INVESTIGATIONS INTO THE MECHANISM OF INTERACTION BETWEEN CISPLATIN AND LOW-DOSES OF X-IRRADIATION IN HYPOXIC CELLS

BY

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B.Sc. (Honors) Simon Fraser University 1986

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE STUDIES

DEPARTMENT OF PATHOLOGY

We accept this thesis as conforming

to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA

May 1991

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Date May 21ST, 1991

DE-6 (2/88)

Abstract

Cisplatin (*cis*-diamminedichloroplatinum(II)) is a highly effective chemotherapeutic drug given either as a single agent or in combination with other drugs to treat several types of tumors. Radiation therapy is also a successful treatment used for many types of tumors. However, many tumors contain hypoxic regions, which are resistant to treatment by radiation and some forms of chemotherapy. Combining cisplatin with radiation may offer advantages to counteract hypoxic resistance. While enhancement of radiation cell killing by cisplatin has been observed, the mechanism of the interaction of these two anti-cancer agents is unknown. In most *in vitro* experiments on radiosensitizers in cultured hypoxic cells extrapolation from high radiation dose experiments is used to predict low dose responses. In this work, clinically relevant doses are used to irradiate both CHO and V79 hypoxic cells. Their survival was assessed by using the Cell Analyzer Imaging System.

Three main approaches were used to investigate the mechanism of interaction. In the first approach, the delivery time of cisplatin relative to irradiation was varied. Results show that enhancement of radiation cell killing was independent of the timing between X-rays and cisplatin administration. In the second approach a series of cisplatin analogs which varied in their DNA cross-linking ability were investigated. None of these analogs produced as strong an enhancement of radiation effects as did cisplatin. The final approach involved depleting intracellular glutathione

which has been implicated in the chemical repair of the cisplatin-DNA cross-link. The depletion of glutathione by buthionine sulfoximine sensitized hypoxic CHO cells to the cisplatin X-ray combination. Results suggest that the DNA cisplatin cross-link may be involved in the enhancement of the effects of low-doses of X-rays. Chromosome analysis of hypoxic and oxic cells shows that cisplatin modifies the radiation dose response of chromosome aberrations. Finally, the results from all experiments suggest that misrepair of damage caused by X-rays and cisplatin may be important in the mechanism of their interaction.

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BSO	L-Buthionine-sulfoximine
cDDP	Cisplatin (*cis*-diamminedichloroplatinum(II))
CHO	Chinese hamster ovary (cell line)
DNA	Deoxyribonucleic acid
DSB	Double-strand break
ER	Enhancement Ratio
GSH	Glutathione (reduced)
Gy	Gray (a measure of radiation, 100 Rads = 1 Gy)
OER	Oxygen Enhancement Ratio
P.E.	Plating Efficiency
SF	Surviving Fraction
SE	Standard error
SSB	Single-strand break
trans-DDP	*trans*-diamminedichloroplatinum(II)
V79	V79-171B (a Chinese hamster lung fibroblast cell line)

Acknowledgements

I would like to thank the following people for all their help and understanding: Dr. Kirsten Skov, my Supervisor; my Supervisory Committee: Dr. Peggy Olive, Dr. Don Brooks, Dr. Dave Dolphin, Dr. Shirley Gillam, and Dr. Janet Chantler (Chairman); and all the people at B.C. Cancer Research Centre who were so helpful: Dr. Lloyd Skarsgard; Dr. Mladen Korbelik, Dr. Branko Palcic, Dr. S.S. Tsang, Hans Adomat, Susan MacPhail, Dr. Ingrid Spadinger, Raija White, Haibo Zhou, Mark Swarchuk, Sio Jao; Terri Felton, Dr. Jiri Kristel, Veronica Antony, and John Fee.

1. Introduction

Radiation is an important tool in the treatment of cancer, and it is estimated that approximately half of all cancer patients receive radiation treatment. Resistance of some tumor cells has limited radiotherapy possibly due to regions of hypoxia in the tumor (Gray et al 1953, Howes 1969). Therefore there has been a search for compounds or regimes to sensitize these resistant cells to radiation therapy. An era for the development of radiosensitizers began, with oxygen-mimetic or electron-affinic nitroimidazoles developed to sensitize hypoxic cells to radiation therapy (Adams 1979, Brown 1982). Use of some of these early drugs was limited in the clinic because of neurotoxicity which resulted from drug concentrations too low to be therapeutically effective (Hall 1988).

Certain platinum complexes which are already clinically useful chemotherapy drugs, (notably cisplatin), also modify the action of radiation (Zak and Drobnik 1971). Furthermore cisplatin not only interacts with radiation preferentially in hypoxic cells (Douple et al 1979), but appears more effective at clinically relevant doses of 2 to 3 Gy (Korbelik and Skov 1989), which are referred to as low-doses in this thesis. The means by which cisplatin complexes interact with clinical doses of radiation in hypoxic cells is not known. It is the object of this thesis to investigate the mechanism of interaction between low doses of radiation and cisplatin in hypoxic cells.

1.1 Introduction to Radiation Biology (von Sonntag 1987, Hall 1988)

In 1895, the German physicist Wilhelm Conrad Roentgen first discovered X-rays. Less than one year later, on Jan. 29, 1896, E.H. Gruppe a physicist in Chicago treated a cancer patient using X-rays from Crookes tubes. In 1897, Professor Freud showed his class the therapeutic effects of X-rays used to eradicate a mole. Since these early findings that radiation could damage biological tissue, it has had a dramatic impact on the treatment of certain diseases, and the means by which radiation damages living cells is still an area of active research.

1.1.1 Types of Ionizing Radiation Absorption of energy from radiation may lead to excitation or ionization. The important characteristic of ionizing radiation is the release of large amounts of energy. Ionizing radiation can be further classified into electromagnetic or particulate. Particulate radiation is directly ionizing. γ-Rays and X-rays are electromagnetic. To generate X-rays, which are produced extranuclearly, an electrical device accelerates electrons to high velocity (and thus high energy), and then stops them abruptly at a target usually made of tungsten or gold. Part of the kinetic energy of the electrons is converted to X-rays. X-rays have a wavelength of 10^{-8} cm to 10^{-6} cm, and their biological effects are usually considered ionizing if they have a photon energy in excess of 124 eV, (wavelength > 10^{-6} cm). X-rays and γ-rays are indirectly ionizing: when absorbed by water or

other abundant molecules, they give up their energy to produce fast moving ejected (recoil) electrons.

1.1.2 Energy Deposition Mechanisms Ionizing radiation produces changes in the material through which it passes via solvated electrons, intermediary radical ions, free radicals, and excited states. These are very reactive species, having high second-order rate constants for reaction, and therefore have very short half-lives within a typical mammalian cell (Ward 1988). Inside cells there is a high density of molecules present, which are all potential targets. When ionizing radiation hits these targets it results in ionizations that release energetic electrons (so-called "recoil electron"). After the initial interaction of radiation with H_2O, a single positive ion is produced, while most of the energy is carried away as kinetic energy by the recoil electron. The energy loss of these electrons, is the energy from which most of the damage is done in the cell. Thus the ionization processes in water are:

$$H_2O \rightarrow H_2O^+ + e^- \qquad (1)$$

$$H_2O^+ + H_2O \rightarrow \cdot OH + H_2O^+ \qquad (2)$$

$$e^- + H_2O \rightarrow e^-_{(aq)} \qquad (3)$$

These radicals interact immediately with each other, producing H_2, H_2O and H_2O_2. In Chinese hamster cells, the protective ability of different ·OH scavengers indicates that the half-life of the ·OH in these cells is 10^{-9} seconds (Reuvers et al 1973).

1.1.3 Absorption of Radiation Energy in Biological Targets Ejected electrons can have direct or indirect action on biomolecules, ie. the recoil electron acts directly on the target, or interacts with H_2O to produce ·OH radical which then attacks the target molecule (DNA, proteins, or lipids of the cellular membrane). It is mainly the radicals formed inside the cell (and not those of the cell suspension) which are considered as damaging (Jacobs et al 1985). Most researchers agree that of the possible targets causing reproductive cell death, DNA must be considered as the prime candidate. Membranes have not yet been ruled out as targets which have lethal effects when damaged, but there have been a number of different independent experiments which implicate DNA as the main target for the lethal action of radiation.

Table 1. Measured Numbers of Damaged Sites per Cell/Gy (von Sonntag 1987).

Type	Yield
SSB	1000
8-Hydroxyadenosine	700
Thymine Damage	250
DSB	40
DNA-protein X-links	150

DNA will be considered as the prime target in this study. Whether the initial damage is produced by direct or indirect action, the attack is distributed randomly among the DNA bases and deoxyribose phosphate moieties (Ward 1988).

1.1.4 Base Damage Base damage is a potentially destructive and mutagenic lesion. However, the seriousness of single base modification has been questioned from studies of thymine glycol formation (typically formed during radiation damage) where it has been noted that single base damage is not necessarily lethal for biologically active DNA. It has been found that osmium tetroxide induced thymine glycol formation is only 33% lethal for biologically active single-stranded DNA (Hariharan et al 1977). This is possibly because thymine often appears in positions where base uniqueness is not strictly required (Watson et al 1987). Furthermore, there have been reports that X-irradiation can induce lethal point mutations (Liber et al, 1986), and it has been concluded by a recent review that radiation can induce mutations in mammalian cells through single base-pair changes (Breimer 1988).

1.1.5 Singly Damaged Sites When a unit nucleotide of DNA is damaged in isolation, without damage to neighboring nucleotides, it is called a singly damaged site. The implications of this are important if one considers the types of damage caused by H_2O_2 and ionizing radiation. They are similar, but measurements reveal that most of the intracellular damage caused by

hydrogen peroxide (H_2O_2) is limited to single-strand breaks. To cause these strand breaks, H_2O_2 reacts with transition metal ions in Fenton reactions generating ·OH radicals near DNA. Yet, more of these strand breaks are necessary for H_2O_2 to kill cells than the DNA damage caused by ionizing radiation (Ward, 1988). Thus, there must be a difference in the way H_2O_2 (and other oxidizing agents) use ·OH to damage DNA when compared to ionizing radiation. Ionizing radiation has a higher efficiency of inducing DNA damage, but this is not the reason that radiation is more efficient than H_2O_2 (Ward 1988). Ward has suggested it is the ability of ionizing radiation to cause damage in more than one moiety of a localized region of DNA. He has called this damage locally multiply damaged sites (LMDS). When cells are irradiated in the presence of oxygen there is a higher efficiency for some types of DNA damage and for cell killing, than when cells are irradiated in hypoxia. Furthermore the sensitizing behavior of some drugs is different in the presence of oxygen. LMDS could be important in the mechanism of X-ray interactions with drugs in hypoxic cells, in comparison to X-ray interactions with drugs in oxic cells.

1.1.6 Single-Strand Breaks Single strand-breaks are rapidly repaired in CHO cells (half-time aproximately 7 min, Skov 1984), but these rates of DNA repair are usually species dependent (von Sonntag 1987). Thus in lymphocytes, as compared to CHO's, 70% of the single-strand breaks are repaired with a $t_{1/2}$ of 3 minutes, and these breaks are not lethal to these cells. A delay in SSB repair does not result in an enhanced cytogenic

damage; hence the rate of repair appears not to influence the conversion of single strand-breaks to double-strand breaks or lethal lesions (von Sonntag 1987).

1.1.7 *Double-Strand Breaks* DNA double-strand breaks are the most likely lesion to be the cause of lethal effects of ionizing radiation (Ward 1988). A linear yield of 70 dsb/Gy/cell was found from 5 to 2000 Gy using a modified neutral velocity sedimentation procedure (Blöcher 1982). The OER for double-strand breaks is 3.6, which is similar to the OER found for cell kill at high doses (usually about 3 (Hall 1988)).

1.1.8 DNA Repair The repair of double-strand breaks in V79 and *E. coli* cells has a $t_{1/2}$ of 8 minutes at 37°C (Weibezahn and Coquerelle 1981). However, some of these double-strand breaks are repaired slowly (von Sonntag 1987). The fast process requires DNA ligase. The slow process utilizes DNA recombination systems, which require the unique enzymatic properties of Rec A protein. Rec A protein is responsible for matching the damaged ends of the DNA strand to a strand of an undamaged complementary duplex. The resulting gaps are then filled by DNA polymerase, and sealed by DNA ligase (Watson et al 1987). However, there may be more damage after repair caused by γ- endonucleases (Bresler 1979). There is almost no enzymatically flawless repair of radiation-induced strand breaks (von Sonntag 1987). Thus, after enzymatic or chemical repair of radiation damage, there is usually some type of damage still present. Frame shifts from a deleted nucleotide may be

an important lethal event in some circumstances if close to a reading area of an important DNA gene sequence. The repair of double-strand breaks after irradiation in hypoxia has not been reported.

1.2 The Oxygen Effect

Although the radiochemistry of H_2O has been well described, the description of the mechanism for the oxygen effect has remained elusive. The oxygen effect was first observed by Schwartz in 1909. By 1923 Petry had firmly established oxygen as a chemical sensitizer, in his work on the effects of radiation on vegetable seeds. Results obtained with different mammalian cell lines indicates that the survival of cells irradiated in hypoxia has a 2.5 to 3 times larger lethal dose needed in comparison to aerobically irradiated cells. It is because of this radioresistance of hypoxic cells in tumors that radiobiologists are interested in the oxygen effect.

1.2.1 Radicals and Types of Damage Because of the presence of oxygen under natural conditions, peroxyl radicals play an important role whenever free radicals are generated in biological systems. The 2 electron reduction product of superoxide is ·OH radical. The ·OH radical's most classically known reactions are easy abstraction of carbon bound hydrogen atoms, or addition to carbon-carbon and carbon-nitrogen double bonds (von Sonntag 1987). The resulting radicals will usually react with O_2 to give the corresponding peroxyl radicals (Adams and Wilson 1969). Some attempts to explain the oxygen effect have been made on the presence of the simplest

peroxyl radical, the superoxide anion radical O_2^-. What is the difference between radicals produced in hypoxia versus radicals produced in oxygen when X-rays are delivered? One hypothesis is that O_2 and/or O_2^- are toxic to cells and enzymes (superoxide dismutases) have evolved which are capable of dismutating superoxide to protect against these radicals (Fridovich 1981). When oxygen is present during irradiation, O_2 readily scavenges solvated electrons and H atoms:

$$e^-_{(aq)} + O_2 \rightarrow O_2^- \qquad K = 2 \times 10^{10}\ dm^3\ mol^{-1}\ s^{-1}$$

$$H\cdot + O_2 \rightarrow HO_2\cdot \qquad K = 2 \times 10^{10}\ dm^3\ mol^{-1}\ s^{-1}$$

Therefore $HO_2\cdot/O_2^-$ are present in aerated irradiated solutions. However, O_2^- is unreactive with many biomolecules. But O_2^- does go through redox reactions, such as the Haber-Weiss reaction, and Fenton reaction to yield ·OH, a very reactive radical. Carbon centered radicals formed from reaction with ·OH, react with O_2 at almost diffusion controlled rates.

$$RH + \cdot OH \rightarrow R\cdot + H_2O$$

$$R\cdot + O_2 \rightarrow RO\cdot_2 \qquad t_{1/2} - 2.5\ s\ \text{(oxygen fixation)}$$

The chain reactions of these peroxyl radicals are very important in propagating damage (von Sonntag 1987).

Termination of the chain reactions yields:

$$RO_2\cdot + RO_2\cdot \rightarrow \text{Products}$$

Or there may be possible chemical repair by, reducing antioxidants such as alpha-tocopherol (AT):

$$RO_2\cdot + AT \rightarrow RO_2^- + AT\cdot$$

The formation of $RO_2\cdot$ can be prevented by O_2 competing with glutathione (GSH) (O_2 is more reactive with R· than GSH, therefore in radiations of oxic cells this probably plays a small role):

$$R\cdot + GSH \rightarrow RH + GS\cdot$$

Hydrogen atom donation by GSH is probably a prominent pathway in chemical repair of hypoxic radiation damage (Ward 1983, Hutchinson 1961).

In hypoxia because of the very small amounts of O_2 there is very little O_2^-. With ionizing radiation:

$$H_2O \xrightarrow{\text{Radiation}} H_2O\cdot^+ + e^-$$

$$H_2O\cdot^+ + H_2O \rightarrow H_3O^+ + \cdot OH$$

The ·OH formed radical is possibly the major damaging species of indirect action produced in hypoxic cells when irradiated with X-rays. Whether in oxygen or hypoxia, the ·OH radical attack of various carbon bound H-atoms is random. When O_2 is not present there will be no organic alkyl peroxide radicals formed (because little or to O_2 present). Traditional theory suggests many alkyl radicals will correctly and quickly repair by chemical means, as outlined above.

1.2.2 Radiation Damage in Hypoxic Cells Because of the differences in the environment and the radicals produced in air and hypoxia during irradiation, there is a difference in the type of molecular damage produced. For example, DNA single-strand breaks are much more numerous in cells irradiated in oxygen versus cells irradiated in hypoxia.

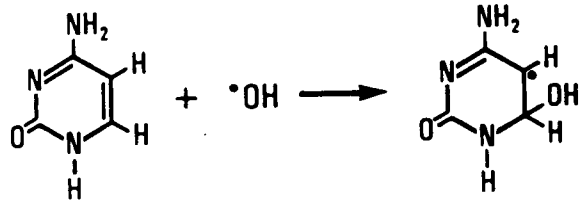
1.2.3 DNA-Protein Cross-links in Hypoxic Cells DNA protein cross-links caused by ionizing radiation are more extensive in hypoxia (Fornace and Little 1977, Oleinick 1990). Also, the yield of DNA-protein cross-links has a similar magnitude to the yield of double-strand breaks produced in cells irradiated in air (see table 1). The creation of DNA-protein cross-links preferentially involves a type of DNA-binding protein of the nuclear matrix, and their attached DNA sequences. The repair of DNA-protein cross-links is slower than the rejoining of DNA strand breaks, occurs at a slower rate in glutathione-depleted cells, and is present in mitotic cells. Chemical repair in hypoxia may also lead to the formation of different bases, and thus mutations in cells irradiated in hypoxia (see section 1.4.1)(Ward 1983).

1.2.4 The Role of Thiols in Repair (Ward 1983) Chemical repair reactions of glutathione easily act on free radicals formed on the deoxyribose moieties of DNA. Repair of DNA base damage is not so simple. When DNA damaging radicals are formed, most product radicals formed between the free radicals

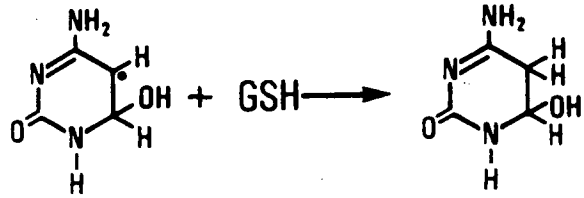
from water radiolysis and DNA bases are from addition reactions (Ward 1988).

For example:

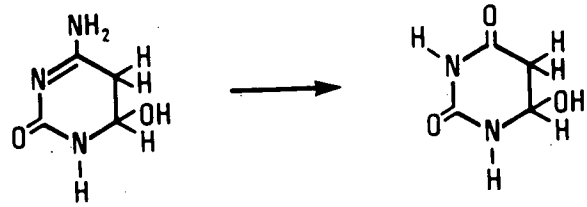
Reaction of ·OH with cytosine:



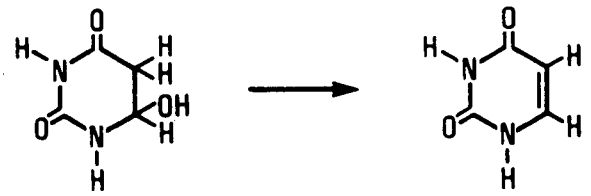
Under aerobic conditions O_2 is in competition with GSH for these radicals; in hypoxia GSH will quickly react with these types of radicals:



Thus donation of an H atom from GSH does not constitute repair, but rather formation of the hydrate. These types of reactions have been found in isolated DNA (Holian and Garrison 1969). The hydrate formed can decay via two routes, either decay to the parent compound, or decay with deamination to a new compound:



The uracil hydrate can then dehydrate to form uracil:



Thus in hypoxia there is the possibility for an increase in potentially mutagenic reactions. Ward (1983) concludes that GSH probably uses a composite of both ·OH scavenging and H-atom donation.

1.3 Effects of Low Versus High Doses of X-Rays

In past years, *in vitro* experiments used high doses of X-rays to study the radiation response, with clinically relevant (2 Gy, low-dose) responses predicted by extrapolation from the high dose results. This extrapolation procedure is dependent upon the mathematical model used to characterize the surviving fraction versus dose relationship, and it can be shown that this gives enhancement ratio (ER) estimations with large uncertainties. Furthermore, this lead to disappointing outcomes when radiosensitizers were first being tested, because these nitroimidazoles did not sensitize as well at the X-ray doses used in the 2 Gy regimen, as they do at high doses. Thus low-dose experiments are desirable to assess ERs and/or mechanisms of these drugs. In this study the cell analyzer imaging system facilitated the assessment of cell survival at low-doses (Palcic and Jaggi 1990).

When referring to survival curves, the ratio of doses, in hypoxic vs oxic conditions, needed to achieve the same biological effect (for example, a 0.01 or 0.80 Surviving Fraction (SF)) is called the OER (oxygen enhancement ratio). In this study the chosen effects are survial fractions of 0.01 at high doses (more than 5 Gy) and 0.80 at low doses (0 to 5 Gy). A typical survival curve from the standard experiment is shown in figure 1. For X-rays and γ-rays the OER at high doses is usually close to 3 (Hall 1988). However, the OER at low doses been found by some to be approximately 2 (Palcic et al 1984), although there is still controversy over the OER for cell killing, at low doses. The OER for strand-breaks is 2.91, and is constant from 1 Gy to 12 Gy (Berger 1982). One of the current mathematical models of radiation cell killing to model these effects is a quadratic equation (figure 1) that includes two components: a linear (i.e., exponential α component), and a bending component (β component). Repair of radiation damage has been reported to affect only the β component (Hall 1988).

