

**RADIOSENSITIZATION OF ANOXIC
MAMMALIAN CELLS**

RADIOSENSITIZATION OF ANOXIC
MAMMALIAN CELLS

by

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SCOPE AND CONTENTS:

The purpose of the work described in this thesis was to (a) better understand the mechanisms of chemical radiosensitization of mammalian cells so that the most effective compounds may be selected for radiotherapeutic use, and (b) to possibly explain the failure of early attempts to sensitize mammalian cells in vivo using chemical radiosensitizers. The effect of triacetoneamine N-oxyl (TAN) and several electron-affinic compounds on the colony forming ability of mammalian cells irradiated at both high and low cell concentrations was studied. A possible mechanism of sensitization for TAN was proposed.

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1. INTRODUCTION

1.1 CANCER AND RADIATION THERAPY

The unlimited cell proliferation associated with malignant tumour growth often leads to a relative radioresistance not encountered in normal healthy tissue. This resistance is conferred by the reduced oxygen tension that exists when the inner core of the tumour has outgrown its vascular supply (Thompson et al., 1971; Thomlinson, 1971). It is known that cells with a very low oxygen tension, such as the hypoxic tumour cells, are much more resistant to the lethal effects of ionizing radiation than well oxygenated cells, such as those occurring in normal tissue. This is referred to as the "oxygen effect". Since the radiation dose delivered to a tumour is limited by the dose which the surrounding healthy tissue can tolerate, any means of effectively reducing the relative radioresistance of the hypoxic tumour cells will increase the effectiveness of the radiotherapy, and thus should improve the cure rate.

Radiotherapy of tumours in patients exposed to oxygen under high pressure (~ 3 atmospheres) has been performed in an effort to increase the O_2 supply to the hypoxic centres

of the tumours. With the improvement in cure rate being only marginal and with the dangers to the patient under high pressure oxygen being very real, this approach is not currently viewed with much enthusiasm.

The treatment of malignant tumours with neutron beams has also been studied. One advantage of neutrons is that they show a reduced oxygen effect (Broerse and Barendsen, 1966), and thus the relative radioresistance of the hypoxic tumour cells is less than is observed using conventional gamma ray therapy. Thomlinson (1971) has postulated that at the beginning of a treatment, neutrons may be more effective than gamma rays not only because they kill more of the hypoxic cells but also because a more rapid reoxygenation of the tumour occurs, presumably as a result of the loss of some hypoxic cells. However, he concludes that in general, neutrons will not be significantly more effective than x- or γ -rays. In addition, the small number of suitable neutron sources now available would limit this form of therapy to a few treatment centres.

A third possible solution to the problem created by tumour hypoxia is to bring the radiosensitivity of all cells up to that for oxygenated cells through the use of chemical radiosensitizers. Many compounds have been found to preferentially sensitize hypoxic bacteria and mammalian cells to ionizing radiation in in vitro systems, and the hope is

that some of the more suitable compounds will be used in the radiotherapy of human tumours.

The properties of an ideal radiosensitizer are:

- 1) an ability to preferentially sensitize anoxic cells to the lethal effects of ionizing radiation,
- 2) metabolic stability, so that the sensitizer is not consumed by the cell prior to irradiation,
- 3) acceptable toxicity,
- 4) appropriate cell cycle dependence, so that hypoxic cells which may stop at some stage of the cell cycle are effectively sensitized at this stage.

The purpose of the work to be described in this thesis was (a) to better understand the mechanisms of chemical radiosensitization so that the most effective compounds may be selected for therapeutic use, and (b) to possibly explain the failure of early attempts to sensitize mammalian cells in vivo (Emmerson and Montana, 1967; Hewitt and Blake, 1970).

1.2 RADIATION CHEMISTRY

The study of the molecular events occurring in intact bacteria or mammalian cells following an exposure to ionizing radiation is often difficult to make directly owing to the great complexity of even the simplest cell systems. To overcome this, simple, well defined chemical systems have been studied in an attempt to understand the molecular events occurring during and immediately following irradiation.

In this section we will discuss some of the radiation chemistry that is believed to be relevant to cell survival.

1.2.1 Direct versus Indirect Damage

Damage to biological molecules occurring when cells are exposed to ionizing radiation can result from absorption of energy by the target molecule directly (direct action), or by the transfer of energy to the target molecule from a reactive molecular species produced during irradiation (indirect action). It has been established that both direct and indirect mechanisms contribute to radiation effects in simple chemical systems, however, there is much disagreement concerning the relative significance of these mechanisms in cells.

Peter Alexander et al. (1970) have taken an extreme view, maintaining that in cells direct action is largely

responsible for the production of single strand breaks in DNA. Their conclusion is based, in part, on experiments performed in their own laboratory with Micrococcus radiodurans and murine lymphoma cells. Using the technique of alkaline sucrose gradients (McGrath and Williams, 1966) to study the production of single strand breaks in irradiated DNA, they observed that the presence of oxygen or free radical scavengers did not affect the production of single strand breaks. However, in more recent and more complete studies, Palcic and Skarsgard (1972) have demonstrated that the presence of oxygen during the irradiation of mammalian cells increases the production of single strand breaks. Therefore, the sedimentation results of Alexander et al. are not consistent with the results of other workers.

In an attempt to substantiate their conclusion that direct action is the principal mechanism in the production of DNA breaks, Alexander et al. refer to the results of Friefelder. However, Friefelder (1966) has shown that the production of single strand breaks in the DNA of B3 bacteriophage is decreased when free DNA is irradiated in the presence of radical scavengers or when the DNA is irradiated while incorporated in the phage head. In both of these cases, the damage to the DNA from indirect action should be reduced, indicating that, in fact, indirect action is involved in single strand DNA breaks. The results of Friefelder do not substantiate the claim by Alexander et al. that indirect action

is mainly responsible for single strand DNA breaks.

Other workers believe that the indirect mechanism of action plays a significant role in cellular damage. This view is supported by Sanner and Pihl (1969) who have concluded that indirect action accounts for approximately 50% of the lethal damage in their E. coli B bacterial system, and by Ebert, Dodd and Nias (1970) who have evidence that indirect action accounts for 70% of the lethal damage in mammalian (HeLa) cells.

The differing opinions of the various experimenters may be due to the different organisms used and the various end points that are studied. The techniques used to differentiate between the direct and indirect actions usually involve looking at the reduced effect of radiation when the organism is frozen, dried or irradiated in the presence of high concentrations of radical scavengers. In all three cases, indirect effect is greatly reduced. These drastic procedures are not really suitable for mammalian cells, however. As well, the study of chemical systems has shown that although these procedures primarily affect indirect mechanisms, they may also influence direct mechanisms of action (Henriksen, 1966; Copeland et al., 1967).

It appears, then, that the relative importance of direct and indirect mechanisms of action in cellular damage has yet to be determined.

1.2.2 Direct Mechanism of Action

Direct action implies the direct absorption of radiation energy by the target molecule resulting in the initial production of an excitation or ionization. This initial energy absorption by target molecules results in the formation of free radicals (for a review of free radical formation in amino acids, nucleotides and bacteriophage DNA see Zimmer and Müller, 1965) which can further react to irreversibly alter the target molecule (Rexroad and Gordy, 1959). This permanent alteration of the target molecule is referred to as damage fixation.

Experiments with dry DNA have been performed to determine the consequences of direct action on these large biological molecules. The direct action of ionizing radiation on DNA can result in the formation of free radicals (for a review see A. Müller, 1967) and the production of single strand breaks (Hagen and Wellstein, 1965). In addition to these, other forms of damage, such as the alteration of bases, probably occur.

The irradiation of dry DNA (Farcasiu, Istratouiu and Milvy, 1971) in the presence of radiosensitizers results in an increase in the yield of DNA free radicals, which suggests that damage resulting from the direct effect can be influenced by the presence of radiosensitizers in the environment of the target molecule. As we will show later (Section 1.3.3),

the radiosensitizer in this case probably acts by scavenging electrons which arise due to ionizations produced in the DNA. The sensitizing effect of O_2 on survival in both dry spores of Osmunda regalis and HeLa cells at $-196^\circ C$ (Ebert, Dodd and Nias, 1970) is further evidence that radiosensitizers can increase the damage produced by direct action.

Similar studies with dry organic polymers (for a discussion see Charlesby, 1968) have confirmed the results obtained using dry biological molecules. The formation of radicals and strand breaks which occur during the irradiation of dry organic polymers can also be altered by the presence of radiosensitizers.

Another consideration relevant to the direct effect is the molecular structure of the target molecule. ESR measurements of dry sulphur compounds and amino acid isomers following irradiation (Koch and Mönig, 1968) have shown that there is an important influence of molecular structure on the behaviour and localization of free radicals produced following irradiation. Similar experiments with dry nucleic acids (Ormerod, 1965) have shown that small changes in the base composition of DNA can greatly alter the production of free radicals. The fact that similar organic compounds exhibit, after irradiation with x- or γ -rays, very different, radical yields indicates that there is an important influence of molecular configuration on direct action.

1.2.3 Indirect Mechanism of Action

Indirect action is the process whereby a molecule absorbs energy by an interaction with a reactive chemical species produced during irradiation. As approximately 80% of the cell is composed of water, it is of interest to know what reactive species are produced when energy is absorbed by water, and what effect, if any, these species have on biological molecules.

When water is irradiated at a moderate dose rate, in the absence of high solute concentrations and in the range of normal pH, the principle products are the radical species $\text{OH}\cdot$, e_{aq}^- and $\text{H}\cdot$ and the molecular species H_2 and H_2O_2 (Henley and Johnson, 1969, Chapter 8). These products arise by one or more of several different possible reaction schemes usually within 10^{-8} seconds or less after the initial energy absorption. The free radicals are short-lived, and generally disappear within times in the microsecond to millisecond range. The molecular product, H_2O_2 , is a strong oxidizing agent but at normal dose rates it is produced in very low yields.

The $\text{OH}\cdot$ radical is also a strong oxidizing agent (for reaction rates with various biological molecules see Scholes, Shaw, Willson and Ebert, 1965) and has been found to play an important part in biological damage. Blok et al. (1967) have looked at inactivation and mutation in an aerobic

suspension of ϕ X174 DNA in the presence of various OH \cdot scavengers. They found that the extent of inactivation and mutation decreased with increasing concentrations of OH \cdot scavenger and thus they concluded that the OH \cdot radicals are responsible for all of the inactivation and mutations produced by the indirect effect.

Roots and Okada (1972) irradiated aerobic mammalian cells in the presence of a variety of hydroxyl radical scavengers. Using alkaline sucrose gradients to study single strand breaks in DNA they observed a reduction in the yield of single strand breaks when the cells were irradiated in the presence of the radical scavengers. They concluded that many of the breaks are a result of indirect action, with the OH \cdot radical being primarily responsible for the indirect effect.

It should be pointed out, however, that in the experiments of Blok et al. and those of Roots and Okada, irradiations were performed in an aerobic environment, a condition which eliminates the reducing species e_{aq}^- and H \cdot . Oxygen scavenges e_{aq}^- and H \cdot to produce O_2^- and HO_2 (Adams, 1967), and since the pK of the equilibrium $HO_2 \rightleftharpoons O_2^- + H^+$ is 4.4, it seems likely that the only important reducing species produced in the presence of oxygen and in the region of physiological pH is the radical O_2^- .

The results of these two groups implicating the OH \cdot

radical as being primarily responsible for the indirect effect in aerobic systems shows, at least, that the O_2^- species does not contribute to single strand DNA breaks. This is consistent with the results of Blok, Luthjens and Roos (1967) which show that O_2^- does not react with $\phi X174$ DNA in aqueous solution. It should be emphasized, however, that these studies do not allow one to conclude that e_{aq}^- and $H\cdot$ are not involved in anoxic irradiations.

Sanner and Pihl (1969) have looked at the indirect action involved in the death of E. coli by irradiating anoxic bacteria at low temperatures and in the presence of various radical scavengers. They conclude from their results that the $OH\cdot$ radical is the only species involved in the indirect mechanisms responsible for cell death. This conclusion was based on the fact that the radiation sensitivity of the bacteria decreased with increasing rates of interaction of radical scavengers with the $OH\cdot$ radical, while no such relationship was found between radiosensitivity and the rates of interactions of the scavengers and e_{aq}^- . However, as has been pointed out by Adams and Michael (1968), a simple relationship between electron rate constants for scavenger molecules (i.e., the rate constant for the reaction between the electron and the scavenger) and the modification of cellular dose response in the presence of these compounds may not exist. Instead, Adams and Michael have proposed that the sensitizing ability of a

molecule is proportional to its electron affinity.

In other words, the importance of the $\text{OH}\cdot$ in the indirect action is acknowledged, however, the failure of Sanner and Pihl to find a simple relationship between electron rate constants and reduced sensitivity in E. coli does not necessarily exclude the involvement of the reducing species e_{aq}^- and $\text{H}\cdot$ in the indirect action responsible for cell death.

The rate constants for the reactions between the reducing species, e_{aq}^- and $\text{H}\cdot$, and various compounds of biological interest have been reported by a number of workers (Neta and Schuler, 1971; Simic and Hayon, 1971). Although the importance of the reducing species in the indirect mechanism of action has not been established for cellular systems, some results are available from studies performed with chemical systems.

Hydrogen atoms in an anoxic aqueous environment have been shown to inactivate ϕX174 and produce single strand breaks and base damage in calf thymus DNA (Jung et al., 1969). $\text{OH}\cdot$ radicals produced in irradiated aqueous solutions have about equal probabilities of reacting with bases and with sugar residues in DNA. H atoms have a particular affinity for the bases (Scholes and Simic, 1968) with which they probably undergo addition reactions, in most cases hydrating the 5-6 double bond.

It would appear that the reducing species are not

important for indirect damage under aerobic conditions due to their removal by O_2 (Blok et al., 1967; Roots and Okada, 1972), however, under anoxic conditions they may play a significant role in cell damage.

Results from chemical systems (Blok et al., 1967), aqueous polymer systems (Charlesby, 1968) and biological systems with whole cells (Sanner and Pihl, 1969; Roots and Okada, 1972) have shown that the magnitude of the indirect effect can be altered by the presence of radical scavengers.

The effect of the secondary structure of biological molecules on the indirect effect has been studied by Shragge, Michaels and Hunt (1971). By studying the reactivity of polynucleotides with e_{aq}^- as a function of pH, they have concluded that the variation in the rate of reaction between the polynucleotide and e_{aq}^- is due mainly to the secondary structure of the biological molecule.

1.2.4 Summary of Radiation Chemistry

Radiation damage in biological systems then, is a result of both direct and indirect mechanisms of action, with their relative importance in cell killing yet to be determined. Indirect action results mainly from the oxidizing species $OH\cdot$, however, under anoxia, the reducing species

e_{aq}^- and $H\cdot$ may contribute to biological damage. The destructive effects of both the direct and indirect actions are dependent on the immediate environment and the configuration of the target molecule.

1.3 CHEMICAL RADIOSENSITIZERS

1.3.1 SH Binding Compounds

One of the mechanisms which has been proposed to explain the sensitizing effect of certain compounds assumes that the sensitizer acts by binding free sulfhydryl groups which normally have a protective effect in irradiated cells. In order to understand this mechanism, it is necessary to describe the process of SH protection.

