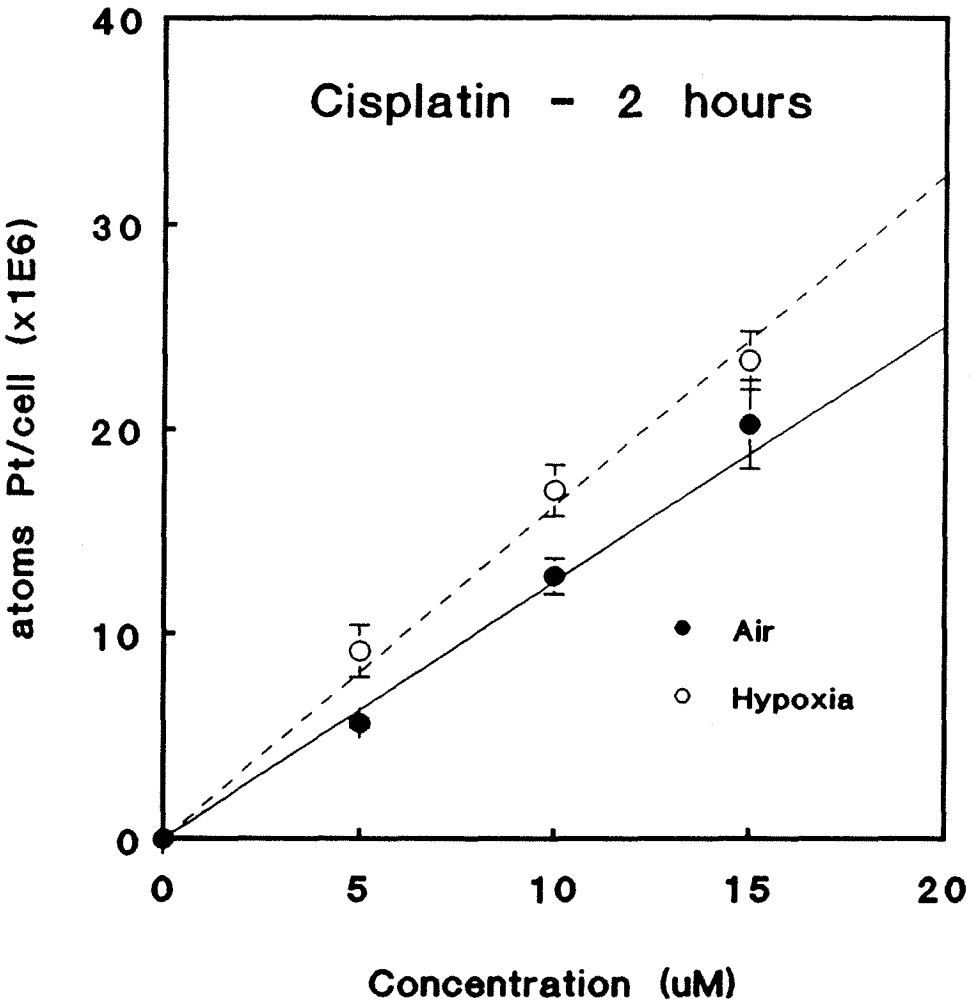


Fig. 3-8 : Comparison of whole cell uptake of cisplatin in exponentially growing CHO cells incubated in air and hypoxia at 5, 10 and 15 μM for 2 hours. Error bars represent standard error from four separate experiments. The hypoxic uptake ratio is 1.30±0.08 .

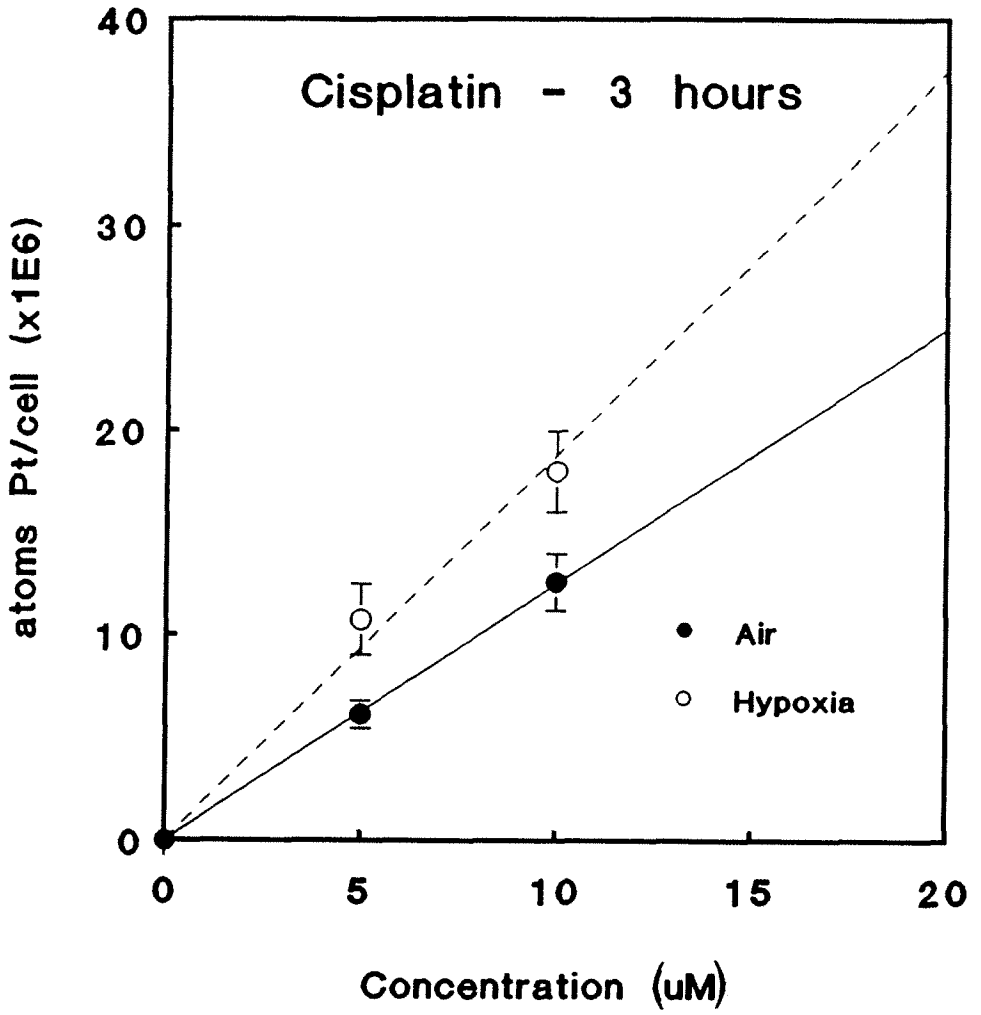


Fig. 3-9 : Comparison of whole cell uptake of cisplatin in exponentially growing CHO cells incubated in air and hypoxia at 5 and 10 μM for 3 hours. Error bars represent standard error from four separate experiments. The hypoxic uptake ratio is 1.50±0.15 .

time (hr)	atoms Pt $cell^{-1}$ μM^{-1}		HUR	p
	Air	Hypoxia		
1.0	0.790±.050	0.994±.057	1.19±.11	.004
1.5	1.068±.066	1.259±.070	1.18±.09	.39
2.0	1.251±.056	1.621±.062	1.30±.08	.004
3.0	1.248±.096	1.877±.124	1.50±.15	.05

Table 3-2 : Hypoxic uptake ratios (HURs) and molar uptake (expressed in atoms of drug per cell per μM exposure) for cisplatin in CHO cells. Calculated from uptake graphs, figures 3-5 through 3-8.

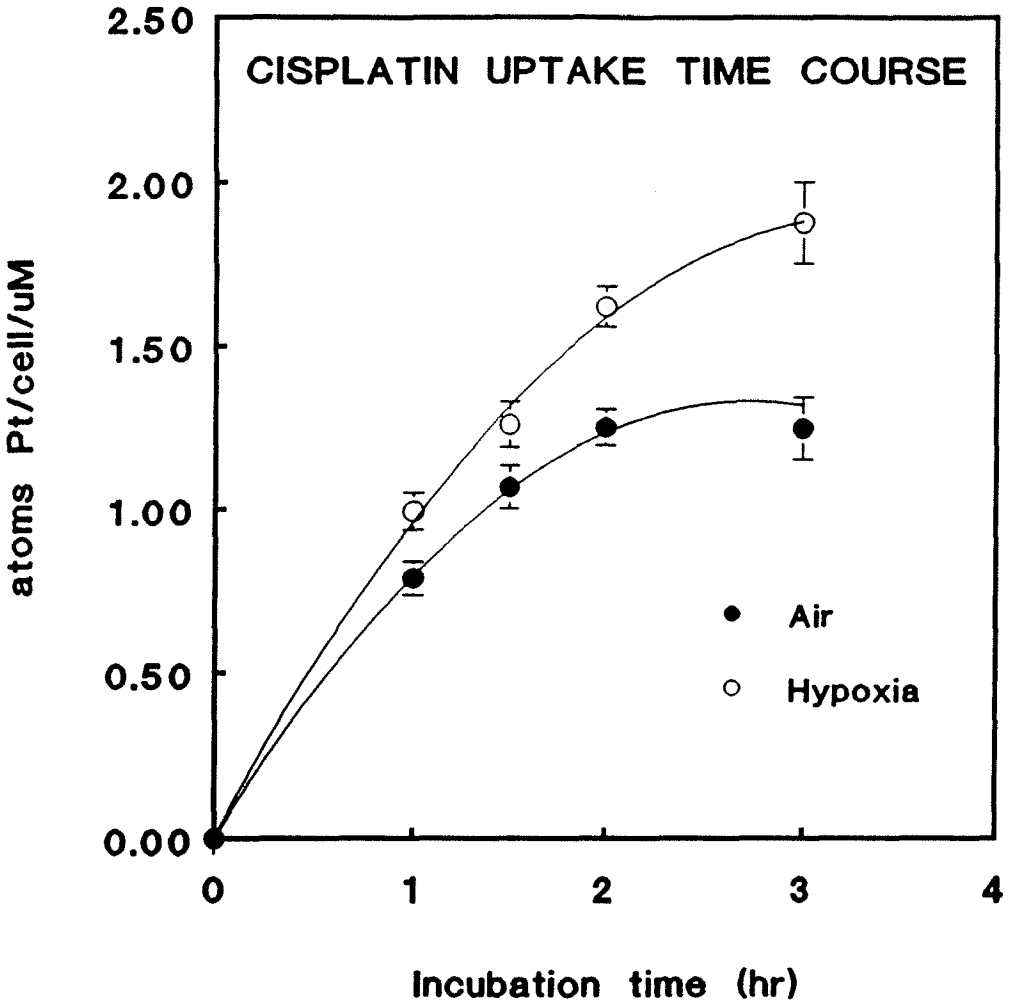


Fig. 3-10 : The variation of the whole cell uptake per mole of cisplatin with length of incubation. Note that as incubation time increases, so does the difference in uptake between aerobic and hypoxic cells.

3.1.3 **DNA Binding**

Cells used in DNA binding experiments for cisplatin were incubated under identical conditions to those stated above prior to phenol extraction of the DNA. The level of binding is plotted for each time interval in figures 3-11 through 3-14. The number of cisplatin molecules per 10^5 DNA base pairs varied linearly with the drug concentration for each incubation period up to three hours, with levels for cells treated under hypoxia consistently greater than those for aerobic cells. To quantitate this difference, the "Hypoxic Binding Ratio" (HBR) was defined as the ratio of slopes for hypoxic and aerobic binding dose response curves. Again, t-tests were performed for each incubation period. The results are listed in table 3-3. The detection limit of this assay is approximately 0.75 atoms Pt/10^5 base pairs of DNA. Since samples for 1 hour incubations were close to this limit, not all measurements returned t-test P values which indicated that hypoxic and aerobic binding are different with statistical certainty. Higher cisplatin concentrations may have yielded better statistics. However, in order to ascertain the relationship between cisplatin toxicity and total DNA binding, both measurements were made at similar concentrations.

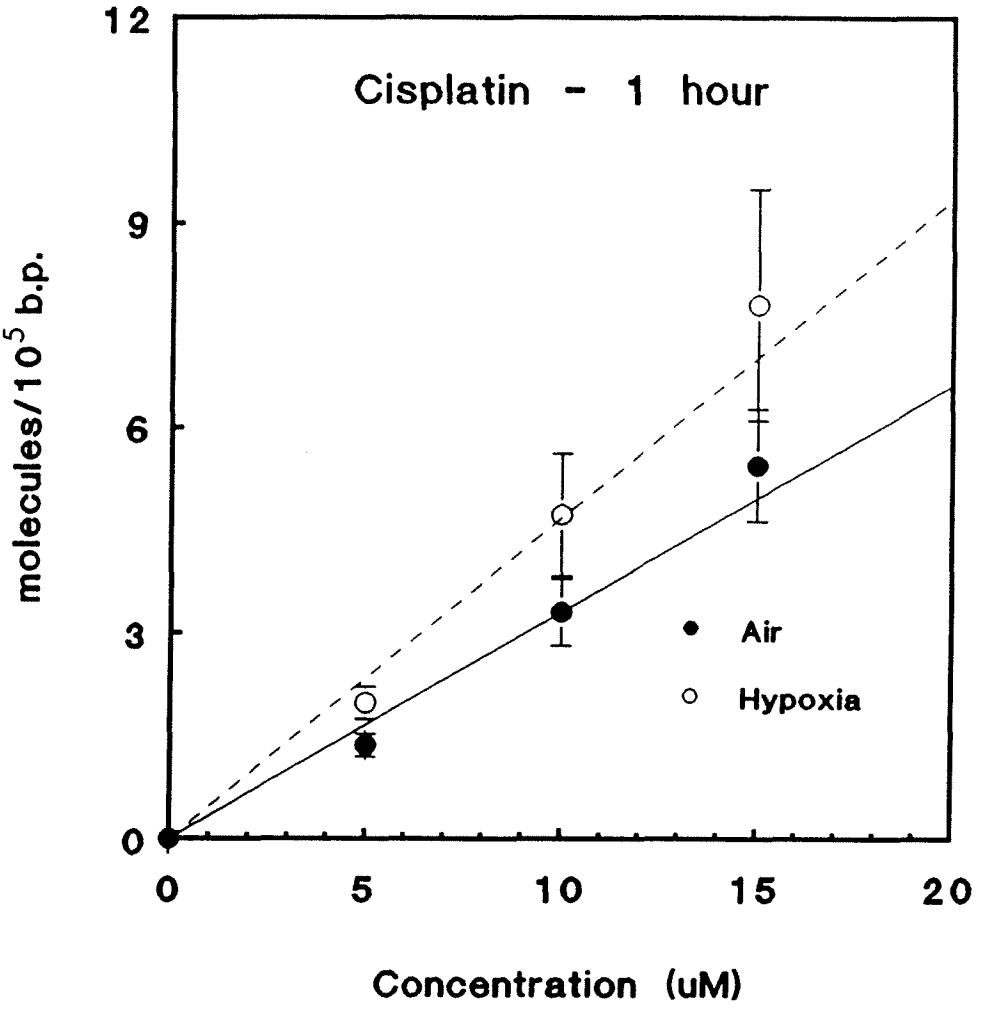


Fig. 3-11 : Comparison of Pt bound to DNA in exponentially growing CHO cells incubated in air and hypoxia at 5, 10 and 15 μM cisplatin for 1 hour. Error bars represent standard error from four separate experiments. The hypoxic binding ratio is 1.41±0.24 .

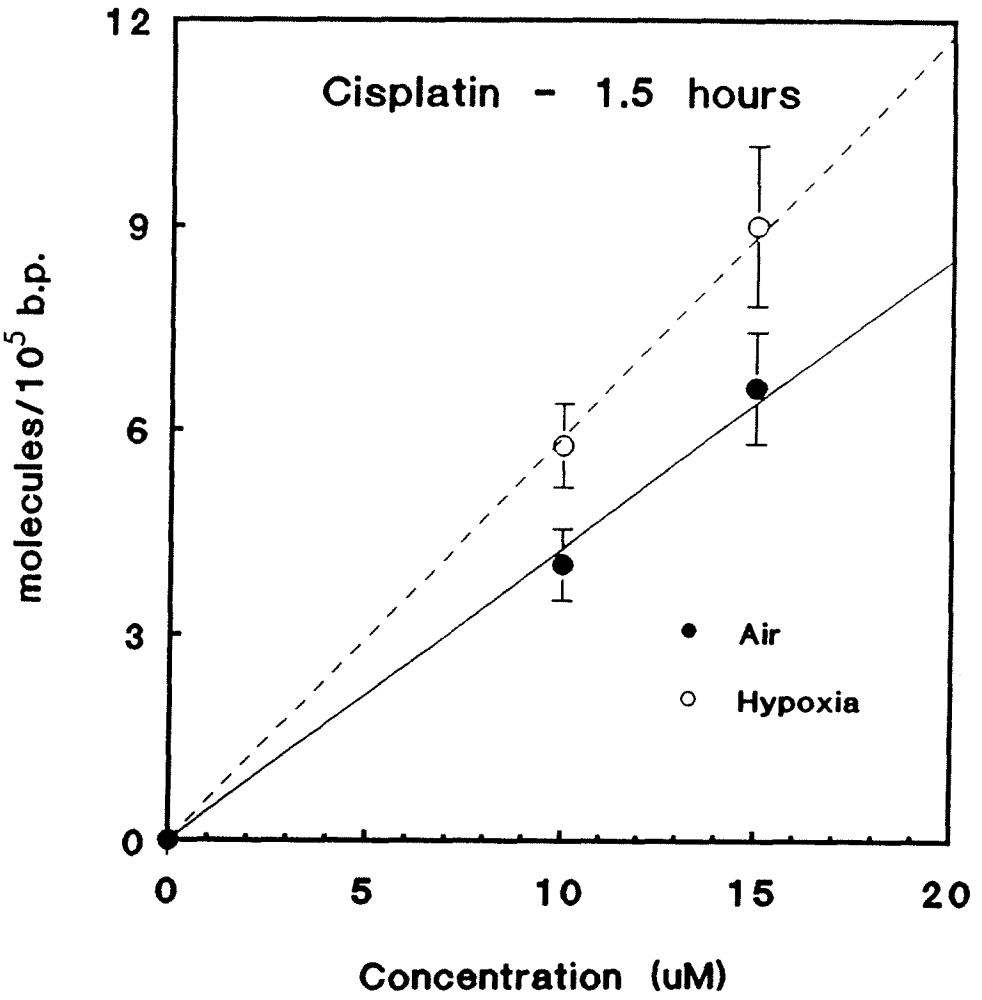


Fig. 3-12 : Comparison of platinum bound to DNA in exponentially growing CHO cells incubated in air and hypoxia at 10 and 15 μM cisplatin for 1.5 hours. Error bars represent standard error from four separate experiments. The hypoxic binding ratio is 1.38±0.19 .