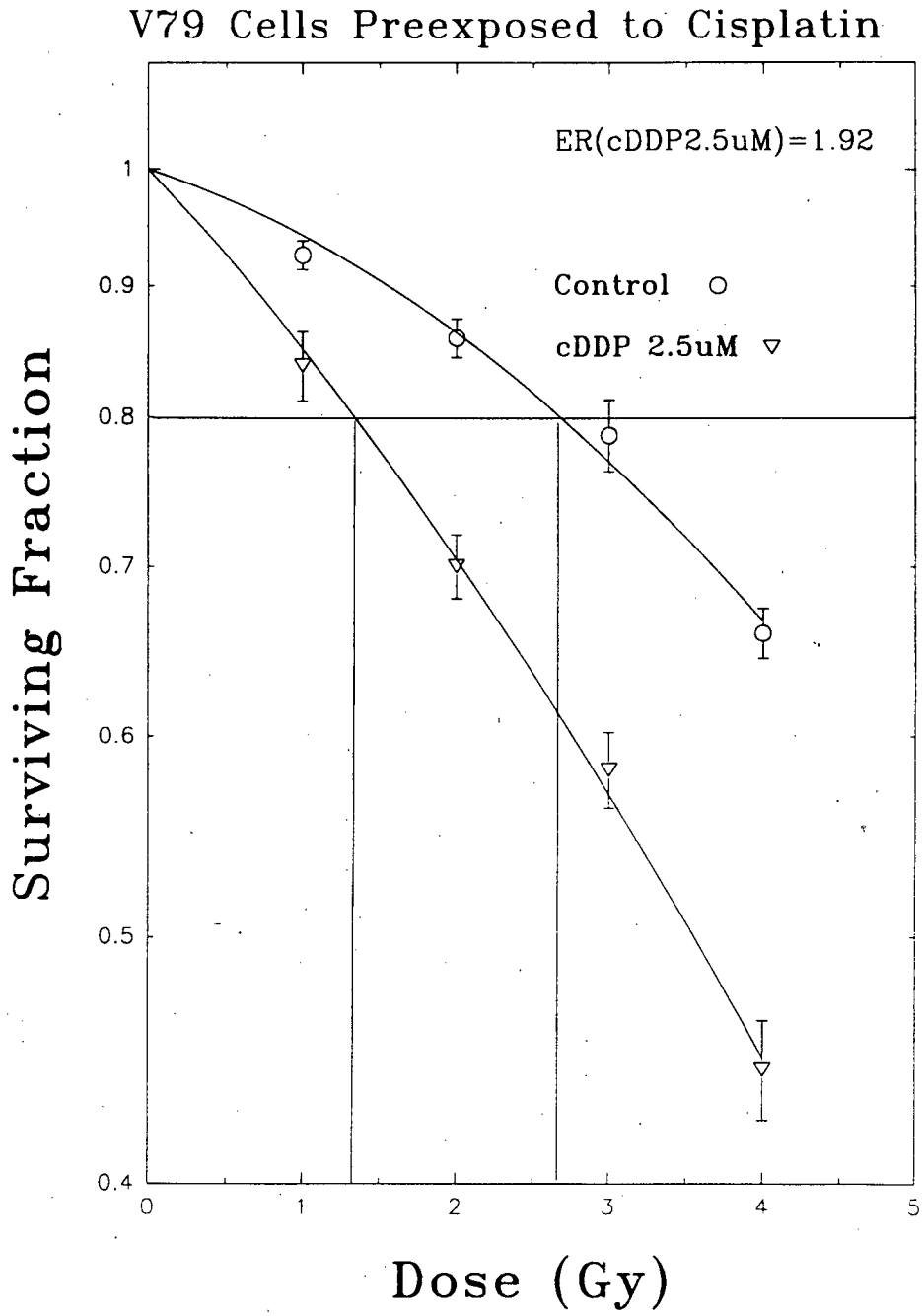


Figure 1. Survival curves from the standard experiment showing the calculation of an enhancement ratio for cisplatin at a surviving fraction (SF) of 0.8 in hypoxic cells.

Dose for 0.8 SF for control cells is 2.59 Gy. Dose for 0.8 SF for cisplatin treated cells is 1.35 Gy. 2.59/1.35 = 1.92. These data were fitted to the quadratic equation $-\ln S=\alpha D + \beta D^2$.

1.4 Radioresistant Cells in Tumors

As mentioned in introductory remarks, tumors are thought to contain hypoxic cells, (Thomlinson and Gray 1953) perhaps because they out-grow their capillaries. At the limits of oxygen diffusion within a tumor there will be enough oxygen for the cells to remain alive, but a low enough oxygen level for the cells to be resistant to radiation. Thus hypoxic cells could be a limiting factor in radiotherapy success. Cells in the hypoxic region could be a focus for regrowth of the tumor. Also, it has been shown that after irradiation there may be reoxygenation, where the hypoxic cells become oxygenated because the oxic cells are lethally damaged by radiation, and no longer take in oxygen (Van Putten and Kallman 1968). Thus oxygen is able to diffuse to the hypoxic cells. Therefore the oxygen status of cells in a tumor is not static, it is transient. (Hall, 1988).

It is the goal of fractionated radiotherapy to time the fractions so that more of the tumor is destroyed during each dose of radiation. Because of the extent of reoxygenation and the time with which it occurs varies immensely for different types of tumors the development of a better approach to the hypoxic problem is still needed. In the 1950s and early 1960s much effort was made to find another solution to the problem of hypoxic cells in tumors. One solution is to use Radiosensitizers.

1.5 Radiosensitizers and Cisplatin

1.5.1 Radiosensitizers One form of radiosensitizer is the hypoxic cell sensitizer. Hypoxic cell sensitizers increase the sensitivity of hypoxic cells but not aerated cells to radiation. These radiosensitizers are designed to mimic oxygen. One of these new drugs designed was misonidazole. When misonidazole was evaluated in clinical trials, however, it found limited use because it caused peripheral neuropathy. Also, recent *in vitro* work has shown drugs such as misonidazole to be less effective in the low dose region (Palcic 1984). Thus, drugs which were more effective in the clinical dose region were needed, and one possibility being tested is DNA-targeted sensitizers.

1.5.2 Cisplatin was First Introduced as a Chemotherapeutic Agent In 1965, Rosenberg first found inhibition of cell growth and antitumor activity for cisplatin (Rosenberg et al 1965). Cisplatin soon became an effective chemotherapy agent for solid tumors (testicular, ovarian, and head and neck tumors) (Hill et al 1972). In 1971, *in vivo* studies showed that cisplatin also enhances radiation effects (Zak and Drobnik 1971).

1.5.3 *Chemistry of Cisplatin* Cisplatin is an inorganic complex formed by an atom of platinum surrounded by 2 chloride and 2 ammonium ligands in the cis position of a square planar structure. Cisplatin also has a geometric isomer, *trans*-DDP. In aqueous solution the two Cl^- ions of cisplatin can be

displaced by H_2O (Lippard 1982). This increases the acidity of H_2O upon ligation to the platinum complex. In the blood the Cl^- ion concentration is high (100 mM) enough so that the Pt(II) structure maintains its chloride ligands. However, once it crosses the cell membrane it hydrolyzes to the aquated complex, because the Cl^- ion concentration is about 4 mM. Pt(II) has a high affinity for nitrogen donor atoms, and at physiological pH the potential donor atoms in DNA are guanine N7, cytosine N3, and adenosine N1 and N7. The importance of this is shown in the type of DNA adducts cisplatin can form with DNA. The binding of cisplatin to DNA perturbs the structure of DNA, which causes a decrease in the melting temperature, shortening (Cohen et al 1979), unwinding (Butour and Macquet 1978), and local denaturation (Scovell et al 1982).

At present, there are six different types of reaction products between cisplatin and DNA that are known (figure 2). Two of these are specific for cisplatin (no. 4 and 6), while the other four can be formed by *trans*-DDP (Lempers, 1990). The intrastrand crosslink between two adjacent guanines (no. 4, figure 2) has been found to be the major adduct of cisplatin DNA interactions (Fichtinger-Schepman et al 1985). In the six products, binding is only observed at guanine N7, adenine N7, or at a protein (through a sulfur atom). In these products, intra and inter-strand crosslinks are observed, as well as DNA-protein cross-links. It seems likely that one of the adducts specific for cisplatin is important for the antitumor activity (Lempers 1990).

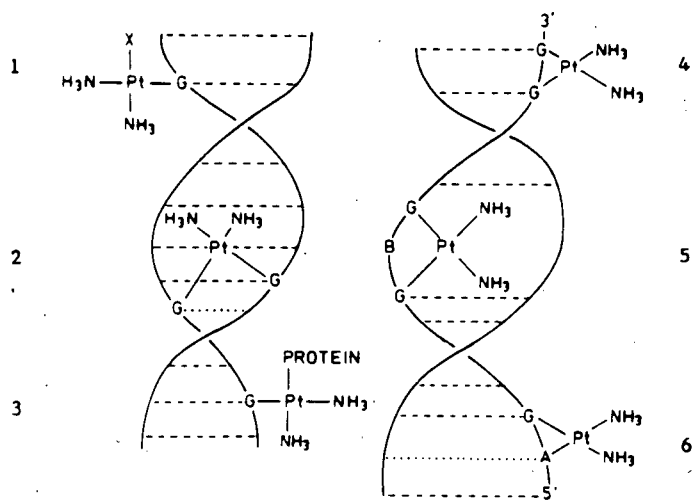


Figure 2a. A schematic diagram of the known cisplatin-DNA interactions. x = Cl-, -OH, H_2O. (From Lempers 1990).

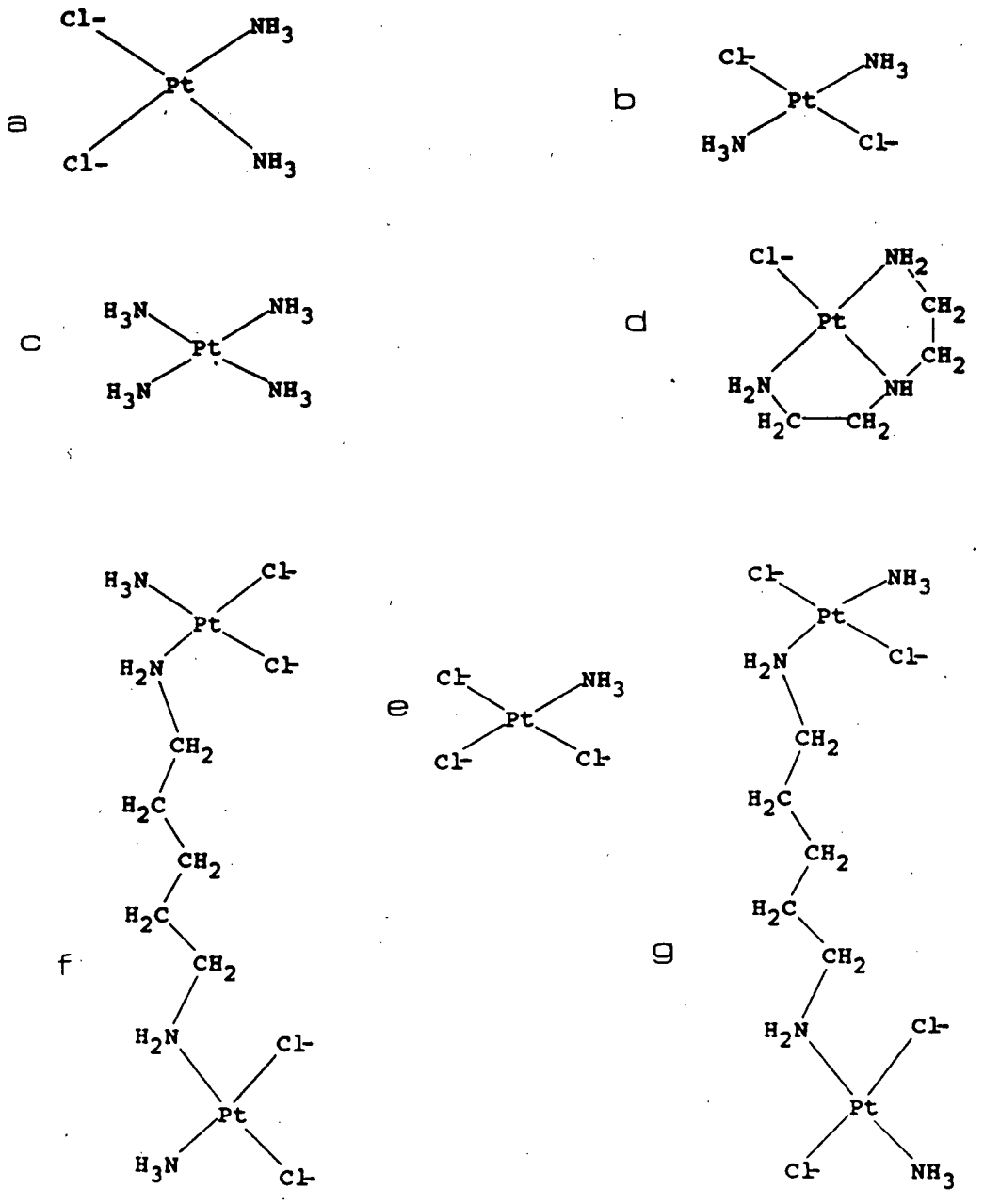


Figure 2B Structures of Cisplatin, trans-DDP, and Analogs
A: Cisplatin, B: trans-DDP, C: tetrammineplatinum(II), D: chloro(diethylenetriammine)platinum(II), E: monoamminetrichloroplatinum(II), F: Bis(platinum)cis, G: Bis(platinum)trans

1.5.4 Types of Damage Caused by Cisplatin and *Anti-tumor Activity* Cisplatin interactions with DNA are thought to be responsible for cytotoxic lesions. Cisplatin is known to inhibit DNA synthesis (Eastman, 1988). Flow cytometric analysis of cellular DNA exposed to cisplatin, has demonstrated a slowed DNA synthesis phase that progresses to a block in G2 phase of the cell cycle (Eastman, 1988). Eastman suggests that cells which live repair the damaged DNA, cells that die are unable to transcribe damaged DNA due to problems in DNA repair and are terminal in G2. He concludes that cytotoxicity of cisplatin may be due to the inability to recover transcription after G2 block. Enhanced DNA repair has been shown to be part of cisplatin resistance. In the corollary to cisplatin resistant cells, bacteria and human cell lines which are DNA repair deficient (cross-links) are more sensitive to cisplatin (Browuver 1981, Plooy et al 1984). Cisplatin is capable of producing permanent damage to DNA, and the mutagenic properties of cisplatin result in frame shift and base substitution mutations (Browuwer et al 1981). It is proposed that these mutagenic events may be important in the interactions of cisplatin with low doses of X-rays in hypoxic cells.

1.5.5 Repair of Cisplatin DNA Lesions After cisplatin damage, DNA is repaired by excision repair enzymes or post-replication repair processes (Dewitt, 1987). The $t_{1/2}$ for excision repair of cisplatin interstrand cross-links is 24-60 hours (Zwelling et al 1979). From the description presented on

cisplatin damage to DNA, it is evident that there are two main types of cisplatin DNA binding, monofunctional, and bifunctional. The monofunctional binding appears to be unlikely to cause the observed cytotoxic, tumorocidal, or radiation enhancement, since *trans*-DDP is at least as effective as the cisplatin in forming monofunctional adducts (Munchhausen et al 1975). The formation of bifunctional adducts is a 2-step reaction where the last step is rate limiting (Lempers 1990), and may be prevented by GSH (Lempers 1990). Intrastrand cross-links are the major type of bifunctional adducts formed by cisplatin *in vitro* and *in vivo* in human cells, with the main adduct being cis-Pt-$(NH_3)_2$-(pGpG), i.e., an intrastrand cross-link between 2 adjacent guanines. *In vivo*, about 75% of the cross-links are excised and removed within 24 hours. *In vitro*, and *in vivo* work by Fichtinger-Schepman, has shown that both the number of cross-links formed and the ability of cells to repair the cross-links differ. She concludes that the differences in repair capacity of different cells will determine the susceptibility of these cells to the toxic action of cisplatin (Fichtinger-Schepman 1988).

Although the *trans* isomer of cisplatin, *trans*-DDP, can also form DNA cross-links (an interstrand cross-link), there is a difference in the way the cell repairs cisplatin versus *trans*-DDP adducts. In CHO cells the *trans*-DDP induced interstrand cross-links are created faster, but also are repaired more quickly than cisplatin (Plooy et al 1984). This quality of *trans*-DDP may be the reason for the rapid recovery from DNA synthesis inhibition in CHO cells after *trans*-DDP treatment. Persistent cisplatin interstrand cross-links can

be very toxic to mammalian cells, especially during DNA replication (Plooy et al 1984). Whether the repair or misrepair of the G-G intrastrand cross-link is involved in cisplatin's enhancement of radiosensitivity remains to be proven, but is a likely to be involved in some way, and is a consideration in this present study.

V79 cells in G_1 phase (the phase preceding S phase) are more sensitive to cisplatin than are asynchronously growing cells (approx. 30% S phase) and DNA synthesis (S phase) is required for cisplatin to cause its cellular toxicity (Fraval and Roberts 1979). The DNA repair processes that attempt to repair these lesions in CHO cells are important in modulating the cytotoxic effects of cisplatin that produce cross-link damage, and these repair pathways also repair UV damage (Meyn et al 1982).

1.5.6 Potentiation of Radiation Damage by Cisplatin In 1971 Zak and Drobnik found that cisplatin enhanced radiation effects in mice. Since this there has been an effort to study this interaction. Early work in bacteria suggested that cisplatin may be a hypoxic cell sensitizer (Richmond and Powers 1976). In mammalian cells enhancement ratios varied from 1.0 to 1.3, and the effects of the combination were usually more pronounced in hypoxic than in aerobic cells (Dewit 1987), however occasionally the reverse or no effect was found (Melvik 1988, Dewit 1987). Because both cisplatin and X-rays target DNA, it has been suggested that there may be an interaction between them leading to a greater than additive cell kill. Cisplatin changes the slope of the radiation-dose response curve *in vitro*, where there is a

reduction in the initial shoulder or an increase in the final slope of the curve (Dewit 1987). Promising results of cisplatin combined with X-rays are emerging from clinical trials (Dewit 1987, Coughlin and Richmond 1989).

Cisplatin enhances low dose radiation effects in hypoxic cells *in vitro* (Korbelik and Skov 1989). Platinum compounds unlike electron-affinic sensitizers (misonidazole) are more effective at low doses of X-rays in hypoxic cells. It should be generally noted that the ability of a platinum drug to modify radiation damage does not always parallel the antitumor activity of the drug, or its cytotoxic activity (Skov and MacPhail 1991). For example, the *trans* isomer of cisplatin, as noted earlier, is not an effective antitumor agent, but is a reasonably effective enhancer particularly at low doses (hypoxia, *trans*-DDP 60 μM, ER = 1.8) (Skov et al 1989).

The optimal time interval for drug delivery has not been found in single dose experiments. With fractionated X-ray doses most have found that the drug causes the largest effect, ie. greater than additive, if it is given a short time before irradiation (Douple et al 1977, Douple and Richmond 1979, Douple and Richmond 1982, Lelieveld 1985). While some *in vivo* studies have found no timing dependency, the discrepancy seems to be in fractionated doses versus single doses. In fractionated X-ray experiments it is possible to produce a large ER if cisplatin is given in close timing with the X-ray fraction, while in single dose studies no dependence on timing has been found.

1.5.7 Proposed Mechanisms of Interaction Between Cisplatin and X-Rays

a) In pulse radiolysis experiments, cisplatin reacts with free solvated electrons, giving rise to Pt(I) intermediates which disproportionate to platinum metal and hydrolyzed Pt(II) complexes (Dewit 1987). Thus, when ionizing radiation induces free electrons from target molecules they could react with cisplatin, thereby interacting with electrons that could otherwise attack the closest biological molecule. Classic radiosensitizers act by electron capture, and must be present during irradiation or very shortly thereafter to react with radiation formed free radicals. Although it has been postulated by some (for example Richmond, or Powers) that platinum complexes may radiosensitize by a similar mechanism involving Pt(I) formation, this seems unlikely in view of the work by Korbelik and Skov and others who find that the ER for cisplatin added after irradiation does not require the presence of cisplatin during irradiation[9]. Classic radiosensitizers are "electron-affinic", ie. they are strong oxidizing agents, which act by a form of an oxidative free radical mechanism. Since the one electron reduction potential of cisplatin is considerably lower (-1000 mV) than that of oxygen (-155mV) or metronidazole (-486 mV), it would seem unlikely that cisplatin acts by such a mechanism.

b) Another possibility is that cisplatin reacts with ·OH radicals (Dewit, 1987), but this again seems unlikely because cisplatin reacts 10X slower with ·OH radical than with free solvated electrons. Thus most hypotheses suggest cisplatin would preferentially react with solvated electrons rather than with ·OH.

c) Depletion of non-protein thiols increases X-ray sensitivity, particularly of hypoxic cells. Transient depletion of these radioprotectors by cisplatin has been shown in golden hamster cells but not V79 cells. The mechanism of this could be by cisplatin scavenging a hydrogen radical from the sulphydryl group that otherwise would react with ·OH radical or the ·R of a critical target molecule, or cisplatin would bind GSH directly (Lempers 1990).

d) Inhibition of cellular repair processes is also another possible mechanism. For example, it has been postulated by Chadwick et al that a greater than additive interaction of radiation and cisplatin would occur if a radiation induced SSB were present opposite a cisplatin induced cross-link, and one lesion would prevent the repair of the other.

More specifically there has been suggestion that the reduction of the shoulder of the X-ray dose response curve is caused by an inhibition of sublethal damage repair (SLDR) (Dewit 1987). Cisplatin has also been found to inhibit potentially lethal damage repair (PLDR) in plateau phase cells (Dritschilo et al 1979). Conversely, sublethal damage is due to toxicity, and at above 5 mM Cisplatin is a cytotoxic agent. Whether such damage can be detected depends on the sensitivity of the assay used. Damage that remains below the threshold of detection may become noticeable when cisplatin is combined with radiation, without the fact any processes of interaction or potentiation of effect of the cisplatin is involved. In other words the increased damage could simply be the result of independent action, even though given independently the two agents do not cause a high degree of cell kill. This

possibility could be overlooked, and has led some to label certain drugs as radioenhancers. Therefore there should be a correction for the amount of cytotoxicity of the drug if there is any. In this study, 2.5 μM concentrations of cisplatin were used which causes only a marginal toxicity (<20% cell kill), and no significant ER at high doses of radiation.

1.6 Approaches Used in this Study

Cisplatin's ability to inhibit cell division by formation of DNA intra or interstrand cross links is generally believed related to its cytotoxic action. Several indirect approaches were used in this study to investigate the possible role of cross-links in the mechanism of interaction with low-doses of X-rays.

1.6.1 The Effect of Timing Cisplatin and X-Rays The first experimental approach used an indirect investigation of the possible role of the cisplatin-DNA cross-link in the radiation interaction by varying the delivery time of cisplatin with X-rays. Here the assessment of the interaction of the two cancer agents was based on the two different types of DNA repair occurring. The repair of DNA strand breaks occurs after X-ray treatment, or repair of DNA cross-links occurs after cisplatin exposure. Investigations into the mechanisms of the influence of cisplatin on radiation effects are complex when both agents are given at the same time, because of the two different

DNA repair processes going on simultaneously for X-ray and cisplatin damage.