When whole cells are irradiated it has been shown that the presence of molecular oxygen enhances the damage produced as measured by many different end-points:

- (1) Inactivation of DNA as measured by transforming ability (Hutchinson and Arena, 1960).
- (2) The production of single strand breaks as measured by the alkaline sucrose gradient technique (Palcic and Skarsgard, 1972).
- (3) Inactivation of enzymes (Hutchinson, 1961).
- (4) Cell death (Howard-Flanders, 1958),

to name just a few. However, if biological molecules such as T₂ bacteriophage (Howard-Flanders, 1960), DNA (Hutchinson, 1961) and trypsin (Hutchinson, 1961) are removed from the normal cell environment and irradiated while suspended in buffer, then no O₂ effect is observed. If sulfhydryl compounds are then added to the buffer, an oxygen effect

similar in magnitude to that found in intact cells is obtained.

To explain the reduced sensitivity found under anoxia in the presence of sulfhydryl compounds, a hydrogen donation model was first proposed by Alexander and Charlesby (1954), to explain the results obtained with organic polymers, and this was later applied to biological systems. It was postulated that a free radical is produced in the target molecule by the radiation and that subsequent reactions of this free radical lead to permanent biological damage. In the presence of a sulfhydryl compound, a hydrogen atom is transferred from the sulfhydryl compound to the radical before it has time to decompose, and thus the chemical lesion is repaired.



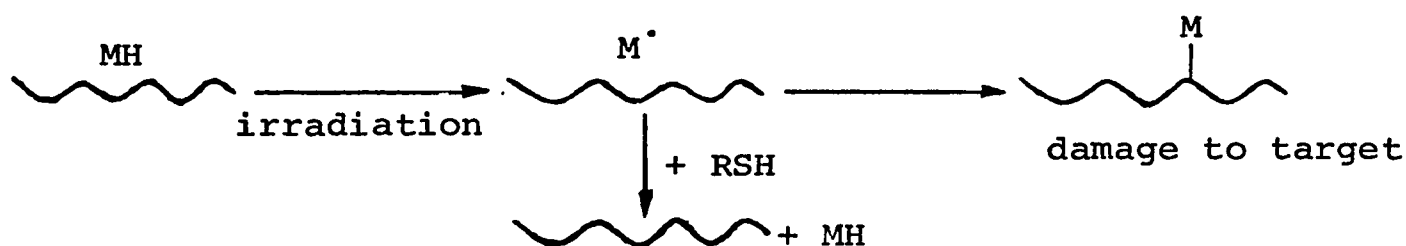
Repair of Target Molecule

In the presence of oxygen, however, the free radical of the target molecule combines with an oxygen molecule to produce a peroxy radical which cannot be repaired by SH, and therefore permanent biological damage is produced. Such an H· transfer has been demonstrated by Adams et al. (1967) using simple organic free radicals such as alcohol and amino acid radicals produced by OH· radicals in the presence of cysteamine.

More recently, Loman, Voogd and Blok (1970) have proposed an alternative mechanism of SH protection that could

complement the H donor model of Alexander and Charlesby.

During irradiation, organic free radicals arise in the cell by both direct and indirect mechanisms (Zimmer and Müller, 1965; Adams, 1967). The second model proposes that these organic free radicals are eliminated by SH compounds through a process of hydrogen donation. Thus the essential difference between these two models is that in the first we have repair of the target molecule, whereas in the second we have a process whereby free radical intermediates are eliminated and indirect damage to the target molecule is prevented.



Prevention of Damage to the Target

The importance of intracellular sulfhydryl compounds in normal cell survival has been the subject of considerable speculation and experimentation. Révész and Bergstrand (1963) investigating the free non-protein sulfhydryl (NPSH) content of ascites tumour cells after incubation with cysteamine have shown that the increase in NPSH resulting from cysteamine incubation parallels the relative protective effect of cysteamine. Sinclair (1968, 1969) also implicates available sulfhydryls as an important intracellular factor governing radiation response.

Harris, Painter and Hahn (1969), on the other hand, have reduced the NPSH content of Chinese hamster cells to 20% of the normal value, using methyl phenyldiazene-carboxylate, and found no change in the cell radiosensitivity. When the intracellular NPSH content was reduced to only 10% of the normal level, some sensitization was found, although these treatment conditions were quite toxic. They conclude that the bulk of endogenous NPSH does not act as a radioprotector.

The importance of intracellular NPSH has not been unequivocally demonstrated, however, the available data suggests that NPSH does play some part in normal cell protection against the damage of ionizing radiation.

Bridges (1960) was the first to suggest that radiosensitization can be attributed to the binding of intracellular SH, thereby eliminating their normal radioprotective effect.

The most thoroughly studied sulfhydryl binding radiosensitizer is N-ethylmaleimide (NEM). Experiments with E. coli (Bridges and Munson, 1967; Moroson and Tenney, 1968) and Micrococcus sodonensis (Mullenger and Ormerod, 1969) have yielded results which are consistent with an SH binding mechanism. On the other hand, Adams, Cooke and Michael (1968), using a rapid mixing technique have demonstrated sensitization in Serratia marcescens under conditions that eliminate SH binding as a possible mechanism. Mullenger

and Ormerod (1969) have suggested that NEM sensitizes by the following two mechanisms:

- (i) NEM reacts with the cell sulfhydryl groups which would otherwise protect biologically important sites, and
- (ii) NEM reacts with radiolytic species to produce an activated molecule which in turn interacts with the target molecule to cause biological damage.

These two mechanisms are consistent with the results from chemical studies using ESR (Farcasiu, Istratoiu and Milvy, 1971) and spectrophotometric (Ward, Johansen and Aasen, 1969) techniques.

The difference between the observations of Adams et al. and those of Bridges and Munson, Moroson and Tenney, and Mullenger and Ormerod might simply reflect differences in the relative importance of these two mechanisms in the various organisms used.

1.3.2 Free Radical Sensitizers

The compounds first known to sensitize living organisms to the lethal effects of ionizing radiation included oxygen (Gray et al., 1953) and nitric oxide (Howard-Flanders, 1958), both of which are stable free radicals. This prompted Emmerson and Howard-Flanders (1964,1965) to investigate the stable organic free radicals such as di-t-butyl nitroxide and its analogues which were found to be effective

sensitizers in E. coli B/r. Subsequent work has centred on the organic nitroxide free radical triacetoneamine N-oxyl [2,2,6,6,-tetramethyl-4-piperidone-N-oxyl (TAN)] which at low concentrations is less toxic and a more effective sensitizer than the other nitroxide free radicals (Emmerson, 1967).

Studies in chemical systems have shown TAN to exhibit the following properties:

- (i) TAN is highly electron affinic (Willson, 1971).
- (ii) TAN reacts rapidly with e_{aq}^- (Willson, 1971) and $OH\cdot$ (Willson, 1972).
- (iii) TAN reacts rapidly with a variety of organic free radicals (Willson, 1971) including $OH\cdot$ adducts of both thymine (Emmerson and Willson, 1968) and DNA (Willson and Emmerson, 1970).
- (iv) TAN binds covalently to $OH\cdot$ produced adducts of DNA under anoxic conditions but not under aerobic conditions (Nakken, Sikkenland and Brustrad, 1970).
- (v) TAN does bind to DNA in aqueous solution under anoxic conditions when TAN is added within a second (Brustad and Nakken, 1970) after irradiation, but no binding occurs when irradiated TAN is added to unirradiated DNA (Nakken, Sikkenland and Brustrad, 1970).

In bacteria TAN must be present during irradiation to be effective, as removal of TAN just prior to irradiation

(Emmerson, 1968) or the addition of TAN as soon as 4 msec after irradiation (Adams, 1970) produces no detectable sensitization. Emmerson (1970) has shown that TAN, when present during the anoxic irradiation of an E. coli donor strain, modifies the episomal DNA and affects its ability to function on subsequent transfer to a recipient cell. He has also shown that TAN present during anoxic irradiation of E. coli superinfected with λ phage increases the yield of single strand breaks in the λ DNA, but concludes that the increase in the DNA single strand breaks probably does not sensitize the episome to irradiation to any great extent.

Rupp et al. (1970) using alkaline sucrose gradient techniques with normal, excision deficient and recombination deficient mutants of E. coli have shown that TAN present during anoxic irradiation results in the post-irradiation synthesis of DNA that is of smaller molecular weight than is found after irradiation in the absence of TAN. The smaller DNA fragments appear to result from the formation of radiation induced TAN-DNA adducts. The results of Rupp et al. also suggest that the degree of sensitization produced by TAN is inversely related to the ability of the bacterium to repair these TAN-DNA adducts.

The evidence suggests that in bacteria, at least one component of TAN sensitization involves increasing the damage to DNA and that one form of this damage is the formation of a TAN-DNA adduct.

TAN preferentially sensitizes anoxic suspensions of mammalian cells to the lethal effects of radiation (Parker, Skarsgard and Emmerson, 1969), although the sensitization is less than that obtained in bacterial systems. Révész and Littbrand (1970) have observed that TAN sensitizes mammalian cells at low drug concentrations but at high concentrations they observed a protective effect. The protection afforded by high TAN concentrations could be expected in view of its ability to effectively scavenge the reducing (Willson, 1971) and oxidizing (Willson, 1972) radical species.

Hewitt and Blake (1970) attempted to demonstrate a sensitizing effect of TAN on anoxic leukaemia cells irradiated in situ in the infiltrated livers of leukaemic mice. The attempt was unsuccessful. The explanations offered for the failure are that the drug did not accumulate in the liver to a sufficiently high concentration to produce significant sensitization, or that the drug was metabolized in the liver and so was not available in a useful form during irradiation.

The practical application of these sensitizers to the radiotherapy of malignant tumours clearly necessitates the use of such compounds in high cell concentration situations, analogous to the experiment of Hewitt and Blake. Radiosensitization at high cell concentrations was therefore

examined carefully in this thesis and possible explanations of the results of Hewitt and Blake will be presented later.

Recently another nitroxide free radical has been studied (Emmerson, Fielden and Johansen, 1971) which produces in bacteria the same sensitization as TAN, but at a much lower drug concentration. The increased effectiveness of this compound, norpseudopelletierine N-oxyl, is attributed to the reduced steric hindrance of the nitroxide group in this molecule. It is assumed that the nitroxide group is involved in the mechanism of action of these compounds.

1.3.3 Electron Affinic Sensitizers

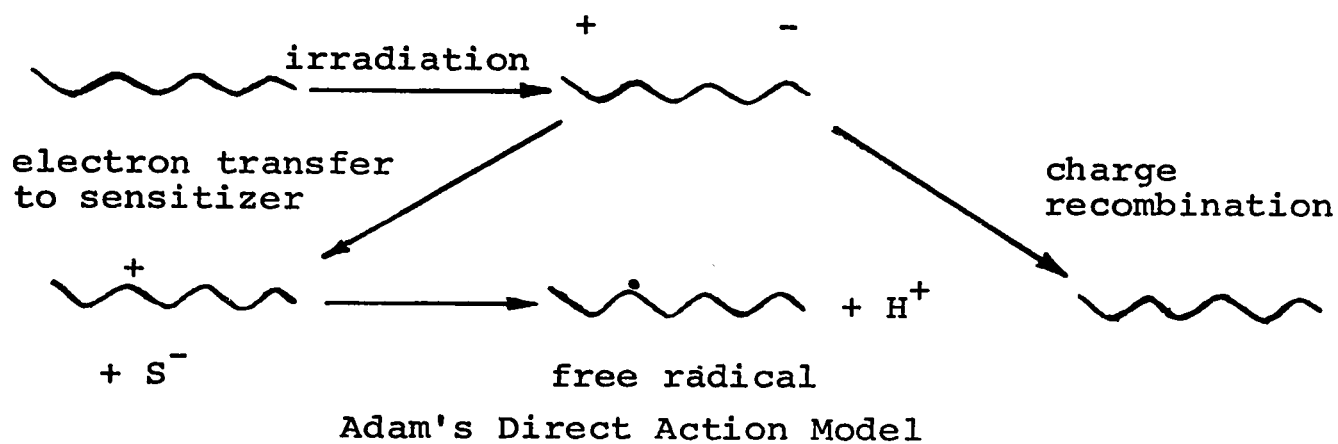
The hydrated electron is normally the principle reducing species produced during the irradiation of an aqueous system (Henley and Johnson, 1969, Chapter 8) and is known to react rapidly with a variety of biological molecules (Anbar and Neta, 1965; Adams, 1967). If the interaction of the hydrated electron with biological molecules within the cell leads to expressed biological damage, then the extent of the damage would depend upon the competition for the electrons by the target molecules and the other electrophilic centres in the immediate vicinity. Any means of increasing the probability that the e_{aq}^- will react with the target molecule should increase the quantity of biological damage and thus increase cellular radiosensitivity.

Adams and Dewey (1963) pointed out that the radiosensitization by NEM of E. coli B/r observed by Bridges (1960) could also be explained via the electron affinity model because NEM was known to have a high electron affinity (Matheson, 1962). In addition, all known sensitizers at the time were either known to be, or were expected to be efficient electron acceptors. Adams and Dewey then postulated a mechanism of sensitization that involved the alteration of the indirect effect by the sensitizing compound; this mechanism is therefore referred to as the Indirect Model of Sensitization. It was suggested that a molecule containing a resonance linkage between electron-acceptor groups would possess a high electron affinity. The model proposed that the capture of an e_{aq}^- by the electron affinic compound would result in the formation of a negatively charged radical anion which would be resonance-stabilized, and relatively long-lived. The greater stability of the electron would increase the probability of its interacting with the target molecule. The argument presupposes, of course, that the target site of the vital molecule has a higher electron affinity than the electron carrier itself.

This model was empirically successful in that the ideas derived from it led to the characterization of some new sensitizers. However, the model failed to explain the

oxygen effect. Owing to its high electron affinity relative to most other molecules, oxygen almost invariably acts as an electron acceptor (Adams, 1967), consequently the electron transfer from O_2^- to a target molecule in a biological system is very unlikely. In particular Blok, Luthjens and Roos (1967) have shown that O_2^- does not react with $\phi X174$ DNA in aqueous solution.

In 1969 Adams and Cook introduced a new model in which the sensitizer increases the damage resulting from a direct action mechanism; this model is thus termed the Direct Action Model. In this case direct ionization of the target molecule produces a positive ion and as well an electron which, after thermalization, migrates to some electron-affinic site on the molecule. If there is a molecule of higher electron affinity available either as a complex or as a free molecule, electron transfer from the polarized target molecule to the electron-affinic molecule could occur. This process would compete with charge recombination in the target molecule, a process which would contribute to self healing. Electron transfer to the sensitizer would reduce the chance of recombination and favour the decay of the positive ion to a free radical.



In support of this mechanism some experiments (reviewed by Michael, 1968) using the electron spin resonance technique, have shown directly that a charge transfer complex is formed in irradiated solid mixtures of DNA and menadione, a process which leads to a substantial increase in the free radical yield. Also, Adams et al. (1972) have demonstrated rapid, one-electron transfer between nucleotides in solution, implying that intramolecular electron transfer along a polynucleotide should occur readily. Also, they have clearly demonstrated that known radiosensitizers can transfer electrons rapidly and quantitatively from the one-electron adducts of simple pyrimidine and purine bases.