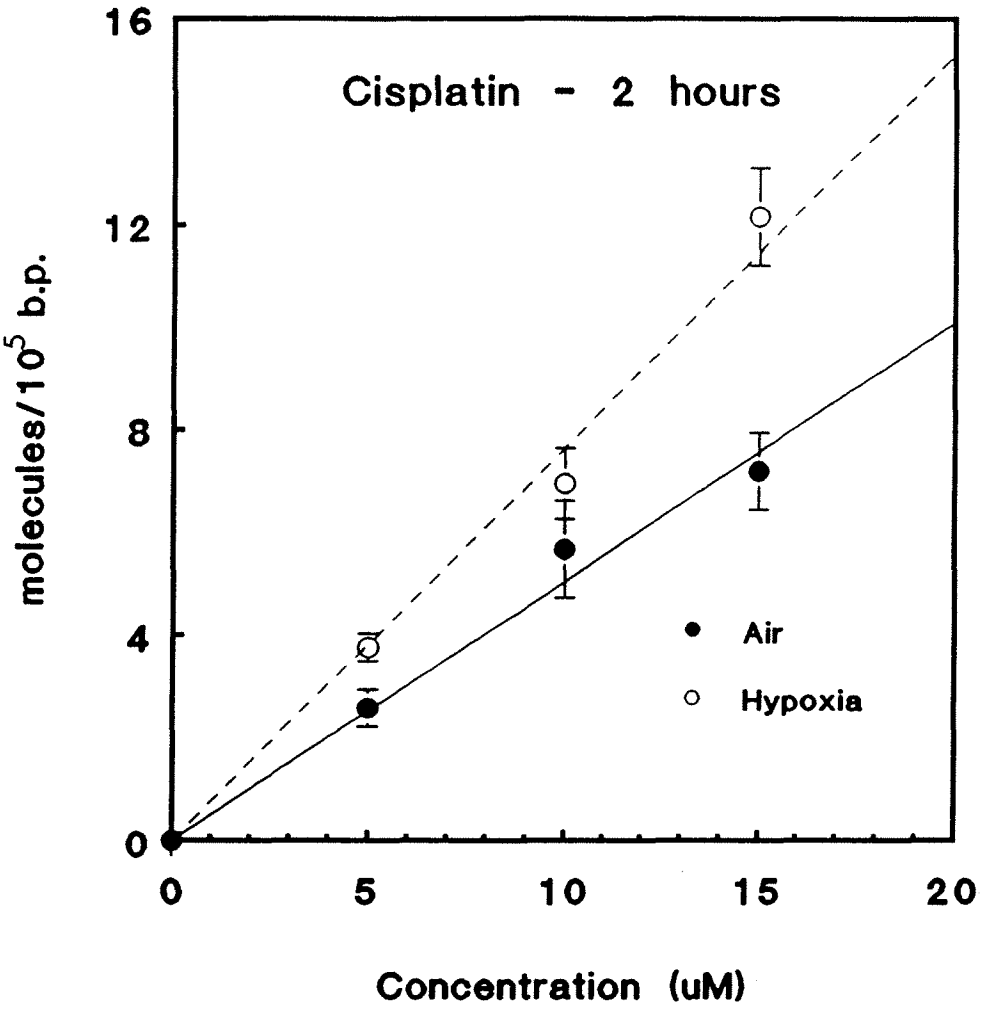


Fig. 3-13 : Comparison of Pt bound to DNA exponentially growing CHO cells incubated in air and hypoxia at 5, 10 and 15 μM cisplatin for 2 hours. Error bars represent standard error from four separate experiments. The hypoxic binding ratio is 1.52±0.17 .

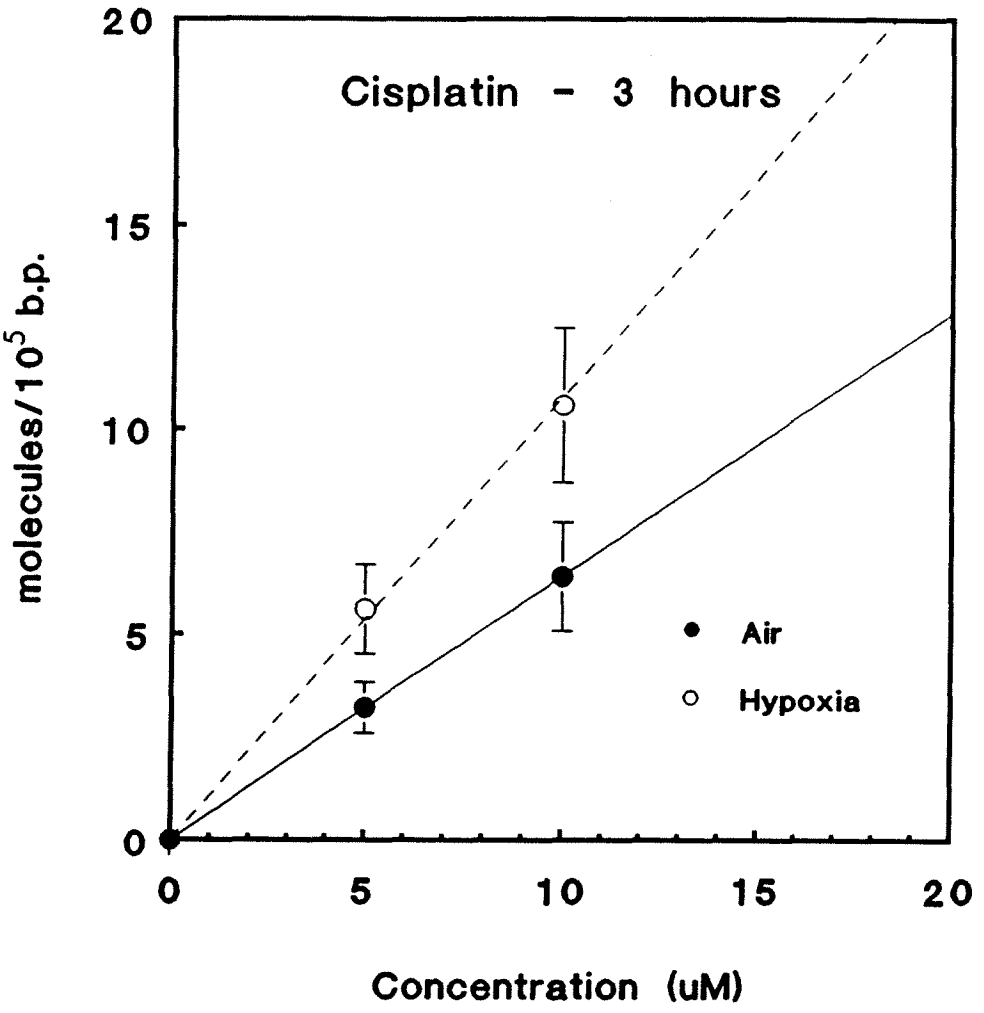


Fig. 3-14 : Comparison of Pt binding to DNA in exponentially growing CHO cells incubated in air and hypoxia at 5 and 10 μM cisplatin for 3 hours. Error bars represent standard error from four separate experiments. The hypoxic binding ratio is 1.68±0.30 .

time (hr)	atoms Pt/10^5 b.p./μM		HBR	p
	Air	Hypoxia		
1.0	0.331±.040	0.468±.054	1.41±.14	.29
1.5	0.426±.046	0.589±.053	1.38±.19	.01
2.0	0.504±.046	0.764±.046	1.52±.17	.01
3.0	0.642±.093	1.078±.115	1.68±.30	.04

Table 3-3 : Hypoxic binding ratios and DNA binding coefficients calculated from data presented in figures 3-9 to 3-12.

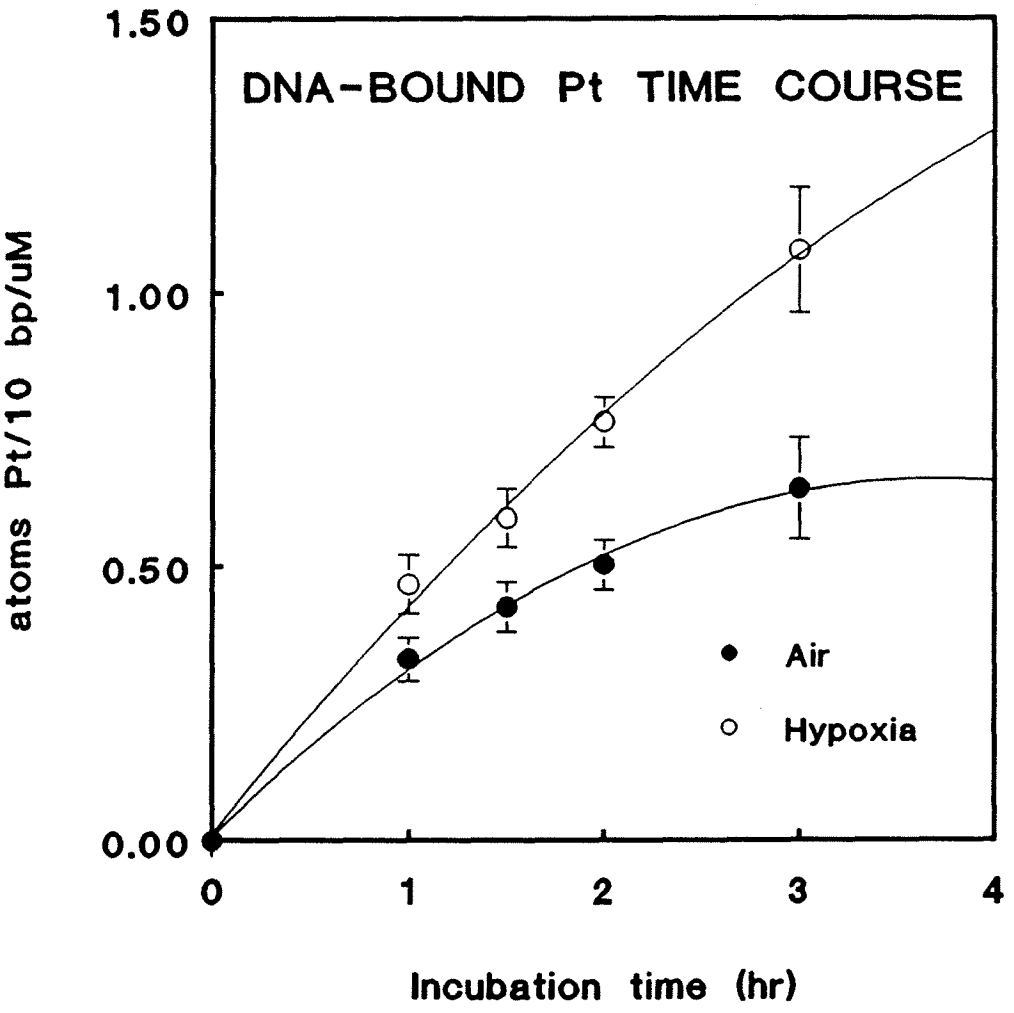


Fig. 3-15 : The variation of the DNA bound platinum per μM of cisplatin exposure with the length of the incubation period. As with toxicity and uptake, the difference between aerobic and hypoxic values increases with incubation time. Note the similarity to fig 3-5.

3.1.4 Effect of pH on Toxicity and Uptake

In the above experiments with cisplatin, the pH of the α media in the hypoxic vessels was found to decrease from 7.4 to 7.0 over the three hours of the experiment. To ascertain whether it was, in fact, this decrease in pH which accounted for the observed increase in cytotoxicity to hypoxic cells, CHO cells were tested for 1 hour incubations in air and hypoxia in media adjusted to pH 6.4 and 7.4 with small amounts of concentrated HCl. The pH changed only negligibly over the one hour of these experiments. Were the apparent hypoxic cytotoxicity of cisplatin due to the effects of decreased pH, one would expect toxicity in air at low pH (6.4) to approach that of hypoxia at normal pH(7.4). In fact, from figures 3-16 and 3-17, the opposite is true. Lowering pH actually decreases the toxic effects of cisplatin, an effect which would counter the action of hypoxia. Therefore, the true HCRs in these experiments could actually be higher than calculated.

The whole cell uptake of cisplatin changes does not change over the same pH range, as illustrated in figures 3-18 and 3-19. DNA binding was measured in only one experiment, and was found to decrease only slightly at lower pH, again, the opposite of what would be expected if the pH change were responsible for the observed increase in cisplatin toxicity in hypoxia.

Two notes must be made regarding these studies of pH. First, ideally, a buffer should have been used to control the pH change in the cisplatin studies. However, the inclusion of Bis-Tris buffer (Gibco) was found to dramatically decrease the toxicity of cisplatin in both air and hypoxia. This being the usual buffer for the pH range of interest,

it was decided that including it would have caused a greater inaccuracy.

Second, figures 3-16 and 3-17 (from experiments performed in the absence of the Bis-Tris buffer) show somewhat greater toxicity from cisplatin than previously measured (see Figs. 3-1 to 3-4) and the measured uptake also exceeded those values presented above. As has been documented, studies of cisplatin can produce a wide range of toxicities, even within the same laboratory. However, it is important to note that increased toxicity and uptake are still observed in hypoxia (table 3-4).

(a)

pH	$D_{0.01}$ (μM)		HCR	HCR from table 3-1
	Air	Hypoxia		
6.4	14.7	12.3	1.18	1.26 ±.49
7.4	12.2	9.5	1.28	

(b)

pH	atom Pt cell^{-1} μM^{-1}		HUR	HUR from table 3-2
	Air	Hypoxia		
6.4	1.37±.04	1.92±.09	1.40±.08	1.27 ±.08
7.4	1.32±.06	1.95±.06	1.46±.08	

Table 3-4 : The effect of pH on aerobic and hypoxic toxicity (a) and whole cell uptake (b). Since aerobic data did not fall on a straight line, the HCR was calculated from the ratio of doses required for a 0.01 survival ($D_{.01}$).There is no effect on the HCR or HUR within the given pH range, and values remain quite consistent with other experiments.

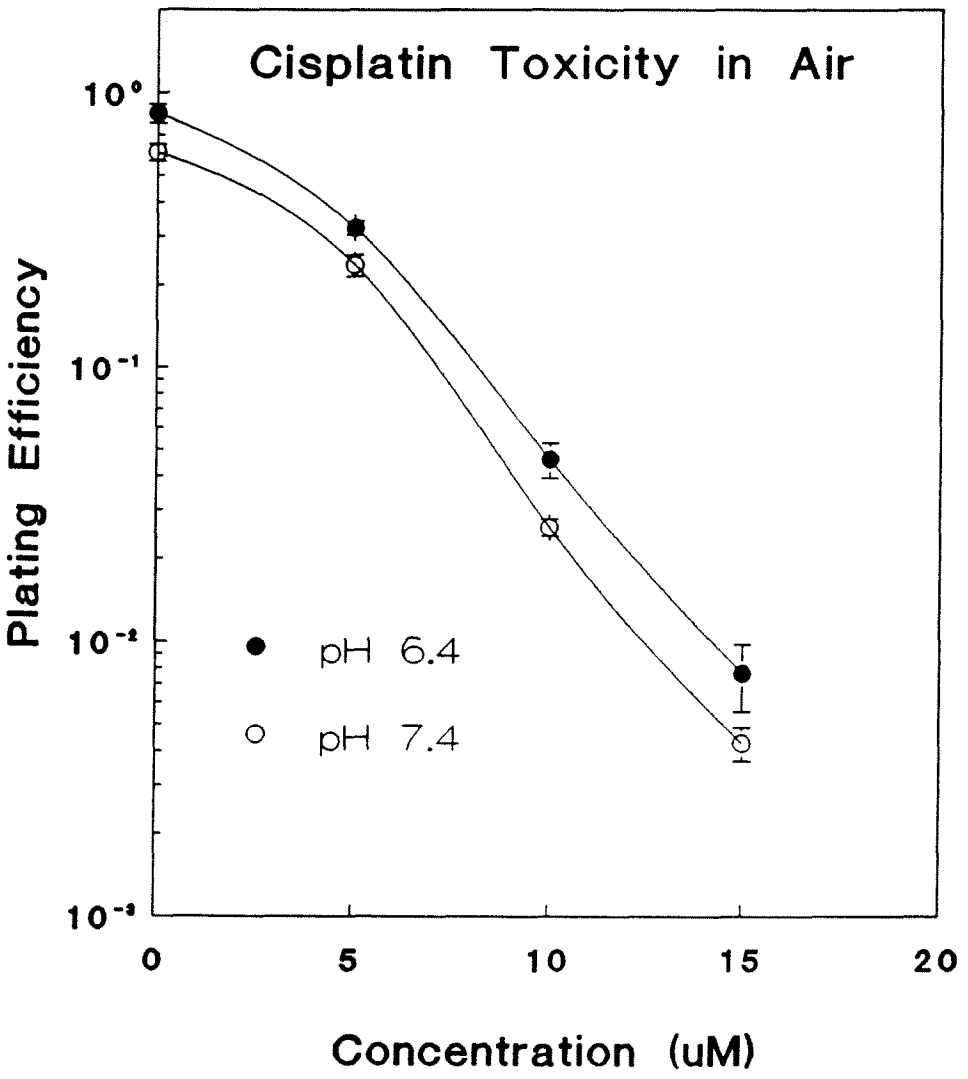


Fig. 3-16 : Toxicity of cisplatin in air at pH 6.4 and pH 7.4 in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 1 hour. Error bars represent standard error from three separate experiments. Lower pH appears to decrease cisplatin toxicity, the opposite effect of hypoxia, and thus is not a contributing factor to the increased hypoxic cytotoxicity of cisplatin.