When X-rays are used alone, repair of DNA single- strand breaks occur very rapidly, with about 85% being complete in 60 minutes (Berger 1982, Skov 1984). If the drug is added to the cells after selected periods of repair of breaks caused by X-rays, the cells will have the opportunity to repair the X-ray damage, and an assessment of the interaction between the totally, or partially repaired X-ray damage, and the cross links formed by cisplatin can be made. After these selected periods of repair of X-ray induced breaks, the possible role of single- strand breaks in the interaction with cisplatin may become apparent.

The DNA repair processes of cisplatin treatment are much slower than single strand break repair. The half time for this repair by excision enzymes is approximately 24 to 60 hours. Thus, if cisplatin treatment precedes irradiation then some of the cisplatin cross-links can be repaired before the administration of X-rays, and their possible role can be evaluated. It is acknowledged that one type of DNA damage may enhance the potential for damage, or prevent the repair of the other. Using this timing approach the evaluation of an interaction between cisplatin and X-rays can be made. An interactive effect is where a radiation induced single strand break or double strand break may be opposite a cisplatin-induced intrastrand cross-link causing an inhibition of repair of one or the other.

Therefore, allowing different time periods between the delivery of the drug and X-rays may facilitate an interpretation of parts of the mechanism.

For example if there were a decrease in the ER when the cells were allowed to repair breaks after X-rays, one could postulate that an interaction between the X-ray induced strand breaks and cisplatin damage was somehow involved in the mechanism.

1.6.2 Colony Size Analysis As will be discussed later, the misrepaired lesion may be responsible for the interaction between cisplatin and X-rays. Thus, a method was needed to demonstrate the possible existence of a sensitive subpopulation of cells which may have this misrepaired lesion but still maintain growth viability. Actual investigation of misrepair was beyond the scope of this project, but the analysis of the size of the surviving colonies would show if the surviving colonies were all similar. This could show the presence of surviving cells which retained the ability to divide, but not at normal growth rates. Therefore this information could indicate the existence of living but still damaged cells, which may be sensitive to cisplatin.

Surviving colonies from radiation experiments have been examined under dissecting microscopes, and the number of cells per colony has been established (Nias 1968). These experiments show the distribution of clone sizes from radiation doses given to CHO or HeLa cells, where the mean clone size decreases with increasing radiation dose. It makes it quite clear then that in the picture of radiation damage there is no simple criteria of radiation damage; rather there is a spectrum of damage.

Increasing radiation doses given to CHO cell caused fewer large clones to form. These higher radiation doses cause the cells to divide only once or

twice and to form giant cells which do not divide at all. Intermediate doses (5 to 10 Gy) have clones of intermediate size, which would have the ability to reproduce but grow more slowly than controls. These cells have suffered non-lethal damage. This is a heritable form of damage that is characterized by slower growth, lower plating efficiency (P.E.), and, by increased sensitivity to repeated treatment (Nias 1990). The reason for measuring colony size in these experiments was to see whether the X-ray treated hypoxic cells are in fact showing signs of increased sensitivity to further treatments.

1.6.3 Chromosome Aberrations Caused by Cisplatin and X-Rays Alone or in Combination In a further effort to see if irradiated hypoxic cells have a type of permanent damage causing sensitivity to cisplatin, chromosomes were analyzed for aberrations.

1.6.3.1 X-Ray Damage When cells are irradiated breaks will be produced in the chromosomes, which may lead to exchanges (Hall 1988). These induced breaks have the possibility for incorrect rejoining. If this occurs in prereplication chromosomes (G_1) this may lead to chromosome aberrations (Hall 1988). If this radiation-induced breakage and incorrect rejoining occurs in postreplication chromosomes (S or G_2) it may lead to chromatid aberrations. The general aberrations discussed in this thesis will be dicentries, rings, and eccentric fragments. It has been found that the incidence of most radiation-induced aberrations is a linear-quadratic function of dose (Hall 1988). When a stained preparation is made at the first

metaphase (or anaphase) after exposure to ionizing radiation, a study of the structure of the chromosome can be made. It is unfortunate that in many mammalian cell lines this study is made difficult by the large numbers of chromosomes each cell possess. Exceptions are some hamster cell lines (Chinese hamster Ovary (CHO), Chinese hamster lung fibroblasts (V79), and Golden Hamster embryo (GHE), with appoximately 17 to 22 chromosomes). The CHO cells used here contained from 17 to 21 chromosomes; aberrations were expressed as frequencies per cell.

Aberrations in this thesis were categorized as chromosome or chromatid, then subcategorized as acromatic gaps, breaks, and intra-arm intrachanges, inter-arm intrachanges or interchanges, according to the classification of Savage (1975).

1.6.3.2 Chromsome Aberrations can Also be Caused by Cisplatin Cisplatin causes chromosome aberrations (Chan et al 1986, Plesková et al 1984), also there is a correlation between the frequency of aberrations and the degree of cell growth inhibition caused by cisplatin (Plesková et al 1984). Furthermore, one of the most frequent types of chromosome aberrations found was the chromatid interchange in the dicentric chromosome (Plesková 1984). Aberrations are sure to occur from exposure to cisplatin and low-dose irradiation, the combination yet unreported was studied in this project.

1.6.4 Effect of Thiol Depletion Glutathione (GSH) is the major intracellular non-protein thiol. At a concentration of 0.5 to 10 mM it protects cells from

damage caused by radiation, and from many drugs including cisplatin. L-buthionine-SR-sulfoximine (BSO) is a selective inhibitor of γ-glutamylcysteine synthesis (Griffith et al 1979). The administration of BSO, and thus the depletion of cellular GSH does not influence rate of cell growth, amount of cell protein, and chromosome structure during a 24 hour cell culture (Ochi et al 1988).

The interaction of GSH with platinum-amines is of increasing research focus, because changes in cellular GSH concentration may be correlated with platinum resistance, and reduced toxicity in cells. Two possible mechanisms have been postulated for detoxification of platinum coordination complexes by glutathione. First, GSH may inhibit the reaction of cisplatin with DNA by binding to it before it reaches the DNA. Second, GSH may bind to monofunctional DNA adducts, and prevent them from arranging to toxic bifunctional adducts (Lempers 1990).

GSH is also known to be involved in cellular protection from X-rays, where it is one of the important non-protein thiols involved in the protection against radical anions formed during irradiation. Cells treated with BSO have a significant sensitization to X-rays, particularly under hypoxic conditions. Furthermore, it has also been shown that cells with higher GSH levels were protected from X-rays. In addition to other functions GSH may be involved with radiation by preventing DNA-protein cross-links. Thus, GSH depletion was used to further study the possible involvement of cross-links in the interaction of cisplatin with X-rays.

1.6.5 The Interaction of Cisplatin Analogs and Low Doses of X-Rays To further explore the mechanism of cisplatin's enhancement of X-ray damage, and to focus on the role of cross-links, 5 complexes with different cross-linking potential were used. A number of experiments with *trans*-DDP have been done in this laboratory (Skov et al 1989). It should also be noted that clinically used cisplatin analogs have been studied (MacPhail and Skov 1991). The 5 analogs used were (see figure 2b for structures):

1. Monoamminetrichloroplatinum(II) $[Pt\,Cl_3\,(NH_3)]^-$

2. Chloro(diethylenetriamine)platinum(II) chloride $[PtCl(dien)]^+Cl^-$

3. Tetraammineplatinum(II) $[(NH_3)_4\,Pt]Cl_2$

4. Bis(Platinum)*trans* $[\{trans\text{-}PtCl_2(NH_3)\}_2NH_2(CH_2)_5NH_2]$

5. Bis(Platinum)*cis* $[\{cis\text{-}PtCl_2(NH_3)\}_2NH_2(CH_2)_5NH_2]$

2. Materials and Methods

2.1 Drug Preparation

Cisplatin was a gift from Bull Laboratories (Mulgrave, Victoria, Australia). Cisplatin analogs were from the laboratory of Dr. N. Farrell. Drugs were always dissolved by stir bar stirring for 30 minutes in cell culture media (described below); bis(platinum) drugs needed longer stirring (up to 45 to 50 minutes). All drugs were then filtered using a sterile MILLEX-GV 0.22 μm filter unit. Drugs were then considered ready for cell culture use, and were used within 8 hours. However, bis(platinum) drugs were stored at -35°C, for future use.

2.2 Treatment of Cells

2.2.1 V79 Cells V79-171B cells were obtained from Dr. R.E. Durand. Cells were maintained as exponential cultures by twice weekly subculture, and grown as monolayers on Falcon plastic culture flasks in Eagle's MEM with 10% fetal bovine serum (both from GIBCO, Burlington, Ont.). Two days before irradiation, 1 x 10^6 cells were passaged into a large plastic tissue culture flask (75 mm^3, Falcon), where they were grown as monolayers in Eagle's MEM with 10% fetal bovine serum. On the day of irradiation, cells were trypsinized as exponentially growing cultures; if cisplatin were to be

added before irradiation, the cells were then maintained in spinner flasks (at 1.5 x 10^5 cells/ml) under optimal growth conditions for 1 hour at 37° C to recover from trypsinization. For cells irradiated prior to drug exposure, samples were taken after each radiation exposure, and cisplatin treatment was then given in separate suspensions at 37° C. Before irradiation, and during the recovery time, glass irradiation vessels with 14.5 ml of bicarbonate-free medium with 10% fetal bovine serum were kept in a 37° C water bath, and gassed with humidified oxygen free nitrogen for at least 45 minutes before the addition of cells. Oxygen electrode measurements show that sufficient radiobiological hypoxia (oxygen < 1 μM) is achieved under these conditions (Palcic and Skarsgard 1984). When the hour recovery was over, cells were taken from the growth spinner and concentrated by centrifuging for 5 minutes at 93 x g. The control tube was resuspended with 0.5 ml of media, while the cells to be treated with cisplatin were resuspended in 0.5 ml of media with 2.5 mM of drug. These cells were then added to the irradiation vessels (1.5 x 10^5 cells/ml), and incubated for 1 hour at 37° C. Cell stirring and nitrogen flow was continued throughout the experiment. Immediately after this treatment, vessels were placed on ice to minimize cisplatin toxicity and prevent repair during and/or after the exposure to graded doses of X-rays. The vessel and cell suspensions used for low doses of X-rays were also used for high doses of X-rays. The source of X-rays was a Philips RT250, with 250 kVp, using a 0.5 mm Cu filter, to give 3.12 Gy/minute. After a particular X-ray dose, approximately 1 ml was removed from the irradiation vessel and diluted into ice cold media. This mixture was

then centrifuged to remove unbound cisplatin, and was resuspended again in ice cold media to minimize clumping and/or further toxicity. Immediately following this, cells were plated in Eagle's MEM with 10% fetal bovine serum. For aerobic conditions, cells were treated in the same manner, except nitrogen gassing was omitted. The typical experimental results from this type of standard experiment are shown in figure 1.

2.2.2 CHO Cells CHO cells were obtained from Dr. G.F. Whitmore, Ontario Cancer Institute and have been used by members of the Medical Biophysics Unit in Vancouver for over 20 years. Cells were grown in spinner culture at 37^{o} C in alpha medium (GIBCO) supplemented with 10% fetal bovine serum (GIBCO). Exponential growth of the cells was maintained by diluting them daily to approximately 1 x 10^5 cells/ml. The doubling time for these cells was 12 to 13 hours. On the day of each experiment prior to irradiation cells were removed from spinners, and concentrated by centrifugation at 93 x g for 5 minutes. Cells were then resuspended at 2 x 10^5 cells/ml in alpha growth medium containing either medium alone or medium plus 2.5 μM cisplatin, and treated as above (V79 cells).

2.2.3 Survival Assays The survival of cells irradiated with low doses of X-rays were evaluated with the use of the Cell Analyzer Imaging System. This system has been described in detail elsewhere (Palcic and Jaggi, 1990), and facilitates the following of approximately 300 to 500 initially plated distinct

cells, which grow or do not grow into colonies. In contrast, high doses of X-rays up to 30 Gy were assayed by the conventional clonogenic assay (Moore et al 1976). In both assays, fifty or more cells in a colony were considered to be derived from a surviving cell. The plating efficiency (PE) was calculated as the number of survivors divided by the number of cells plated. For assessing the effects of various drug treatments, a compound can be considered "non-toxic" if the PE is the same as control. However, as it will be shown later (sections 3 and 4), some effects are masked by this arbitrary definition of survival.

2.2.4 Colony Size The size of colonies that resulted from cells exposed to high doses of X-rays were estimated to be either large or small, by following earlier procedures used by Sinclair (1964). He found that 13 days after irradiation V79 control colonies (doubling time about 11 hours) had a mean diameter of approximately 2 mm. In the present experiments CHO cells (mean (n = 6) doubling time 11.45 (SE = 0.62) were only allowed 7 days before staining with malachite green. By inspection of CHO colonies under a microscope the mean size of control colonies was approximately 1 mm. Thus small colonies were considered less than 1 mm in diameter, and large colonies were considered greater than or equal to 1 mm.

The estimation of the average size of colonies exposed to low-doses of radiation was performed using the cell analyzer imaging sytem, using additional softwear programs writen by Dr. Ingrid Spadinger. Features of

surviving colonies were stored and then analyzed by Dr. Spadinger's programs, giving the mean colony size of survivors, and also the size of the largest and smallest colonies.

2.3 Procedures for Timing Studies

The same cell handling procedures were used as above, except for as follows: During the defined repair periods, cells were maintained in a cell suspension at 37°C using a water thermostated cell shaker bath.

A) When cisplatin was to be given after X-rays, the cells were diluted into ice cold media immediately following X-ray treatment and then centrifuged. Following this the cells were resuspended in 10 ml of media and added to 50 ml erlenmeyer flasks fitted with rubber stoppers allowing air flow. These flasks were then placed in the cell shaker bath for the desired time period to allow for repair before cisplatin treatment. Cisplatin was then added to the cell suspensions by pipette, and when the one hour exposure was over the cells were centrifuged and plated as described in section 2.2.1.

B) When cisplatin was given before X-rays, immediately following cell preparation cells were added to erlenmeyer flasks were they were exposed to 2.5 μM cisplatin for 1 hour in the cell shaker bath. After cisplatin exposure, cells were diluted in ice cold media. This mixture was centrifuged to remove unbound cisplatin, and resuspended in media. These cells were then added

to the erlenmeyer flasks and placed in the cell shaker bath at 37°C for the desired repair time period. After this the cells were centrifuged, and added to irradiation vessels in preparation for X-ray treatment as described in section 2.2.1.

2.4 Chromosome Analysis

For the preparation of chromosome samples, CHO cell suspensions from irradiation experiments (as described in section 2.2) were grown on glass cover slips placed at the bottom of plasic culture dishes (Nunc). At approximately 16 hours post radiation treatment, 0.2 ml of 0.01% colchicine (Sigma) was added to each culture dish, and incubated for 2 hours. Cells were then harvested by the following method: Cover slips with cells attached were removed and immersed in a 1% sodium citrate solution for 20 minutes. The cover slips were then removed and placed in a Carnoy's solution (200 ml acetic acid and 600 ml ethanol) for 10 minutes. The Carnoy's fixative was then drained off, and allowed to air dry for 30 minutes before staining. For staining, the cover slips were placed in metal staining racks and stained for 5 minutes with a 2% acetic orcein solution. To study chromosomal aberrations, 20 metaphases were analysed (Suzuki et al 1990), while the determination of type of aberration was scored by the method of Savage (1975). Samples were scored without knowing what treatment the slide in question had recieved (blind).

2.5 Use of BSO to Deplete GSH

Cells for GSH depletion studies were pre-exposed to 100 μM BSO for 20 hours. Otherwise, cell handling and drug exposure procedures were the same as described above. To assess the effectiveness of the BSO pretreatment in lowering GSH levels, cells treated with BSO were examined for non-protein sulfhydryl (NPSH) content using a spectrophotometric assay, encorporating 5,5-dithiobis(2-nitrobenzoic acid) (DNTB, Ellman's reagent) which binds non-protein sulfhydryls. The resulting change in optical density was read at 412 nm. Results showed that non-protein thiol levels were reduced to concentrations that were less than 10% of control cells.

2.6 Analysis of Data

The experimental design of these low radiation dose experiments follows the pioneering work of Palcic, who (among others) found that there is an accurate way to measure small numbers of non-survivors. In classically performed radiation experiments, accurate data can be obtained by measuring colony forming ability. However, at low-doses where most cells survive this method does not yield accurate data due to errors made during counting, pipetting, plating and sampling. The use of the cell analyzer imaging system eliminates the need to be concerned about errors made in counting, pipetting, and plating, because it accurately determines the number and location of cells plated, and then the survivors and non-survivors

from this group. Sampling errors can be minimized only by the inclusion of large numbers of identical cells (Palcic and Jaggi 1986). Thus for these experiments 15 000 cells were followed for a typical experiment.

Statistical methods used in this thesis to examine the difference between treatments in the averaged survival response of a particular experiment include:

1) range
2) standard deviation
3) variance
4) standard error
5) students t-test

These tests were applied to sets of averaged data (n greater than 3 unless indicated). These data were then fitted to parameter values alpha and beta of the quadratic model, $S=\exp(-\alpha D -\beta D^2)$, obtained by fitting the model to each of the averaged dose responses.

3. Results

3.1 The Effect of Timing Cisplatin and X-Rays

3.1.1 Cisplatin given after X-rays The response of hypoxic V79 cells to low doses of X-rays and a one hour repair period before exposing to 2.5 μM cisplatin is shown in figure 3. The results show that after one hour the enhancement is not changed from that of cells which did receive repair time. The ERs were 1.02 ± 0.01 at SF = 0.01 and 1.92 ± 0.07 at SF = 0.8, the enhancements for no repair time are 1.92 ± 0.03 at SF = 0.8, and 1.06 ± 0.02 at SF = 0.01, which are similar to studies by Korbelik and Skov (1990). These results were obtained at 2.5 μM cisplatin, where the toxicity in hypoxia or air due to cisplatin is minimal. CHO cells were also used in identical experiments, and again the ER remained unchanged. The ERs for CHO cells were 1.6 ± 0.02 at SF = 0.8, and 1.03 ± 0.01 at SF = 0.01.

Longer repair periods of 2 and 3 hours after radiation were also examined. The results (figures 4 and 5) are similar to those obtained for the 1 hour repair period. Again there is no change in the ER in hypoxic V79 cells, and the same result is found for CHO cells (figures 7 and 8).

The next experiments extended the repair period after x-rays for 1 hour intervals up to 8 hours. Full dose response curves were not done, but 0 and 2 Gy plating efficiencies were calculated at each hour. Cisplatin at 2.5 μM and control treated cells were used. The surviving fraction (SF) of cells at

each time point was calculated. For cells exposed to cisplatin after the specified repair time, the 0 or 2 Gy surviving fraction did not significantly change for time periods of 4 to 8 hours (figures 7 and 8). SF at 2 Gy for these cells was 0.8 $\pm$ 0.02 for CHO cells, and 0.7 $\pm$ 0.06 for V79 cells.

3.1.2 Cisplatin given before X-rays Repair time was also allowed to cells treated with cisplatin before irradiation. These experiments (figures 7, 8, 9, 10 and 11) gave up to 3 hours of repair of cisplatin damage and did not show a change in the ER as compared to hypoxic cells exposed to cisplatin immediately before or after X-rays.

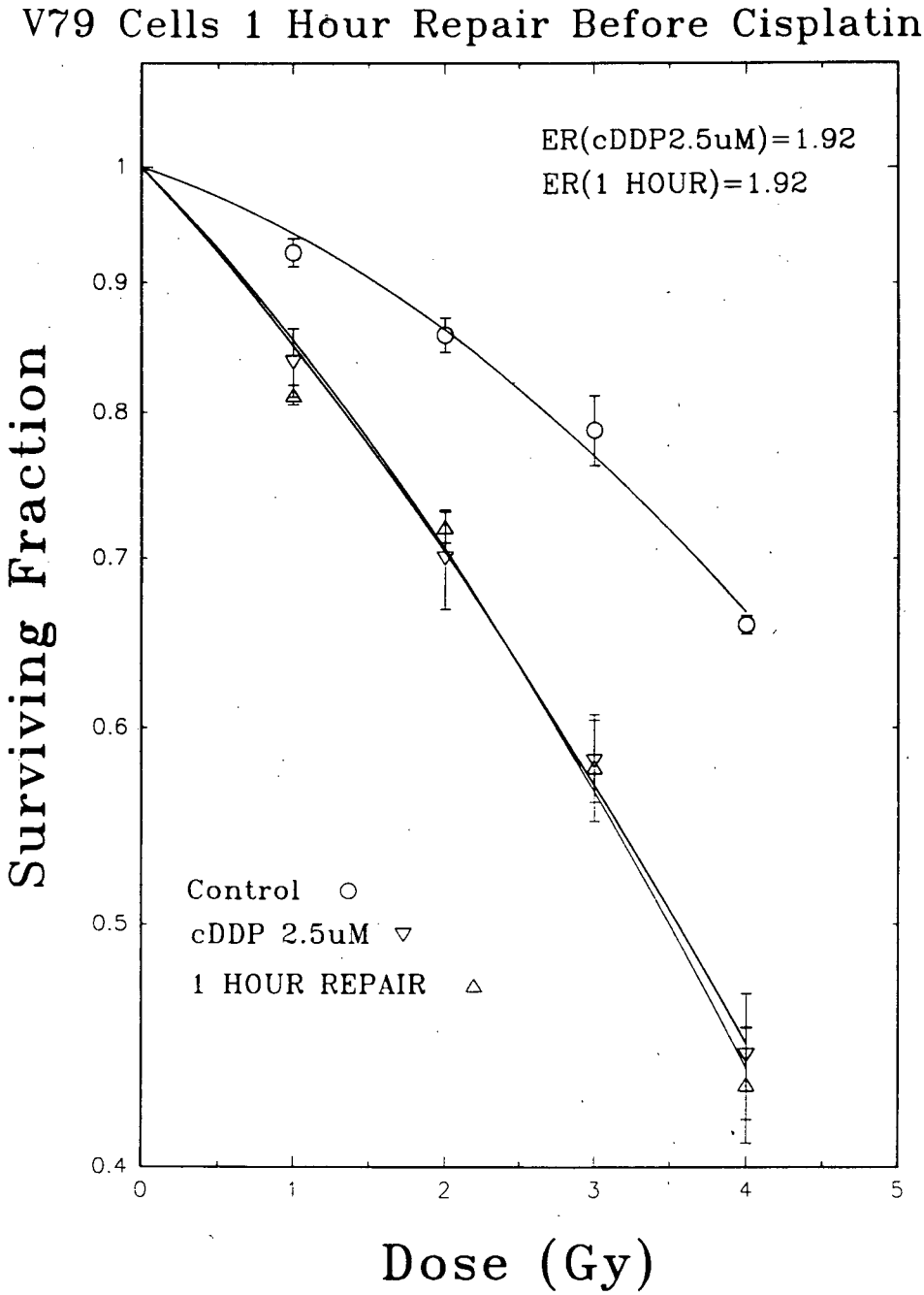


Figure 3. Cisplatin enhancement of hypoxic V79 cell inactivation by X-rays with or without a 1 hour repair interval.