The direct-action model is more consistent with recent experimental data and has the advantage of accommodating oxygen in a general class of sensitizers.

2. MATERIALS AND METHODS

2.1 TISSUE CULTURE TECHNIQUES

2.1.1 Cell Lines

Two mammalian cell lines have been used in these experiments, a Chinese hamster cell line CH2B₂ (Prescott and Bender, 1963), and the mouse cell line L-60, both of which were obtained from Dr. G. F. Whitmore (University of Toronto).

2.1.2 CH2B₂ Tissue Culture

The Chinese hamster cells which have a doubling time of approximately 14 hours were grown attached to glass in 4 ounce prescription bottles (Browns Bottle and Supplies Inc., Montreal) or to plastic in 20 x 100 mm plastic tissue culture petri dishes (Falcon Plastics, Oxnard, California) in growth medium which consisted of synthetic nutrient medium CMRL 1066 (Parker, Castor and McCulloch, 1957) supplemented with 10% newborn agamma calf serum (North American Biological Inc., California) and incubated under standard conditions (37°C in an atmosphere of 5% CO₂, 95% air and 100% humidity).

In order to maintain the cell line in log phase

growth (cells actively growing and dividing), the cells in monolayers were subcultured by trypsinization every 3 to 4 days. For cells growing in prescription bottles, this involved treating the cells at 23°C for 8 minutes with 4 ml of 0.1% trypsin (Bacto-trypsin, Difco Laboratories, Detroit, Michigan) in citrate saline that had been warmed to 37°C, shaking the cells free of the surface and immediately neutralizing the trypsin action by adding the trypsin-cell suspension to an equal volume of growth medium. The cells were then centrifuged for 6 minutes at 40 g, resuspended in growth medium and aliquots of from 2×10^5 to 4×10^5 cells were plated into new bottles containing 8 ml of growth medium. Cells to be used in experiments were plated into 20 x 100 mm plastic tissue culture petris containing 15 ml of growth medium at 1.2×10^6 cells/petri and grown for 2 days under standard conditions. The cells were removed from the plastic surface using 10 ml of trypsin in the manner described above. In all cases fresh growth medium was added to the cells approximately 18 hours prior to trypsinization.

2.1.3 L-60 Tissue Culture

The mouse L-60 cells which have a doubling time of approximately 18 hours were grown at 37°C in suspension in spinner culture in CMRL 1066 lacking nucleic acids and coenzyme and supplemented with 10% fetal calf serum (Grand

Island Biological Company, Grand Island, New York). In the sedimentation experiments (2.6), however, the L-60 cells were grown in Minimal Essential Medium (MEM F-13, Gibco) supplemented with 10% undialysed fetal calf serum (Gibco). The cells were maintained in log phase growth (cell concentrations between 5×10^4 and 2×10^5 cells/ml) by regular dilution with fresh medium. In all experiments only log phase cells were used.

2.2 RADIATION SOURCE

2.2.1 Dose Rate

Irradiations were performed with 0.66 MeV gamma rays from a ^{137}Cs source at a dose rate of 330 rads/min for cells irradiated while suspended in medium, 700 rads/min for cells irradiated at high cell concentrations (cell pellets). One exception is the experiment described in Section 2.4 for which the dose rate was 420 rads/min.

2.2.2 Dosimetry

Dosimetry measurements were performed by:

- (a) Ficke's method (ferrous ammonium sulphate) using the procedure outlined by the American Society for Testing and Materials (Annual Book of ASTM Standards, 1971), and
- (b) ionization measurements using a Victoreen model 570 condenser r-meter.

Measurements of radiation dose using both techniques varied by less than one percent.

2.3 CELL SUSPENSION EXPERIMENTS

2.3.1 Preparation of the Cell Suspension

Cells that were to be irradiated while in suspension were harvested from log phase monolayers in the case of the CH2B₂ line (see 2.1.2), and from suspensions of log phase cells when using the L-60 line. Both the Chinese hamster cells which were centrifuged following trypsinization (see 2.1.2) and the L-60 cells which were centrifuged (40 g for 8 minutes) upon removal from spinner culture were resuspended at a concentration of 2×10^5 cells/ml in growth medium (CMRL 1066) from which the sodium bicarbonate had been omitted. The pH was adjusted to 7.2. Fifteen millilitre aliquots of cell suspension were placed in special glass irradiation vessels (Figure 1) and stirred with a magnetic stirrer.

2.3.2 Anoxia

Since our interest in these experiments is related to the increased survival of cells when they are irradiated in the absence of oxygen, we have defined the anoxic state in our experimental system to be that for which radiation survival is a maximum.

An experiment was performed to determine the duration of gassing with nitrogen (at 0.7 litres/min) required to

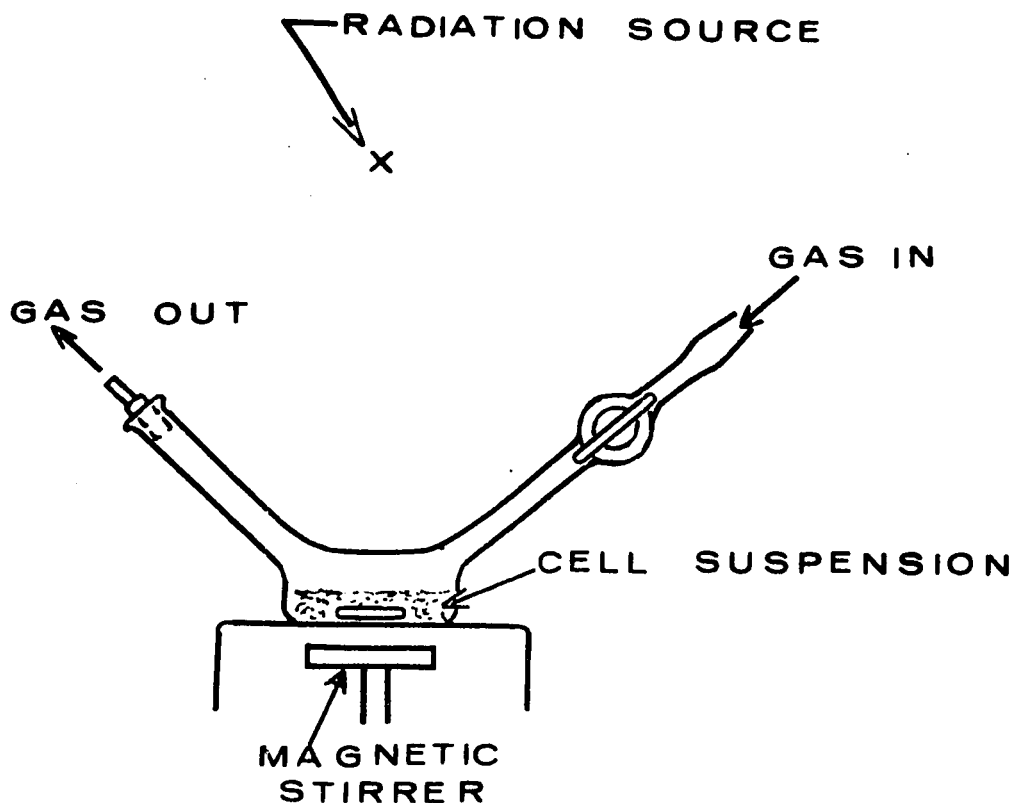


FIGURE 1. The Glass Irradiation Vessel in which Anoxic and Aerobic, Dilute Cell Suspensions are Irradiated
 Dilute cell suspensions can be made anoxic or aerobic by flushing the vessel with nitrogen or oxygen, respectively. Anoxia in the vessel can be maintained even during the removal of aliquots of cell suspension if the pipet used to remove the cells from the vessel is first flushed with nitrogen. The mixing action of the magnetic stirrer maintains a relatively constant cell concentration in the suspension.

achieve anoxia and the results are shown in Figure 2. The time between the start of the gas flow and the beginning of irradiation is shown on the graph. The survival curve for an 8 minute gassing interval has not yet reached maximum resistance probably due to oxygen still present in the solution, but for 15 minutes gassing the survival curve has reached a maximum level that remains constant for an additional 30 minutes of gassing. In all cell suspension experiments then, anoxia was obtained by flowing certified, pre-purified nitrogen containing less than 5 ppm of O₂ (Canadian Liquid Air, Hamilton) at 0.7 litres/min over the suspension for 45 minutes prior to irradiation. Aerobic conditions were obtained by flowing O₂ (Canadian Liquid Air, Hamilton) rather than N₂.

2.3.3 Irradiation and Colony Forming Ability

Following the required accumulated doses of radiation, 1 ml aliquots were taken from the vessel, diluted in normal growth medium and plated into five 60 x 15 mm plastic tissue culture petris (Falcon Plastics) containing 6 ml of cloning medium (see 2.3.4). The cells were then incubated under standard conditions in order that each surviving cell could proliferate and form a tight cluster of cells or colony. After an incubation period of 8 days for the CH2B₂ line and 15 days for the L-60 line, the cloning medium was poured

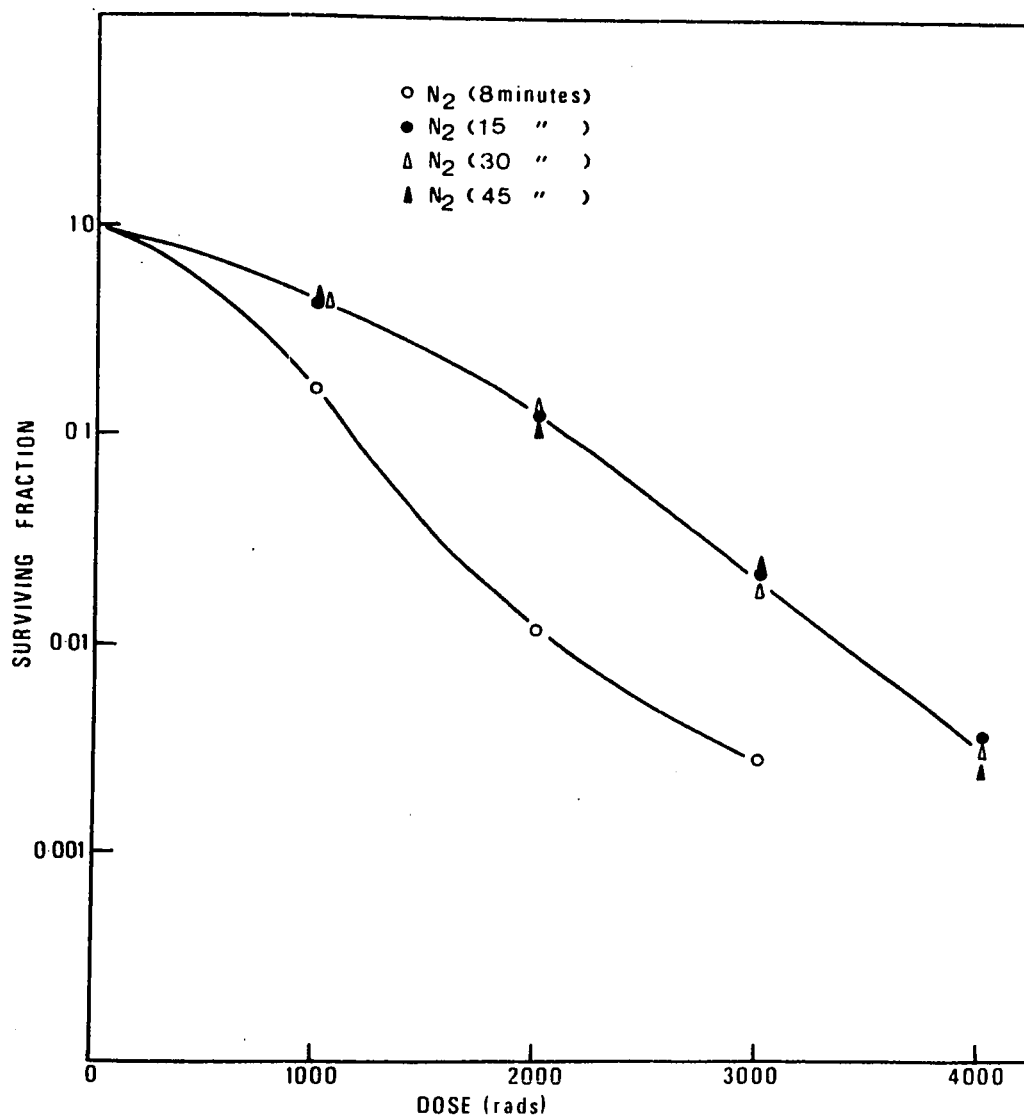


FIGURE 2. Survival Curves of Chinese Hamster Cells Irradiated in Glass Irradiation Vessels Following Various Gassing Intervals Prior to Irradiation

Chinese hamster cells suspended in growth medium (minus sodium bicarbonate) were irradiated in glass irradiation vessels following 8 minute (○), 15 minutes (●), 30 minutes (△) and 45 minutes (▲) of gassing with N₂ prior to the start of irradiation. Following irradiation the cells were diluted and plated in order to determine colony forming ability (3.2.2).

from each petri and the colonies were stained for 6 minutes at room temperature with 2 ml of a methylene blue solution (2 gm/litre). The number of colonies was then counted with the criterion of 50 cells being used to define colony forming ability (i.e., only colonies with more than 50 cells were counted).

2.3.4 Cloning Media

For the CH2B₂ line the cloning medium consisted of normal growth medium plus 4% fetal calf serum (Gibco) and 10⁵ heavily irradiated (6000 rads - under aerobic conditions) feeder cells per petri. Cloning medium for the L-60 line contained normal growth medium plus 8% horse serum (Gibco) and 10⁵ heavily irradiated (6000 rads) feeder cells per petri.

2.3.5 Plating Efficiency

In experiments where the end point measured is colony forming ability, we define a survivor as a cell that will form a colony of 50 or more cells in the allotted incubation time (see 2.3.3). The fraction of cells plated (determined using a Coulter Counter) that will form a colony is referred to as the 'surviving fraction'.

In a population of untreated cells (controls) not all of the cells will form colonies because some may divide a limited number of times or not at all. The term 'plating

efficiency' is used to indicate what percentage of the control cells will grow into colonies.

2.3.6 Normalized Surviving Fraction

The surviving fraction of cells exposed to some treatment is corrected for those cells that will not form colonies in any case. This is done by dividing the 'surviving fraction' by the plating efficiency to yield the 'normalized surviving fraction'. In this thesis, the term 'surviving fraction' refers to the normalized surviving fraction.

2.3.7 Survival Curves

When mammalian cells are irradiated and the log of the surviving fraction is plotted as a linear function of dose, generally the resulting survival curve has a threshold (shoulder) followed by a region of exponential survival. If the straight line portion of the survival curve is extrapolated back to zero dose, its intercept with the ordinate is referred to as the extrapolation number. The reciprocal of the slope of the straight line region is denoted D_0 .

When the effects of irradiation are qualitatively similar, one can superimpose one survival curve onto the other by the application of a dose scale factor called a dose modifying factor (DMF).

In this thesis the DMF is determined by calculating the ratio of the D_0 values of the 2 curves.