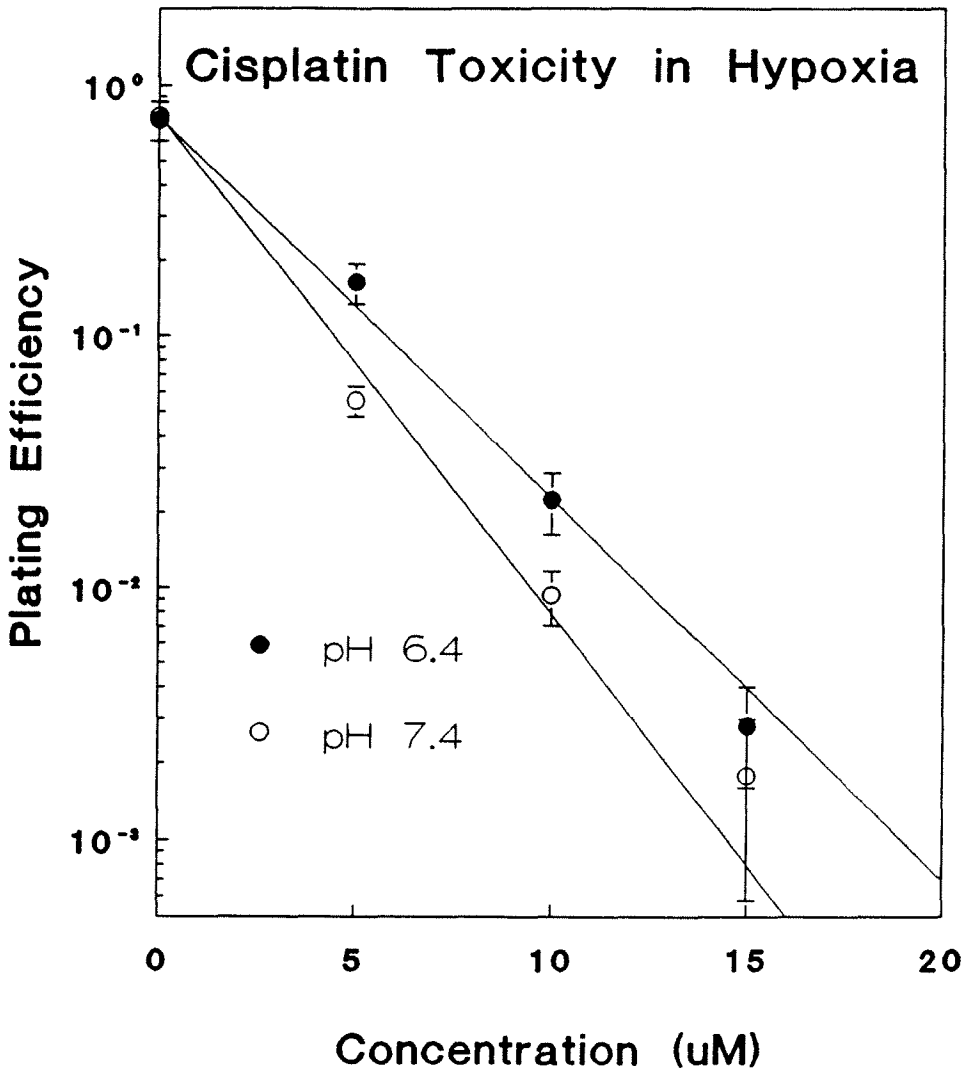


Fig. 3-17 : Toxicity of cisplatin in hypoxia at pH 6.4 and pH 7.4 in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 1 hour. Error bars represent standard error from three separate experiments. Again, as in aerobically treated cells, lowering the pH decreases cisplatin toxicity.

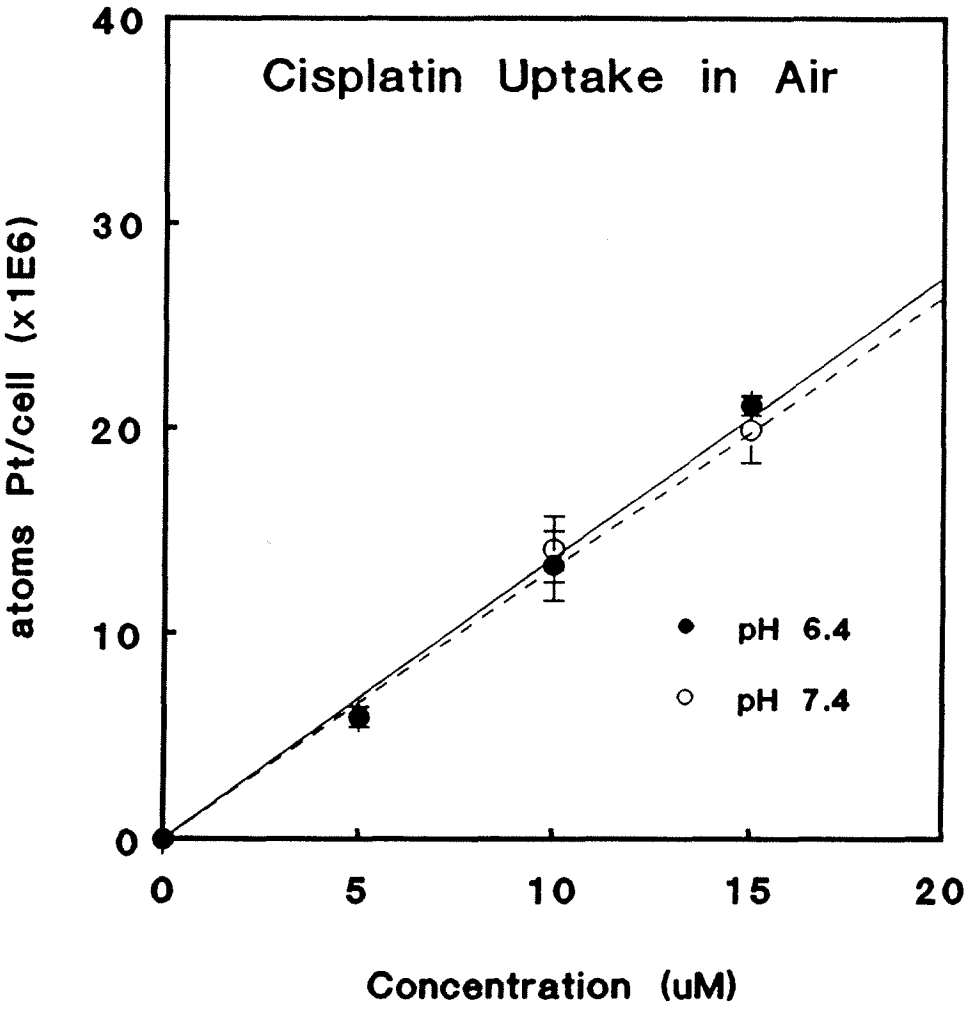


Fig. 3-18 : Whole cell uptake of cisplatin in air at pH 6.4 and pH 7.4 in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 1 hour. Error bars represent standard error from four separate experiments, performed simultaneously with toxicity measurements. Over the range studied, pH does not affect the aerobic uptake of cisplatin.

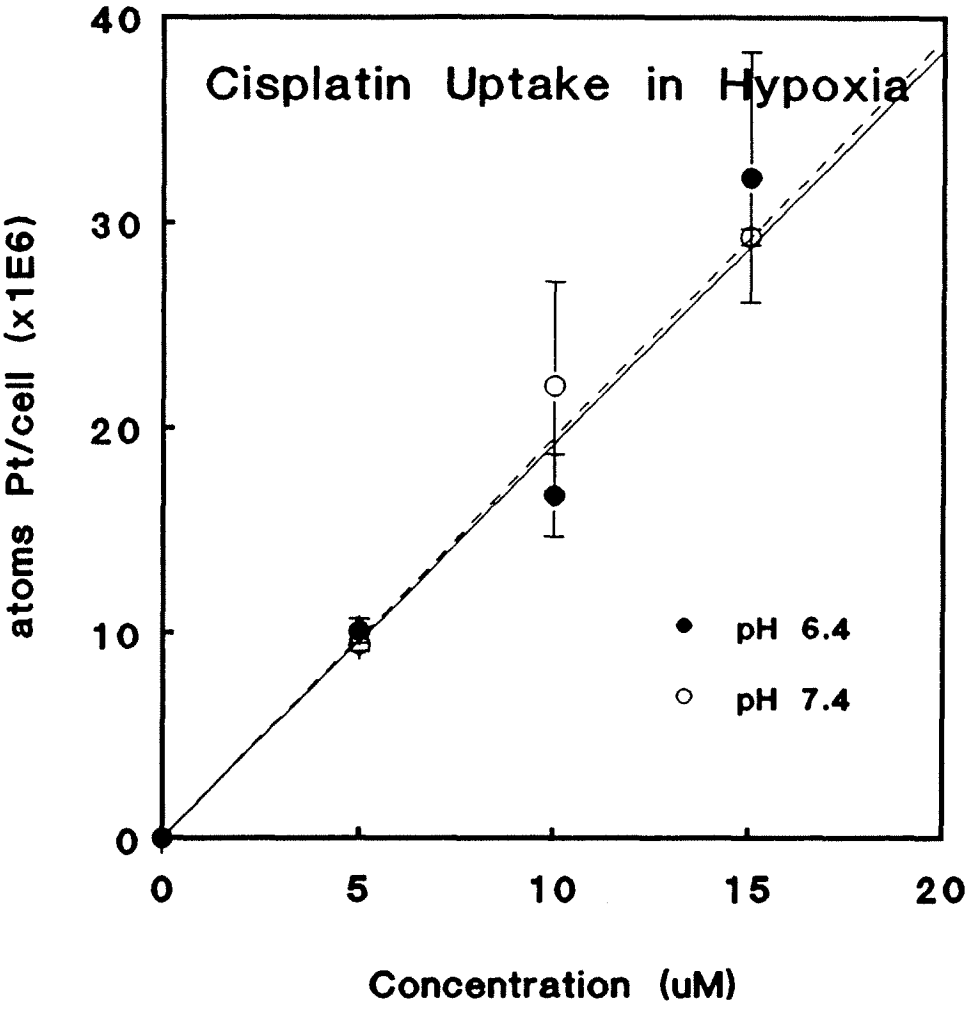


Fig. 3-19 : Whole cell uptake of cisplatin in hypoxia at pH 6.4 and pH 7.4 in exponentially growing CHO cells incubated in 5, 10 and 15 μM for 1 hour. Error bars represent standard error from four separate experiments, performed simultaneously with toxicity measurements. As in aerobic studies, there is no significant change in cisplatin uptake over the investigated pH range.

3.2 **Studies with the Bisplatinum AN-38 (n=6)** (see Fig. 1-4 for structure)

3.2.1 **Toxicity**

Cells were incubated in air and hypoxia in 15, 30 and 45 μM AN-38 for incubation times of up to 4 hours. Figures 3-20 to 3-23 show cell survival plotted against AN-38 dose for each incubation period. All points are the average of three experiments unless otherwise noted, and straight-line fits were performed as with cisplatin toxicity (section 3.1.1). Toxicity of 10 μM cisplatin was also measured during each experiment to ensure that conditions were consistent with previous experiments.

AN-38 is far less toxic than cisplatin (see figure 3-1 to 3-4). Toxicity generally appears greater in hypoxic cells than in aerobic, with the HCR increasing significantly with longer incubation periods. Table 3-5 shows the HCR values for each of the incubation periods, and figure 3-24 illustrates the variation of HCR with time.

As was previously noted, hypoxic incubations tended to slightly increase the acidity of the incubating medium, changing the pH from 7.4 to 7.0 over a three hour period. The same effect was noted for incubations with the bis(platinum) compounds. To ascertain the effect of lowered pH on AN-38 toxicity, a single experiment was performed in aerobic and hypoxic media, at levels of acidity ranging from pH 6.5 to 7.5. As discovered with cisplatin, over the specified pH range, the hypoxic cytotoxicity ratio remained consistent with vallues presented in table 3-5.

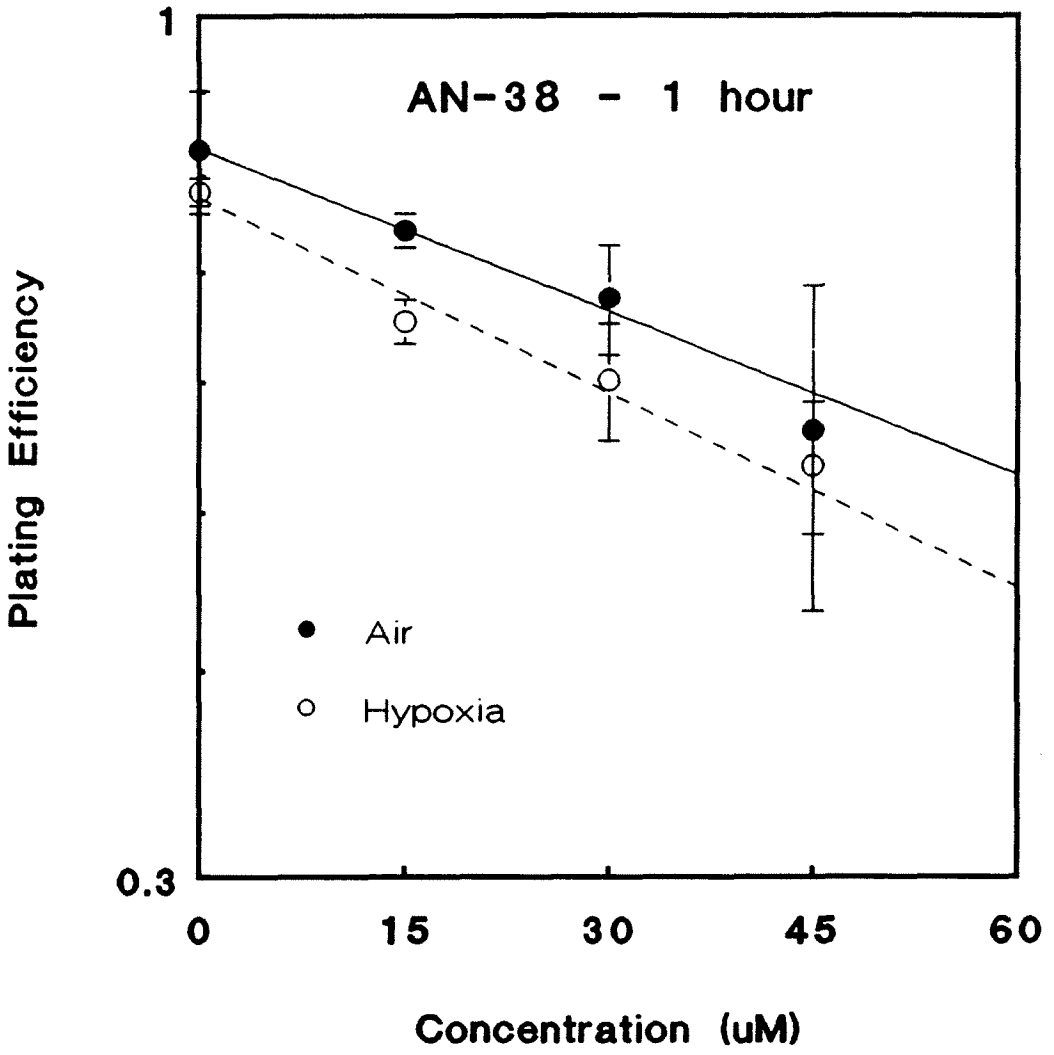


Fig. 3-20 : Comparison of AN-38 toxicity in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 1 hour. Error bars represent standard error from three separate experiments. The hypoxic cytotoxicity ratio is 1.20±0.91 .