Exponentially growing V79 cells were made hypoxic and exposed to 2.5 μM cisplatin before irradiation (standard experiment, as in figure 1), or allowed 1 hour repair after X-rays before cisplatin treatment. Cisplatin exposure was for 1 hour at 37° C in air. Repair was in air. All irradiations were done at 0°C. The ER was measured at SF = 0.8. Data for drug treatment and control cells were normalized, drug toxicity was very low at this concentration (see introduction section, and materials and methods). Error bars are for standard error (SE) of 3 independent experiments.

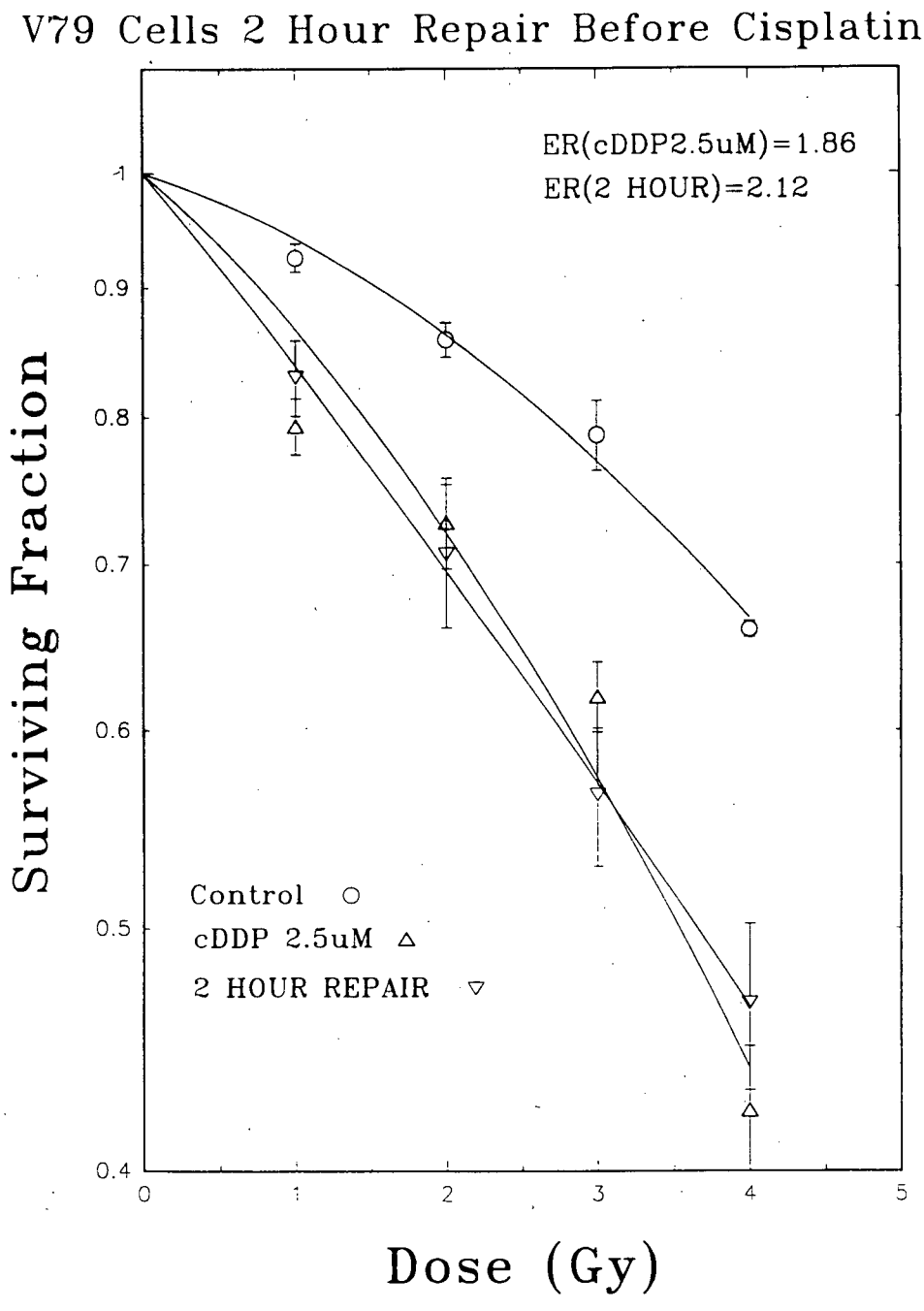


Figure 4. Cisplatin enhancement of hypoxic V79 cell inactivation by X-rays, after 2 hour repair.

V79 Cells were treated as in figure 3, except 2 hours of repair was allowed after X-rays

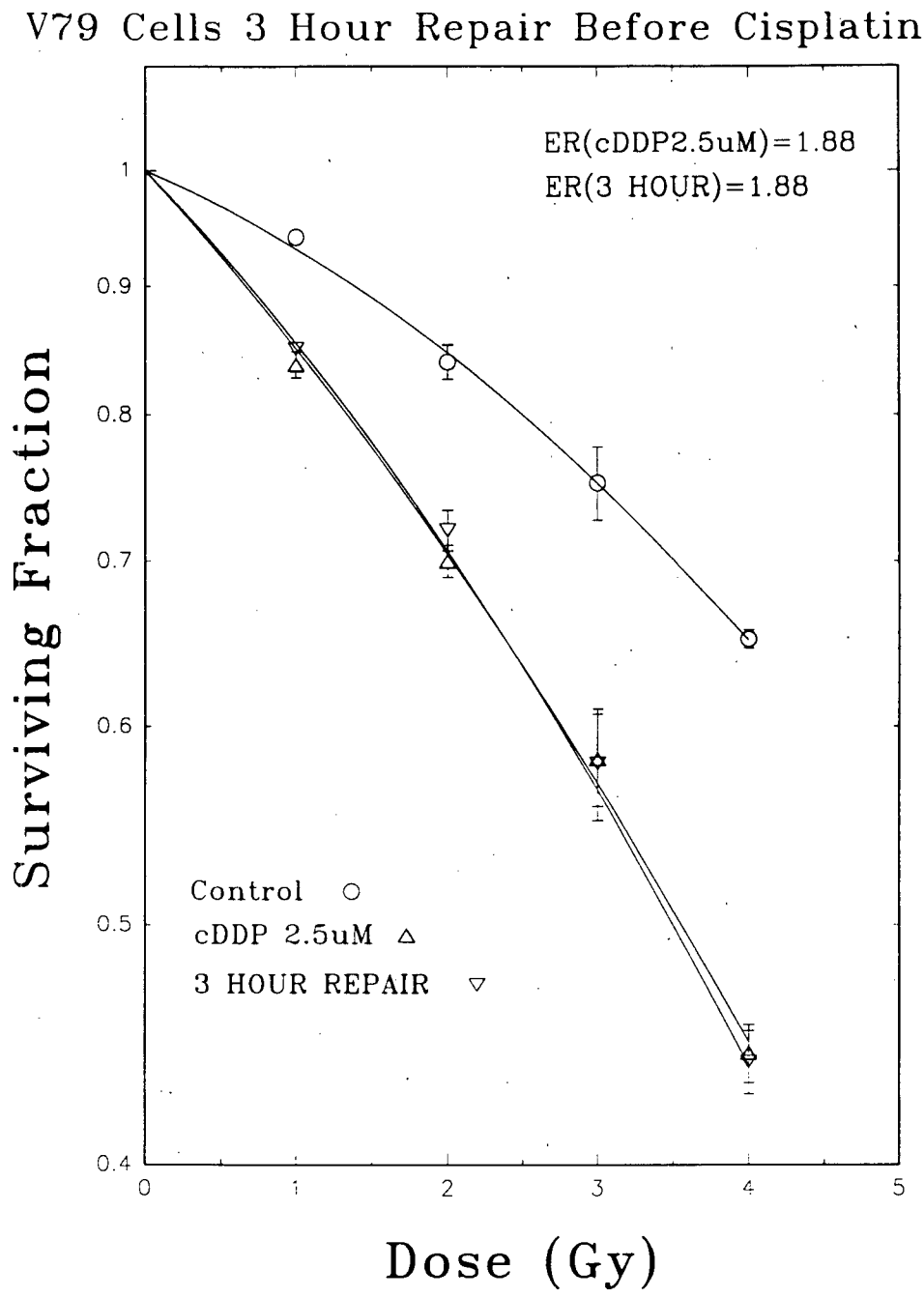


Figure 5. Cisplatin enhancement of hypoxic V79 cell inactivation by X-rays, after 3 hours of repair.

V79 cells were treated as in figure 3, except 3 hours of repair time was given.

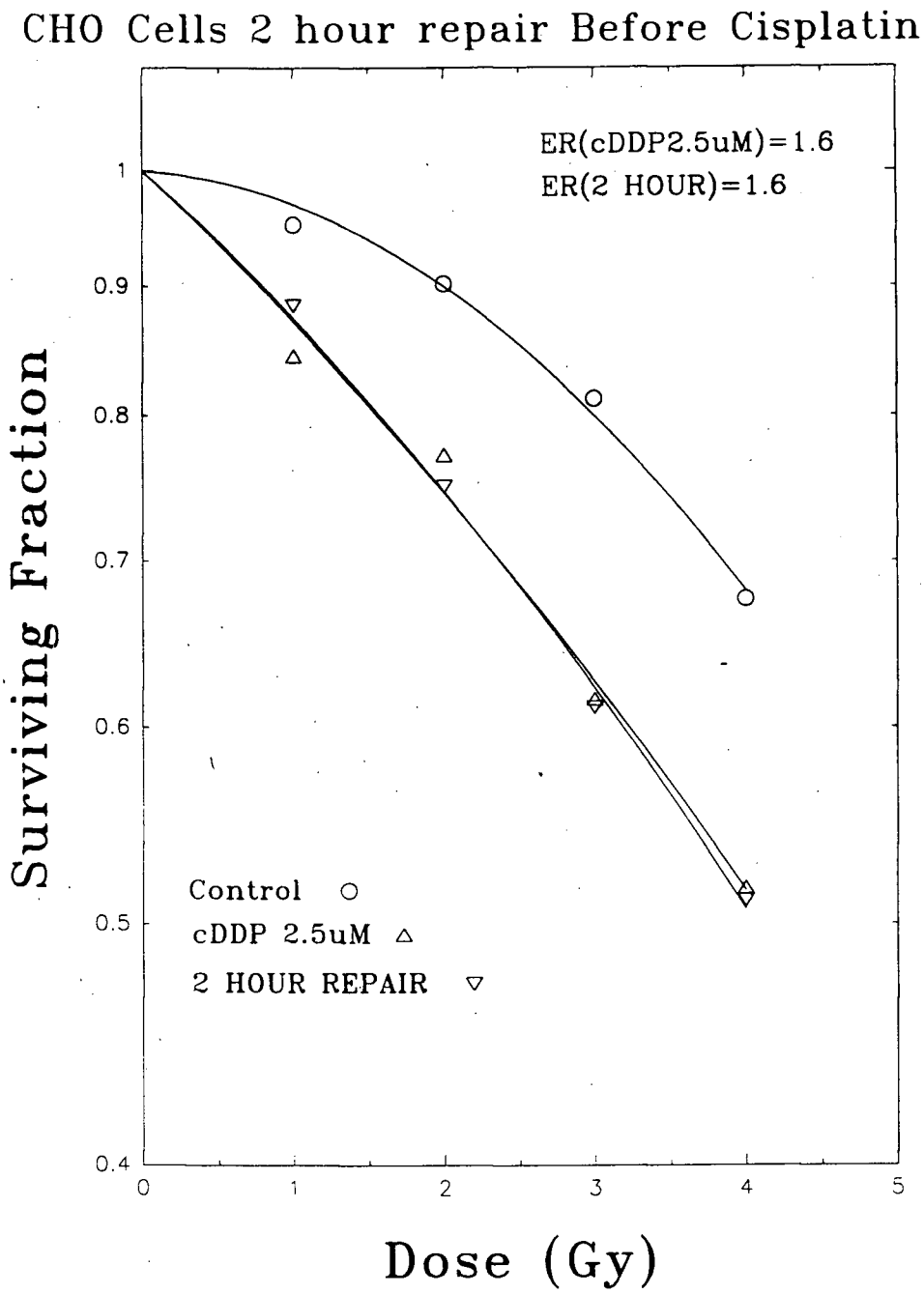


Figure 6. The effect of 2 hours of repair after X-rays with CHO Cells
Exponentially growing CHO cells were treated the same as V79 cells in figure 4, except they were allowed 2 hours of repair before cisplatin treatment.

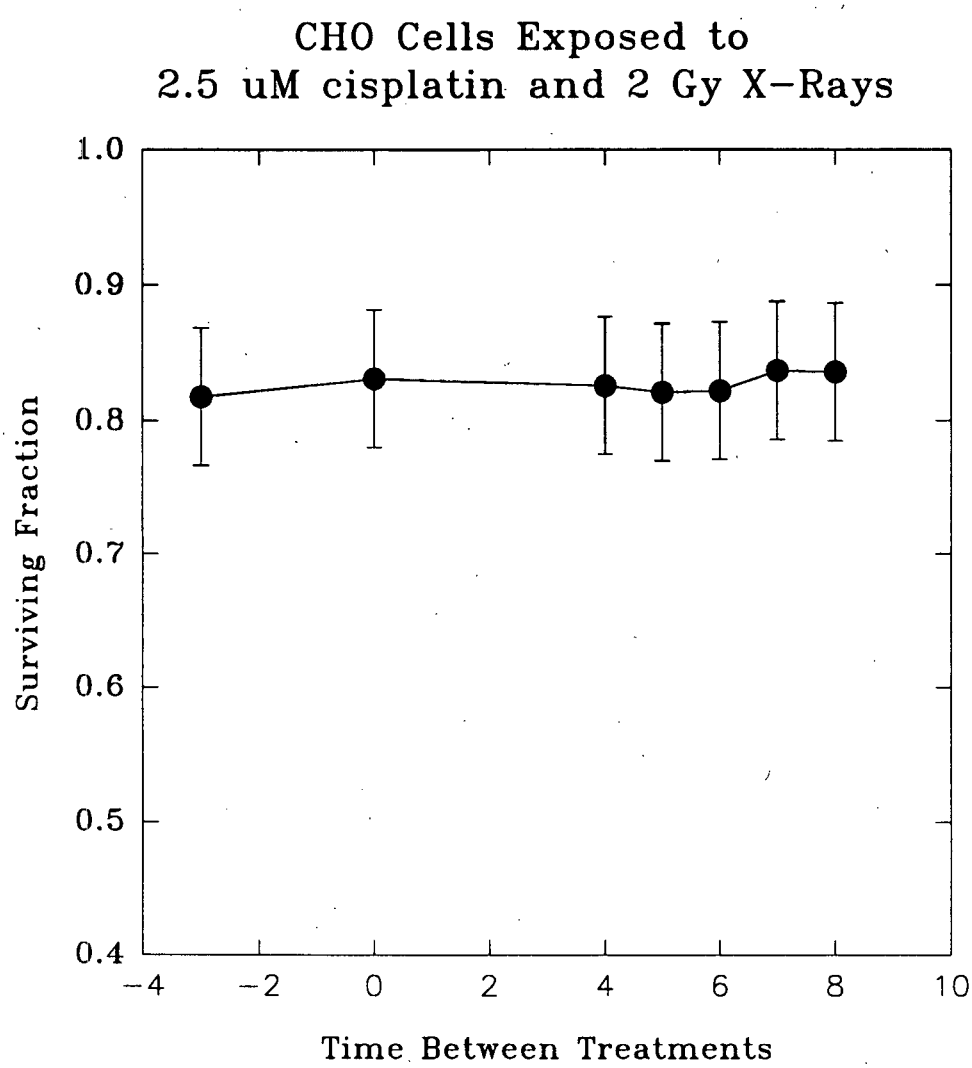


Figure 7. Summary of the effect of repair time on cisplatin enhancement of radiation inactivation of hypoxic CHO cells.

Cells were treated as in figure 3, except the repair interval was extended to 8 hours.

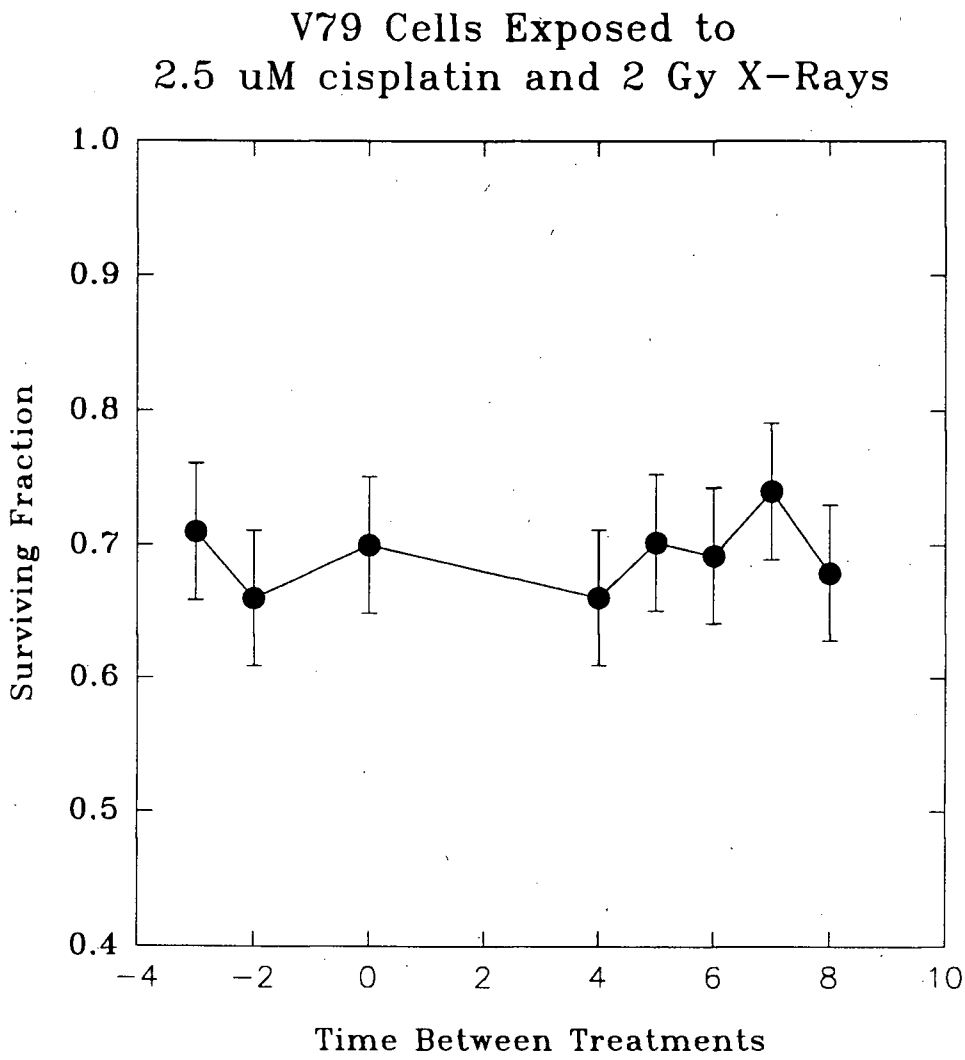


Figure 8. Summary of the effects of repair time on the cisplatin inactivation of hypoxic V79 cells.

V79 Cells were treated as in figure 7.

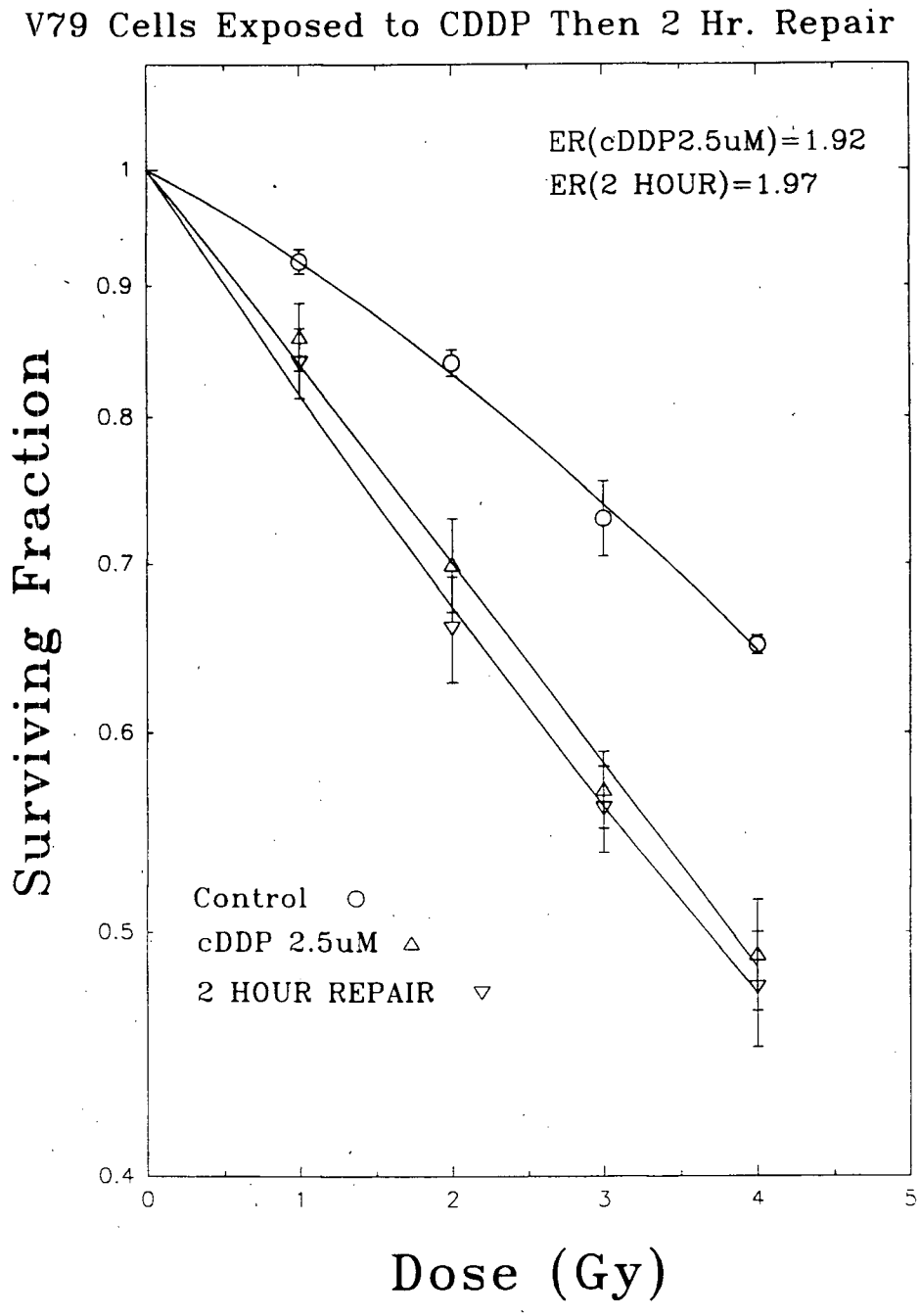


Figure 9. The effect of 2 hour repair after cisplatin treatment prior to irradiation on the cisplatin enhancement of hypoxic V79 cell inactivation by X-rays.