2.4 THE CELL PELLET SURVIVAL EXPERIMENTS

2.4.1 Formation of the Cell Pellet

Chinese hamster cells were trypsinized (2.1.2) and resuspended in normal growth medium lacking sodium bicarbonate at a cell concentration of approximately 5×10^6 cells/ml. Twenty millilitres of suspension was placed into each of two 50 ml glass centrifuge tubes at which time the sensitizer to be tested (see Section 2.7) was added to one tube. Anoxia was obtained by flowing nitrogen at 0.7 litres/min through each tube (see Figure 3 (a)) for 60 minutes at 20°C during which time the tubes were regularly shaken. Both centrifuge tubes were then sealed to maintain anoxia and centrifuged for 8 minutes at 120 g. Following centrifugation nitrogen was again flushed through the tubes as the supernatant was drawn off with an aspirator (see Figure 3 (b)).

2.4.2 Irradiation of Pellet Samples

The cell pellets were incubated at 20°C and at various times after the end of centrifugation samples of the pellets (2×10^5 - 5×10^5 cells) were drawn into the ends of 1 ml plastic disposable pipets (Falcon Plastics) and (a) resuspended in normal growth medium to give the drug treated and untreated zero dose controls, or (b) transferred

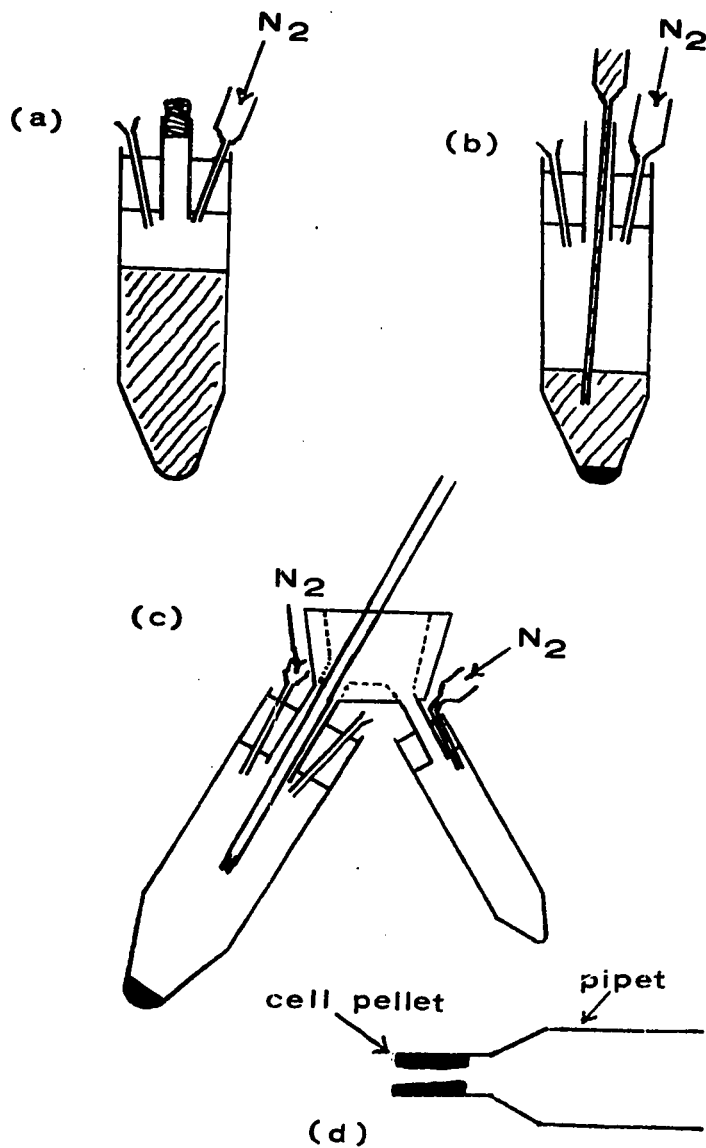


FIGURE 3. The Apparatus for Preparing the Cell Pellet Experiment Showing the Techniques for Achieving Anoxia, Removing the Supernatant while Maintaining Cell Anoxia and Preparing the Pellet Sample for Irradiation

The apparatus shown above is designed to keep the cell pellets and pellet samples anoxic until irradiation is completed.

into a 12 ml pyrex centrifuge tube while keeping the cells anoxic (see Figure 3 (c)) and irradiated while in the pipet with 2500 rads. Once the sample was taken from the pellet, nitrogen continued to flow over the cells (see Figure 3 (d)) until the sample was resuspended. After resuspension of the pellet samples in aerobic growth medium, the cells were counted, diluted and plated into 15 x 60 mm plastic petris containing 6 ml of cloning medium. Cell survival was determined as described in Section 2.3.3.

2.4.3 Pellet Experiments at 0°C

In order to greatly reduce the metabolic activity in the cells the pellet experiment was performed (see Sections 2.4.1 and 2.4.2) keeping the cells at 0°C from a few minutes after the drug was added until the termination of irradiation.

The cells were immersed in an ice bath both prior to and following centrifugation at 0°C. Samples to be irradiated were removed from the pellet with a precooled plastic pipet and placed into a 12 ml centrifuge tube that was also kept at 0°C both prior to and during irradiation. Following irradiation the samples were resuspended in normal growth medium, counted, diluted and plated as described in Section 2.3.3.

2.4.4 The Cell Concentration Experiment

An experiment was performed in order to determine the sensitization of 10 mM TAN at cell concentrations between 2×10^5 cells/ml and 7×10^8 cells/ml (cell pellet). The experimental procedure is described in Section 3.1.12.

2.5 ELECTRON SPIN RESONANCE (ESR) MEASUREMENTS

2.5.1 Preparation of Cell Sample

Chinese hamster cells were suspended in normal growth medium lacking sodium bicarbonate at a cell concentration of 2×10^6 cells/ml and were incubated under aerobic conditions in the presence of 10 mM TAN for 60 minutes at 37°C. Following incubation the cells were centrifuged at 120 g for 8 minutes, the supernatant was drawn off, and known weights of both supernatant and cell pellet were placed into separate quartz tubes where they were kept at -196°C until the ESR measurements were taken.

2.5.2 ESR Measurements

The change with time in the number of spins in the pellet relative to the number of spins in an equal mass of supernatant was determined by taking the derivative of the complete ESR absorption spectrum of the pellet every few minutes at a specific temperature (20°C or 37°C). The number of spins in the pellet relative to the number of spins in the supernatant was determined by comparing the double integral of the derivatives of the spectrums, as this quantity is proportional to the number of spins. The derivative of the absorption spectrum was obtained using a JEOL spectrometer model number JES-3BS-X.

2.6 SEDIMENTATION STUDIES

The technique described for alkaline sucrose gradients is essentially that described by B. Palcic and L. D. Skarsgard (1972) with the only changes being those necessary to accommodate the larger 17 ml tubes for the Beckman SW27 rotor.

2.6.1 Radioactive Labelling

For labelling purposes 20 x 100 mm plastic petri dishes containing 20 ml of medium (see Section 2.1.3) were seeded with 5.6×10^6 mouse L-60 cells and allowed to attach to the plastic during a 1 hour incubation under standard conditions. The medium was then drawn off and replaced with 20 ml of radioactive medium (2 $\mu\text{C}/\text{ml}$) containing 19.6 ml of growth medium, and 0.4 ml of 100 $\mu\text{C}/\text{ml}$ of $^3\text{HTdR}$ (tritiated thymidine, 15.5 curies/mole, Amersham-Searle, Don Mills, Ontario). The cultures were then incubated for 24 hours under standard conditions. The labelling was terminated by an additional 1 hour incubation in non-radioactive medium.

2.6.2 Irradiation Procedure

Following the one hour cold chase the cells were trypsinized as described in Section 2.1.2 and resuspended in CMRL 1066 nutrient medium (lacking sodium bicarbonate) at a concentration of 2×10^5 cells/ml. The cells were loaded

into the glass irradiation vessels and gassed with N₂ or O₂ at 0.7 litres/min for 1 hour at 0°C. Immediately following irradiation the gas flow was discontinued but the cells were kept at 0°C while they were centrifuged, resuspended in medium at 2 x 10⁶ cells/ml and then lysed on top of the alkaline sucrose gradient.

2.6.3 Gradients

An automatic gradient former (ISCO Model 570) was used to prepare 5-20% alkaline sucrose gradients in 17 ml cellulose nitrate tubes (Beckman Instruments). The gradient solutions contained 0.3 molar sodium hydroxide, 0.01% sodium dodecyl sulphate (SDS), and 0.001 molar ethylenediamine tetraacetate (EDTA) and appropriate concentrations of sucrose. On top of a prepared gradient 0.5 ml of lysing solution containing 0.5 molar sodium hydroxide, 0.2% SDS and 0.01 molar EDTA was carefully layered just prior to lysis. A 50 microlitre microsyringe, precooled to 0°C was used to dispense 0.02 ml of cell suspension (containing 2 x 10⁴ to 3 x 10⁴ cells in normal medium) into the lysing layer. Cells were lysed on top of the gradient for 12-14 hours at room temperature after which time they were centrifuged at 20°C using an SW27 rotor in a Beckman L-65 preparative ultracentrifuge.

2.6.4 Collecting and Counting

After centrifugation, 25 fractions from each tube were collected in scintillation vials using an ISCO Model D fraction collector. The 0.75 ml fractions were made acidic with the addition of 0.2 ml of 4 molar HCl following which 5 ml of Aquasol scintillation fluid (New England Nuclear, Boston, Massachusetts) was added. The radioactivity was measured in a Beckman scintillation counter (Model LS-233).

2.7 SENSITIZING DRUGS

2.7.1 Triacetoneamine N-Oxyl (TAN)

Samples of TAN, a highly water soluble nitroxide free radical, were obtained from Dr. P. T. Emmerson and Dr. Barrie Jones and were stored at 4°C, generally in a nitrogen atmosphere.

Stock solutions were prepared by dissolving the yellow crystalline material in double distilled water to produce a 0.2 molar solution. After filtration (0.22 μ Millipore filter), the solution was stored at 4°C. Fresh solutions were made up every 2 weeks as prolonged storage resulted in the formation of a precipitate. The sensitizing properties of a 2 week old stock solution were found to be the same as those for freshly prepared samples.

2.7.2 p-Nitroacetophenone (PNAP)

4'-nitroacetophenone, an electron affinic compound, was purchased from Fisher Scientific (Toronto).

Immediately prior to use, a fresh 0.1 molar solution of PNAP was prepared by dissolving the compound in 95% ethanol. The drug was then added to the cell suspension to give a concentration of 500 μ M PNAP. The resulting cell suspension also contained 0.5% ethanol. The controls containing no PNAP also contained 0.5% ethanol, a concentration

that does not affect cell survival.

2.7.3 Dimethyl Fumarate

Dimethyl fumarate was obtained from Fisher Scientific (Toronto). A few hours before use, the drug was dissolved in double distilled water to produce a concentration of 10 mM and then the solution was filtered (0.22 μ Millipore filter).

2.7.4 Nitrofurazone

Nitrofurazone, one of the class of compounds commonly referred to as the nitrofurans, was obtained from Dr. D. R. McCalla (McMaster University) and stored at 4°C in crystalline form. The solutions of nitrofurazone were prepared by dissolving the compound at 23°C in normal growth medium (minus sodium bicarbonate) overnight and then filtering this solution through a millipore filter. The compound was protected from fluorescent light at all times to prevent its decomposition in solution.

2.7.5 A Mannich Derivative of PNAP (PNAP-M)

Two small samples were received from Dr. G. E. Adams via Dr. G. F. Whitmore. PNAP-M was dissolved in a phosphate buffered saline (PBS) solution and filtered prior to use. The first sample was prepared as a 2 mM stock solution and stored in the freezer, but because of irreproducible

results the later samples were prepared fresh about 1 hour prior to use. The difficulties with this compound regarding its instability and toxicity are discussed in Sections 3.5.1 and 3.5.2 (chemical formula: $[\text{NO}_2 \cdot \text{C}_6\text{H}_4\text{CO} \cdot \text{CH}_2\text{CH}_2\text{N}(\text{CH}_3)_2]^+\text{Cl}^-$).

2.7.6 Other Compounds

Four additional compounds which were tested were all dissolved in PBS immediately prior to use (except phenyl pyruvate which was dissolved in normal growth medium lacking sodium bicarbonate) at the following concentrations:

- (a) Phenyl pyruvate - the sodium salt of phenyl pyruvic acid (Calbiochem, Spring Valley, New York), 10 mM.
- (b) 2-Methyl-1,4-napthaquinone (Fisher Scientific Company, Toronto), 50 mM.
- (c) Ethyl pyruvate (Canlab, Toronto), 200 mM.
- (d) Phenyl glyoxal (K and K Laboratories, Inc., Plainsview, New York), 150 mM.

In all cases, the filtration of the solutions (0.22 μ Millipore filter) effectively sterilized them.

3. RESULTS

3.1 TRIACETONEAMINE N-OXYL (TAN) RESULTS

At the time that this work was started, TAN was known to preferentially sensitize dilute suspensions of E. coli (Emmerson, 1967) and Chinese hamster cells (Parker, Skarsgard and Emmerson, 1969) to the lethal effects of ionizing radiation when present during irradiation but failed to sensitize cells in vivo (Emmerson and Montana, 1967). The following experiments were performed to obtain an understanding of the mechanisms of TAN sensitization in mammalian cells and to attempt to discover the reason for its failure to sensitize in vivo.

3.1.1 Treatment of L-60 Cells with TAN

In these experiments (performed at 23°C) TAN was added to the suspension of L-60 cells 60 minutes prior to irradiation and was removed immediately after irradiation by dilution in TAN-free medium. The concentration of TAN in the cloning medium for the CH2B₂ and the L-60 cell lines after inoculation of the petris was always less than 0.1 mM, a concentration found to have no effect on the colony forming ability of irradiated or unirradiated cells.

In anoxic Chinese hamster cells TAN present at the time of irradiation gives dose modifying factors (DMF's) for 1 mM TAN and 10 mM TAN of 1.3 and 1.5 respectively (Parker, Skarsgard and Emmerson, 1969). The DMF for O_2 in those experiments was 2.8 with or without TAN present.

Figure 4 shows the results obtained with 10 mM TAN present during the irradiation of anoxic suspensions of the L-60 line. The DMF's for 10 mM TAN and O_2 are 1.35 and 2.9 respectively, calculated from the slopes of the survival curves. Aerobic suspensions of L-60 cells were irradiated in the presence of 10 mM TAN and the results are shown in Figure 5. TAN does not sensitize aerobic L-60 cells to ionizing radiation, a result which agrees with experiments performed using the CH2B₂ line.

In all experiments using both cell lines, the presence of 10 mM TAN for periods up to 2 hours did not affect the plating efficiency of the unirradiated controls.

Throughout this thesis, the term "TAN treatment" will be used to describe the situation where the sensitizer is present at the time of irradiation. Analogous terms will be used for the other sensitizers.