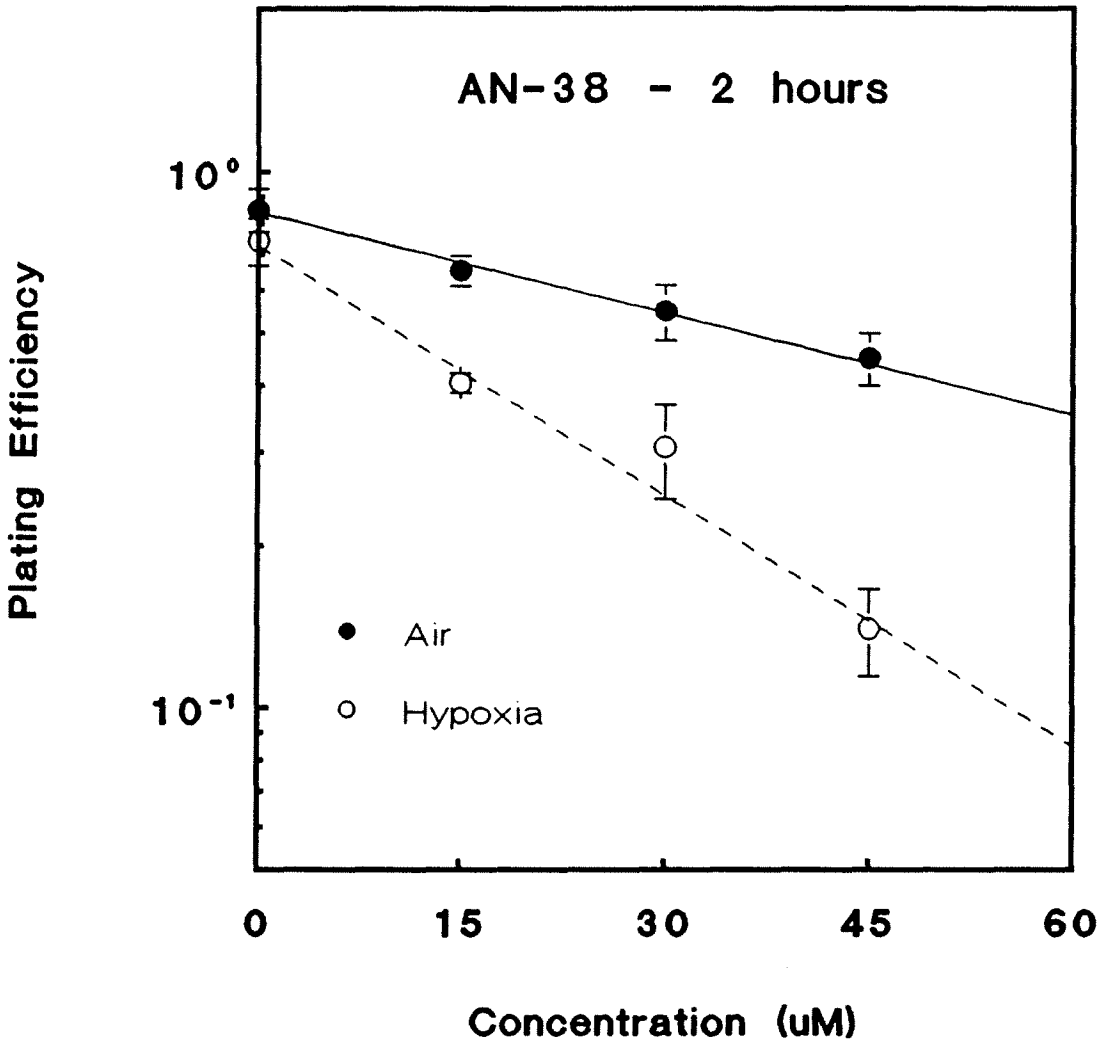


Fig. 3-21 : Comparison of AN-38 toxicity in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 2 hours. Error bars represent standard error from three separate experiments. The hypoxic cytotoxicity ratio is 2.47±0.51 .

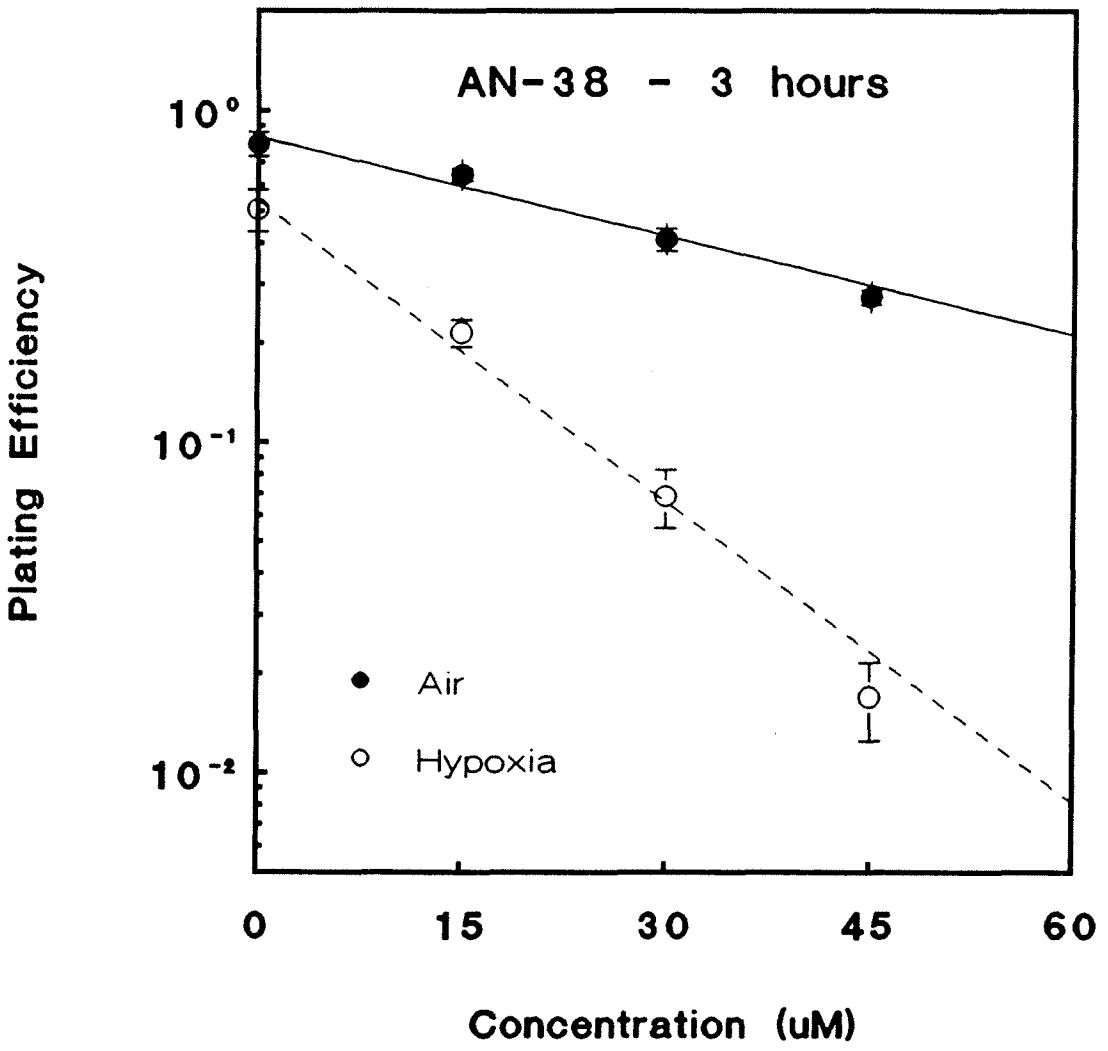


Fig. 3-22 : Comparison of AN-38 toxicity in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 3 hour. Error bars represent standard error from three separate experiments. The hypoxic cytotoxicity ratio is 3.04±0.37 .

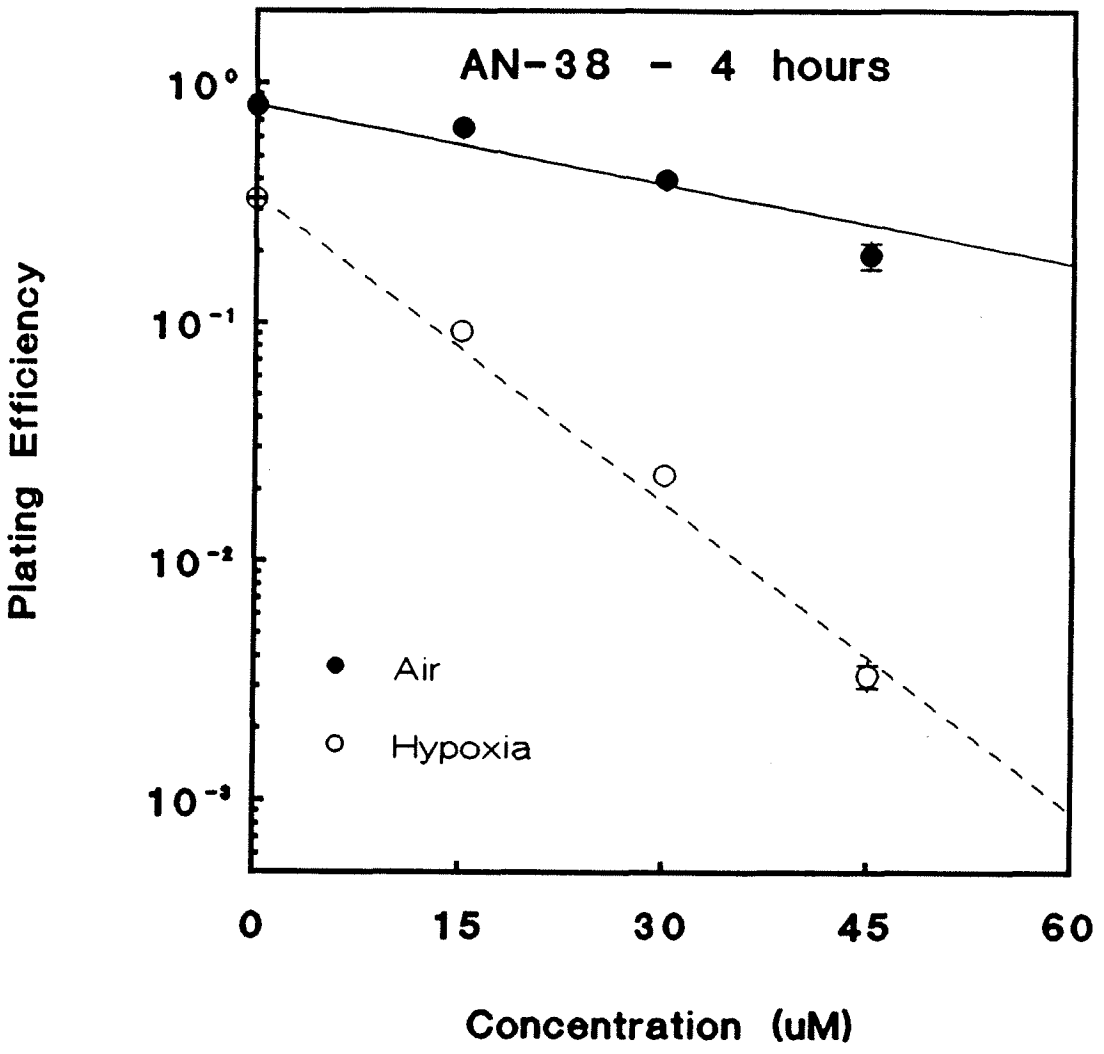


Fig. 3-23 : Comparison of AN-38 toxicity in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 4 hour. Error bars represent standard error from three separate experiments. The hypoxic cytotoxicity ratio is 3.91±0.32 .

time (hr)	τ (μM^{-1})		HCR	p
	Air	Hypoxia		
1	0.0076±.0059	0.0091±.0044	1.20±.91	> 0.5
2	0.0145±.0061	0.0358±.0105	2.47±.51	0.02
3	0.0229±.0060	0.0698±.0185	3.04±.37	<.001
4	0.0254±.0067	0.0993±.0174	3.91±.32	<.001

Table 3-5 : Hypoxic cytotoxicity ratios and τ values for exponentially growing CHO cells incubated in AN-38.

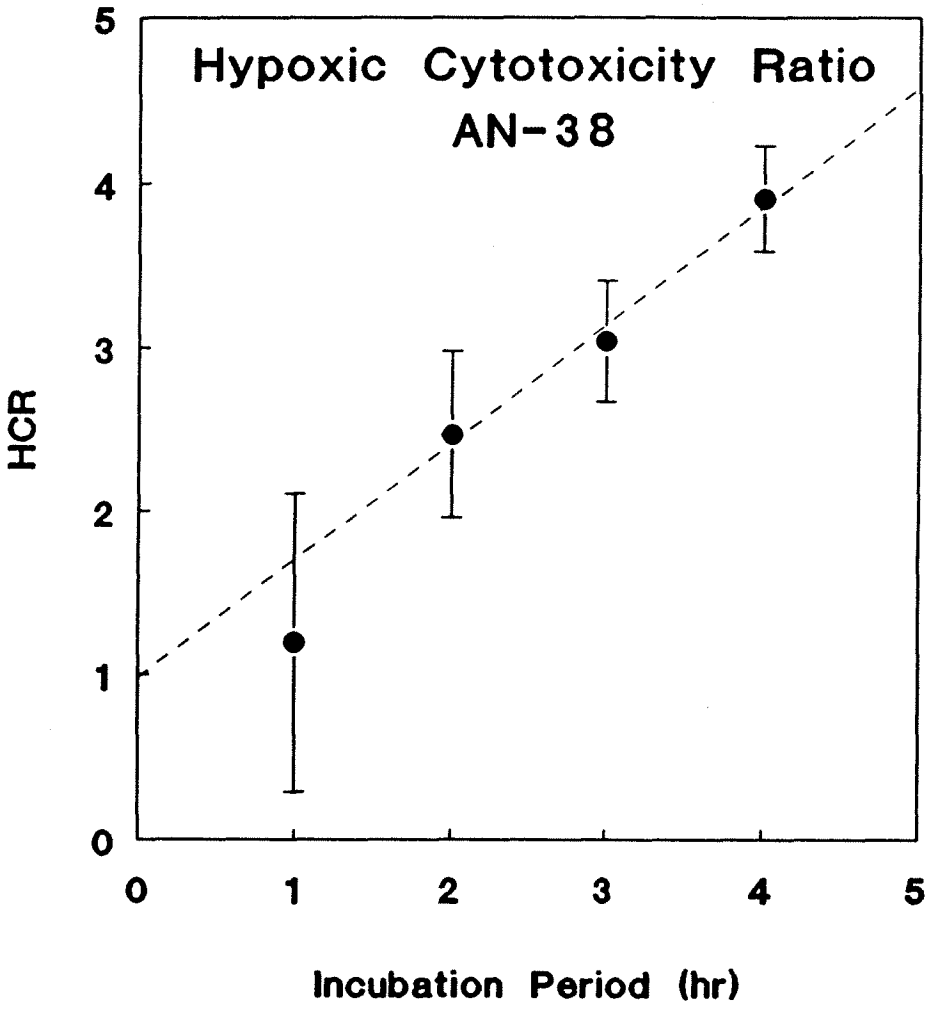


Fig 3-24 : Variation of the hypoxic cytotoxicity ratio for AN-38 with incubation period. Data is taken from table 3-5. The line shown is a straight line fit to the 2, 3 and 4 hour points (the 1 hour point was omitted due to the large experimental uncertainty). The slope of this line is 0.72 hr^{-1}.

3.3.2 **Whole Cell Uptake**

Conditions for whole cell uptake studies with AN-38 matched those for toxicity measurements. Figures 3-25 through 3-28 show average values for 2 experiments. AN-38 uptake appears greater in hypoxia for most incubation periods and doses, but as illustrated in Table 3-6 below, the HUR's are lower than the associated HCR values. As illustrated in Figure 3-29, there is no variation of the HUR with length of incubation.

time (hr)	atoms Pt cell^{-1} μM^{-1}		HUR
	Air	Hypoxia	
1	0.134±.014	0.160±.014	1.19±.16
2	0.219±.017	0.274±.016	1.25±.13
3	0.296±.009	0.402±.018	1.36±.08
4	0.509±.018	0.600±.037	1.18±.11

Table 3-6 : Whole cell uptake expressed in atoms of AN-38 per cell per μM concentration of exposure and corresponding hypoxic uptake ratios. CHO cells were incubated in 15, 30 and 45 μM AN-38.

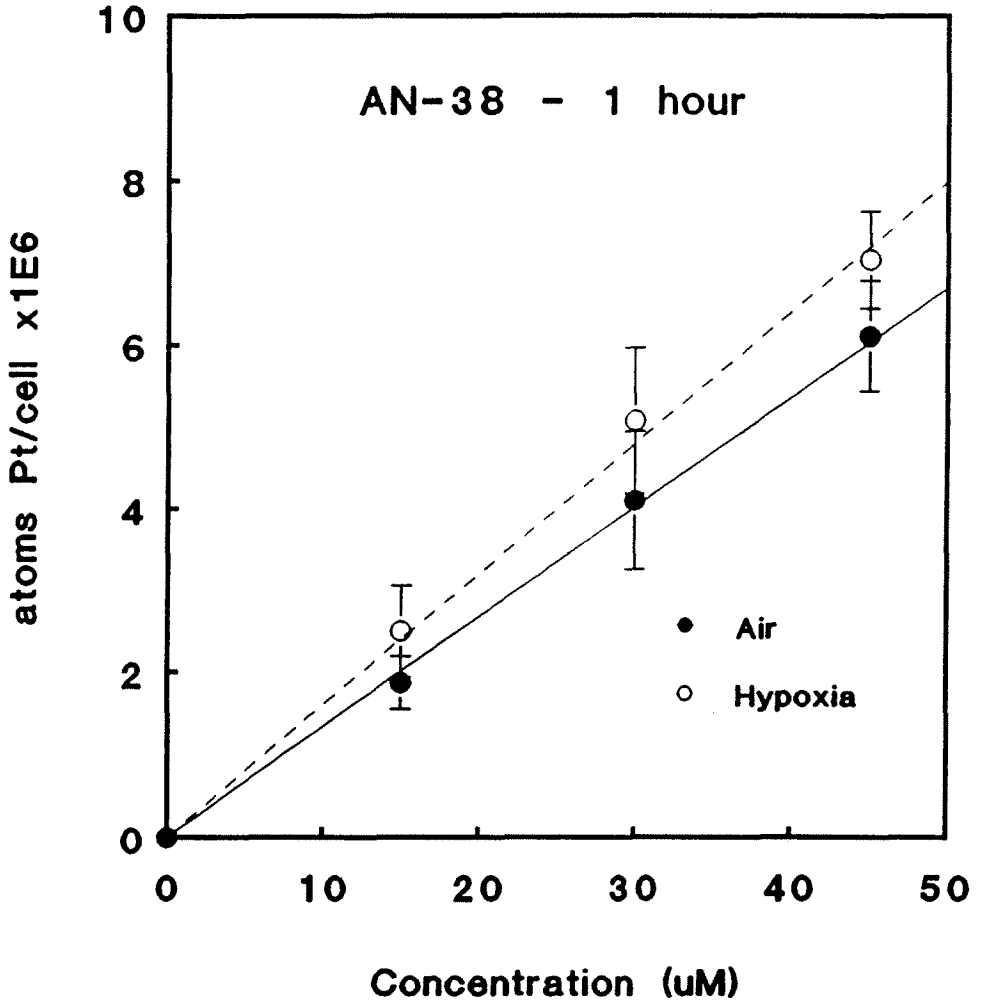


Fig. 3-25 : Comparison of whole cell uptake of AN-38 in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 1 hour. Error bars represent standard error from two experiments. The hypoxic uptake ratio is 1.19±0.14 .