V79 cells were treated as in figure 7, except repair was allowed after cisplatin treatment for 2 hours.

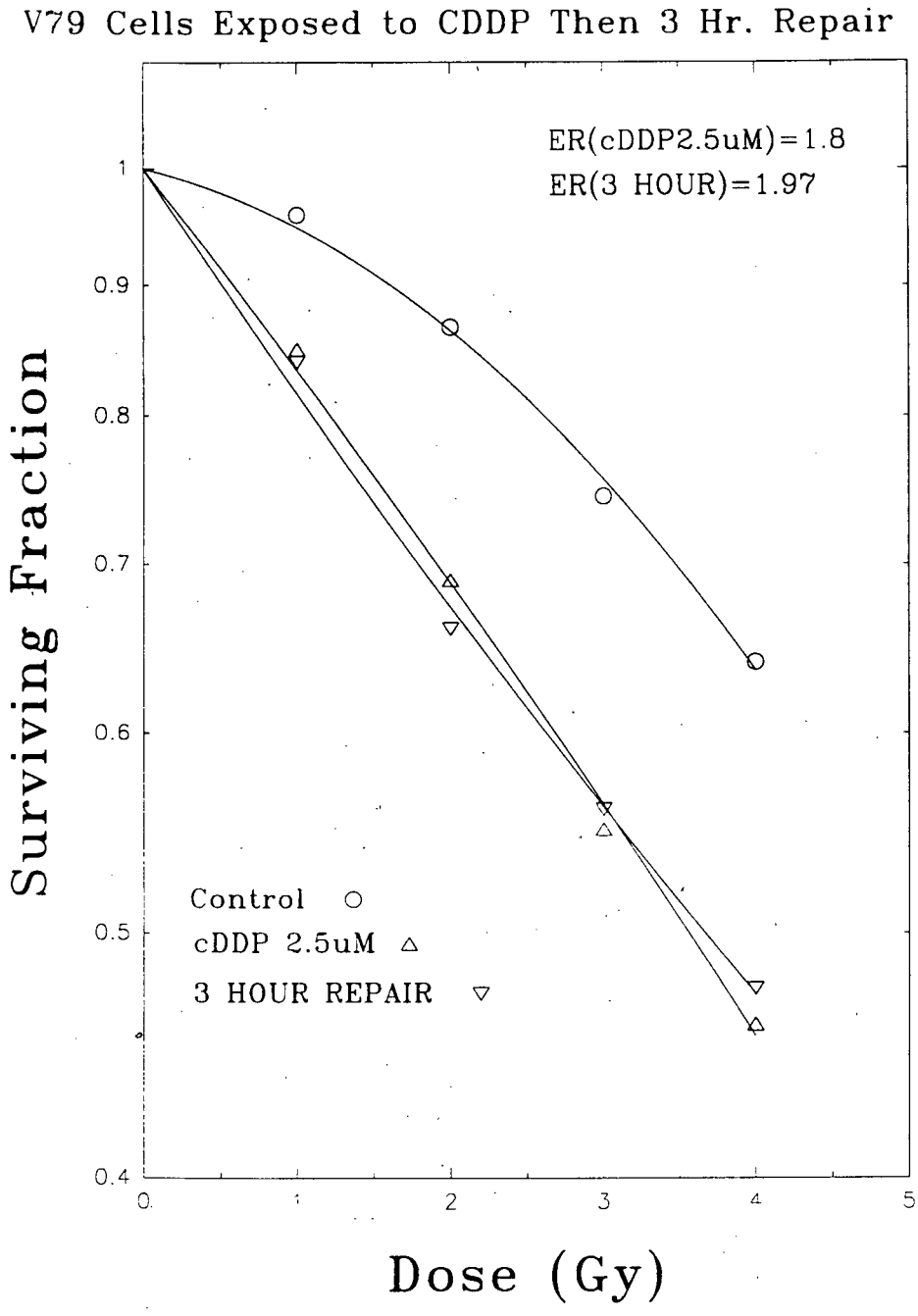


Figure 10. The effect of 3 hours of repair after cisplatin treatment on cisplatin enhancement of hypoxic V79 cell inactivation by X-rays.

V79 cells treated as in figure 9, except 3 hours of repair are allowed after cisplatin treatment.

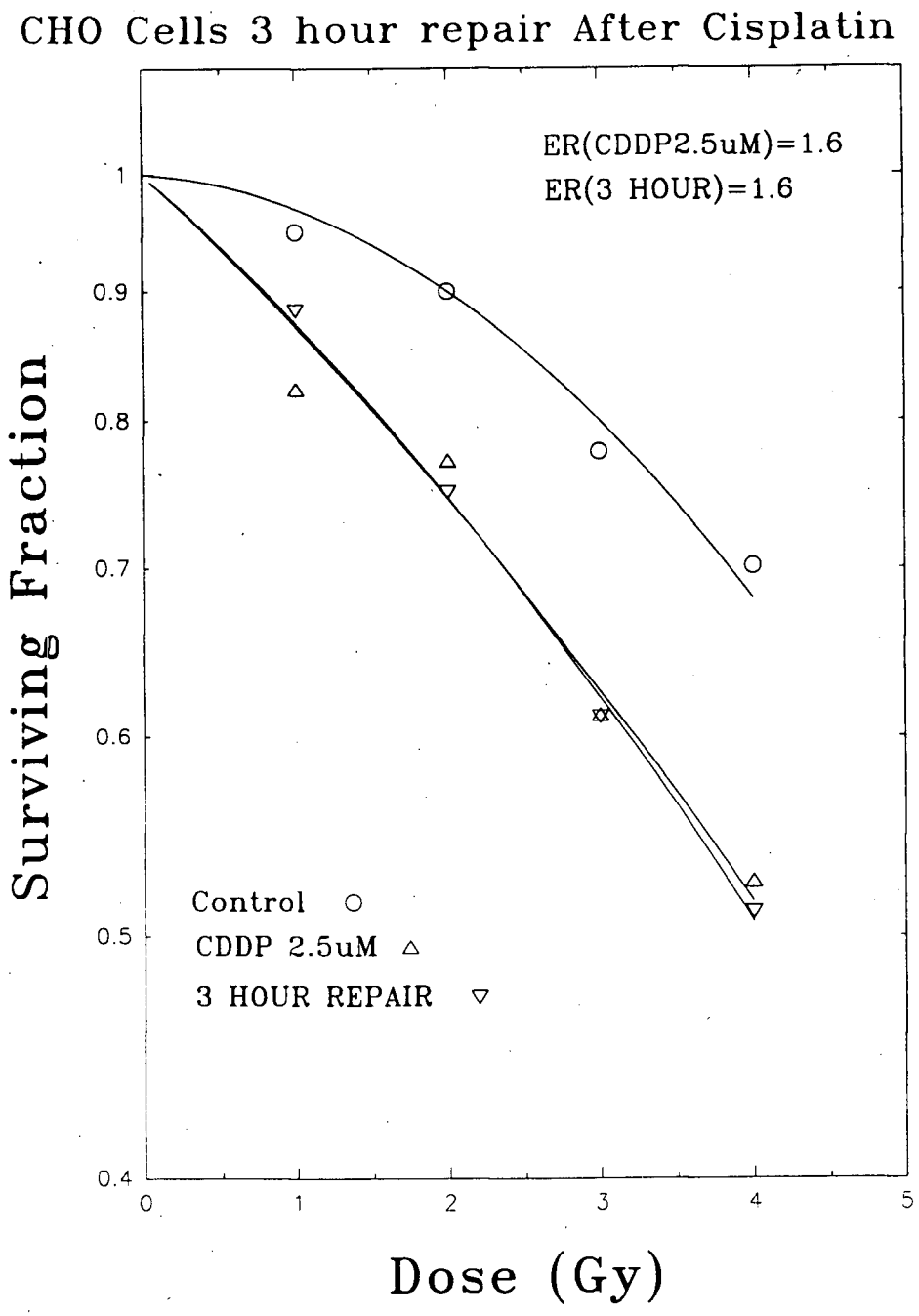


Figure 11. The effect of 3 hours of repair after cisplatin treatment, on the cisplatin enhancement of hypoxic CHO cell inactivation by X-rays.

Cells were treated as in figure 9, except cells were allowed 3 hours of repair after cisplatin treatment.

3.2 Colony Size Analysis

Figure 12 shows two photomicrographs, one of typically large control colonies, and the other of small colonies which occur after irradiation (3Gy). Analysis of colony size for low doses of X-rays shows that there is an increase in the number of small colonies for increasing doses of irradiation (Figure 13). When cells are preincubated with 2.5 μM cisplatin before X-rays, there are still smaller surviving colonies (per dose of irradiation) when compared with controls. Figure 14 shows photographs of malachite green stained colonies from high dose experiments. The top photo shows control colonies were approximately 165 cells were plated to produce the colonies shown. The bottom photo shows colonies which grew from 8 000 cells that had been irradiated in hypoxia with 20 Gy. These photos clearly show the smaller colonies produced from cells irradiated with high doses of X-rays. Figure 15 shows the effect of high doses of X-rays on CHO colony size. This figure clearly shows the decrease in colony size as a function of dose. The cells which gave rise to these colonies were irradiated in hypoxia. These results are similar to those of Sinclair (1964), and Nias (1968) for cells irradiated in air.

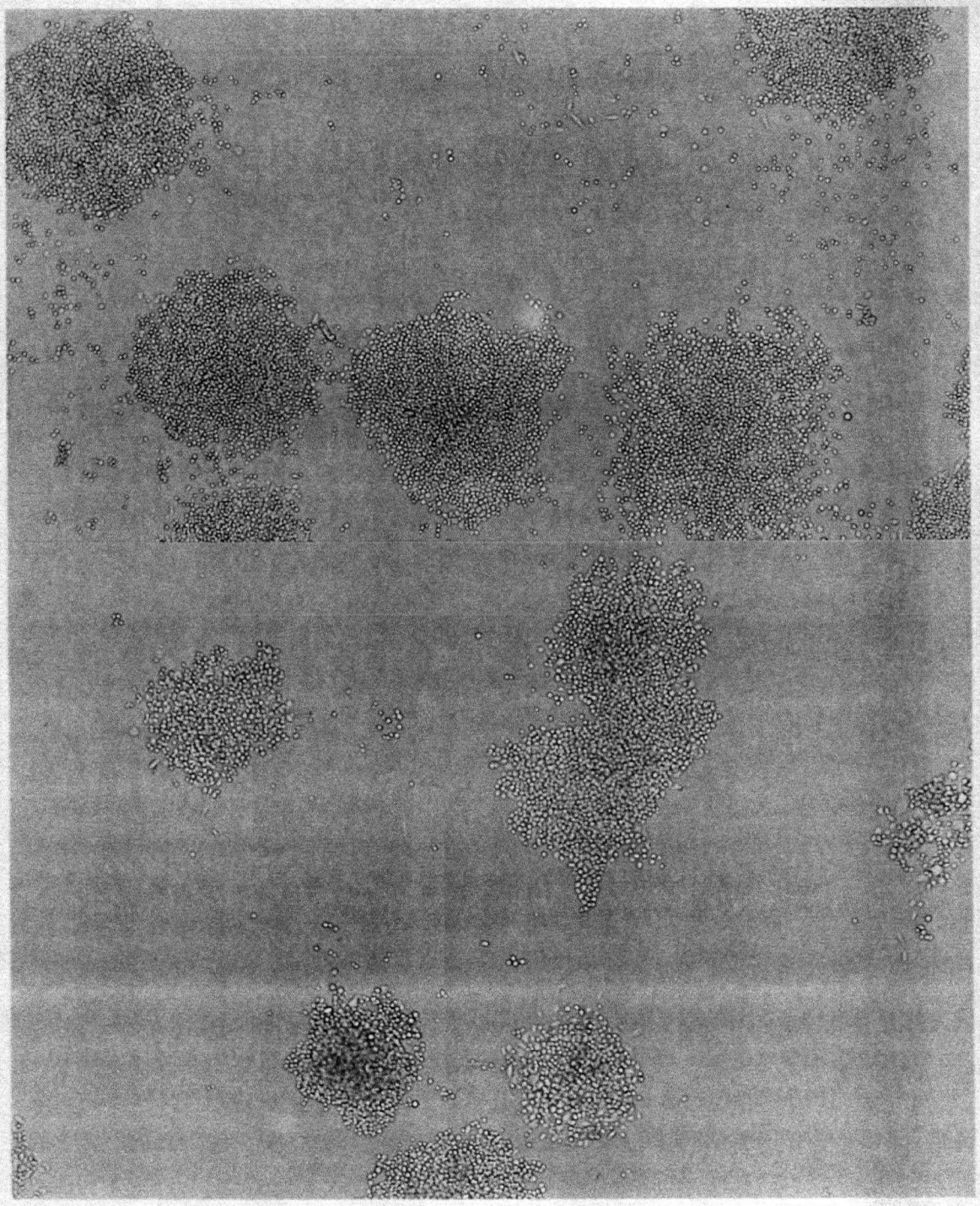


Figure 12. Photomicrographs of surviving colonies after low-doses of X-rays given to hypoxic cells.

Top photo shows control CHO colonies (not exposed to irradiation). Bottom photo shows CHO colonies grown from irradiated cells (3 Gy). 50 X magnification, Nikon microscope.

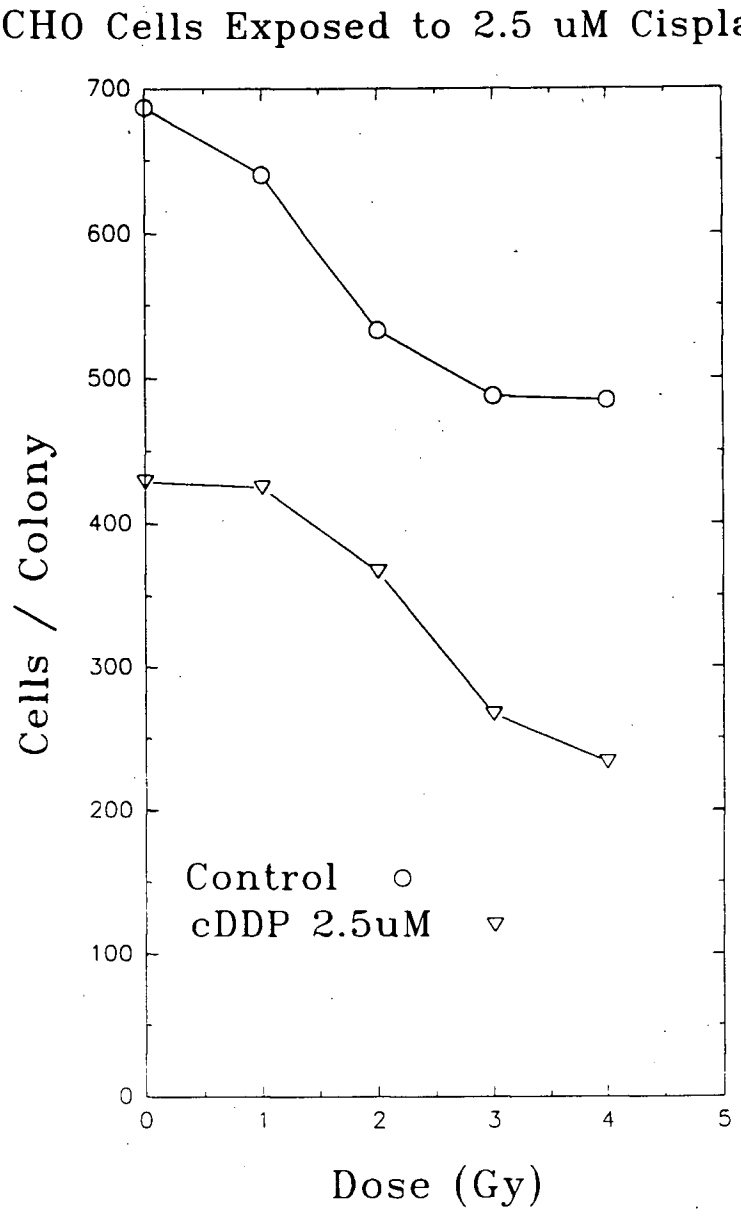


Figure 13. Average colony size of surviving cells having been exposed to low-doses of X-rays in hypoxia with or without cisplatin pretreatment

Colony size was estimated using the cell analyzer imaging system. 300 to 500 colonies were sized per dose point. Results are from a single experiment.

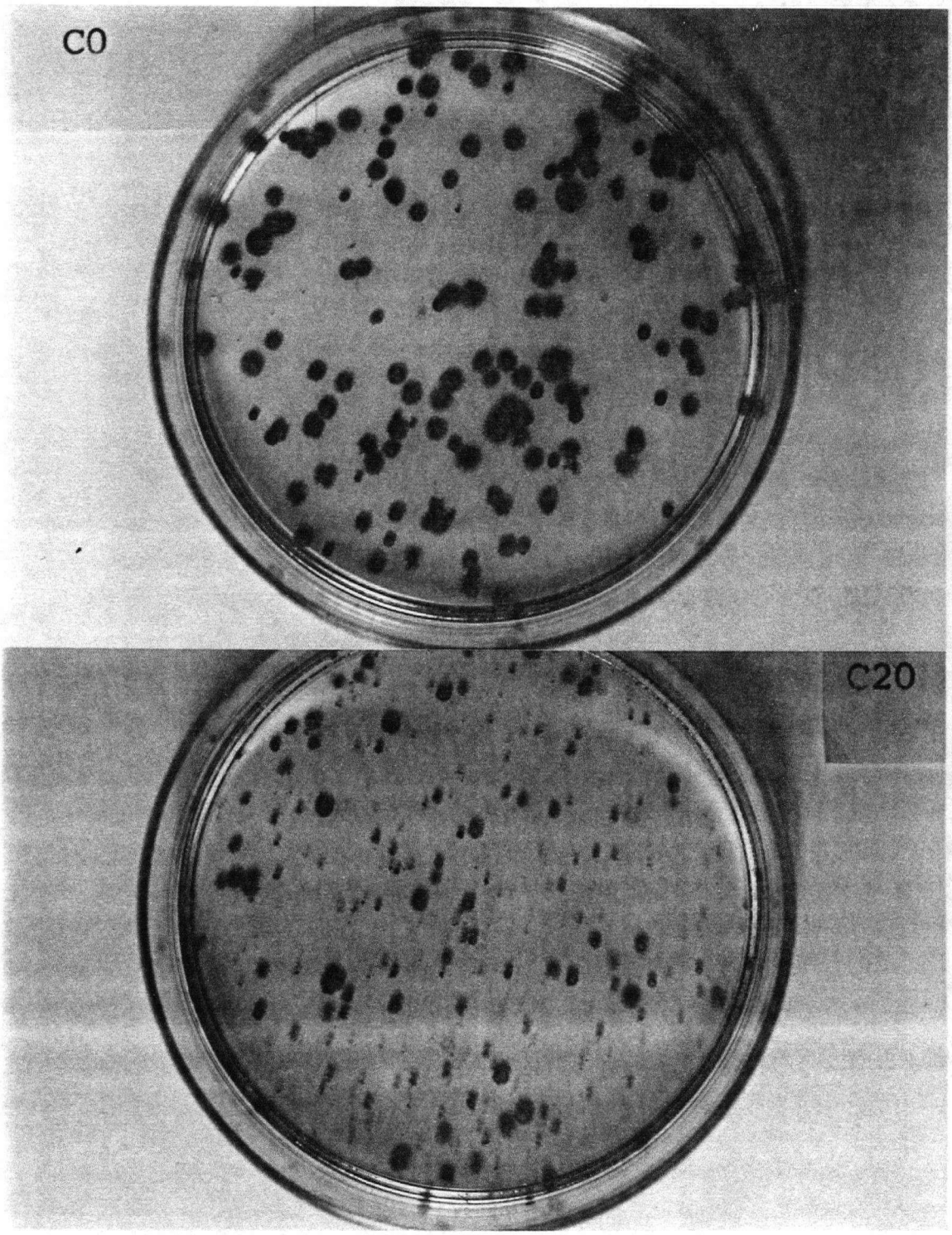


Figure 14. Colonies of CHO cells having been exposed to 0 Gy (top), and 20 Gy (bottom) in hypoxia.

CHO cells were grown for 7 days, before staining with malachite green.