3.1.2 Pretreatment with TAN (23°C throughout)

In experiments with bacteria, it has been found that TAN must be present at the time of irradiation in order to

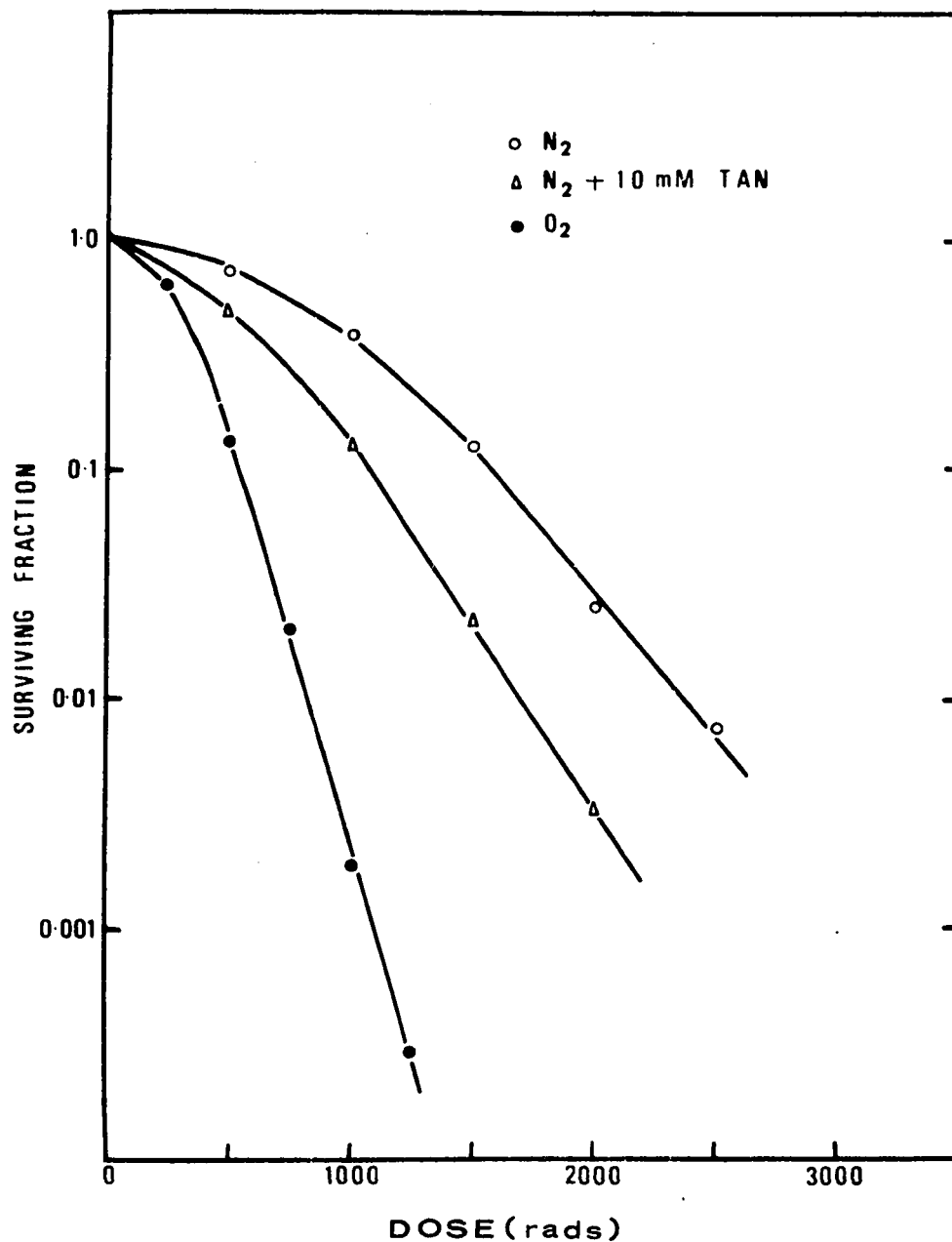


FIGURE 4. Survival Curves for Mouse L-60 Cells Irradiated in the Presence of Nitrogen, Nitrogen Plus 10 mM TAN, and Oxygen

Mouse L-60 cells suspended in normal growth medium minus sodium bicarbonate and at a cell concentration of 2×10^5 cells/ml were gassed for 45 minutes in a glass irradiation vessel in the presence of N₂ (O), N₂ + 10 mM TAN (Δ) or O₂ (\bullet) prior to irradiation. Following irradiation the cells were diluted and plated in order to determine colony forming ability (2.3.3). Cells treated with 10 mM TAN were exposed to the drug 60 minutes prior to irradiation.

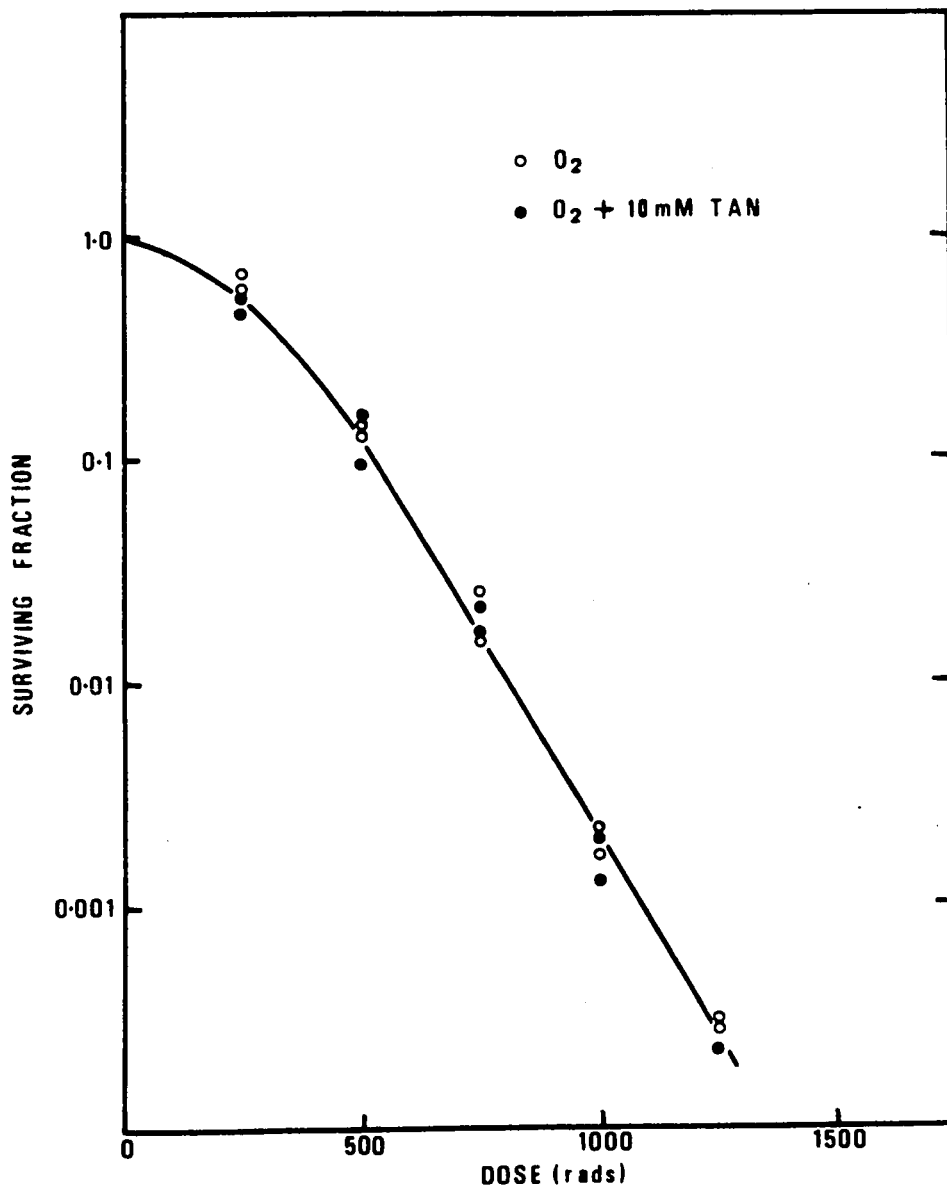


FIGURE 5. Survival Curves for Mouse L-60 Cells Irradiated in the Presence of O₂ and O₂ + 10 mM TAN

Mouse L-60 cells suspended in normal growth medium minus sodium bicarbonate and at a cell concentration of 2×10^5 cells/ml were gassed for 45 minutes in a glass irradiation vessel in the presence of O₂ (O) or O₂ + 10 mM TAN (●) prior to irradiation. Following irradiation the cells were diluted and plated in order to determine colony forming ability. The cells treated with TAN were exposed to the drug 60 minutes prior to irradiation. The results of two experiments are shown.

effect sensitization (Emmerson, 1968; Adams, 1970), indicating that TAN sensitization involves the interaction of the compound with a radiation produced, short-lived, reactive chemical species. The pretreatment experiments with the CH₂B₂ line described below were performed to help determine if interactions with radiation produced, short-lived species are the important events in TAN sensitization of mammalian cells.

Chinese hamster cells suspended at a concentration of 2×10^5 cells/ml in normal growth medium lacking sodium bicarbonate were treated for 1 hour at 23°C with 10 mM TAN under anoxic or aerobic conditions. The TAN was then removed by twice centrifuging the suspension at 40 g for 8 minutes and resuspending the cells at a concentration of 2×10^5 cells/ml in TAN-free medium lacking sodium bicarbonate. The cells were placed in a vessel and flushed with N₂ for 45 minutes at 23°C prior to irradiation. Following irradiation the cells were plated for colony forming ability (see Section 2.3.3). The results in Figure 6 show the sensitization produced by a 1 hour pretreatment with 10 mM TAN, the TAN being removed 2 hours prior to irradiation. The sensitization produced, although less than when 10 mM TAN is present at the time of irradiation, is still significant (DMF = 1.3). The TAN pretreatment experiment was repeated varying the time between the removal

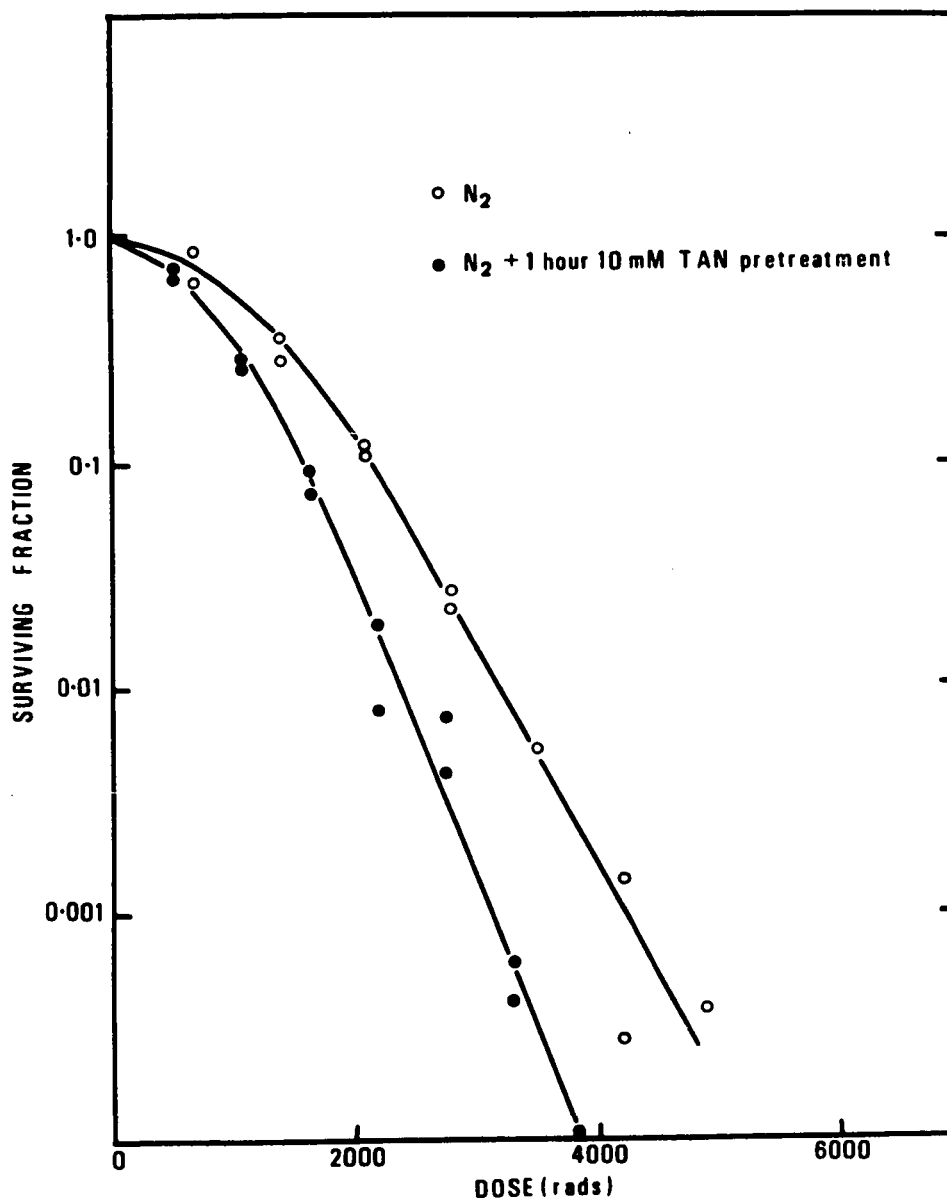


FIGURE 6. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen and Irradiated Following Pretreatment with 10 mM TAN.

Chinese hamster cells suspended in normal growth medium minus sodium bicarbonate were incubated in the absence (○) or presence (●) of 10 mM tan for 60 minutes at 23°C, after which time they were centrifuged twice and resuspended in TAN-free, normal growth medium lacking sodium bicarbonate. The cells were then placed in a glass irradiation vessel, gassed with N₂ for 45 minutes at 23°C, irradiated and assayed for colony forming ability (see 2.3.3). TAN was removed from the cells 2 hours prior to irradiation. The results of two experiments are shown.

of the drug and irradiation and for time intervals of 1 to 4 hours the DMF remained the same.

3.1.3 Pretreatment with TAN (0°C during TAN Pretreatment)

It was of interest to determine whether the sensitization observed following pretreatment with TAN was the result of TAN binding to cellular sulfhydryl groups and thus rendering them inactive as normal cellular radioprotective agents (see Section 1.3.1). Since the reaction of TAN with sulfhydryl groups has been found to be inhibited at 0°C (Emmerson, 1967), the pretreatment experiment was repeated keeping the cells at 0°C from the time prior to the addition of TAN until after the second washing with TAN-free medium. The cells were then warmed to 23°C, placed in a vessel and irradiated under anoxic conditions. The results are shown in Figure 7 and can be compared with TAN pretreatment at 23°C which was performed at the same time. The results show that the pretreatment effect is independent of temperature, yielding a DMF of 1.3 at both 0°C and 23°C. The results then indicate that a sulfhydryl binding mechanism is probably not involved in pretreatment sensitization. This 0°C pretreatment experiment also suggests that neither active membrane transport nor cell metabolism of TAN are necessary events in pretreatment sensitization since most enzymatic activity is greatly reduced at 0°C.