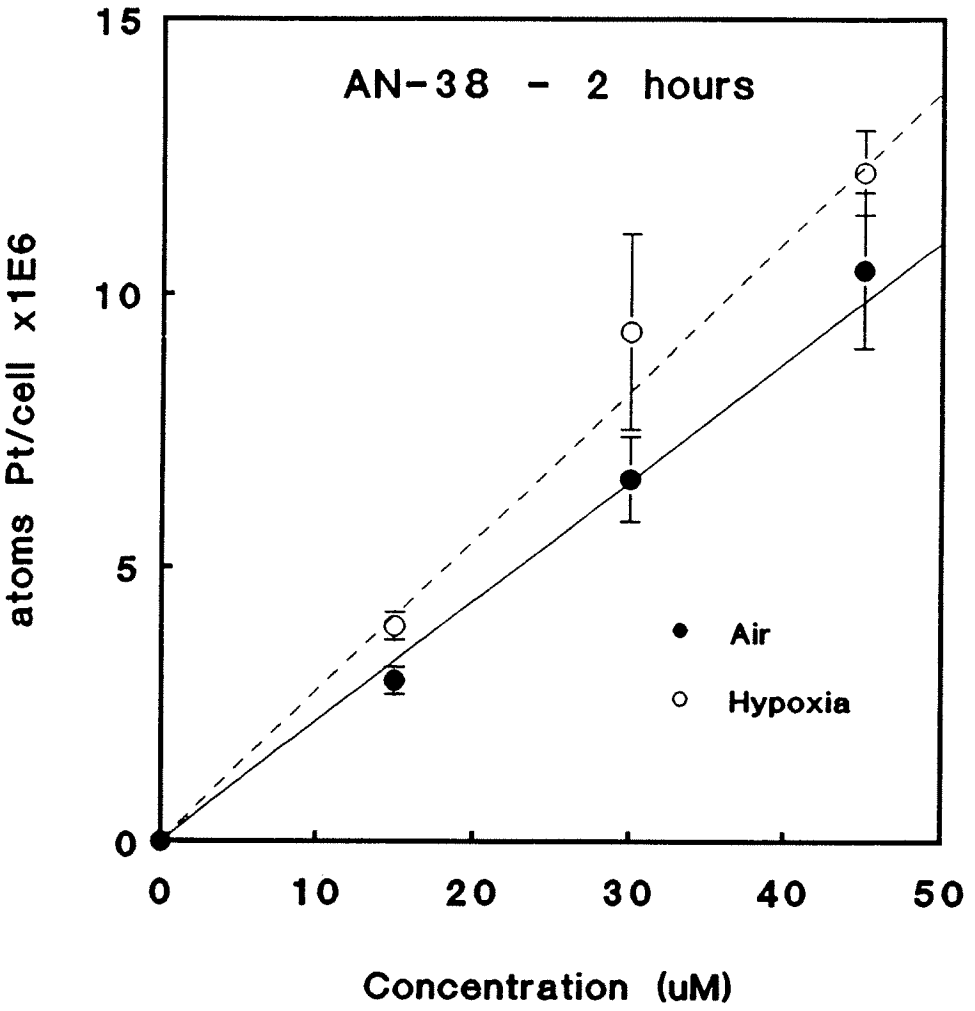


Fig. 3-26 : Comparison of whole cell uptake of AN-38 in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 2 hours. Error bars represent standard error from two experiments. The hypoxic uptake ratio is 1.25±0.13 .

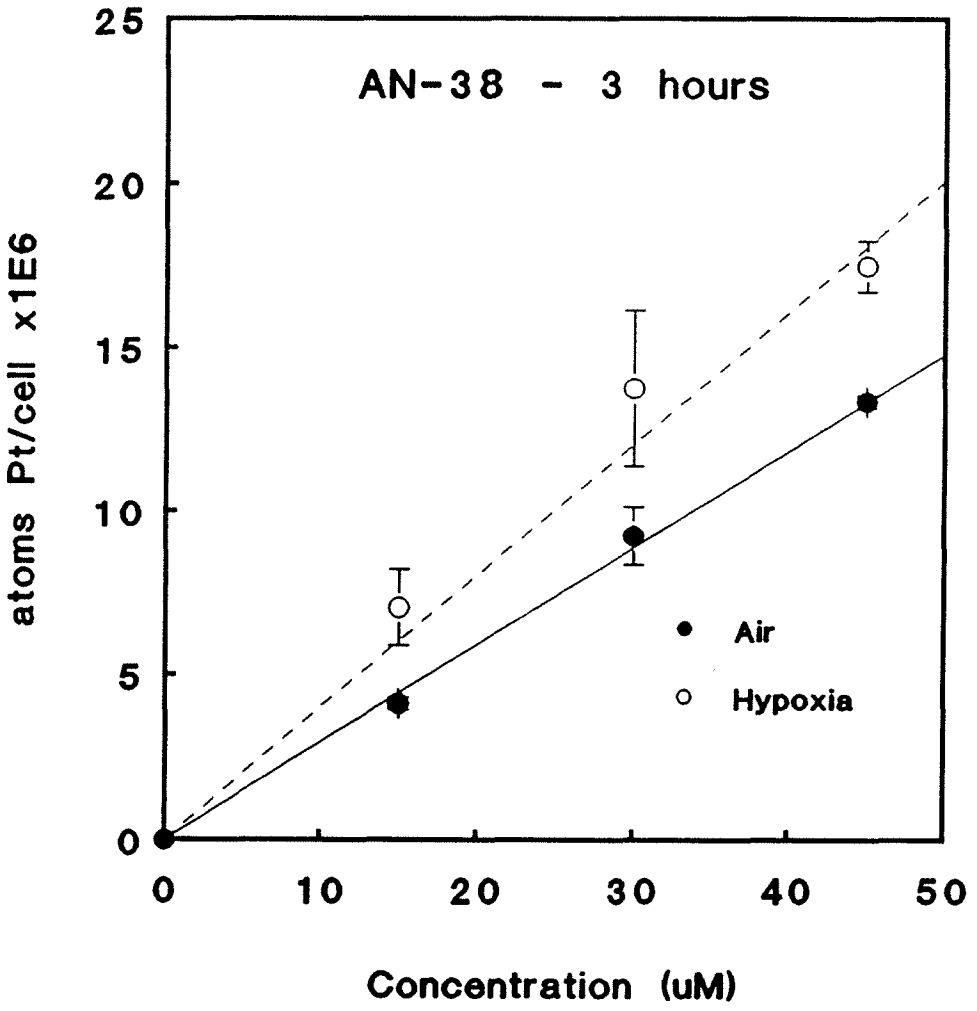


Fig. 3-27 : Comparison of whole cell uptake of AN-38 in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 3 hours. Error bars represent standard error from two experiments. The hypoxic uptake ratio is 1.36±0.08 .

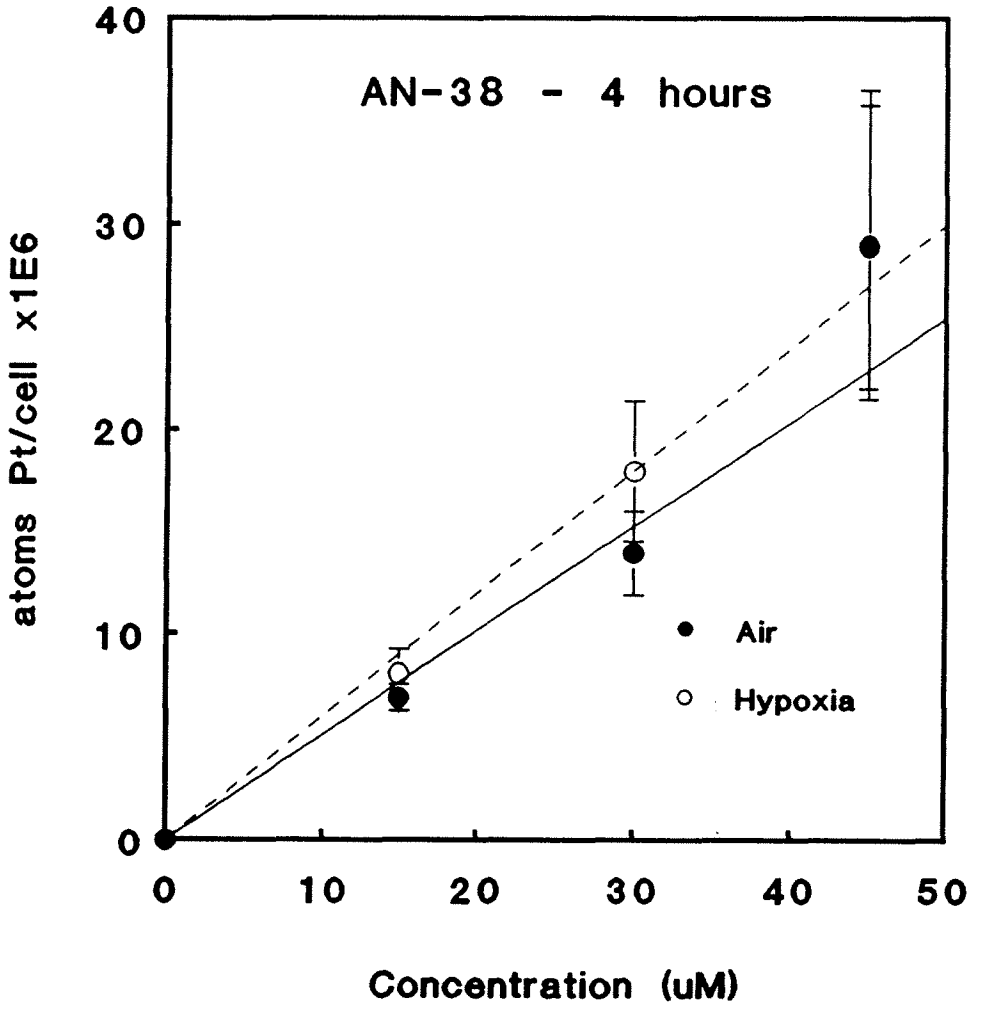


Fig. 3-28 : Comparison of whole cell uptake of AN-38 in exponentially growing CHO cells incubated in air and hypoxia at 15, 30 and 45 μM for 4 hours. Error bars represent standard error from two experiments. The hypoxic uptake ratio is 1.18±0.11 .

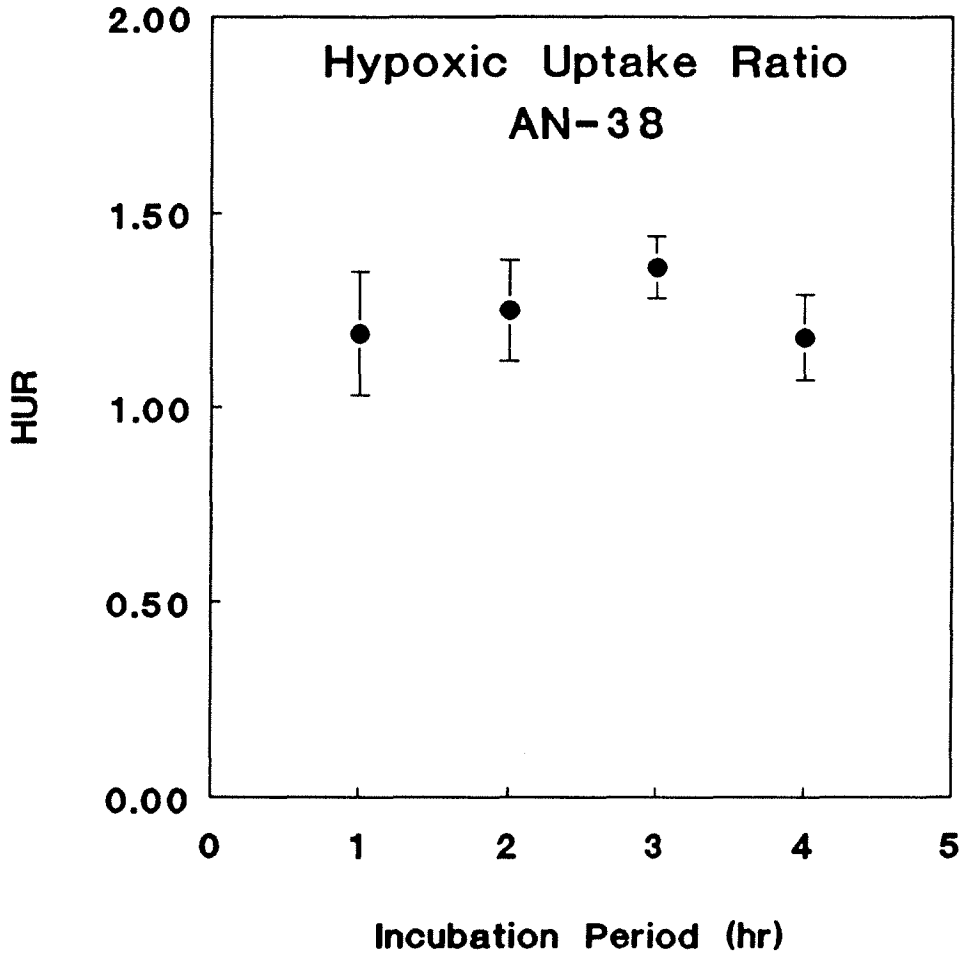


Fig 3-29 : Variation of the hypoxic uptake ratio for AN-38 with incubation period. In contrast to the hypoxic cytotoxicity ratio of AN-38 (see Fig. 3-24) the HUR shows no dependence on the length of the incubation period. Data is taken from table 3-6.

3.3.3 **DNA Binding**

The extent of platination of DNA after 2 and 3 hour incubations in 30 and 45 μM AN-38 is very low. Even at the maximum DNA load in the AAS tube, very low absorbance readings were observed, affecting the accuracy of the data. For this reason, a single experiment was performed to gain, at least, a qualitative assessment of AN-38 binding to DNA. Table 3-7 shows that there is most likely a higher level of binding under hypoxic conditions, although it must be stressed the listed HBR values carry a random error nearing 40%.

time (hr)	Dose (μM)	atoms AN-38/10^5 b.p.		HBR*
		Air	Hypoxia	
2	30	0.86	1.26	1.5
	45	1.02	1.46	1.4
3	30	0.90	1.62	1.8
	45	1.42	1.96	1.4

Table 3-7 : Pt bound to DNA in exponentially growing CHO cells treated with AN-38 in air and hypoxia. Binding results are from a single experiment. The HBR presented here is simply the ratio of hypoxic to aerobic binding for each point. More platinum is bound to DNA in cells treated under hypoxia.

3.3 Studies with AN-36

3.3.1 Toxicity

Due to the possession of a limited quantity of AN-36, only two toxicity experiments were performed, parallel to AN-38 experiments. Insufficient quantities of drug were available for the study of whole cell uptake or DNA binding.

AN-36 toxicity curves are shown in Figures 3-30 through 3-33. The n=4 compound also shows increased toxicity in hypoxia, the effect growing with increased incubation times. The HCR values, listed in Table 3-8, are comparable to that of AN-38.

time (hr)	τ (μM^{-1})		HCR
	Air	Hypoxia	
1	0.014	0.017	1.21
2	0.025	0.055	2.20
3	0.037	0.112	3.03
4	0.047	0.146	3.11

Table 3-8 : Hypoxic cytotoxicity ratios and τ values for exponentially growing CHO cells incubated in AN-36. Values are the average of two experiments.

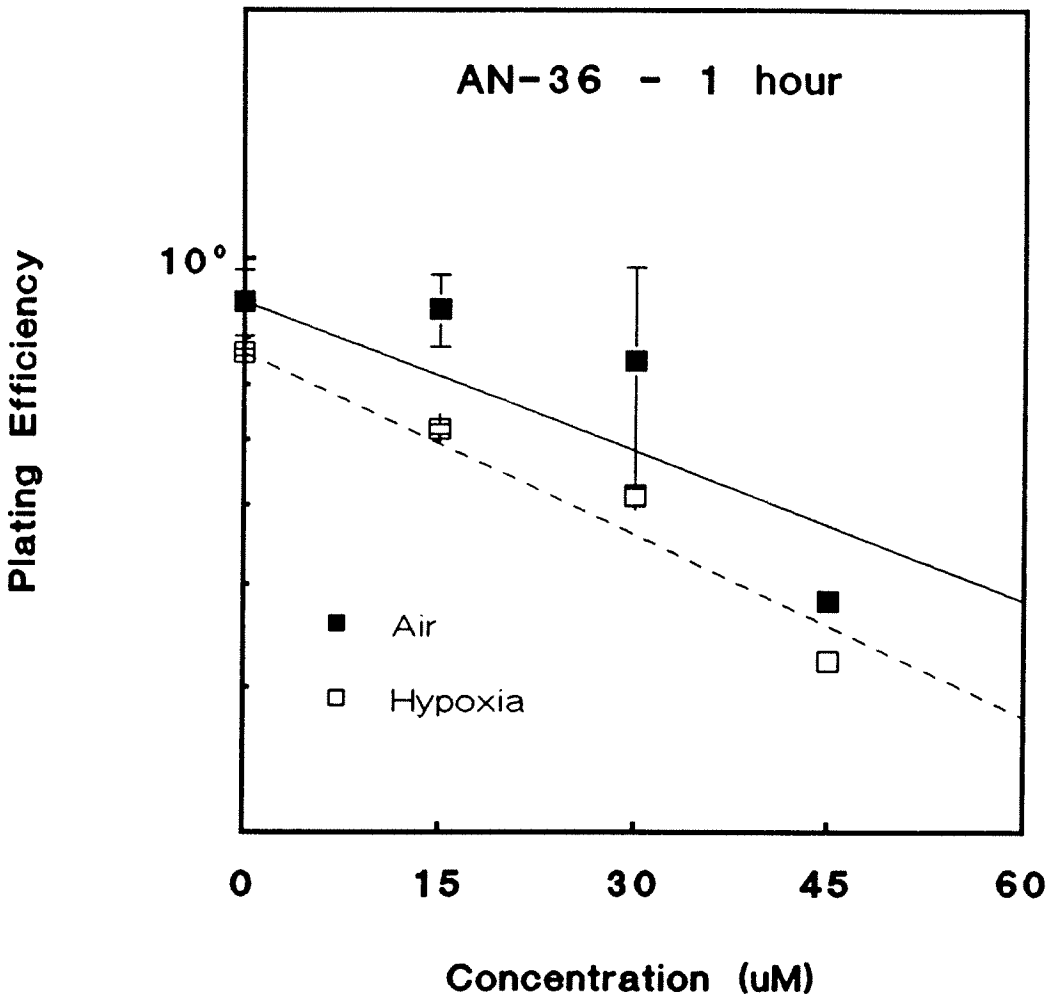


Fig. 3-30 : Comparison of AN-36 toxicity in exponentially growing CHO cells incubated in air and hypoxia for 1 hour. Error bars represent standard error from two separate experiments. The hypoxic cytotoxicity ratio is 1.21.