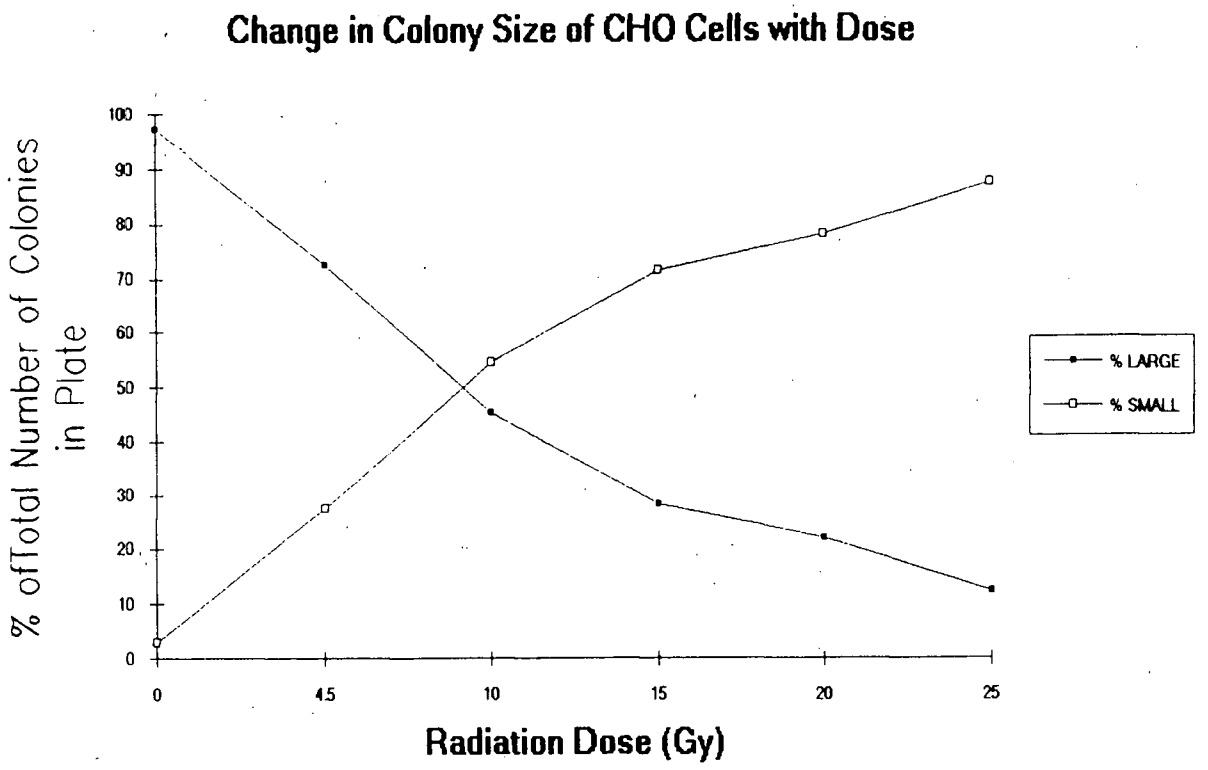


Figure 15. The effect of X-irradiation on surviving colony size after CHO cells were exposed to high doses X-rays in hypoxia.

Colony size was divided as large colonies being greater than 1 mm, and small colonies being smaller than 1 mm. Results are from a single experiment, but were typical for all experiments.

3.3 Induction of Chromosome Aberrations

Figure 17 shows photomicrographs of normal and aberrant chromosomes. All comparisons between treatments were made after the first mitosis post irradiation (approximately 16 hours). Figure 16 shows the formation of the number of gaps (plot A), breaks (plot B), and exchanges per cell (plot C). All plots show the dependence of radiation induced chromosome aberrations on the presence of oxygen (open symbols). The OER for chromosome exchanges was 2.9 at 0.25 exchnges per cell, and 2.5 at 0.5 exchanges per cell. The pre-exposure of cells to cisplatin before irradiation in hypoxia has a clear effect on gaps, breaks and exchanges, and the formation of aberrations in cells not exposed to radiation was expected and agrees with the experiments of Chan et al (1984), and Plesková et al (1984). The interesting finding was that cisplatin caused a dose modifying effect on radiation induced chromosome aberrations. Normalizing for exchanges in chromosomes of cells treated with cisplatin but not exposed to X-rays, the ER for exchanges in hypoxic cells was 1.75 at 0.5 exchanges per cell (approximately 3.4 Gy in hypoxia without cisplatin). In aerobic cells, the ER was 1.0. These results are similar to survival data in this project.

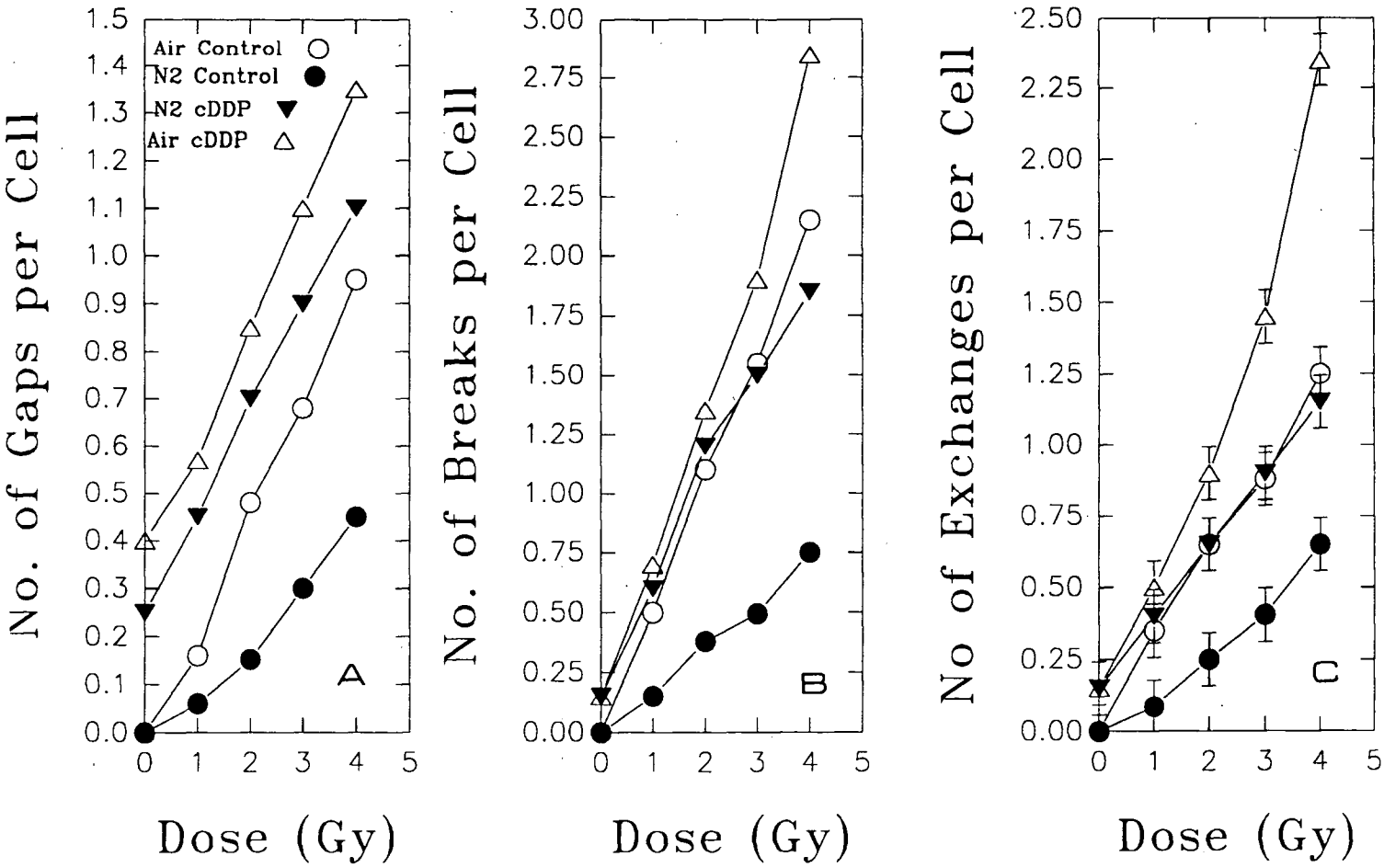


Figure 16. Induction of various types of chromosome aberrations.
CHO cells were treated with or without 2.5 μM cisplatin, then irradiated in air or hypoxia, as indicated.

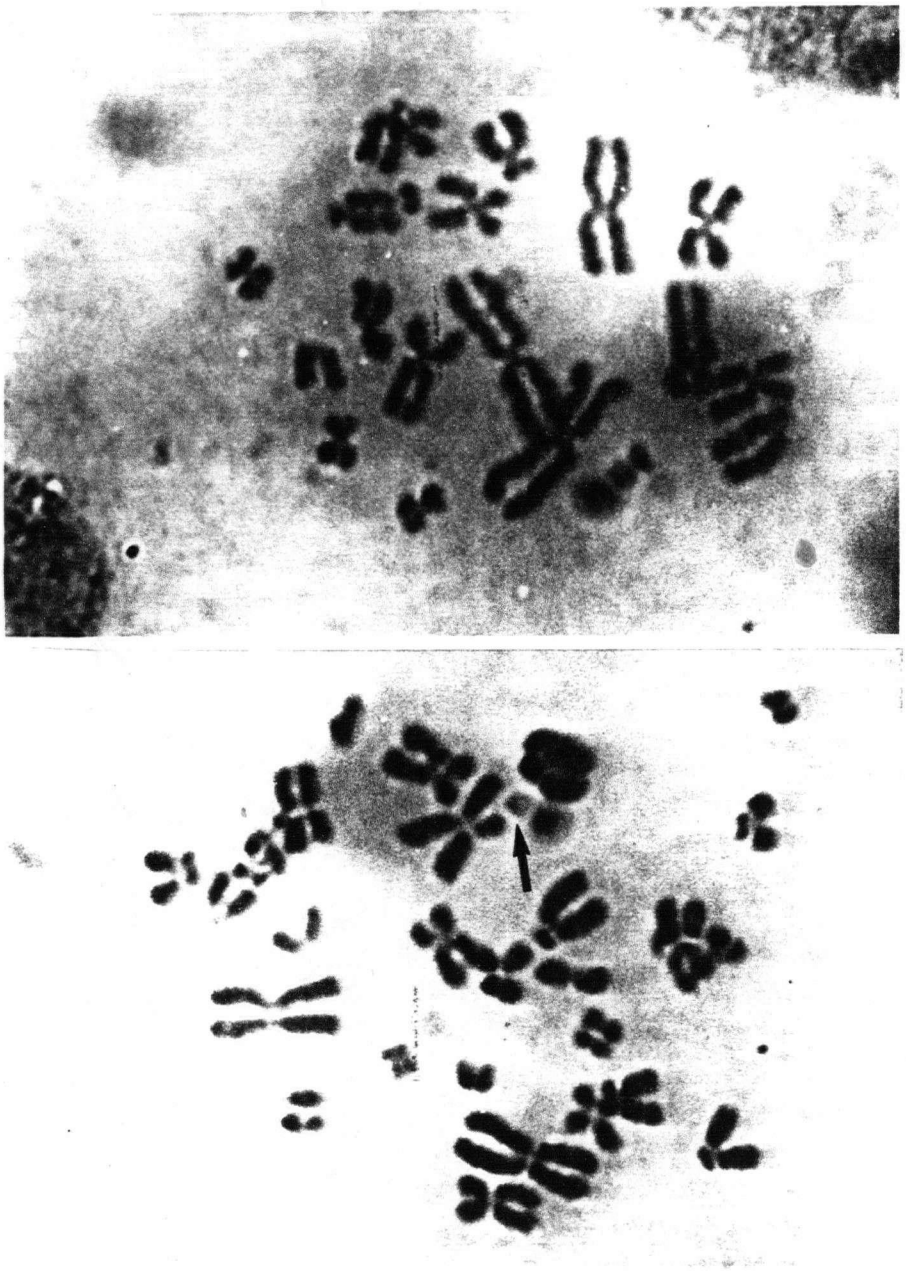


Figure 17. Photomicrogrphs of control (top) and aberrant chromosomes (bottom, 1 Gy X-ray, hypoxic CHO cells)

After treatment with or without X-rays, and cisplatin CHO cell chromosomes were stained with acetic orcein as described in materials and methods. Arrow (bottom photo) shows a gap and fragment.

Table 2

Cell Line Drug (uM)	Conditions of Drug & X-Ray Treatment	Fig No.	Toxicity (PE) (SE)	Low-Dose ER (SE)	High-Dose ER (SE)
V79 Cells:					
cDDP(2.5)	Before I/Hypoxic	1	0.80(0.02)	1.92(0.03)	1.06(0.016)
cDDP(2.5)	Before I/Aerobic		0.75(0.07)	1.20(0.07)	1.03(0.016)
cDDP(5.0)	Before I/Aerobic		0.63(0.09)	1.30(0.06)	
cDDP(2.5)	After 1h/Hypoxic	3	0.80(0.02)	1.92(0.03)	1.02(0.009)
cDDP(2.5)	After 2h/Hypoxic	4	0.60(0.20)	2.12(0.04)	1.07(0.017)
cDDP(2.5)	After 3h/Hypoxic	5	0.71(0.10)	1.88(0.03)	1.07(0.017)
cDDP(2.5)	Before 1h/Hypoxic		0.80(0.02)	1.95(0.03)	
cDDP(2.5)	Before 2h/Hypoxic	9	0.60(0.20)	1.97(0.04)	
cDDP(2.5)	Before 3h/Hypoxic	10	0.71(0.10)	1.97(0.03)	
CHO Cells:					
cDDP(2.5)	Before I/Hypoxic		0.76(0.06)	1.80(0.12)	1.03(0.015)
cDDP(2.5)	Before 3h/Hypoxic	11	0.70(0.05)	1.87(0.03)	
mono(2.5)	Before I/Hypoxic	20	0.60	1.29	1.1
dien(5.0)	Before I/Hypoxic	21	0.65(0.07)	1.20(0.07)	1.04(0.009)
tetra(2.5)	Before I/Hypoxic	22	0.78	1.08	1.1
Bis(t)(2.5)	Before I/Hypoxic	23	0.83(0.05)	1.05(0.004)	1.05(0.009)
Bis(c)(2.5)	Before I/Hypoxic	24	0.76(0.04)	1.44(0.05)	1.03(0.01)
CHO Cells:					
BSO alone	Before I/Hypoxic	18 & 19	0.79(0.10)	1.46(0.09)	1.2(0.03)
BSO + cDDP(2.5)	Before I/Hypoxic	18 & 19	0.68(0.01)	3.03(0.04)	1.3(0.03)
BSO + cDDP(5.0)	Before I/Hypoxic	18 & 19	0.41(0.10)	3.53(0.05)	1.4(0.03)

Table 2. Summary of Enhancement Ratio Data
Before I, Drug immediately before X-rays; h, hours

3.4 The Effects of Glutathione Depletion

Hypoxic CHO cells pretreated with 100 μM BSO for 12 hours, were exposed or not exposed to 2.5 μM or 5 μM cisplatin before doses of low and high X-rays is shown in figures 18 and 19. The results at high doses show a small enhancement of sensitivity of cells pre-exposed to BSO, ER = 1.26 ± 0.08. The sensitization of hypoxic cells with BSO pretreatment is in agreement with recent studies (Wong et al 1991). These cells if also exposed to cisplatin before irradiation show a further enhancement. The ER was 1.31 ± 0.05 for 2.5 μM cisplatin, and 1.37 ± 0.05 for 5 μM cisplatin in BSO pretreated cells.

In contrast, the results for low radiation doses show a very pronounced enhancement of cell killing with BSO pre-exposure, the ER was 1.42 ± 0.08. Recent experiments using hypoxic CHO cells have found similar results (Skov and MacPhail 1991). However, treatment with cisplatin before irradiation in BSO pretreated cells gave very marked increases in cell sensitivity to radiation (figure 19). The ERs were 3.03 ± 0.06 for 2.5 μM cisplatin, and 3.53 ± 0.08 for 5 μM cisplatin, in GSH depleted cells.

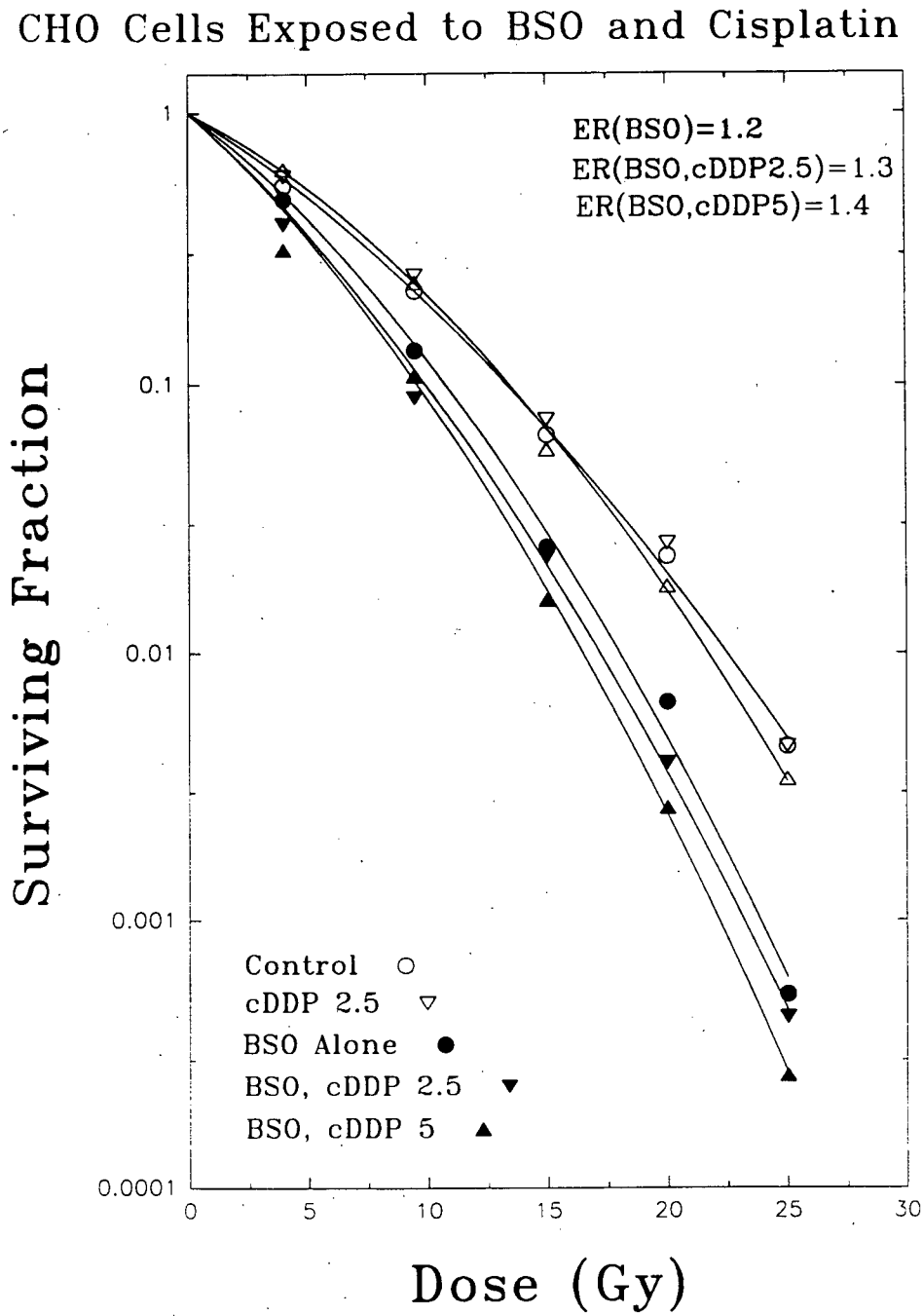


Figure 19. GSH depletion and cisplatin enhancement of hypoxic CHO cell inactivation by high dose X-rays. Cells pretreated with BSO have solid symbols. Results are the average of 2 independent experiments.

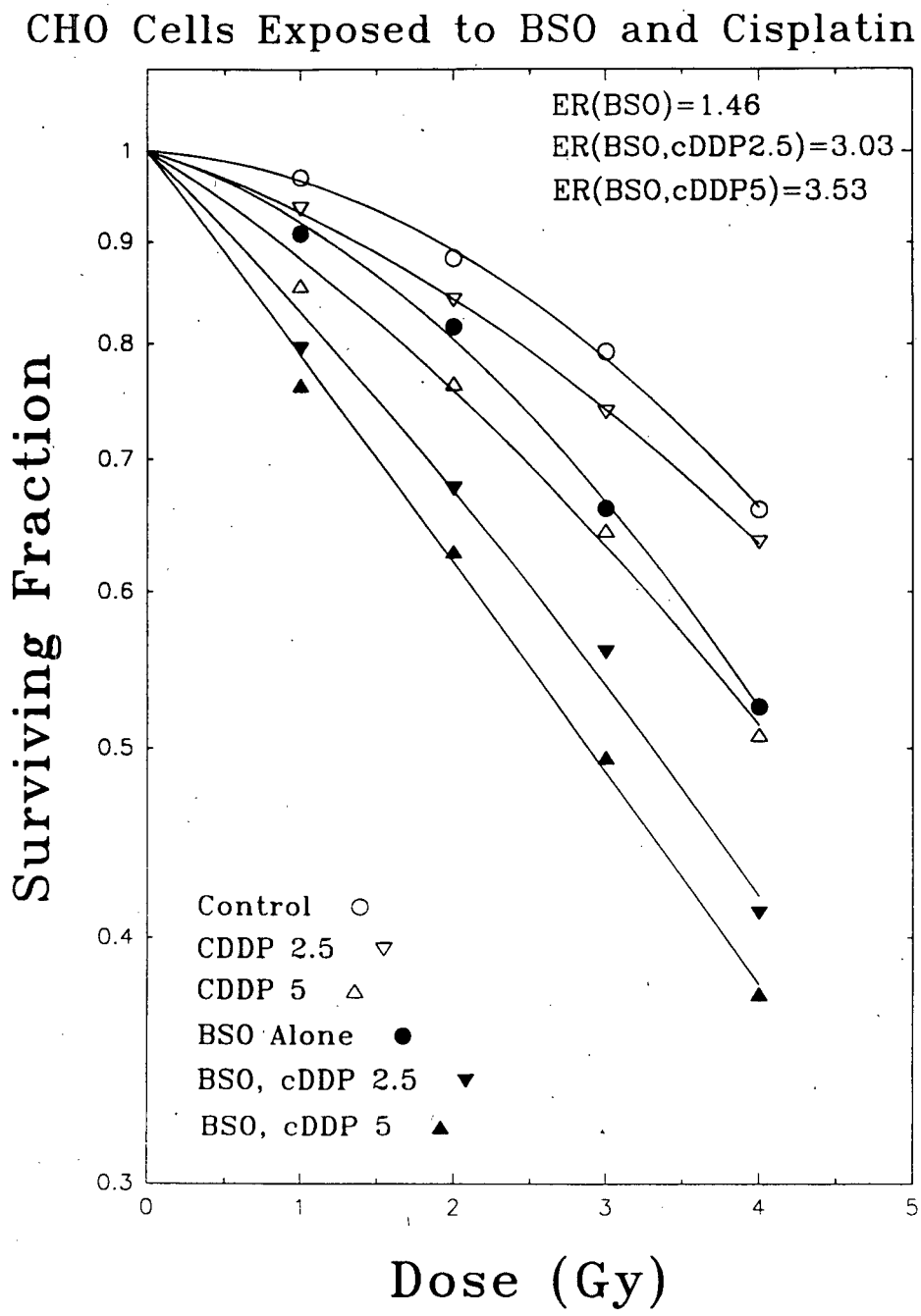


Figure 18. GSH depletion and Cisplatin enhancement of hypoxic CHO cell inactivation by low-doses X-rays. Cells were treated as in figure 3, except that some cells were pretreated with BSO, solid symbols. Average of 2 experiments

3.5 The Interaction of Cisplatin Analogs and X-rays

The response of hypoxic CHO cells pretreated with cisplatin analog Pt-monoammine is shown in figure 20. For Pt-monoamine there is a very slight enhancement at low doses, but not at high doses. the ER was 1.29 at SF = 0.8, and 1.1 at SF = 0.01 (table 2). For cells exposed to Pt-dien before irradiation the ERs were similar as compared to the monoamine (figure 21). Pt-dien has a low toxicity, therefore 5 μM was used, however, still no enhancement comparable to cisplatin was seen. The results for CHO cells preincubated with Pt-$(NH_3)_4$ and bis(platinum)*trans* show no enhancement at low or high doses (figures 22 and 23). In contrast, CHO cells exposed to Bis(platinum)*cis* show an enhancement at low doses (ER = 1.3 at SF = 0.8, figure 24), but none at high doses of X-rays (ER = 1.0 at SF = 0.01, table 2). These analogs have different structures compared to cisplatin (figure 2b), and thus DNA binding modes may differ from those shown in figure 2 for cisplatin. Because of the inability of the analogs to enhance the effects of low radiation (as compared to cisplatin) cisplatin cross-links to DNA could be suggested to be involved in the mechanism of cisplatin's interaction with radiation.