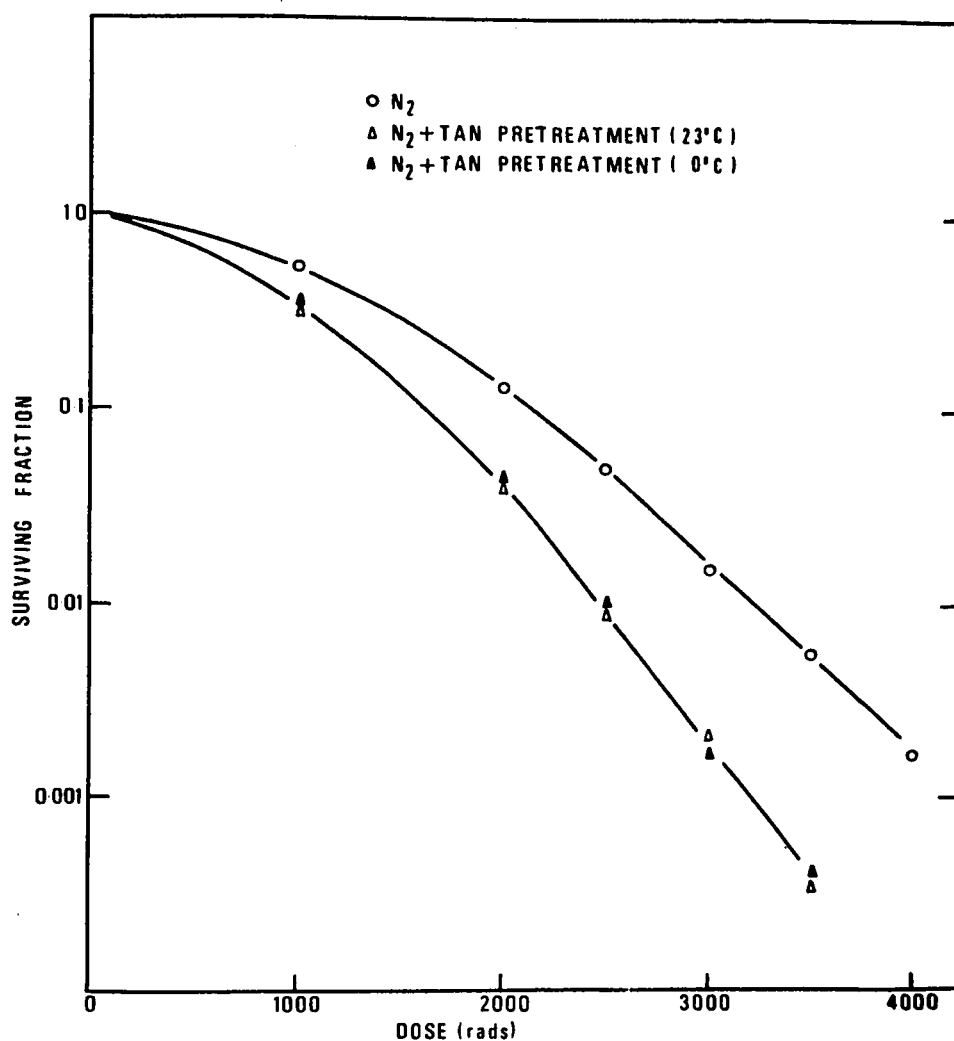


FIGURE 7. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen and Irradiated Following Pretreatment at 0°C or 23°C with 10 mM TAN

Chinese hamster cells suspended in normal growth medium lacking sodium bicarbonate were incubated at 0°C (\blacktriangle) or at 23°C (Δ) during a 1 hour exposure to 10 mM TAN, after which time they were centrifuged twice and resuspended in drug-free medium. The cells were then gassed for 45 minutes with N_2 , irradiated and assayed for colony forming ability (2.3.3). The anoxic survival curve for Chinese hamster cells incubated at 23°C for 1 hour in the absence of TAN is also shown (\circ).

3.1.4 Pretreatment with TAN (0°C following TAN pretreatment)

It is possible that following pretreatment with TAN a small amount of the compound remains associated at crucial sites in the cell, perhaps bound to cell membranes (Hubbel and McConnell, 1968; Griffith and Waggoner, 1969) or to nucleic acid, and is not washed away. If this were the case and if the cell were able to quickly metabolize the drug, then one might expect to see a temperature dependent loss in sensitization in the first hour after removal of the drug as the cell breaks down the remaining compound. With this in mind, the pretreatment experiment was repeated with the cells kept at 0°C from the time that the TAN was removed until the completion of irradiation, to see whether this might result in a larger DMF (perhaps comparable to the DMF obtained when TAN is present during irradiation). The results shown in Figure 8 demonstrate that the low temperature did not alter the DMF of pretreatment with 10 mM TAN. The lower temperature prior to and during irradiation does not alter the normal anoxic response without TAN present (see Figure 9) so that the survival curve in Figure 8 cannot simply be the fortuitous result of a greater pretreatment effect compensated for by a greater inherent cell radioresistance at 0°C. It should be noted that the data shown in Figure 8 represent a reduced sensitization for TAN treatment (TAN present during irradiation) as well as TAN pretreatment.

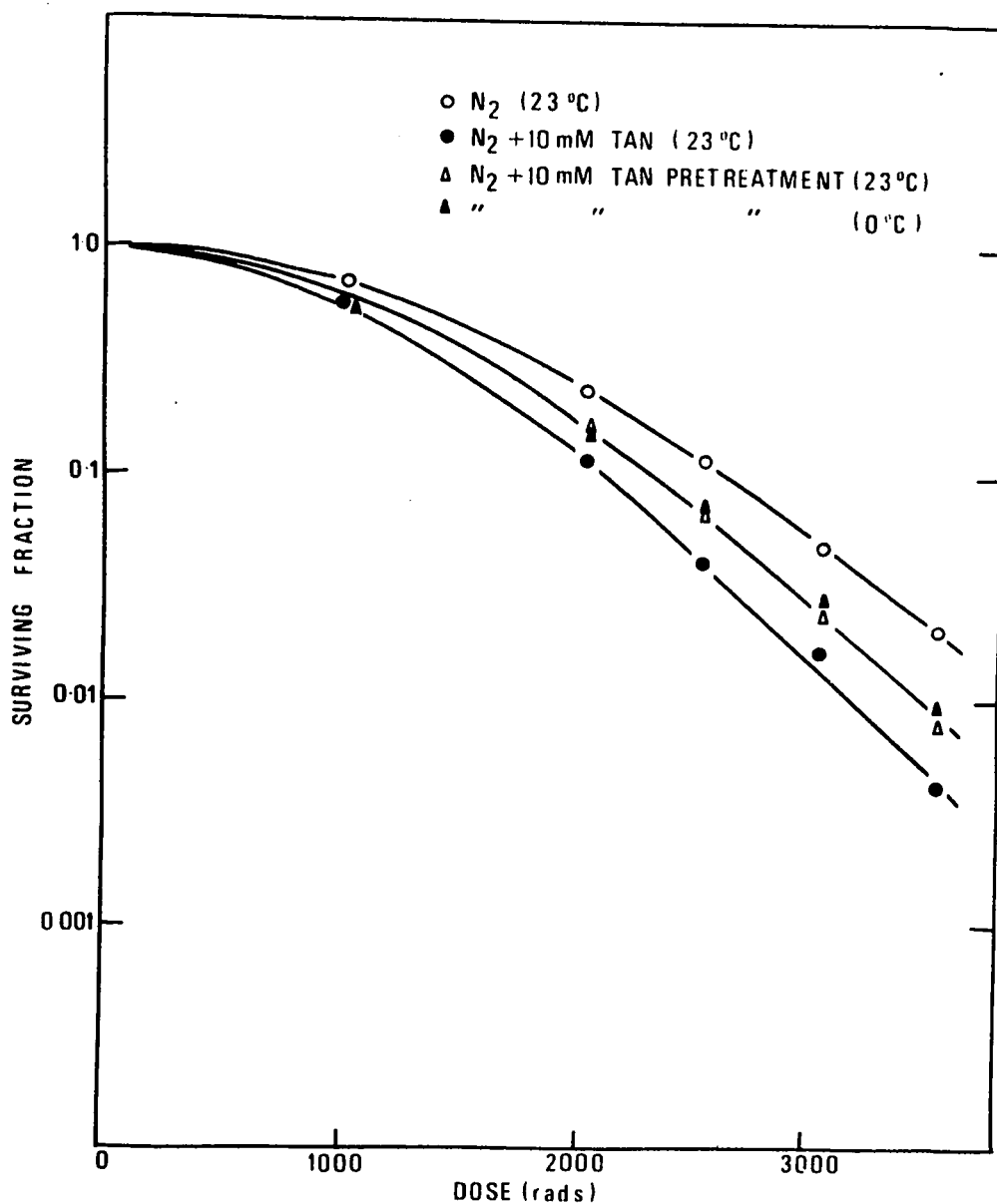


FIGURE 8. Survival Curves of Anoxic Chinese Hamster Cells Following a 1 Hour Pretreatment at 23°C with 10 mM TAN and a Subsequent Incubation at 0°C Until the End of Irradiation

Chinese hamster cells suspended in normal growth medium lacking sodium bicarbonate were incubated for 1 hour at 23°C in the presence of 10 mM TAN. The drug was then removed by centrifuging the cells twice and resuspending them in normal medium lacking sodium bicarbonate. The cells were kept at 0°C (▲) or at 23°C (△) while they were gassed with N_2 and irradiated. Controls were also obtained under anoxia by irradiating TAN untreated cells (○) and cells exposed to 10 mM TAN during irradiation (●).

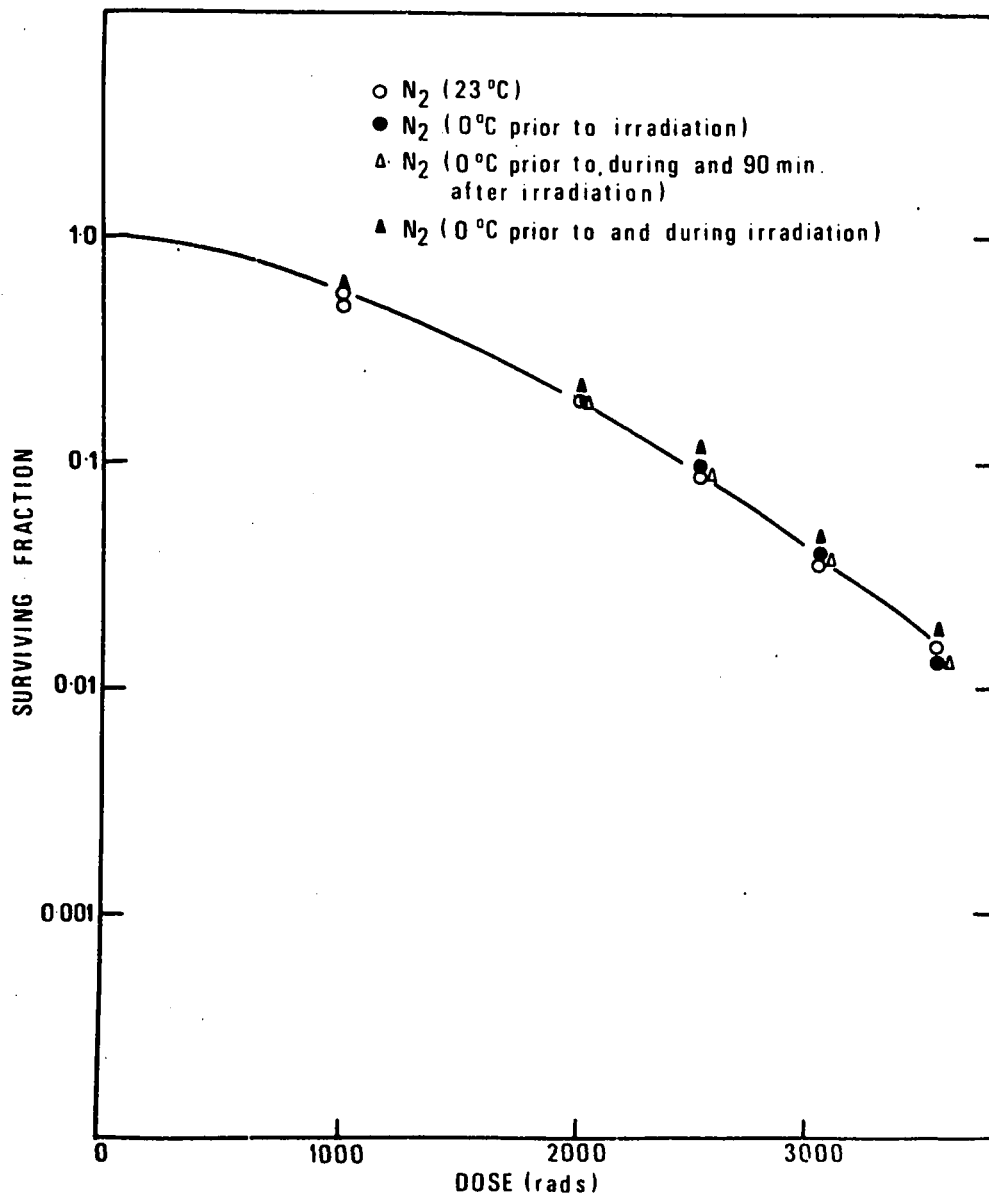


FIGURE 9. Survival Curves of Chinese Hamster Cells Irradiated in the Presence of Nitrogen: The Exposure of Cells to a Temperature of 0°C

Chinese hamster cells suspended in normal growth medium minus sodium bicarbonate were incubated at 0°C over time intervals shown in the above figure. Treatment at 0°C prior to irradiation involved a 45 minute interval during which time the vessel was being gassed with N₂. In all cases, cells were removed from the vessel immediately following irradiation.

This may be the result of impurities which were observed (a small amount of water insoluble residue) in the last sample of TAN we received. However, the identical sensitization seen with 23°C and 0°C pretreatment (Figure 8) suggests that cell metabolism is not involved in the reduced DMF observed in the pretreatment experiment.

3.1.5 Removal of TAN from the Cell in Pretreatment

In order to determine if TAN is effectively washed from the cells prior to irradiation in the pretreatment experiments, a measure was made of the free spins remaining. Cells at a concentration of 2×10^6 cells/ml were treated with TAN for 1 hour at 23°C, centrifuged (40 g for 8 minutes) and washed two times in TAN-free medium. The cells were then centrifuged (120 g for 8 minutes) and the supernatant removed. The cell pellets were placed into quartz tubes and checked immediately for free spins using electron spin resonance (ESR) techniques. No spins could be detected indicating that the TAN concentration in these cells was less than 10^{-5} Molar. We also did an experiment where TAN was added to cells but not washed away, the cells were centrifuged as above, the supernatant was withdrawn and the cells checked immediately for free spins. We found 80% as many spins in the cell pellet as in an equal mass of supernatant taken when the pellet was formed (see cell pellet-

ESR experiment, Figure 13). These results indicate that in the pretreatment experiment, washing the cells prior to irradiation reduces the concentration of TAN as a free radical within the cell to a very low level, and suggests that some mechanism other than the interaction with short-lived reactive species (as occurs for O_2) is operative in this case. It cannot be ruled out, however, that following the pretreatment with TAN, small quantities of the compound remain in the cell for long periods of time to produce a partial sensitization by a mechanism similar to O_2 .