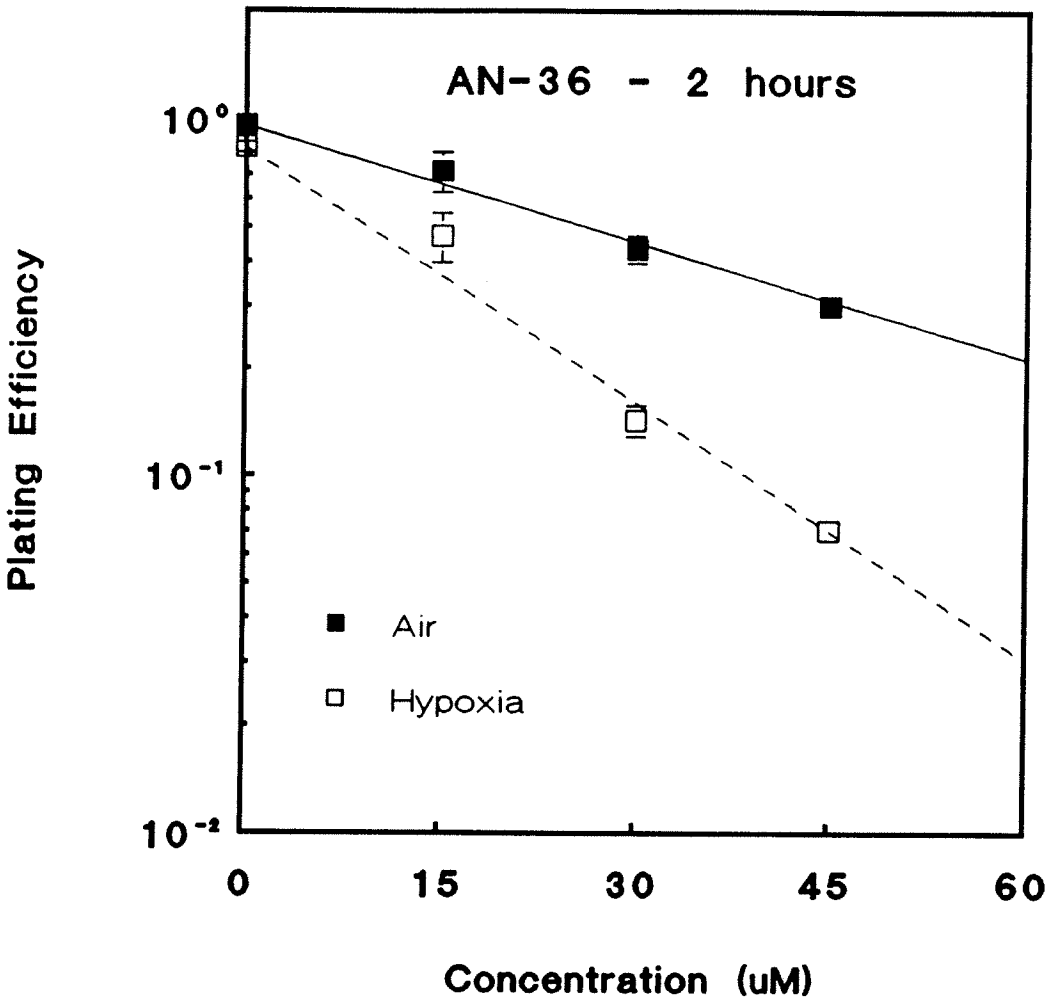


Fig. 3-31 : Comparison of AN-36 toxicity in exponentially growing CHO cells incubated in air and hypoxia for 2 hours. Error bars represent standard error from two separate experiments. The hypoxic cytotoxicity ratio is 2.20 .

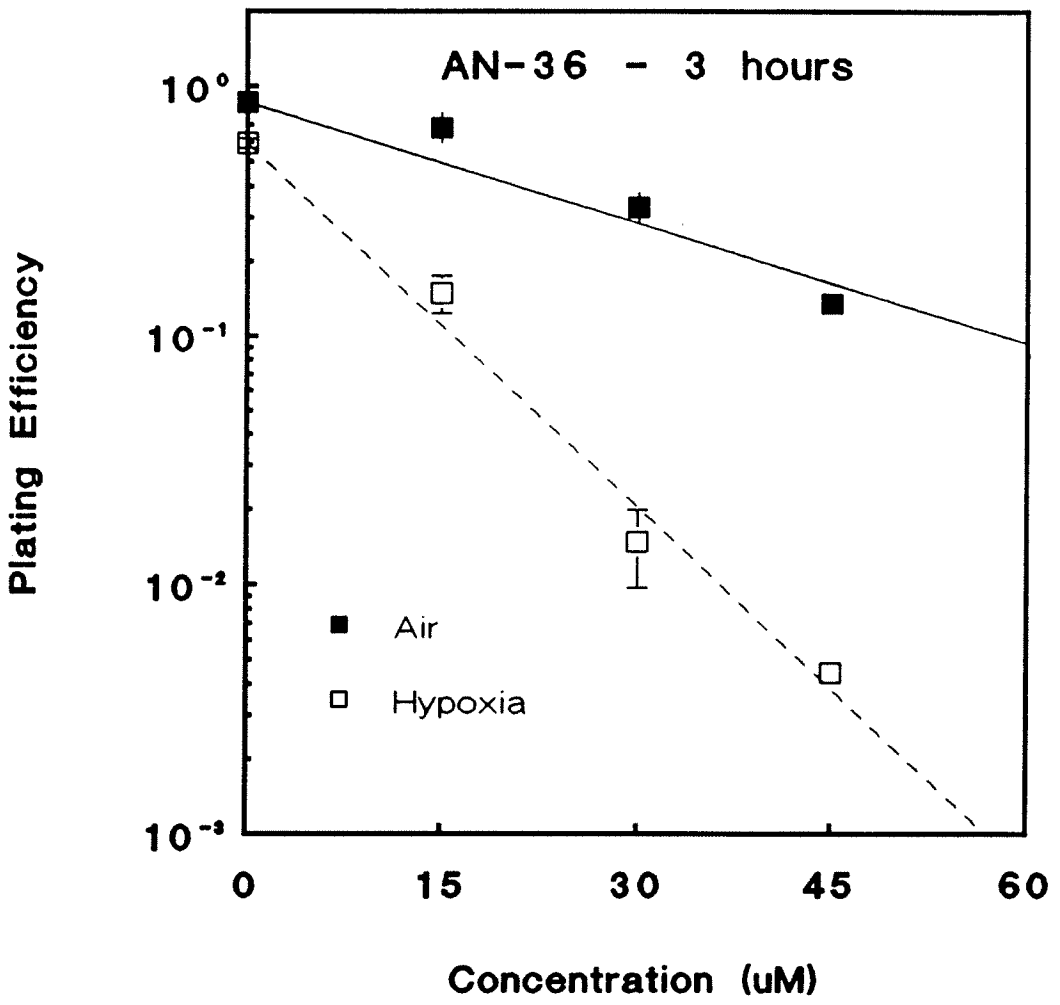


Fig. 3-32 : Comparison of AN-36 toxicity in exponentially growing CHO cells incubated in air and hypoxia for 3 hours. Error bars represent standard error from two separate experiments. The hypoxic cytotoxicity ratio is 3.03 .

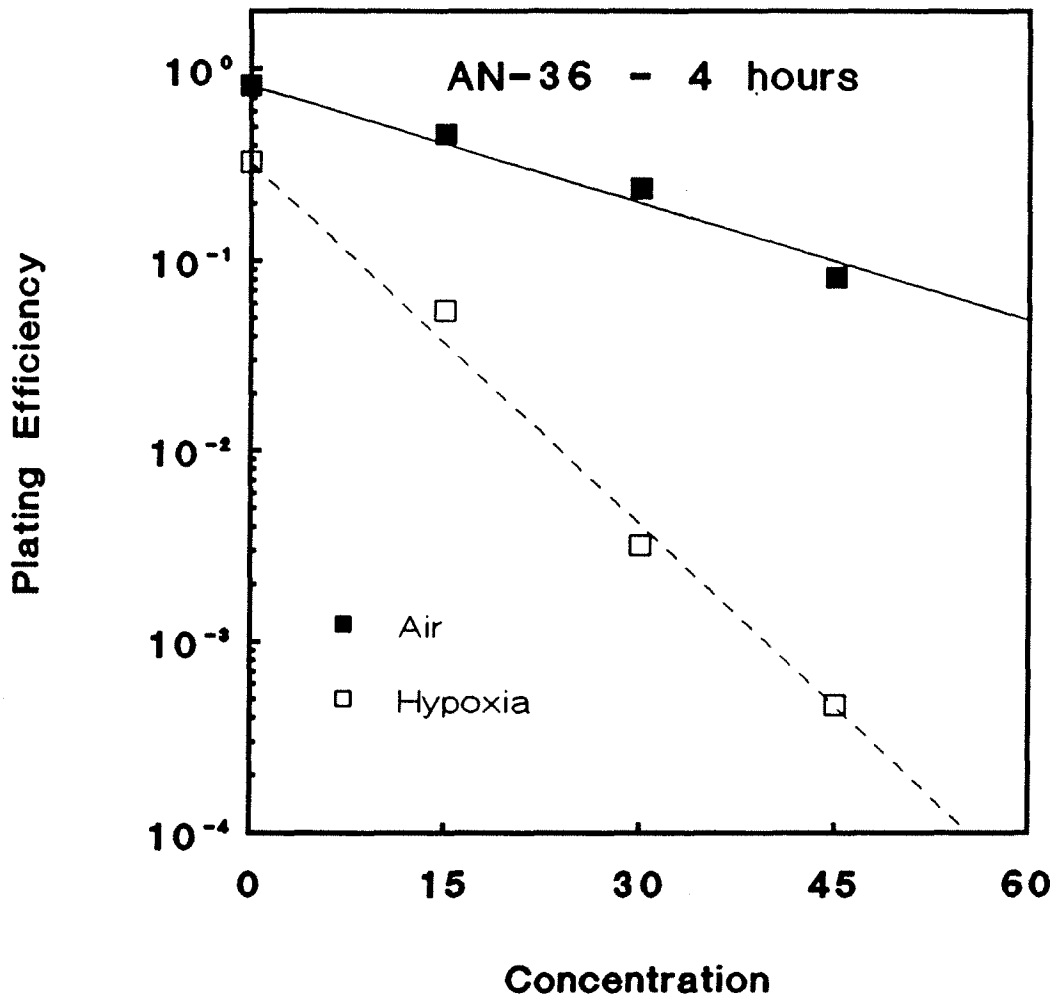


Fig. 3-33 : Comparison of AN-36 toxicity in exponentially growing CHO cells incubated in air and hypoxia for 4 hours. Error bars represent standard error from two separate experiments. The hypoxic cytotoxicity ratio is 3.11

4. DISCUSSION

4.1 Cisplatin

Cisplatin toxicity measurements for aerobic cells are in agreement with other reported values. In this study, the concentration required for a surviving fraction of 0.3 is 5.8 μM for a one hour incubation in air, and lies within the range of values shown in table 1-1 (page 11).

As was previously illustrated in table 1-2, many researchers have measured the cytotoxicity of cisplatin in aerobic and hypoxic cells, and found conflicting results. In this study, cisplatin was found to be up to two times as toxic (on a concentration basis) in hypoxic cells, a result which seems inconsistent with previously published data from this laboratory[82]. In fact, the cisplatin concentrations used in those previous studies were subtoxic (as low as 1 μM), well below the concentrations used here, and were restricted to one hour treatment times. Some of the other differences in results from those presented in table 1-2 may be explained by similar arguments.

Plooy *et al.* studied the DNA-binding properties of cisplatin in CHO cells, for one-hour incubations in various concentrations of drug[29]. They reported that the total number of DNA adducts was proportional to the concentration of cisplatin in the incubating medium, a result consistent with that found here (see figs. 3-11 to 3-14).

Johnson *et al.* have previously investigated the DNA-binding and uptake properties of cisplatin in aerobic CHO cells, for much longer incubation times, from 3 to 24 hours[93], at 2 μM concentration, and have found that DNA-binding and uptake increase proportionally to cisplatin concentration for incubation times up to 3 hours. The data

reported here (Fig. 3-10 and 3-15) show the uptake and DNA-binding to plateau near 3 hours. This difference in findings may be a result of the low concentration of drug used by Johnson (2 μM versus 5 μM, the lowest concentration studied here) requiring a longer time necessary for saturation.

This study has shown that cisplatin is indeed more cytotoxic to CHO cells treated in hypoxia than cells treated in air. Cell uptake and DNA binding are also increased in cells treated in hypoxia. Some of the possible explanations for these effects are examined below, and discussed in the context of the experiments performed in this project.

4.1.1 Cell Growth and Cell Cycle Considerations

There are several considerations in examining the reasons for the observed increase in the hypoxic cytotoxicity, uptake and DNA binding of cisplatin. First, Wilson *et al.* have reported that hypoxia results in an almost immediate cessation of cell growth and progression through the cell cycle[71]. Cells incubated in aerobic conditions suffer no such consequences. Thus one would expect to observe lower DNA-binding and uptake of cisplatin in the aerobically-growing cells due to the synthesis of new strands of DNA and the multiplication of cells. However, even if the hypoxic cells were to cease growth completely, one can calculate that DNA binding and cell uptake would appear less than 1.15 times greater in hypoxic cells (based on the untreated CHO doubling time of 14 hours), a value below what has been observed here (table 3-2 and 3-3). Note that this would have no effect on cytotoxicity, as cell number was counted following the removal of the cells from the incubation medium. However, if there were a sufficiently long

growth lag of hypoxic cells after reoxygenation, cytotoxicity would appear greater in these cell populations as insufficient time would have elapsed for the cells to reach 50 cell colonies compared with cells treated in air, and therefore fewer viable colonies would be scored. A growth lag in reoxygenated cells has been shown to occur, but only for hypoxic incubations of 6 hours or more[73]. Cell cycle effects may also be eliminated as a cause of this result, as it has been shown there is no observable change in the cell cycle distribution of a population of cells when made hypoxic[73].

Hypoxia does have an effect on cell viability[72]. Colony formation of control cell populations in hypoxia decreased with the length of time spent in hypoxia (see Figs. 3-1 to 3-4). This was anticipated, and did not directly affect the interpretation of cytotoxicity measurements, as the calculation of the τ values normalizes the data. However, it is entirely possible that cell uptake of drug and DNA-binding were affected. For example, if changes in membrane permeability to cisplatin occur from damage due to hypoxic stress, it can be hypothesised that cell uptake and hence DNA-binding measurements would be elevated from their true values. It seems unlikely this is a major factor since uptake and DNA binding are greater than the associated aerobic values for one hour incubations (Tables 3-2 and 3-3), when aerobic and hypoxic plating efficiencies were effectively equal.

4.1.2 Cell Uptake and DNA-Binding

Figure 4-1 shows the relationship between DNA-binding and whole cell uptake for cells in air and hypoxia. At higher uptake levels, it appears that the DNA-binding of

cisplatin in aerobic cells reaches a plateau, while in hypoxic cells, DNA-binding continues to increase at a rate proportional to the uptake. However, it is clear that for lower levels of uptake, DNA-binding increases with increasing uptake at the same rate in air and hypoxia. Furthermore, on average, for all the conditions studied, 15.4% ± 6.6% of the total cisplatin bound in cells was attached to the DNA. There was no detectable difference in the percentage of cellular platinum bound to DNA between air and hypoxia. Thus, although more cisplatin is bound to DNA in hypoxic cells, it would appear that the enhanced toxicity in hypoxia is unlikely to be caused by an increased affinity of cisplatin for DNA under hypoxic conditions. From these data, it would seem more likely that increased cisplatin uptake in hypoxia causes an increase in DNA-binding, and hence greater toxicity.