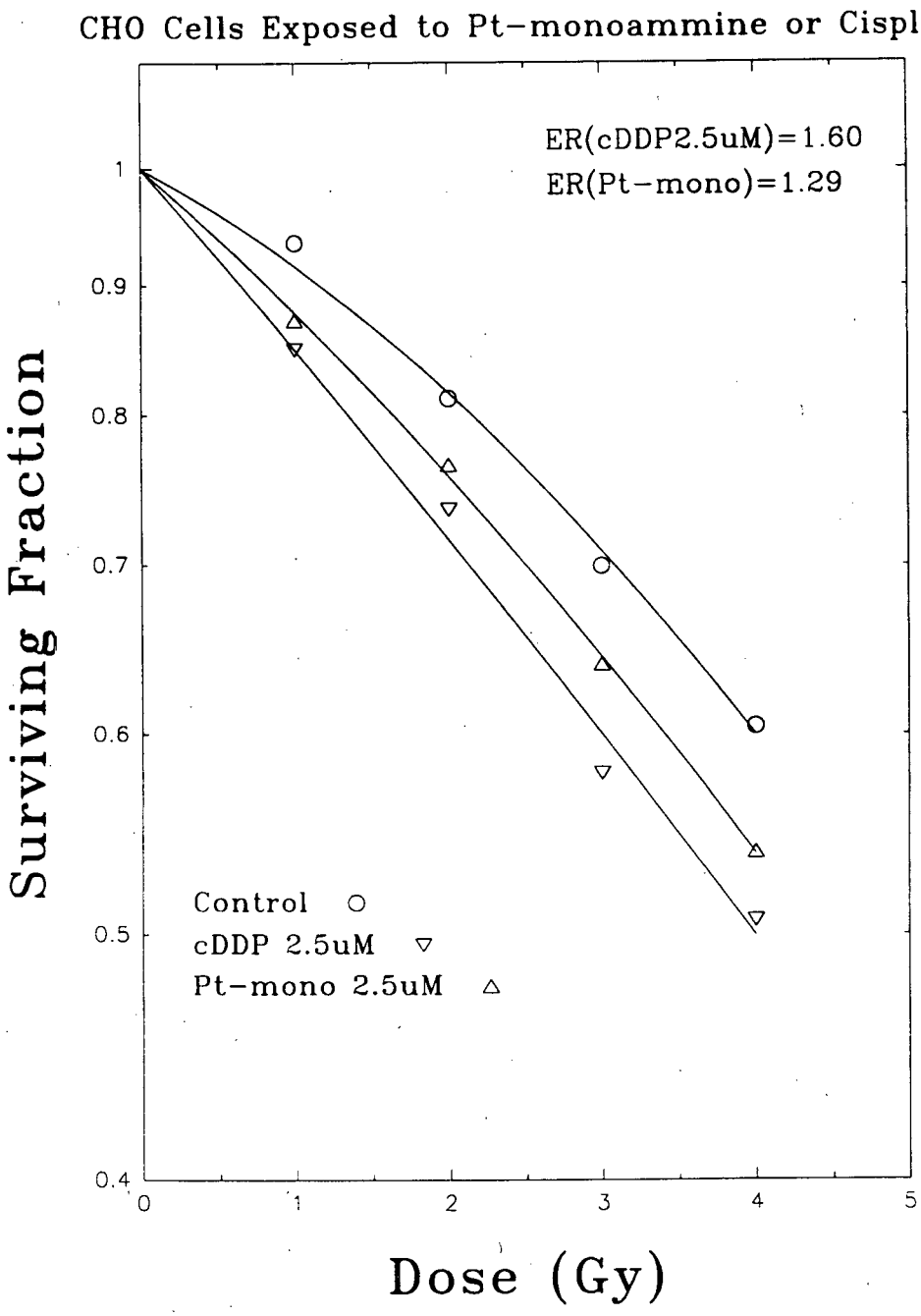


Figure 20. The effect of Pt-monoammine on the inactivation of hypoxic CHO cells treated with low-doses of X-rays.

Hypoxic CHO cells were pretreated with 2.5 μM Pt-monoammine, or 2.5 μM cisplatin before low-doses of irradiation, as in the standard experiment described in materials and methods. Results are from a single experiment.

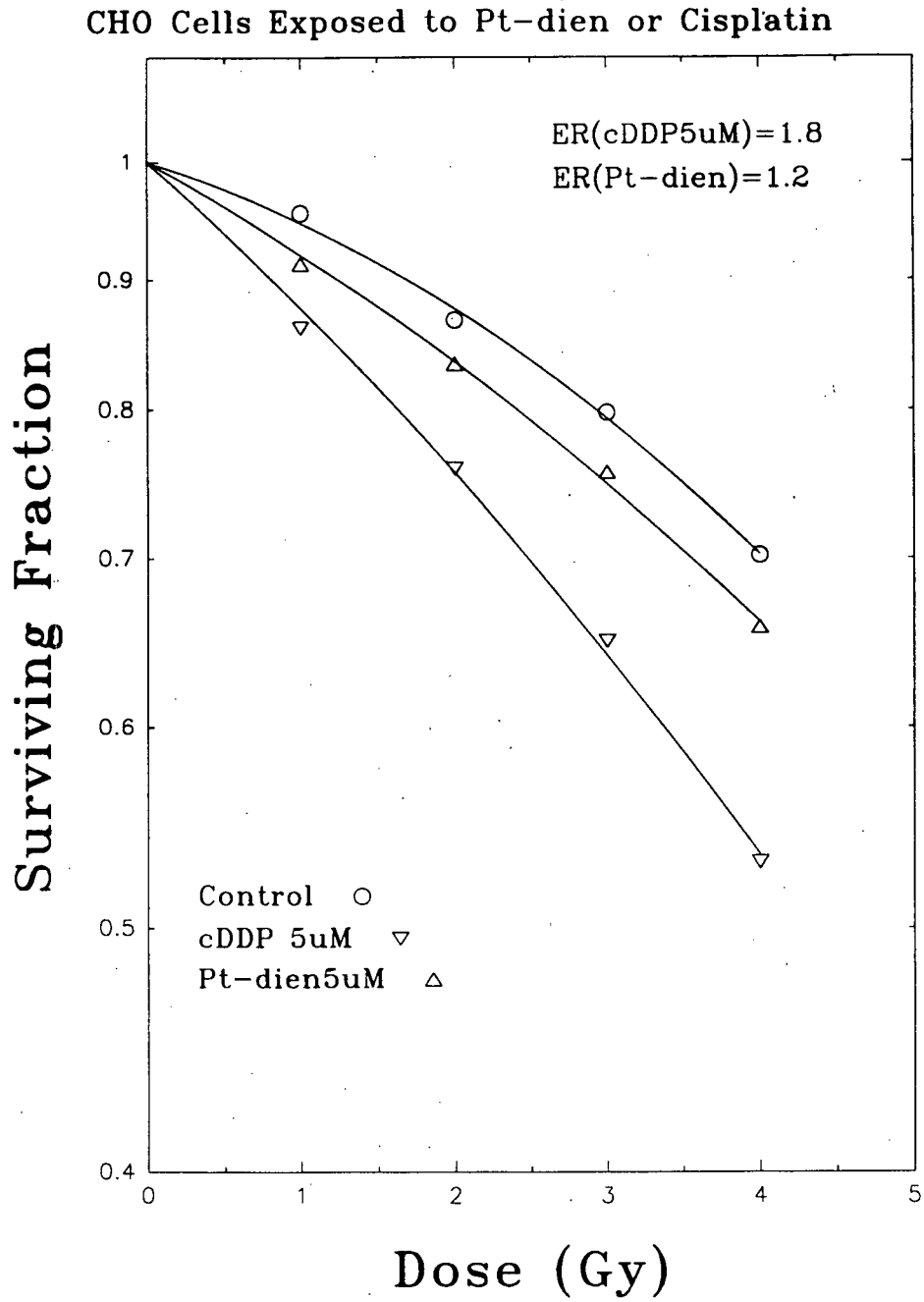


Figure 21. Pt-dien interaction with low-doses of X-rays in hypoxic cells.
Hypoxic CHO cells were pretreated with 5 μM Pt-dien or 5 μM cisplatin. Results are the averages of two independent experiments. Average of 2 experiments.

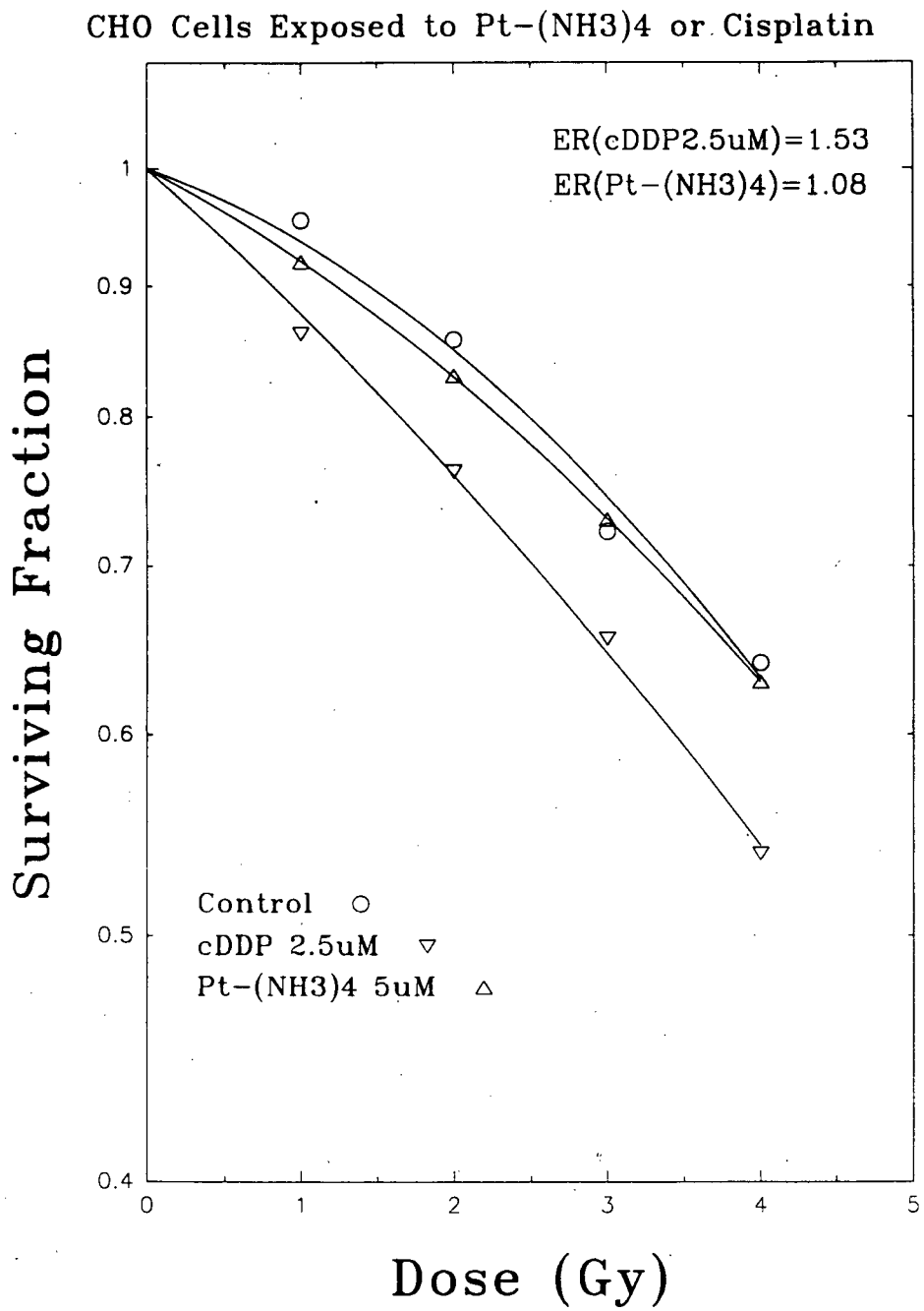


Figure 22. Pt-$(NH_3)_4$ interaction with low-doses of X-rays in hypoxic CHO cells. Hypoxic CHO cells were pretreated with 2.5 μM Pt-$(NH_3)_4$ or 2.5 μM cisplatin. Results are from a single experiment.

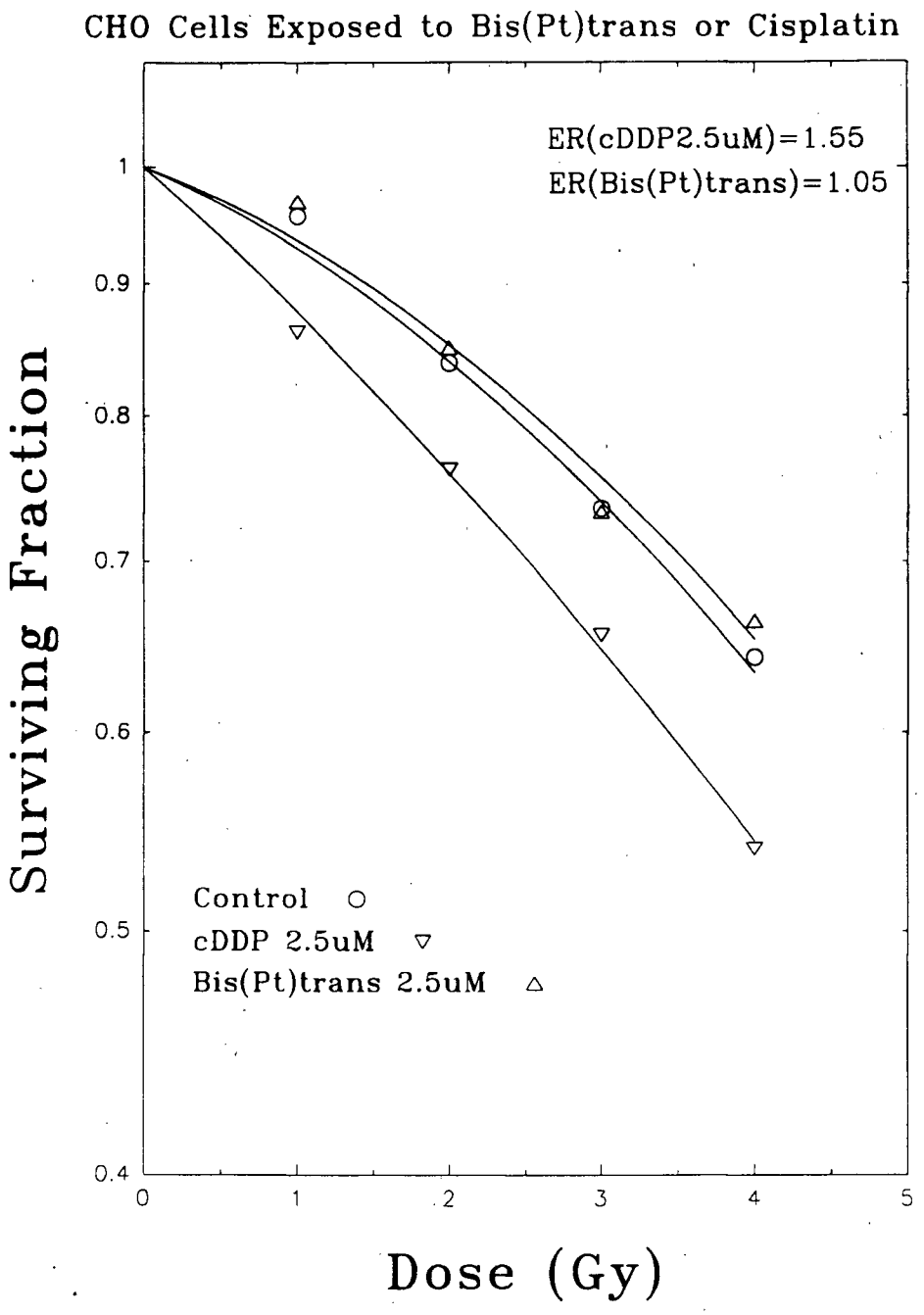


Figure 23. Bis(platinum)*trans* interaction with low-doses of X-rays in hypoxic CHO cells.

Hypoxic CHO cells were pretreated with 2.5 μM Bis(platinum)*trans* or 2.5 μM cisplatin. Results are the averages from two independent experiments.

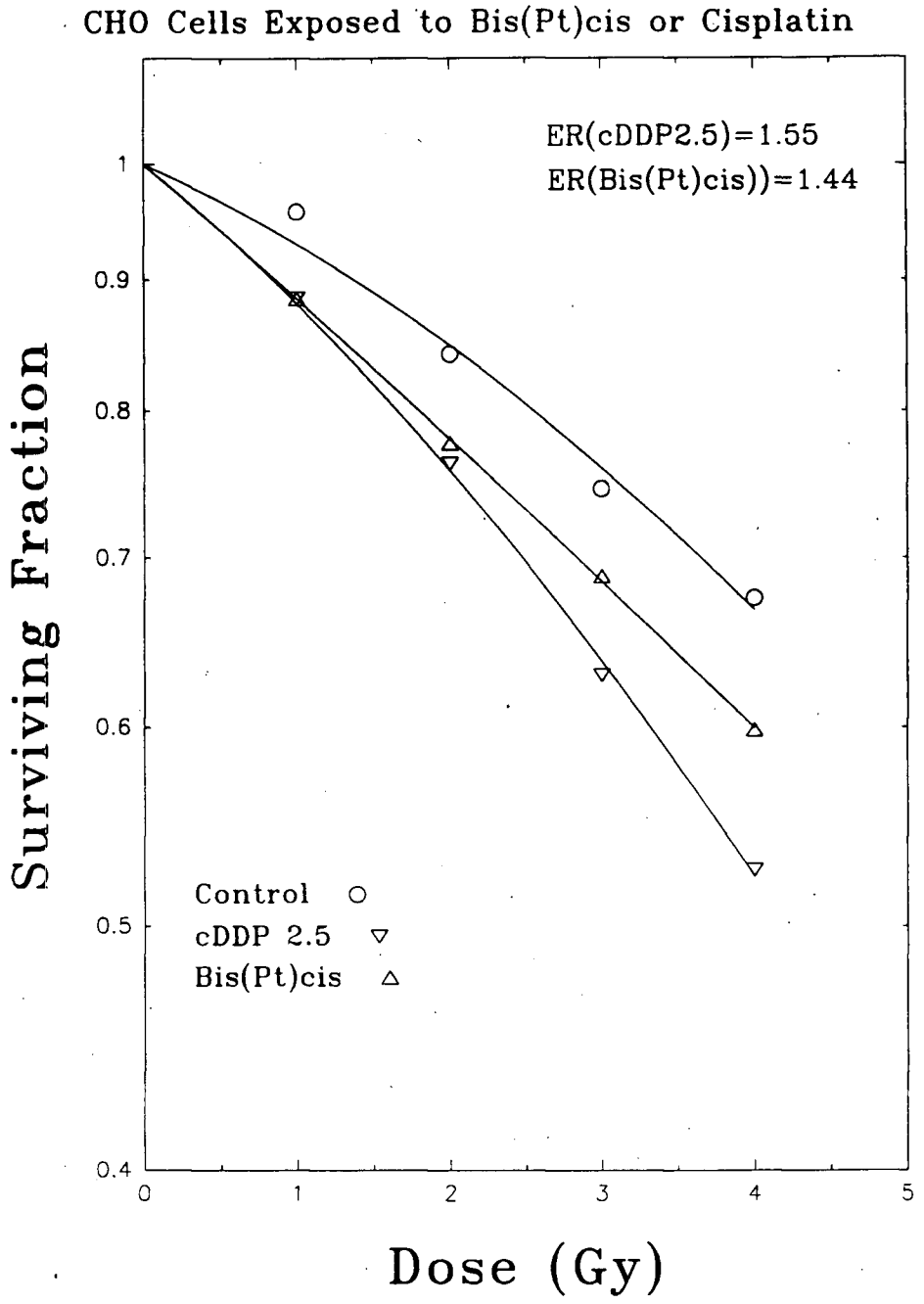


Figure 24. Bis(platinum)*cis* interaction with low-doses of X-rays in hypoxic CHO cells. Hypoxic CHO cells were pretreated with 2.5 μM Bis(platinum)cis or 2.5 μM cisplatin. Results are the averages from two indpendent experiments.

4. Discussion

The fundamental questions left unanswered from previous studies on the interaction between cisplatin and low-doses of X-rays are, 1) why do irradiated hypoxic cells have larger cisplatin enhancement ratios than cells irradiated in air and; 2) why is the enhancement by cisplatin in hypoxic cells more effective at low X-ray doses. Furthermore the overall mechanism of interaction is not yet known. Thus the hypothesis that cisplatin cross-links are involved in this mechanism was investigated in this project using several approaches.