3.1.6 Post-treatment with TAN

Pretreatment experiments with TAN produce significant sensitization suggesting that the interaction of TAN with short-lived species evident in bacteria is not the only mechanism that could be operating in mammalian cells. To further investigate the modes of action of TAN in mammalian cells, post-treatment experiments were performed in which TAN was added to the cells after irradiation, at a time when fast reactions such as those involving radiolytic species would have been completed.

An anoxic suspension of Chinese hamster cells (kept at 33°C, 35°C or 37°C for 1 hour prior to, during and following irradiation) was exposed to a single dose of 3000 rads. At various times after the end of irradiation

1 ml aliquots were taken from the anoxic suspension and added to an aerobic, 9 ml solution of 11.1 mM TAN to give a final TAN concentration of 10 mM. After 60 minutes at 23°C the cells were removed from TAN by centrifuging (40 g for 8 minutes), resuspended in TAN-free medium and plated to determine colony forming ability. In addition, control samples were obtained:

- (i) by repeating the above protocol but omitting TAN from the post-irradiation incubation medium, and
- (ii) by irradiating cells in the presence of 10 mM TAN, centrifuging (40 g for 8 minutes), resuspending in TAN-free medium and then plating immediately.

The combined results of 3 TAN post-treatment experiments are shown in Figure 10. "Incubation time" (abscissa) refers to the time interval between the termination of irradiation and the addition of cells to the aerobic TAN solution. The survival of control cells irradiated under anoxic conditions with no TAN post-treatment (open circles) or irradiated in the presence of 10 mM TAN (enclosed circles) is not altered by the temperature or incubation under anoxia for periods at least up to 2 hours. The results do demonstrate, however, a significant amount of sensitization with TAN post-treatment, the extent of which depends on (a) the time after the end of irradiation at which TAN is added to the cells, and (b) the incubation temperature of the cell suspension.

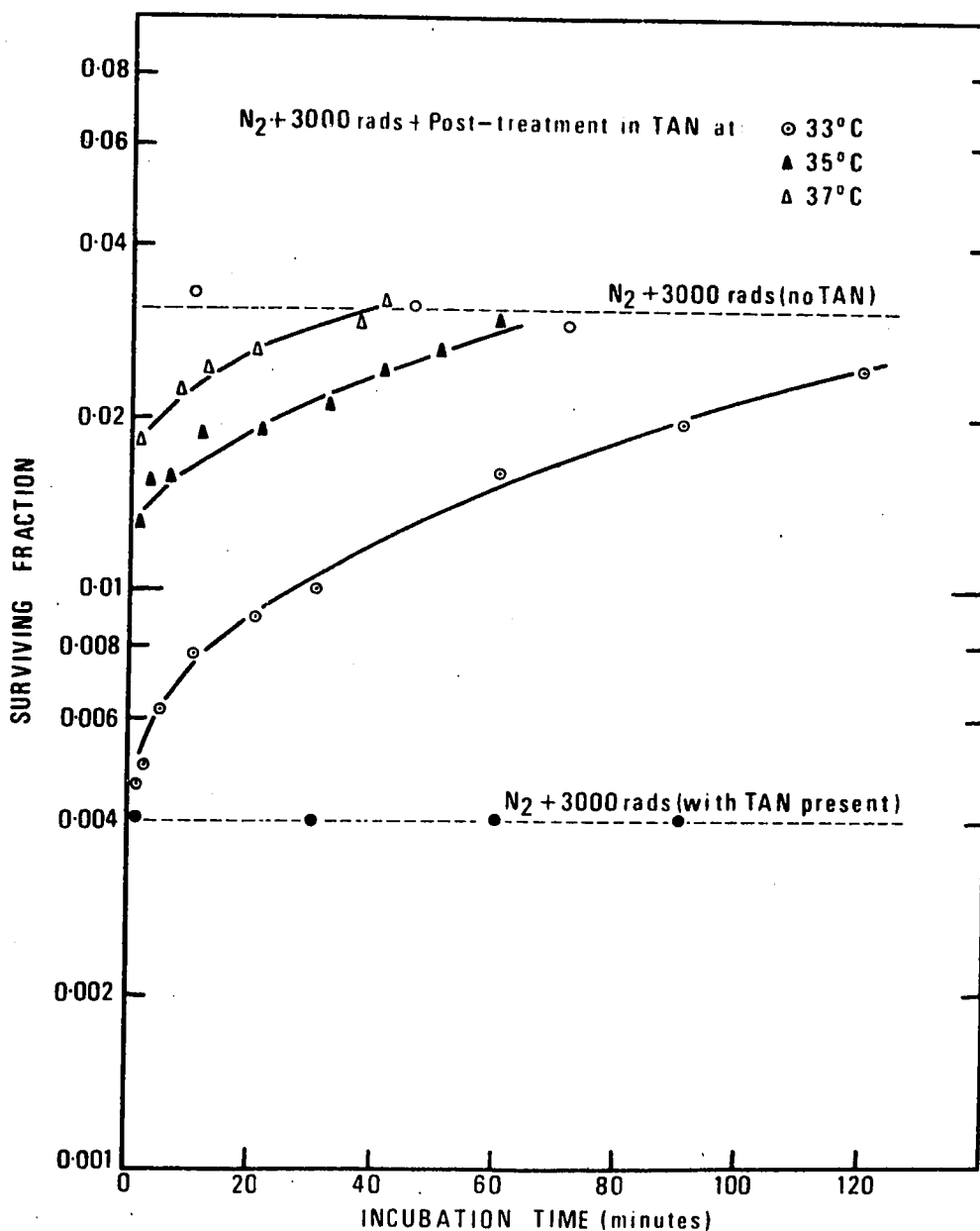


FIGURE 10. Surviving Fraction of Chinese Hamster Cells Subjected to TAN Post-treatment at Various Temperatures as a Function of Incubation Time after the Termination of Irradiation

Chinese hamster cells suspended in growth medium lacking sodium bicarbonate were incubated at 33°C (○), 35°C (▲) or 37°C (△) prior to, during and following irradiation under anoxic conditions. At various times after the end of irradiation, aliquots were removed from the anoxic irradiation vessel and added to 10 mM TAN. After a 1 hour exposure at 23°C to the aerobic TAN solution, the cells were centrifuged twice, resuspended in TAN-free medium and assayed for colony forming ability (2.3.3).

At 33°C and one minute after the end of irradiation, TAN post-treatment gives almost full sensitization, but with longer intervals between irradiation and the addition of TAN, the sensitization decreases. At even the earliest times at which TAN was added to the cells following irradiation (1 minute) the reactions involving the short-lived, radiolytic species would have been completed. It appears then that in Chinese hamster cells TAN is able to interact with relatively long-lived transient states following irradiation and this interaction results in increased cell death. The effect of increasing temperature is to decrease the sensitization of TAN post-treatment at short times after irradiation and hasten the complete disappearance of a post-treatment effect. This temperature dependence could be due to the shortening of a transient state lifetime, perhaps by some cellular metabolic process which would progress more rapidly at the higher temperatures.

These results are in contrast with the situation in bacterial systems where TAN exhibits no post-treatment effect (Adams, 1970). Thus the post-treatment experiments, like the pretreatment, suggest that TAN is able to sensitize by mechanisms other than the one involved in O₂ sensitization, namely the interaction with short-lived species.

3.1.7 Fractionated Dose Experiments

The results of the TAN post-treatment experiments indicate that there may be a component of sensitization involving interference with the enzymatic repair of radiation damage. One experimental means of studying cellular repair of radiation damage is the fractionated dose technique. When a dose of radiation is delivered in two fractions (split dose) separated by a suitable time interval at 37°C, the cell survival is greater than if the dose is delivered all at once. This increased cell survival is attributed to repair of sublethal damage resulting from the first dose (Elkind and Sutton, 1959). Experiments were performed to determine what effect, if any, TAN has on split-dose recovery.

Chinese hamster cells at a concentration of 2×10^5 cells/ml were suspended in normal growth medium minus sodium bicarbonate (plus 10 mM TAN for those cells to be irradiated in the presence of the drug) and gassed with nitrogen for 45 minutes at 37°C prior to irradiation. Single dose survival curves were obtained by removing 1 ml aliquots from the vessel after the required accumulated dose, diluting in TAN-free medium and plating to determine colony forming ability. Cells irradiated with fractionated doses received an initial dose of 2500 rads in the anoxic suspensions and 1000 rads in the aerobic suspensions in order to saturate the mechanism for the repair of sublethal damage. Following

incubation for 60 minutes at 37°C under anoxia the cells were again irradiated, diluted in TAN-free medium and plated to determine colony forming ability.

The results for irradiation under anoxic conditions are shown in Figure 11. The enhanced survival observed in anoxic cells when the dose is fractionated is seen to be suppressed by the presence of 10 mM TAN. It should be mentioned that the amount of recovery under anoxic conditions varied somewhat and may reflect different low level contaminations of oxygen (Koch and Kruuv, 1971). However, in all experiments where split-dose recovery was observed under anoxic conditions, TAN was found to suppress this recovery.

Figure 12 shows that the recovery normally seen in aerobic fractionated dose experiments is also suppressed in the presence of 10 mM TAN. This result is somewhat anomalous in view of the fact that the presence of TAN does not affect the shoulder of the single dose survival curve in O₂. The current concept of the system by which sublethal damage is repaired offers an explanation of this result, however, as will be seen in Section 4.1.1.

It is evident from these results that TAN is able to interfere with the repair of radiation damage although the suppression of the recovery seen in the fractionated dose experiments should be insufficient to explain the degree of

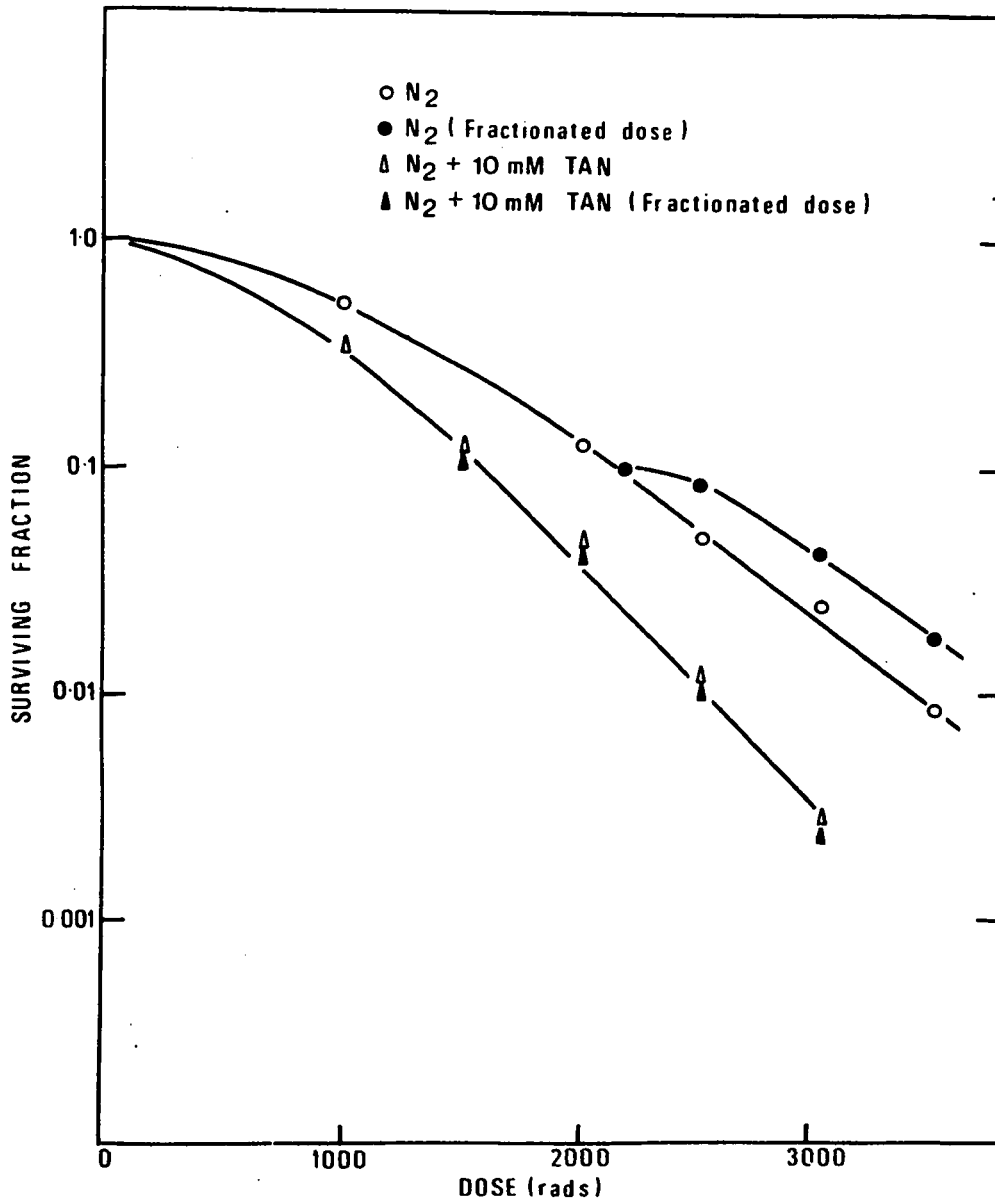


FIGURE 11. Single-dose and Fractionated Dose Survival Curves of Chinese Hamster Cells Suspended in Normal Growth Medium (2×10^5 cells/ml) and Irradiated in the Presence of Nitrogen

Chinese hamster cells incubated in normal growth medium at 37°C were irradiated under anoxic conditions with single-dose (O) and divided dose (●) irradiations and in nitrogen + 10 mM TAN with single-dose (Δ) and divided dose (▲) exposures. The time between fractionated dose exposures was 1 hour and during this time N₂ continued to flow through the vessels.