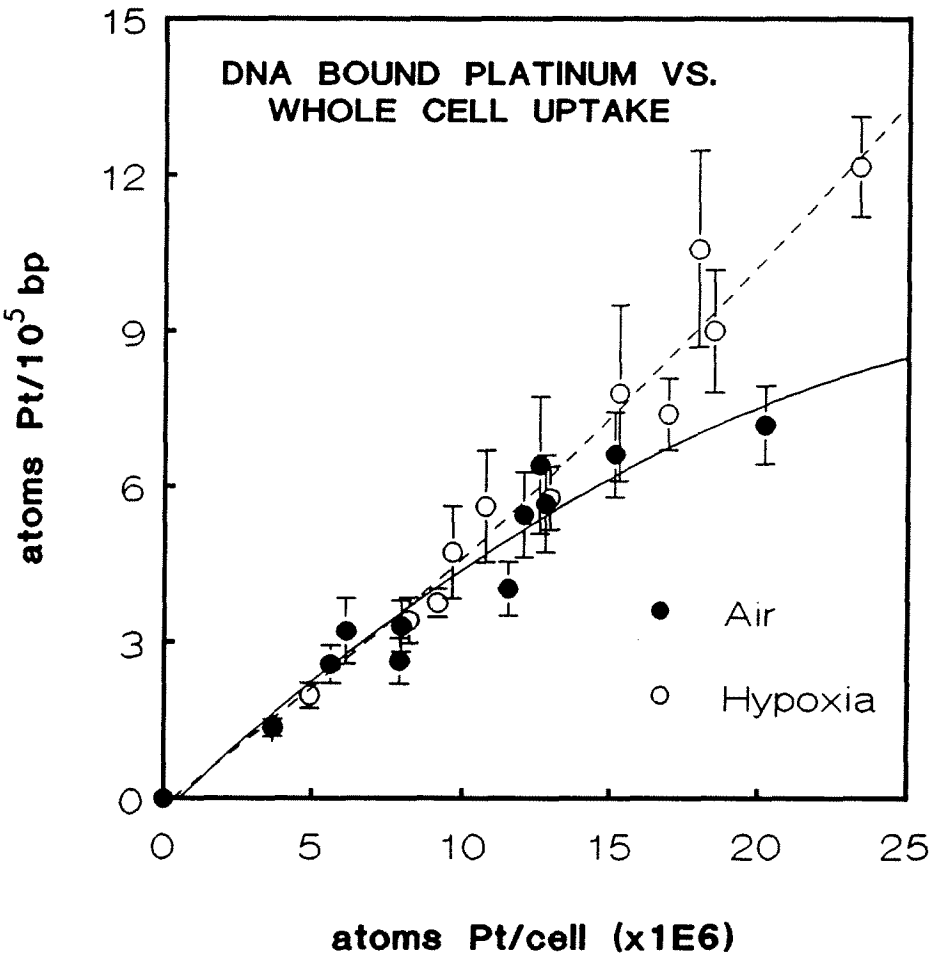


Fig 4-1 : The relationship between whole cell uptake and DNA-binding of cisplatin in CHO cells incubated for up to 3 hours in aerobic and hypoxic environments. At lower levels of platination, the lack of difference in the proportion of cellular platinum bound to DNA in air and hypoxia suggests that hypoxia has no influence on the affinity of cisplatin for DNA

4.1.3 DNA-Repair Processes and Hypoxia

Glutathione may play a role in the observed increased hypoxic cytotoxicity. It is known that glutathione plays a role in resistance to cisplatin of some cell types[37-40] and that its synthesis is impaired in hypoxic cells[95]. It is also possible that GSH plays a role in DNA-repair of cisplatin damage, as suggested by Lai *et al.*[42]. Thus, as time increased, and GSH used for the purpose of detoxification could not be replaced, toxicity in hypoxia would increase, as observed here. Preliminary results in cells depleted of GSH by buthionine sulfoximine(BSO) showed increased cisplatin toxicity in both aerobic and hypoxic cells. Further studies are required to assess the role of GSH in the different toxicities of cisplatin in air and hypoxia.

Cytotoxicity has been compared with whole cell uptake and DNA-binding. Figure 4-2 shows that as time increases, the number of platinum adducts necessary to yield the same survival is lower in hypoxia as compared with air. The same effect is seen with whole cell uptake of cisplatin, shown in figure 4-3. These data imply that cisplatin-DNA adducts are more effective in hypoxia than in air. Cells may have a decreased ability in hypoxia to repair cisplatin-damaged DNA, or alternately, the adducts formed with DNA by cisplatin in hypoxic cells may be of a more damaging type.

DNA-repair processes seem a likely target for toxicity modulation. Note that the cisplatin toxicity curve for air at one hour (fig. 3-1) has an initial shoulder region at lower concentrations. This reflects the ability of cells to withstand cisplatin damage at lower concentrations. After longer incubation times (see fig. 3-2 to 3-4) the shoulder disappears,

possibly because the amount of cisplatin damage overloads the capacity of the repair system. A similar saturable repair process was postulated to explain the shoulder of the survival curve of cells exposed to ionising radiation[96]. The one hour curve for hypoxia (fig. 3-1) shows no such shoulder region, suggesting that the repair of cisplatin-damaged DNA may be impaired in hypoxic cells.

Looking more closely at cisplatin hypoxic toxicity data for longer incubation times, one notices that the slope of the curves at low concentrations may be greater than at higher concentrations (for ease of comparison of aerobic and hypoxic data, previous analyses assumed straight lines for hypoxic toxicity curves within the concentration region studied). This indicates a lesser ability to deal with cisplatin damage in hypoxia. Examining figure 4-2, aerobic toxicity (closed symbols) follows a straight line relationship with the number of platinum lesions in the DNA. However, low levels of platinum adducts in the DNA of hypoxic cells (open symbols) appear to be more effective in causing cell death (fig 4-2) than higher levels. It is possible that a cisplatin-induced repair mechanism, present in all cells, is activated at very low levels of cisplatin adducts in aerobic cells, but only at higher levels in hypoxic cells. Indeed, an induced post-replication repair mechanism has been suggested by Fraval and Roberts[45]. Hypoxia prevents ATP synthesis[97], thereby decreasing energy availability within hypoxic cells. The decreased ATP availability in hypoxic cells may play a role in the repair of cisplatin-DNA damage in hypoxic cells, and should be investigated further. However, repair of cisplatin adducts has been shown to continue for up to 48 hours after treatment[29], long

after the maximum 4 hour duration of hypoxia used in this experiment. This would suggest that there may be another reason for the increased hypoxic cytotoxicity of cisplatin.

Xue et al. have reported that background levels of DNA-protein cross-links(DPCs) are increased in hypoxia[98]. It is thus possible that the "more damaging" type of DNA adduct alluded to on page 95, is the result of an interaction of the DPCs with cisplatin to form a type of adduct which is difficult for the cell to repair.

When CHO cells were incubated in hypoxia in drug-free medium for two hours, and then exposed to cisplatin in aerated medium for up to two hours, these cells still showed greater sensitivity to cisplatin than cells which were pre-incubated in air (toxicity was between 1.3 and 1.5 times greater). Thus, hypoxia need not be present during the treatment with cisplatin. Hypoxia exerts a stress on the cells which renders them more sensitive to cisplatin damage even for a time after the stress has been removed. The proposed explanations for the greater hypoxic toxicity of cisplatin (the role of GSH, energy deficiency, cross-linking, induced/enhanced repair) would all be consistent with this observation.

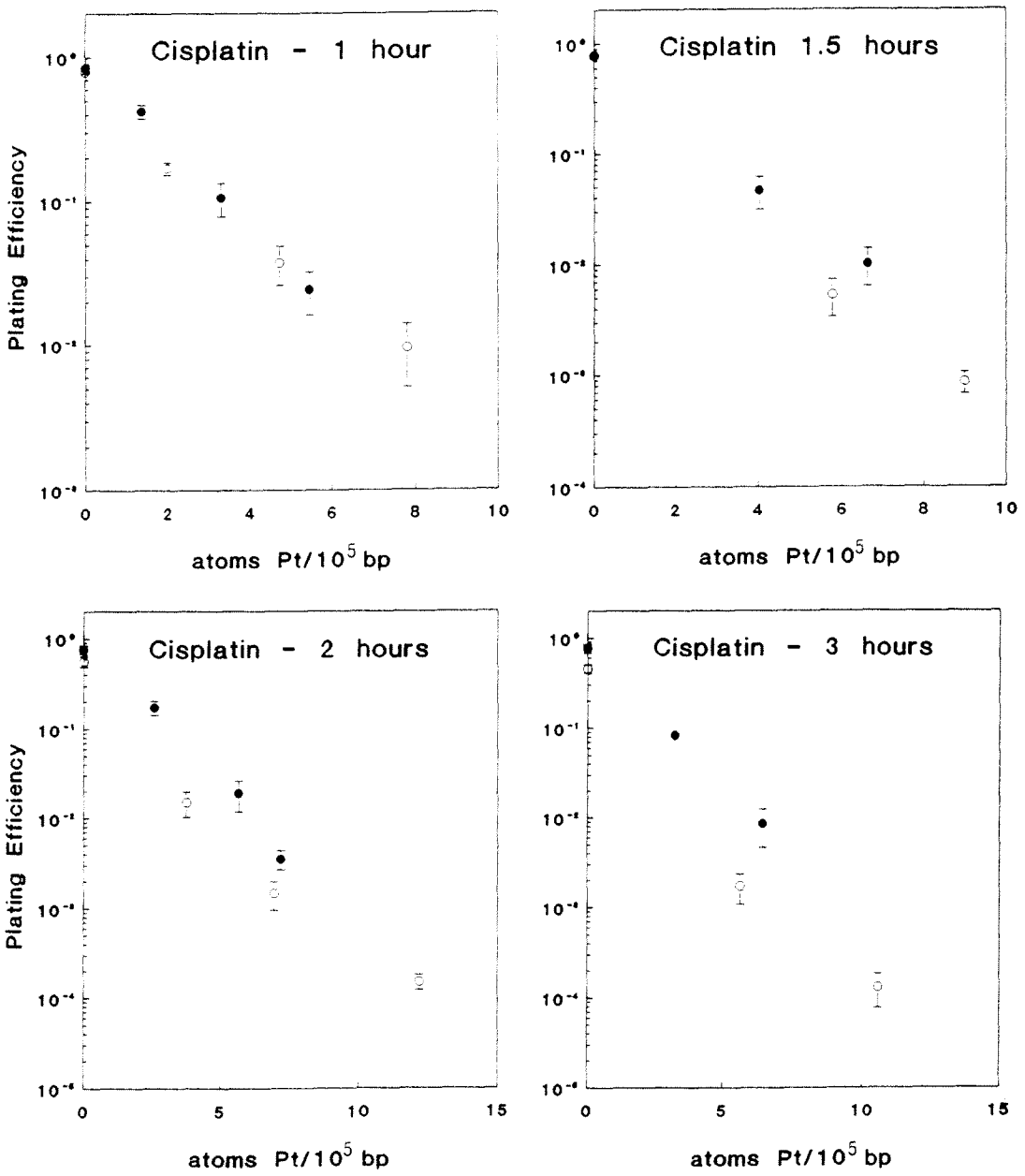


Fig. 4-2 : The variation of cytotoxicity with DNA-binding of cisplatin. Open circles are hypoxic incubations and closed circles are aerobic. An equal number of cisplatin lesions in hypoxia results in greater toxicity than the same number in air.

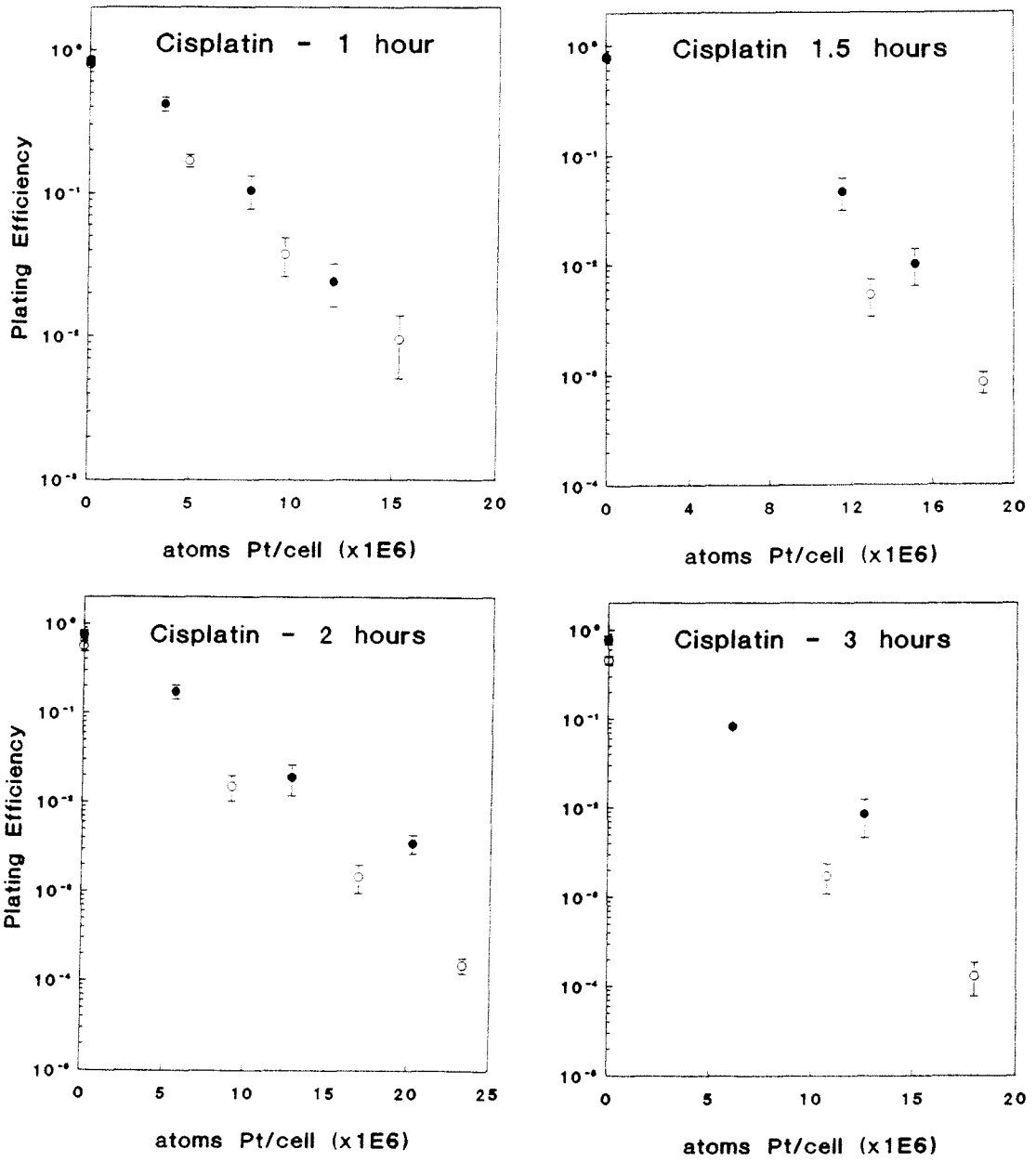


Fig. 4-3 : The variation of cytotoxicity with whole cell uptake of cisplatin. Open circles are hypoxic incubations and closed circles are aerobic. An equivalent amount of platinum bound in the cell causes a greater amount of toxicity in hypoxia than in air.

4.2 **Bis(platinum) Compounds** (see Fig 1-4)

Due to their relatively recent introduction into the laboratory, there exists no published findings regarding the toxicity of the bis(platinum) compounds AN-36 and AN-38 in CHO cells. The majority of studies have concentrated on cisplatin resistant cells[57,58] and were performed for different incubation times. Furthermore, no data were available concerning their behaviour in hypoxic cells.

4.2.1 **Mechanisms of Hypoxic Toxicity**

At first glance, the toxicity of the bis(platinum) complexes is far below that of cisplatin (see tables 3-1, 3-5 and 3-8). This can be at least partially attributed to lower uptake of the bis(platinum) compounds (tables 3-2 and 3-6).

The surprising element of the two bis(platinum) complexes studied here is the marked increase in the hypoxic cytotoxicity ratio as incubation time increases (table 3-5 and 3-8). The HCR values for AN-38 increase from 1.20 to 3.91 with incubation times from 1 to 4 hours. Under the same conditions, the HUR remains approximately constant between 1.19 and 1.34, and shows no such dependence on length of incubation. Thus, AN-38 uptake by CHO cells does not explain the difference between aerobic and hypoxic cytotoxicities. The dependence of AN-38 toxicity on the amount of platinum present in the cells is illustrated in figure 4-4. Although cytotoxicity does not correlate with uptake for longer incubation times, for each incubation period studied, toxicity is proportional to uptake. As incubation time increases, the ratio of hypoxic to aerobic slopes in figure 4-4 increases significantly, indicating that the effectiveness of AN-38 with the cells is

enhanced in hypoxia. Table 4-1 shows a comparison of the ratio of hypoxic to aerobic slopes of toxicity graphs and those calculated from figure 4-4.

As in the case of cisplatin, a possible explanation for the difference between aerobic and hypoxic cytotoxicities of the bis(platinum) agents is an impaired ability to repair lesions in the DNA. Bis(platinum) adducts in plasmid DNA have been shown to be subject to incision by the *E. coli* uvrABC nuclease complex[55], suggesting a role of excision repair in detoxification of these lesions within the cell. A decreased availability of ATP in hypoxic cells, as eluded to earlier, might be the underlying cause of this impairment.

The bis(platinum) series of compounds has been found to exhibit an even greater cross-linking potential than cisplatin[54]. The HCRs found in this study are considerably larger than those found for cisplatin, thus, as was proposed for cisplatin, increased DNA-protein cross-link formation in hypoxia[98] may also play a role in the increased hypoxic cytotoxicity of the bis(platinum) complexes.