4.1 The Effects of Timing Cisplatin and X-Rays

To investigate this question, the different rates of repair of the most suspicious DNA lesions caused by the two agents in question were indirectly investigated: X-ray induced strand breaks, and cisplatin cross-links. This timing approach had not been published previously in the investigation of the low dose enhancement by cisplatin *in vitro*. Thus the *in vitro* results shown herein are in agreement with the *in vivo* experiments of Twentymen et al, (done at 12 Gy) and indicate that whether cisplatin is added after low-doses of X-rays for periods for up to 8 hours, or before X-rays for up to three hours, timing has no effect on the low dose ER. Thus the ER is independent of time in the periods investigated, where cisplatin ERs are about 1.9 in hypoxic V79 cells, and 1.6 in hypoxic CHO cells.

4.1.1 Cisplatin Given After X-Rays Hypoxic cells were irradiated, and then allowed repair time before the delivery of cisplatin. Repair of single-strand breaks have biphasic kinetics, both in hypoxic and oxic irradiated cells, with half-times in the first phase of approximately 7 minutes (Berger 1982, Skov 1984). The various theories used to explain these kinetics usually involve two categories of lesions, those lesions repaired in the rapid phase, and those repaired in the slow phase (Skov 1984, and references therein). DNA of cells irradiated in hypoxia have about twice as many breaks which are repaired in the slow component when compared to cells X-rayed in air (Berger 1982, Skov 1984).

Thus, in the initial kinetic experiments a repair period of 1 hour permits repair of about 85% of the single-strand breaks after low-doses of irradiation (Berger 1982, Skov 1984). Comparison of these experiments (1 hour repair time period) with experiments giving no repair time gave the opportunity to assess the involvement of unrepaired single-strand breaks in the mechanism. If unrepaired single-strand breaks were not being repaired because of interaction with a cisplatin-DNA cross-link (even though the maximum number of cross-links are not formed for 6 to 12 hours, the initially formed cross-links could interact with single-strand breaks) then this interaction created an event that would be a contributing factor in the mechanism of cisplatin's increased cell killing in the low-dose ER. If this had been true one would expect a reduction of the ER, because the possibility of

this interaction was removed due to the DNA repair of 80% of the single strand-breaks.

The next experiments provided repair periods of 2 or 3 hours. By this time over 90% of the single strand breaks caused by low doses of X-rays in hypoxic cells would have been repaired (Berger 1982, Skov 1984). Exposure of these cells to cisplatin still produced ERs that were the same for cells given no repair time. However cells irradiated in hypoxia repair about twice as much (19%) of the damage in the slow phase of repair when compared with cells X-rayed in air which only repair 9% in the slow phase. Furthermore there have been proposals (Dewit 1987), that the lesions which take longer to repair are those that may contribute in some way to lethal events. To investigate the possible involvement of lesions repaired in this slow phase, the time periods for repair were extended by one hour intervals up to eight hours (figures 7 and 8). The surviving fraction of these cells was not significantly different from the previous time periods. Eight hours of repair surpasses the half-time for even the slow phase of repair of SSB in hypoxic cells. Thus it appears doubtful that the X-ray induced damages which interact with cisplatin to produce the significant ER at 80% survival are DNA strand breaks. It is possible that the small fraction of unrepaired breaks are responsible, but it is also possible that the misrepair of breaks is the significant damage (Elkind 1978). Misrepair of initial damages may play a major role of the interaction with cisplatin. Investigations with CHO cells (with an inserted human chromosome) have found a higher frequency for mutations per Gy at low doses of irradiation than high doses of X-rays

(Waldren et al 1986). Thus, misrepair is considered to be important in the mechanism, as will be discussed in section 4.5.6.

4.1.2 Cisplatin Before X-Rays When cisplatin was delivered to cells at different time periods before X-rays, then the exposure of these cells to irradiation will occur at different stages in the completeness of the formation and repair of the cisplatin-DNA aducts. The cisplatin interstrand cross-link has been found to have biphasic repair. The first repair phase has a half-life of 21 hours in V79 cells (Zwelling et al 1979). The first kinetic experiments with cisplatin allowed a one hour repair period before exposure to X-rays which did not affect the ER. A one hour repair period will allow only a small proportion of the cross-links to be repaired, therefore we extended the repair periods for up to two and three hours. These repair periods still did not make any difference in the ER. The *trans* isomer repairs cross-links more quickly than cisplatin and it has been suggested that cisplatin's ability to cause slowly repairing cross-links is part of its mechanism of toxicity. To investigate this possibility future experiments could be done which allow much longer (8 to 48 hours) repair time. Interpretation of these would be difficult, however, because of the complicated nature of cisplatin adduct formation and removal. Furthermore, cell division occurring in such long experiments would augment the problems in interpretating such experiments.

4.2 Colony Size Analysis

Hypoxic cells irradiated with high doses of X-rays give rise to colonies that have an increasing frequency to be smaller than 1 mm as the dose is increased (figure 15). This result parallels those of Nias with CHO cells and Sinclair with V79 cells irradiated in air (Sinclair 1964, Nias 1968). At low-doses of X-rays given to hypoxic cells the results are more subtle but still there is a decrease in colony size as the dose is increased (figure 13). This indicates that even though a cell survives X-ray treatment, and gives rise to a colony, it has not repaired all of the X-ray damage. Thus there is probably a population of cells which is sensitive to further damaging agents, such as cisplatin.

4.3 Chromosome Aberrations

Chromosome analysis shows that hypoxic CHO cells exposed to low-doses of X-rays experience a type of permanent damage in the form of chromosome exchanges. Although there is repair of some of these aberrations (Rowley 1990), exchanges are the most permanent type of aberration (Hall 1990), and there is general agreement that exchanges are the most significant type of aberration formed (Stich 1984). But, cells irradiated in air also had significant chromosome damage, and the OER for exchanges was 2.9 (figure 16), in agreement with a previous finding (Bushong et al 1967). Furthermore this OER value is similar to the OER for cell survival experiments. Thus, because cells irradiated in air have relatively

more chromosome damage, chromosome aberrations do not provide the evidence that irradiated hypoxic cells sustain a type of permanent damage that makes them sensitive to cisplatin. However, it was interesting that cisplatin had a significant ability to enhance the dose response of radiation induced chromosome aberrations, resulting in an ER of 1.75 for exchanges, compared to 1.9 (V79 cells) or 1.6 (CHO cells) for survival. Furthermore cisplatin had a clear ability to also increase the formation of radiation induced gaps, and breaks (figure 16). These findings may indicate that cisplatin does cause more than an additive effect with radiation.

4.4 Glutathione Depletion

The question of thiol involvement was investigated using BSO which is a potent and specific inhibitor of gamma-glutamylcysteine synthetase, an enzyme of the biosynthetic pathway of glutathione. The role of glutathione was investigated because it has the capacity to prevent the cisplatin-DNA interaction, or the bifunctional cisplatin-DNA cross-link can be prevented by blocking it as a monofunctional cisplatin-DNA adduct. These protective roles can therefore be evaluated in their importance to the low dose enhancement mechanism. There is complication to this, however, because glutathione plays an important role in the chemical repair of radiation damage especially in hypoxic cells. Thus GSH depletion sensitizes hypoxic cells.

Results (figures 18 and 19) show that in agreement with others there is significant enhancement of radiation damage to hypoxic cells pre-exposed to BSO then irradiated with high doses of X-rays (Clark et al 1984), or low-

doses of X-rays (Skov and MacPhail 1991). When cells which have been depleted of glutathione are exposed to cisplatin before X-rays there is considerably more enhancement of the low dose X-ray cell kill. The ER is 3.03 when the cells are also pre-exposed to BSO. This positive change in the ER is far greater than the enhancement to cells irradiated without cisplatin but with pre-exposure to 100 μM BSO, ER = 1.46. This suggests that glutathione is an important repair constituent for these hypoxic cells. But it also suggests that because the ER for BSO + cisplatin (ER = 3.03) is more than the ER for BSO treatment (ER BSO = 1.46), that perhaps the loss of GSH has allowed more cisplatin to effectively bind and cross-link DNA.

These data are suggestive that the ability to form cross-links is important to the mechanism that causes the substantial cell kill with low doses of X-rays in hypoxic cells. Thus, cross-links and their repair by GSH may be relevant in the mechanism of cisplatin's enhancement of low doses of X-rays in hypoxic cells.

4.5 Cisplatin Analogs

(see figure 2b)

4.5.1 Monoammineplatinum(II) The monoammine Pt(II) (monoammine) analog of cisplatin has only one NH_3 bound to the platinum(II) ion. This allows binding to DNA at the other three sites on the platinum(II) square planar structure. With low-doses of X-rays the

enhancement ratio for the monoammine analog was modest (1.26, and 1.2 at high doses).

X-ray crystallography by Lippard has shown that not only is the platinum(II) coordination to the Nitrogen (7) of guanine (or Adenine) important, but also important are the hydrogen bonds formed between the NH_3 groups and DNA (Lippard 1982, Van Kralingen et al 1979). This hydrogen bonding with DNA would be impossible for the monoammine analog. It is possible that the monoammine molecule could make bifunctional cross-links, but the DNA distortion would be different from cisplatin because of the different hydrogen bonding. Toxicity studies by our laboratory show the monoammine analog is less toxic than cisplatin. The nominal ER at low-doses compared with cisplatin coupled with the structural differences between the monoammine analog and cisplatin suggest that the hydrogen bonding potential is also important to the mechanism of cisplatin with respect to its radiation enhancing abilities as has been suggested for its cytotoxic effects (Lippard 1982, Van Kraligen et al 1979).

4.5.2 *Platinum(II)-dien* Platinum(II)-diethylenediammine (Pt-dien) allows only a single monofunctional bond to DNA. Pt-dien also has lower toxicity in mammalian cells than cisplatin. We find a considerably smaller enhancement of low doses of hypoxic x-rays when compared to cisplatin (ER = 1.25). Because of Pt-dien's inability to form bifunctional cross-links, the lower ER again supports the hypothesis that the bifunctional cross-link is important to the mechanism of cisplatin's radioenhancement.

4.5.3 Platinum(II)-$(NH_3)_4$ (Pt-$(NH_3)_4$) The Pt-$(NH_3)_4$ analog with four NH_3 groups permits no coordination with DNA, unless one of the NH_3 groups is labile. Pt-$(NH_3)_4$ has a lower toxicity than cisplatin. The low dose ER again was much lower than that of cisplatin (ER = 1.22). Thus, ability to bind DNA through coordination bonds as well as a hydrogen bond via the ammine (section 4.4.1) appears relevant to the mechanism of cisplatin's biological activity, and also important to is radiosensitizing ability.

4.5.4 Bis(platinum)-trans Bis(platinum)-*trans* makes a cross-link in the DNA with 5 alkyl groups linking the two amine groups which coordinate both platinum(II) atoms in a *trans* configuration. The *trans* molecule shows no enhancement at all at high or low doses (ER = 1.0). However, in theory the *trans* configuration has the capacity to make many DNA-interstrand cross-links, and the bis(Pt)-*trans* molecule could have the capacity to make more interstrand cross-links. For bis(Pt)-*trans* this ability was obviously not successfull for enhancing the effects of low doses of X-rays. The bis(Pt)-*trans* analog probably cannot form DNA-intrastrand cross-links, and this may inhibit its capacity to enhance radiation. In summary these results for the bis(Pt)-*trans* analog provide some proof that the cis configuration is important to the mechanism because it allows the intrastrand cross-link to form.

4.5.5 Bis(platinum)-cis The bis(platinum)-*cis* molecule also makes a cross-link in DNA, however the *cis* molecule acted differently from the *trans* isomer. With this analog there was moderate enhancement found. At low doses the ER was 1.44, and at high doses 1.1. This suggests that the cis configuration may somehow be important in the mechanism. However, we might have expected an even greater enhancement than we found, because there are two cisplatins together in this molecule, and one could have suggested that this might have given this bis analog twice the DNA binding ability. However, this capacity did not provide the bis analog with the capability to greatly enhance the effects of low-doses of X-rays in hypoxic cells.

It should be acknowledged that, in the experiments with the analogs equitoxic concentrations of the drugs were not used. However, these experiments with the cisplatin analogs, which do not have the same cross-linking characteristics as cisplatin, have suggested that cisplatin is relatively unique. This uniqueness begins with the particular way it binds to DNA. The analogs bind DNA differently; Pt-$(NH_3)_4$ prevents DNA binding altogether, it has a lower ER; Pt-dien prevents bifunctional cross-links, it has a lower ER; Pt-monoammine allows bifunctional cross-links but prevents the characteristic hydrogen bonding of the NH_3 group, it has a low ER. These experiments thus support the ideas that DNA is targeted, bifunctional cross-links are necessary, and that cisplatin's NH_3 hydrogen bonding is important. This is supported by studies with *trans*-DDP which has much lower efficiency

than cisplatin at radiosensitizing on a concentration basis, and *trans*-DDP as well cannot make the same hydrogen bonding that cisplatin can. Thus cisplatin's unique binding with DNA seems to play an important role in its ability to enhance the effects of low-doses of X-rays in hypoxic cells.

4.6 Discussion of Results with Respect to Recent Theories

4.6.1 Solvated Electron Scavenging by Cisplatin Recent reviews (Dewit 1987, Coughlin and Richmond 1989) still suggest that radiation-induced free electrons may be scavenged by cisplatin, thereby sensitizing hypoxic cells to radiation despite the fact that the reduction potential of cisplatin (-1000 mV) is too high for this to be a likely mechanism. Furthermore, the interaction cannot be explained on this basis because addition of cisplatin after irradiation causes approximately the same low-dose ER as pretreatment with cisplatin shown by results shown in figures 3 to 11, and discussed in section 4.1.1 and 4.1.2. This parallels results by Korbelik and Skov (1990). Thus, cisplatin need not be present during irradiation, and the time periods given for repair are much longer then the half life for the solvated electron, where up to 8 hours gave the same enhancement. The release of toxic ligands as a result of reduction of Pt(I) intermediates would also be impossible for these same reasons.

4.6.2 Hydroxyl Radical Scavenging by Cisplatin It is not known exactly whether cisplatin would react with ·OH radicals in biological systems, but cisplatin has been shown to react with ·OH radicals in aqueous solution

(Butler 1985). Dewit suggests this to be an unlikely mechanism because cisplatin reacts more slowly with ·OH radicals than with free aqueous electrons. Again the results in figures 3 though 11 rule out this mechanism, as repair periods given are much longer than the half-life of the ·OH radical.

4.6.3 Depletion of Non-Protein Thiols by Cisplatin It has been suggested that cisplatin could deplete non-protein thiols, and thus sensitize cells to radiation. Cisplatin has been shown to lower non-protein thiols in golden hamster cells (Alvarez et al 1978). However this has not been supported in V79 cells (Chibber et al 1985). Furthermore, adding cisplatin after X-rays (figures 3 to 11) gives the same ER. Therefore cisplatin's questionable ability to lower thiol levels is probably not part of the mechanism of its low dose X-ray enhancement.

4.6.4 Inhibition of Cellular Repair Systems A model for the interaction of radiation and cisplatin based on their DNA repair processes has been proposed (Chadwick et al 1976). A supra-additive interaction of X-rays and cisplatin would occur if a radiation induced single-strand break were present opposite a cisplatin cross-link, where one lesion would interfere with repair of the other. The timing experiments, which allowed repair of the 95% of the radiation induced breaks do not support this model, unless it is the small proportion of unrepaired breaks which is responsible. The finding of no change in the ER is suggestive that a more permanent type of damage (for example misrepair) is responsible.

4.6.5 The Nature of Hypoxic Damage: *Why are Cells Irradiated in Hypoxia sensitive to Cisplatin?* It was outlined in the introduction that when hypoxic cells are X-irradiated, they suffer an array of damages which are different from the damage caused when oxic cells when irradiated. The idea that repair of DNA strand breaks is less efficient after irradiation in hypoxia than in air was one reason for designing the kinetic experiments as the interaction is more pronounced in hypoxia. Clearly 8 hours of repair is enough time for these strand breaks to be repaired. Therefore, the relatively less efficient repair of DNA strand breaks caused by irradiation is not sensitizing these cells to further damage by cisplatin. Thus, it is probably more likely to be important as to how much fidelity is maintained in the DNA when it is repaired. Thus the hypothesis by Ward in 1983 that hypoxic irradiation may cause cytosine to uracil mutations is an important consideration. Not because it is important to consider it (as it is yet unproven) for a key role in the mechanism, but because it is an area which needs more research: what are the exact differences in DNA damage caused by hypoxic versus aerobic irradiation. This work needs to be performed before an accurate description can be made.

Another relevant damage difference to consider are protein-DNA cross-links. Oleinick has done extensive research in this area. She and others have found that more protein-DNA cross-links are found after hypoxic irradiations, and that they are slower to repair (Oleinick, 1990, Fornace and Little 1977). Cisplatin also creates DNA-protein cross-links, and one

hypothesis is that they could augment those caused by irradiation, thus increasing resistance to repair enzymes. The data in this thesis does not discount this possibility, and experiments should be done to confirm or discount the role of DNA-protein cross-links in the mechanism.

To summarize: cells irradiated in hypoxia may be sensitive to cisplatin because of the different forms of damage caused by hypoxic x-rays. These lesions may interact with the cisplatin cross-links, and/or mutations to produce the enhanced cell kill seen at low-doses of x-rays in hypoxic cells.

4.6.6 Possible Mechanisms Having ruled out some of the previously published explanations for the interaction, some other possibilities are as follows. With respect to exposing cells to cisplatin after X-rays, if the repair of DNA single-strand and double-strand breaks is almost entirely complete in the 8 hour repair period, we can say that the cisplatin cross-link is probably not interacting with unrepaired single- and double-strand breaks, unless it is interacting with the persistent unrepaired breaks. Alternatively we can suggest that cisplatin is interacting with misrepaired DNA breaks. In support of this idea, recent work with CHO cells using an inserted human chromosome has shown that there is an increased frequency of mutations caused by low doses of X-rays when compared to high doses (Waldren et al, 1986). Thus at low doses, cisplatin could be interacting with the more frequent mutations produced compared to high doses where more cells are killed by irradiation rather than producing mutations. This could provide

part of the explanation as to why cisplatin is more efficient at low doses than at high doses of X-rays in enhancing radiation cell kill. The mechanism of enhanced mutation effeciency at low doses of X-rays has been suggested by Waldren to be caused by deletions created by low X-ray doses which are not recognized by DNA repair enzymes. Thus in the time periods when cells are recovering from low doses of X-rays, there may be subtle yet demonstratable mutations that are not injurious to cell replication in surviving cells. These surviving cells may thus be sensitive to any further DNA damaging agents. The colony size experiments show there is a subpopulation of cells which have not fully recovered from radiation treatment. Furthermore, the chromosome experiments show that when cells are irradiated in hypoxia there are permanent changes in the survivors shown as chromosome aberrations. Thus when these cells are exposed to cisplatin they are more easily killed because of the suggested mutations interacting in some way with cisplatin.

On the other hand, the timing results further show that exposing the cells to cisplatin first then allowing repair also gives the same large low dose ER in hypoxic cells. These timing experiments gave 3 hours of repair time after cisplatin exposure. Realizing that the repair time for cisplatin-DNA cross-links is longer, it is still surprising there was not even a small change in the ER. It takes V79 cells longer to repair cisplatin interstrand cross-links than to repair *trans*-DDP cross-links (Plooy et al 1984). It has been suggested by Zwelling that the difficulty of repairing cisplatin cross-links may offer an alternative to the intrastrand cross-link as the mechanism of its

toxicity. In view of this it can be suggested that toxicity may contribute to the interaction of platinum complexes with radiation. However, the relationship of cisplatin toxicity to radiation sensitivity has been addressed. Results show that low concentrations of cisplatin causing negligible toxicity still caused a large ER (Korbelik and Skov 1990), and that the ER is not related to PE (Skov and MacPhail 1991). Thus toxicity would not be expected to be the only explanation for the cisplatin/radiation interaction. In terms of the persistent cisplatin-DNA cross-links, it still could be extended to radiation biology that this difficulty to repair these cross-links may be part of cisplatin's mechanism of radiation enhancement. It could therefore be argued that our experiments should have included longer repair time for cisplatin. While the importance of these experiments is acknowledged, it is possible to extract some useful information from the *in vivo* experiments of Twentymen et al. Their results show that there is no change in the effects of cisplatin and 12 Gy of radiation on tumor cells whether cisplatin is added before or after X-rays for periods up to 72 hours (Twentymen et al 1979). This would also suggest, therefore that the repair or misrepair of the cisplatin cross-links is important in the mechanism, and is independent to the timing of x-rays. Because of the time periods allowed for repair in this work and by Twentymen et al, it would suggest a more permanent type of damage is interacting with x-rays. In support of this, molecular biological experiments with CHO cells have shown the misrepair of the cisplatin bifunctional intrastrand cross-link leads to mutations (deBoer and Glickman 1989). This has also been shown in *E coli* (Mis and Kung 1990), and yeast

(Burnouf et al 1987). Recent X-ray crystallography, NMR, and numerous physical studies (section 1.5.3) have shown that cisplatin bound to DNA creates a change in DNA conformation, and it has been suggested that this may be associated with the misrepair of the cisplatin-DNA cross-link. Thus the ability of the cisplatin-DNA intrastrand cross-link to modulate DNA conformation is possibly part of cisplatin's mechanism of interaction with X-rays. In support of this, the cisplatin analogs, which lack the ability to bind DNA as cisplatin does, had limited capacity to enhance the effects of low-doses of X-rays. Furthermore, the BSO experiments show that when the cell's capacity for cross-link formation is higher due to the absence of GSH (GSH presence inhibits the cisplatin bifunctional cross-link (Lempers 1990)), they are extremely sensitive to radiation, again suggesting that cisplatin's ability to form DNA cross-links is important in its mechanism with X-rays. Thus, cisplatin's ability to cross-link DNA (intra, inter, or DNA-protein) should be considered in the mechanism of its low-dose radioenhancement in hypoxic cells

4.7 Conclusions

To explain the interaction between cisplatin and low-doses of X-rays in hypoxia, it is suggested that there is interaction between the two types of damages created by them. The GSH depletion, and cisplatin analog experiments suggest that a cisplatin-DNA cross-link (intra, inter, or DNA-

protein) may be important. This cross-link may then be misrepaired, and these infidelities may interact with unique types of damages created upon irradiation at low-doses in hypoxia. Specifically these cells may have unique damage such as: 1) In cells irradiated by low-doses of X-rays there is the possibility for increased numbers of mutations in surviving cells, when compared to high doses of X-rays (Waldren 1986). 2) In cells irradiated in hypoxia there is the possibility for misrepair of base damage caused by ·OH radical attack (Ward 1983), and 3) There is enhanced DNA-protein cross-links in cells irradiated in hypoxia (Fornace and Little 1977; Oleinick 1990). In summary, the timing experiments suggest it is possible that the interactions between low-doses of X-rays and cisplatin in hypoxic cells which produce the pronounced ER are permanent changes in the DNA caused by the two agents.

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