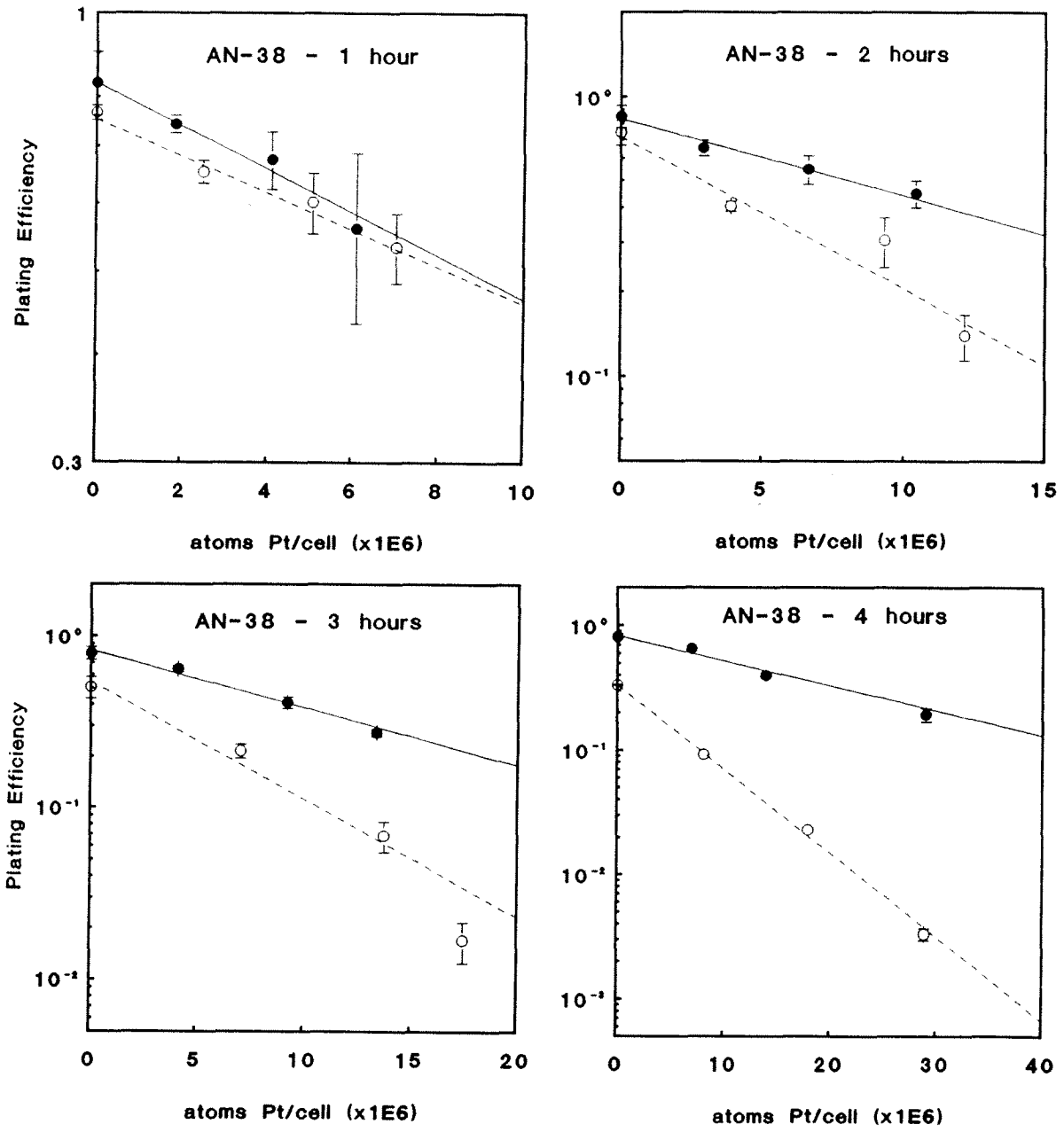


Fig. 4-4 : The variation of cytotoxicity of AN-38 with whole cell uptake. AN-38 exhibits greater toxicity per Pt atom incorporated into the cell in hypoxic(o) versus aerobic(•) cells.

time (hr)	τ_1 (cell atoms Pt^{-1})		$\frac{\tau_1 \text{ (hyp)}}{\tau_1 \text{ (air)}}$	HCR
	Air	Hypoxia		
1	0.0581	0.0495	0.852	1.20±.91
2	0.0636	0.125	1.97	2.47±.51
3	0.0771	0.158	2.05	3.04±.37
4	0.0460	0.155	3.37	3.91±.32

Table 4-1 : Data showing that AN-38 in hypoxic cells is more effective at causing toxicity. Although uptake is only slightly higher in hypoxic cells as compared to aerobic, once inside the cell it appears to cause greater toxicity per molecule. τ_1 is representative of the toxicity per molecule inside each cell (from the equation $SF=Aexp(-\tau_1*D)$, fit to data shown in figure 4-4) and $\tau_1(hyp)/\tau_1(air)$ is the ratio of hypoxic to aerobic toxicity (HCR from table 3-5).

4.3 Further Studies

The data suggest that cisplatin is more cytotoxic to hypoxic CHO cells than it is to aerobic cells. Due to the large variability in response of different cell lines to cisplatin, this work should be expanded to include other cell lines(ie. human), to determine whether the result presented here is specific to CHO cells. Of additional interest would be DNA-repair deficient cell lines such as the UV-20 line, a cross-link repair-deficient CHO mutant[99]. A variety of other methods are available to study the relative importance of each of the types of cisplatin-DNA adducts, including alkaline elution, which may be used to determine the significance of DNA-protein and interstrand cross-links[100]. Furthermore, antibodies specific to cisplatin intrastrand crosslinks have been isolated[101], which would provide a very sensitive probe for quantitating and locating these adducts. Comparison of toxicity, uptake and binding properties of cisplatin with analogues which possess different cross-linking abilities could indicate the relative importance of the various kinds of DNA lesions. The bis(platinum) complexes investigated in this study are an example of two of compounds which exhibit greater cross-linking potential than cisplatin. Other complexes in the bis(platinum) series have shown even greater cross-linking ability than those investigated here[102].

Furthermore, lower concentrations of cisplatin should be used to better characterise any substructure in the toxicity curves, possibly due to an induced repair mechanism (or repair saturation, as alluded to earlier). Measurement of DNA-adduct levels using radio-labelled cisplatin would increase resolution significantly, allowing lower concentrations

of cisplatin to be studied. The aforementioned antibodies would also be sensitive enough to determine the contributions of intrastrand crosslinking at these concentrations.

A more in-depth study using buthionine sulphoximine depletion of glutathione should be carried out to determine the effect of GSH levels on hypoxic toxicity and cisplatin-DNA adduct formation. To study the importance of energy availability, inhibitors of oxidative phosphorylation should also be used in toxicity experiments.

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APPENDIX - PRELIMINARY EXPERIMENTS

In order to be certain that atomic absorption measurements yielded accurate results, several control experiments were performed to assess the effect of the cell preparation techniques.

A.1 Effect of Washing Procedure on Whole Cell Uptake

The cell washing procedure used in whole cell uptake and DNA binding studies was investigated to determine the appropriate number of PBS washes necessary for complete removal of unbound platinum. Cells incubated in 10 μM cisplatin for 1 hour were centrifuged to remove drug solution and resuspended in 10 ml of ice cold PBS. Five fractions were removed and washed from one to five times each with 10 ml PBS. Each was counted for cell number before digestion of the cells in 100 μl concentrated HNO_3. The amount of Pt per cell was determined using AAS.

From figure A-1, it is clear that the initial wash removed the majority of excess platinum from the samples. Subsequent washes appear to have little effect. As a precaution, two washes were normally performed in the cell uptake and DNA-binding studies.

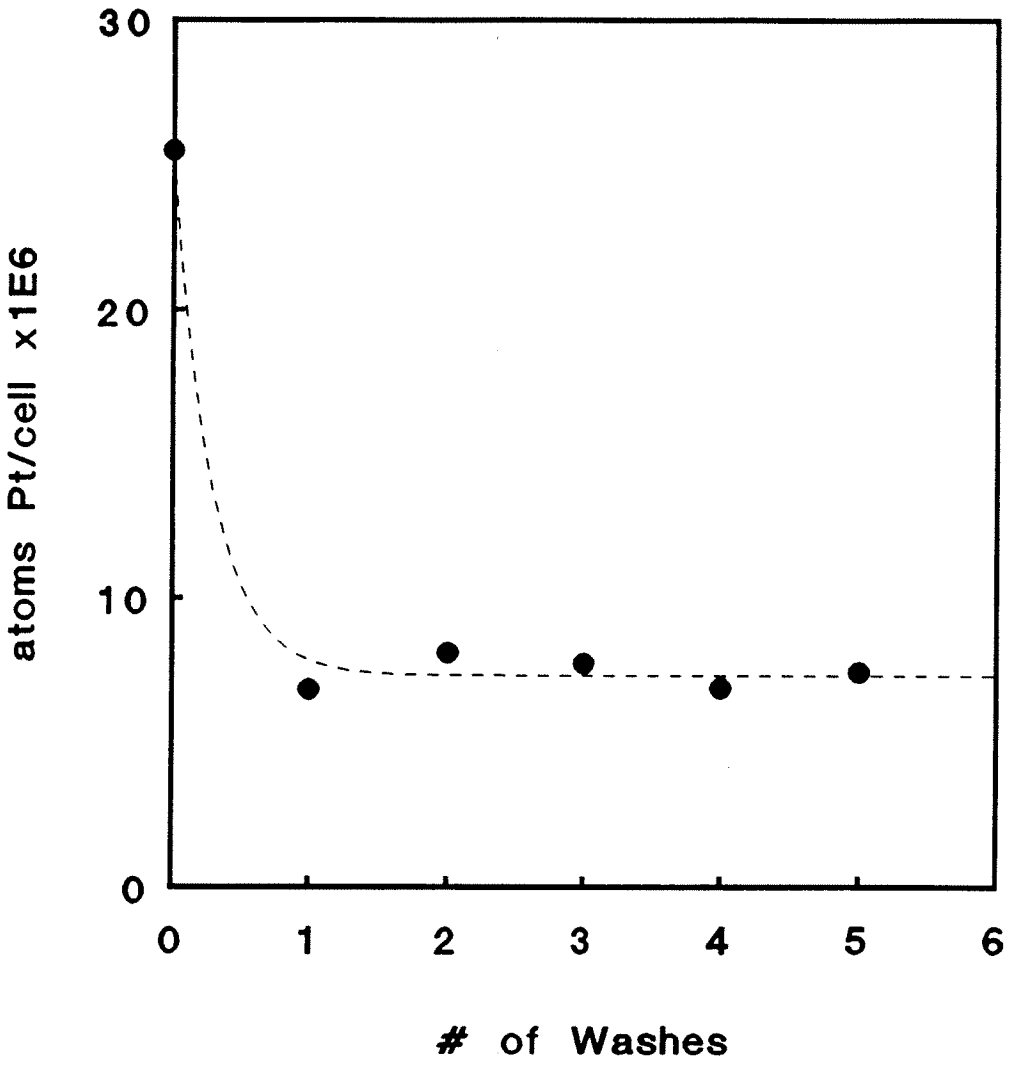

Fig. A-1 : The effect of the washing procedure on whole cell uptake of cisplatin. Exponential phase CHO cells were incubated in 10 μM cisplatin for 1 hour and washed up to five times to determine the number required to remove free platinum. The majority of extracellular platinum was removed by a single wash.

A.2 Effect of Cell Concentration on Platinum Recovery

For whole cell uptake experiments, approximately $2x10^5$ digested cells were placed in the atomizer during atomic absorption analysis. The actual number of cells typically varied within a range of 20% from this value. To ascertain whether this variation might affect sample absorbances, absorbance was measured as a function of cell load within the AAS tube.

Three different volumes of a 400 ng/ml platinum standard solution were added to an untreated digestion of CHO cells, yielding platinum concentrations of 67, 133 and 200 ng/ml. These solutions were injected into the tube with various cell loads up to $1.3x10^5$ cells, the maximum obtainable under the conditions of this study.

Figure A-2 shows that all background due to the cell matrix was eliminated through Zeeman background correction, and variations in the number of cells analyzed did not influence platinum absorbance readings of whole cell uptake.

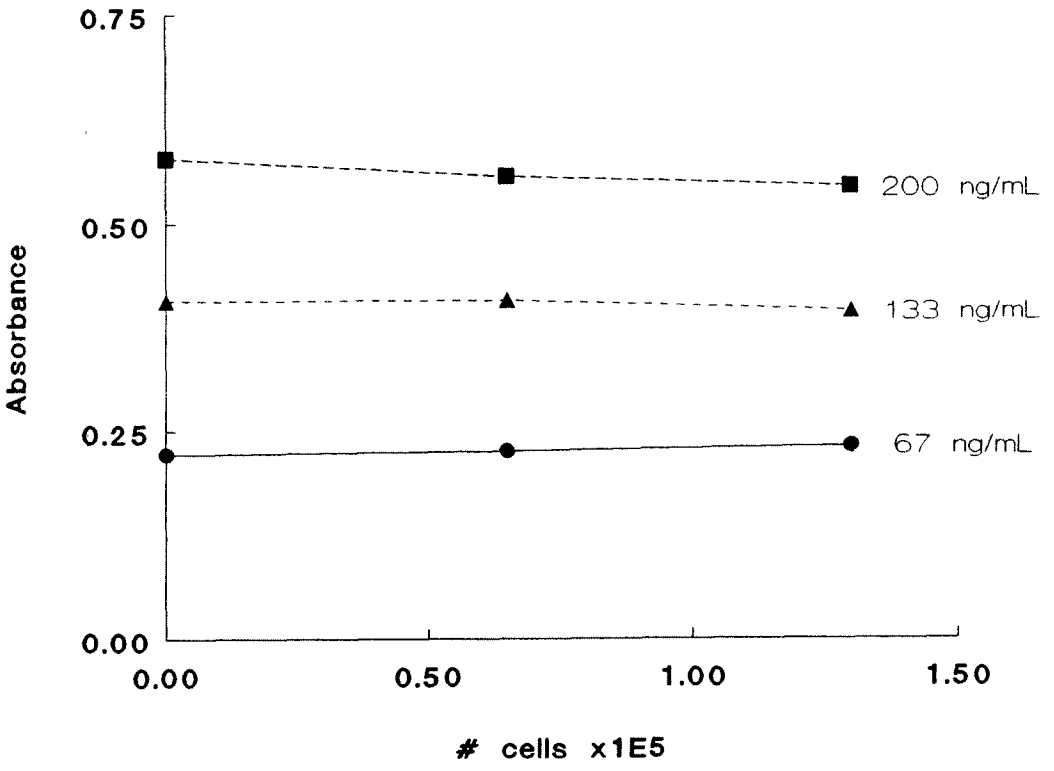

Fig A-2 : Analysis of the effect of cell load on the atomic absorbance signal of a platinum standard solution. Three concentrations of CHO cells digested in concentrated HNO_3 were spiked with three different volumes of a platinum standard, and absorbance was measured according to the temperature program shown in figure 2-2.

A.3 Effect of DNA Load on Platinum Recovery

The DNA extraction procedure typically yielded sample DNA concentrations ranging from 0.8 to 1.2 mg/ml. To examine the effect of these variations on the recovery of platinum from the samples, absorbances were measured at DNA concentrations covering the observed range.

Solutions of 0, 0.66, 1.32 and 1.84 mg DNA/ml of TE were spiked with four different volumes of a platinum standard solution, and their absorbances measured.

The results show, in figure A-3, that variations in DNA concentration affect the absorbance reading by, at most, 7% over the given range. Moreover, there is little, if any, significant difference between these readings and absorbance values when no DNA is present in the sample. This demonstrates that almost all of the matrix components present during the atomization stage were detected by the Zeeman background correction process. Variations in DNA load over the measured range had no effect on resulting DNA-binding measurements.

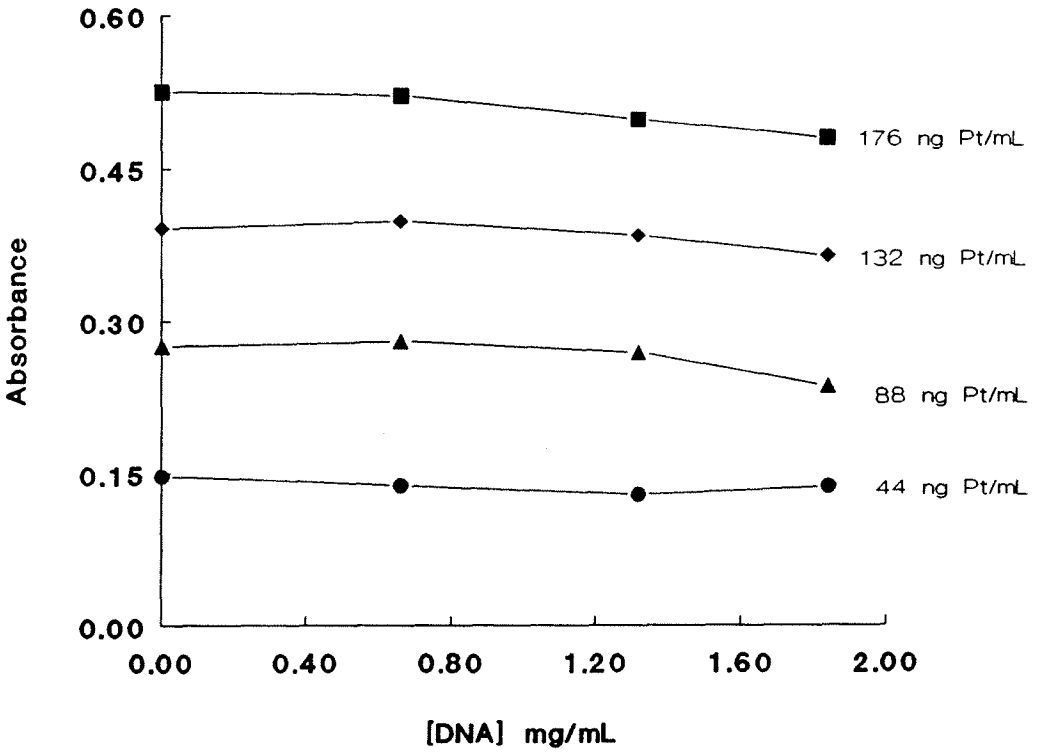

Fig A-3 : Analysis of the effect of DNA load on the atomic absorbance signal of a platinum standard solution. Four concentrations of DNA extracted from CHO cells were spiked with various volumes of a platinum standard, and absorbance was measured according to the temperature program shown in figure 2-2.