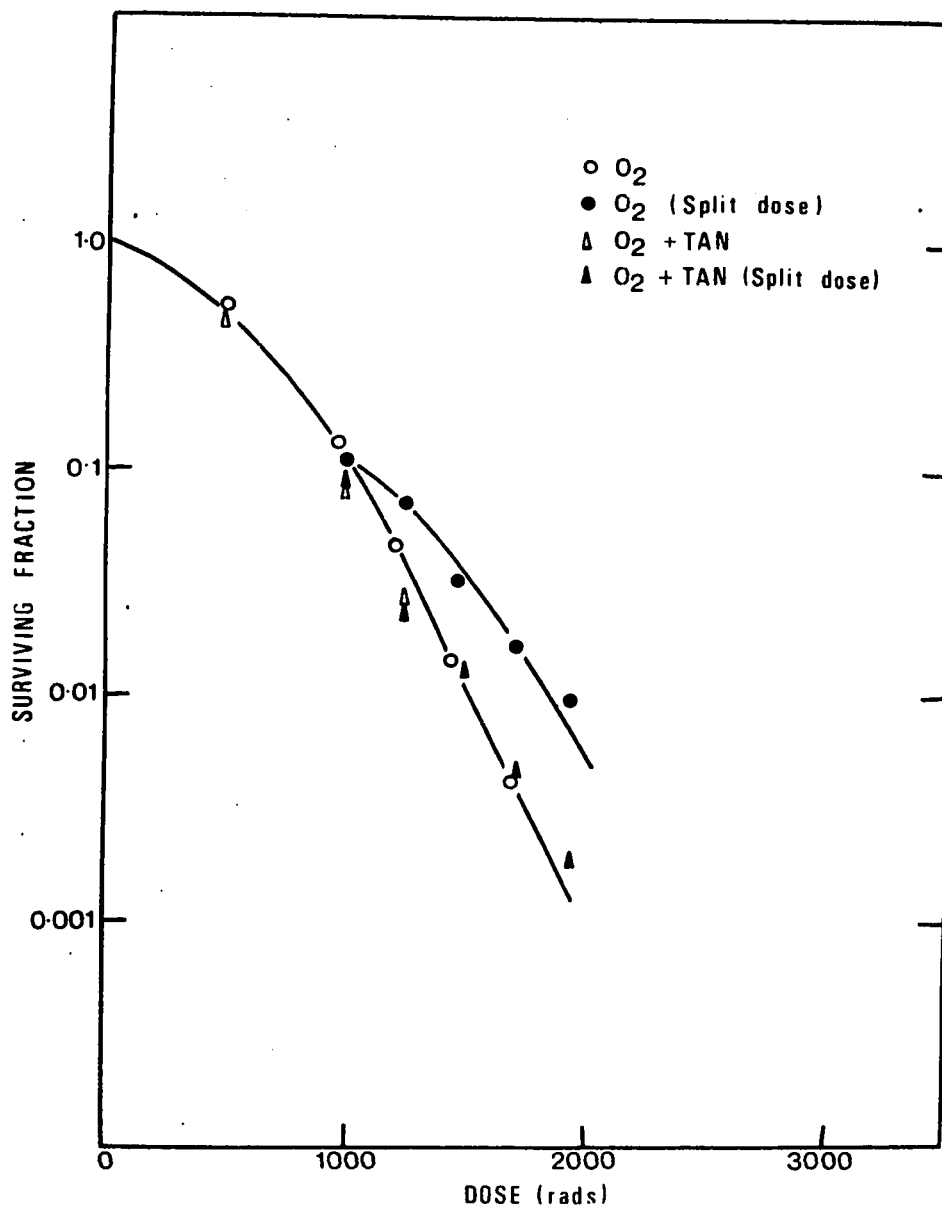


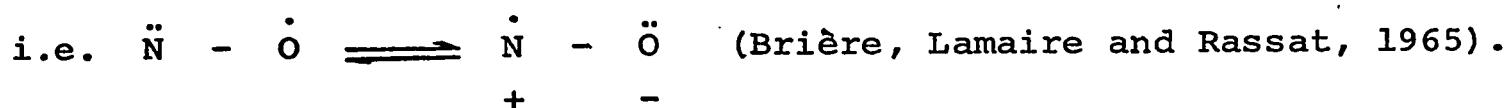
FIGURE 12. Single-Dose and Fractionated Dose Survival Curves of Chinese Hamster Cells Suspended in Normal Growth Medium (2×10^5 cells/ml) and Irradiated in the Presence of Oxygen

Chinese hamster cells incubated in normal growth medium at 37°C were irradiated under aerobic conditions with single-dose (○) and divided dose (●) irradiations and in oxygen plus 10 mM TAN with single dose (△) and fractionated dose (▲) exposures.

sensitization observed in TAN sensitization.

3.1.8 Electron Spin Resonance (ESR) Experiments

TAN is a neutral nitroxide free radical and possesses an unpaired electron in a resonance form in the region of the nitroxide group



The presence of this unpaired electron can be detected in solution or inside the cell using electron spin resonance techniques, and so provides a method for determining the concentration of the TAN free radical within the cell.

Experiments performed with leukaemic cells irradiated in situ in the infiltrated livers of dead leukaemic mice have shown TAN to be ineffective in sensitizing these anoxic cells to irradiation (Hewitt and Blake, 1970). In order to determine if the failure of TAN to sensitize whole animals could be due to metabolism of the drug at high cell concentrations, ESR measurements were made on cells exposed to TAN at high cell concentrations.

TAN-treated cell pellets were prepared as described in 2.6.1. The pellets were warmed to either 20°C or 37°C and the relative number of spins was measured as a function of the time of incubation at these temperatures. Spin

determinations were made on the cell pellet as well as on the supernatant which was withdrawn when the pellets were formed. The results showing the ratio of the number of spins/mg of pellet to the number of spins/mg of supernatant versus time at both 20°C and 37°C are shown in Figure 13. At the earliest measured times the concentration of TAN in the cell pellet was approximately 80% of that in the supernatant (10 mM) and is taken as evidence that TAN does in fact penetrate into the cell. The possibility exists, however, that TAN accumulates on the outside surface of the cell membrane to such an extent that the average TAN concentration in the pellet is, by coincidence, about equivalent to the TAN concentration in the supernatant. Once the pellet is formed, the relative number of spins decreases with time approximately according to zero order kinetics reaching an undetectable level in 40 minutes at 37°C and in 2 hours at 20°C. It appears, then, that TAN is reduced by the cells in pellet form resulting in a loss of free spins by a process that is strongly temperature dependent.

3.1.9 Cell Pellet Survival Experiment (20°C)

If the loss in free spins at high cell concentrations (Figure 13) corresponds to a reduction in the sensitizing capacity of TAN, then cell metabolism of the drug could

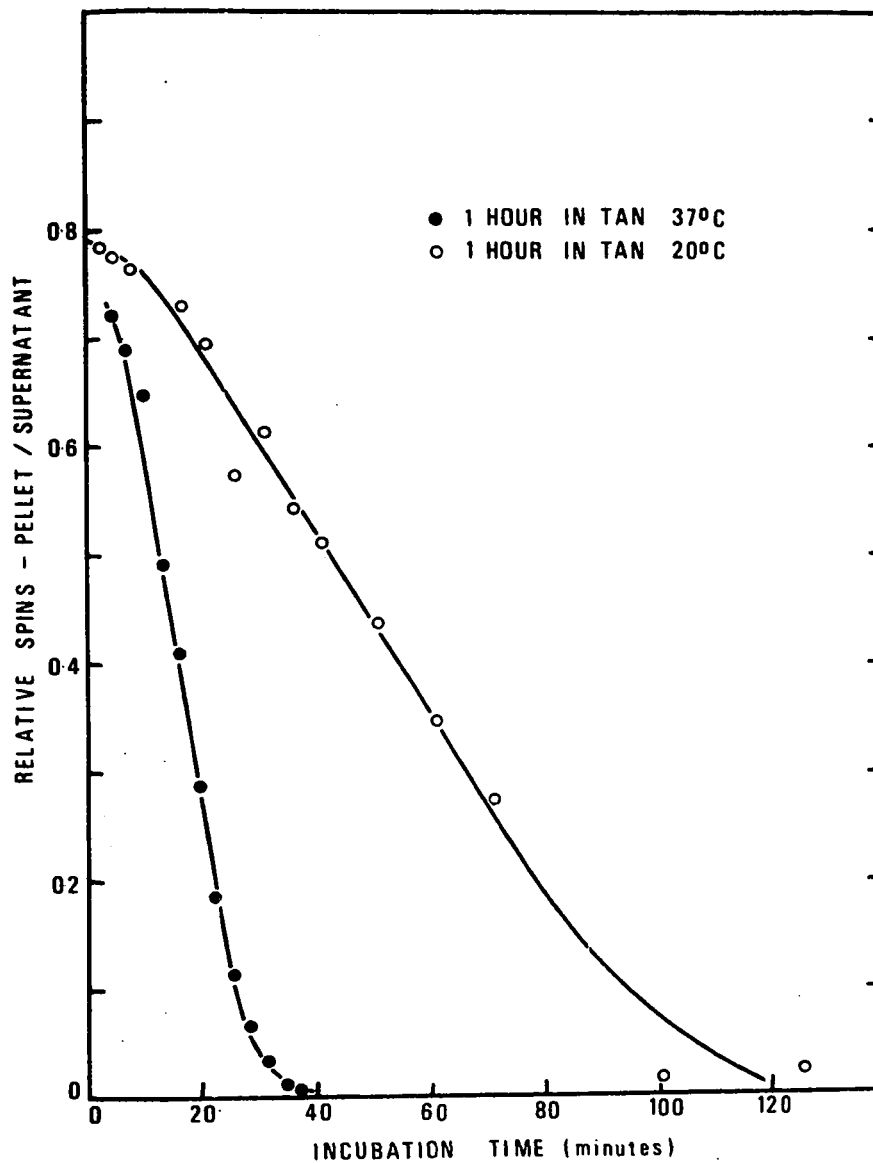


FIGURE 13. ESR Measurements of the Relative Number of Spins in the Pellet and Supernatant of Centrifuged Cells as a Function of the Incubation Time After the End of Centrifugation

Chinese hamster cells were incubated in the presence of 10 mM TAN for 60 minutes at 37°C. The cells were then centrifuged and the relative number of spins in the cell pellet and supernatant as a function of the incubation time after the end of centrifugation was measured at both 20°C and 37°C using electron spin resonance techniques.

explain the failure of TAN to sensitize cells in experiments with whole animals.

Experiments were performed to determine if the loss in free spins observed in the ESR experiment was accompanied by a corresponding loss in sensitization. Thus, cells in pellet form which had been treated for 1 hour with 10 mM TAN at 20°C prior to pellet formation were incubated at 20°C under anoxic conditions for various periods of time after pellet formation and then irradiated as described in Section 2.5.2.

The results shown in Figure 14 reveal a loss in sensitization which is complete after 2 hours of incubation at 20°C. This corresponds to the time required for the disappearance of free spins in the cell pellet at 20°C and demonstrates a correlation between the loss of free spins and a loss of sensitization.

Of perhaps greater importance is the great reduction in sensitization observed with cell pellets irradiated soon after pellet formation. The arrow in Figure 14 indicates the surviving fraction of cells irradiated with 2500 rads in a cell suspension containing 10 mM TAN. This large initial loss in sensitization will be discussed again in Section 3.1.11.

It is important to note that the survival of the anoxic pellet controls (N_2 + 2500 rads) is the same as for

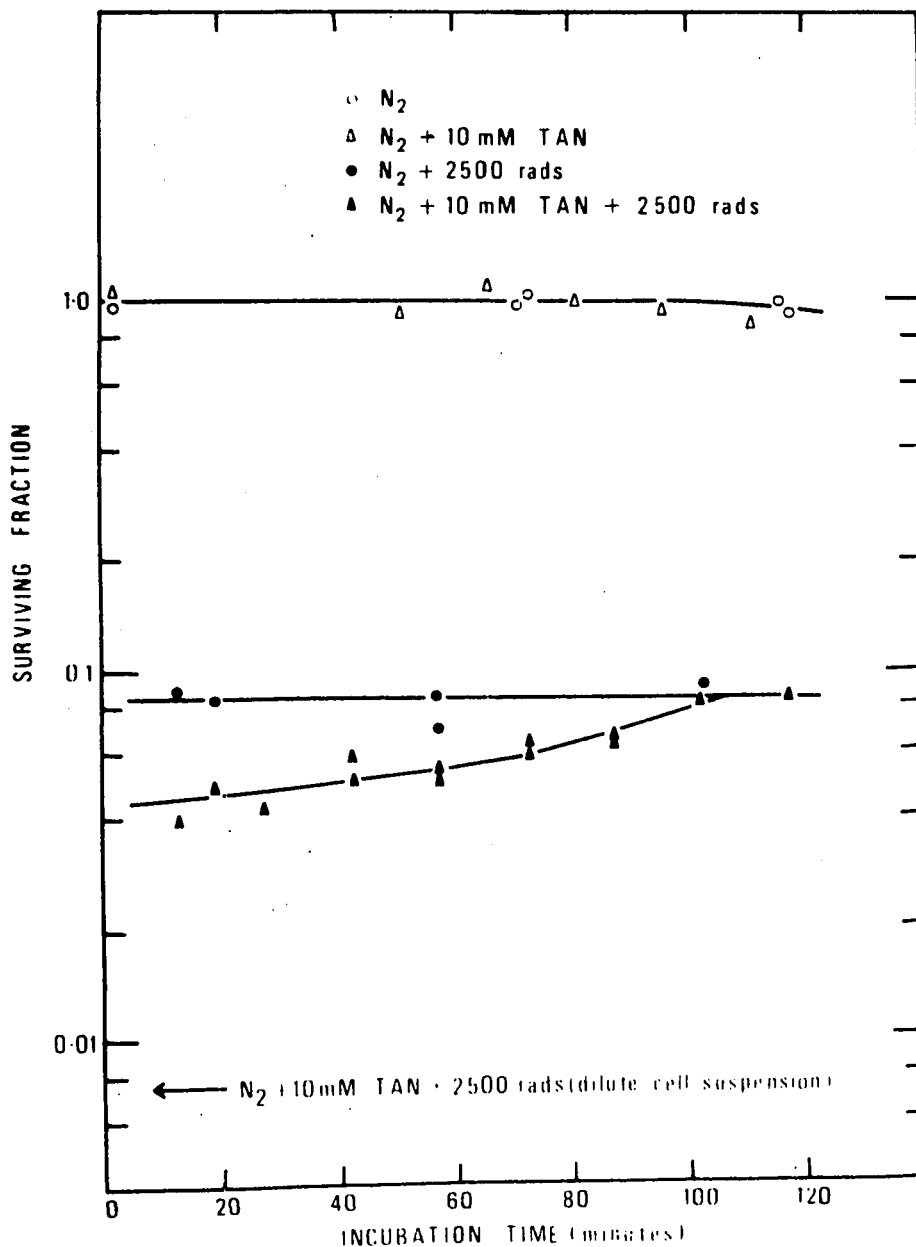


FIGURE 14. Surviving Fraction of TAN Treated Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in growth medium (5×10^6 cells/ml) were incubated under anoxic conditions at 20°C for 1 hour in the presence of 10 mM TAN. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (▲) and untreated (●) samples, unirradiated controls were taken from the drug treated (△) and untreated pellets (○). Cells were kept anoxic throughout the experiment.

anoxic cells irradiated in suspension (2500 rads).

3.1.10 Pretreatment Cell Pellet Survival Experiment

Before concluding that the loss in sensitization in the pellet resulted from the loss of free spins seen using the ESR technique, it was necessary to determine whether the loss in sensitization was just a disappearance of the pretreatment effect.

A suspension of Chinese hamster cells at 5×10^6 cells/ml was incubated at 20°C in the presence of 10 mM TAN for 60 minutes. The cells were then centrifuged twice (8 minutes at 40 g), resuspended in TAN-free medium lacking sodium bicarbonate and gassed for 45 minutes with nitrogen in 50 ml centrifuge tubes. The method then proceeded as described in 2.4.1 and 2.4.2, with the results shown in Figure 15 demonstrating a sensitization similar to those in Figure 14. Therefore it may well be that the loss in sensitization observed in Figure 14 occurs mainly from the loss of a pretreatment effect.

Resuspending cells twice in TAN-free medium following a 1 hour treatment with 10 mM TAN reduces the concentration of TAN free radicals in the cell to a very low level (see Section 3.1.5). Therefore, the sensitization obtained in the pretreatment pellet experiment (Figure 15) suggests, as did the pretreatment suspension experiment of 3.1.2,

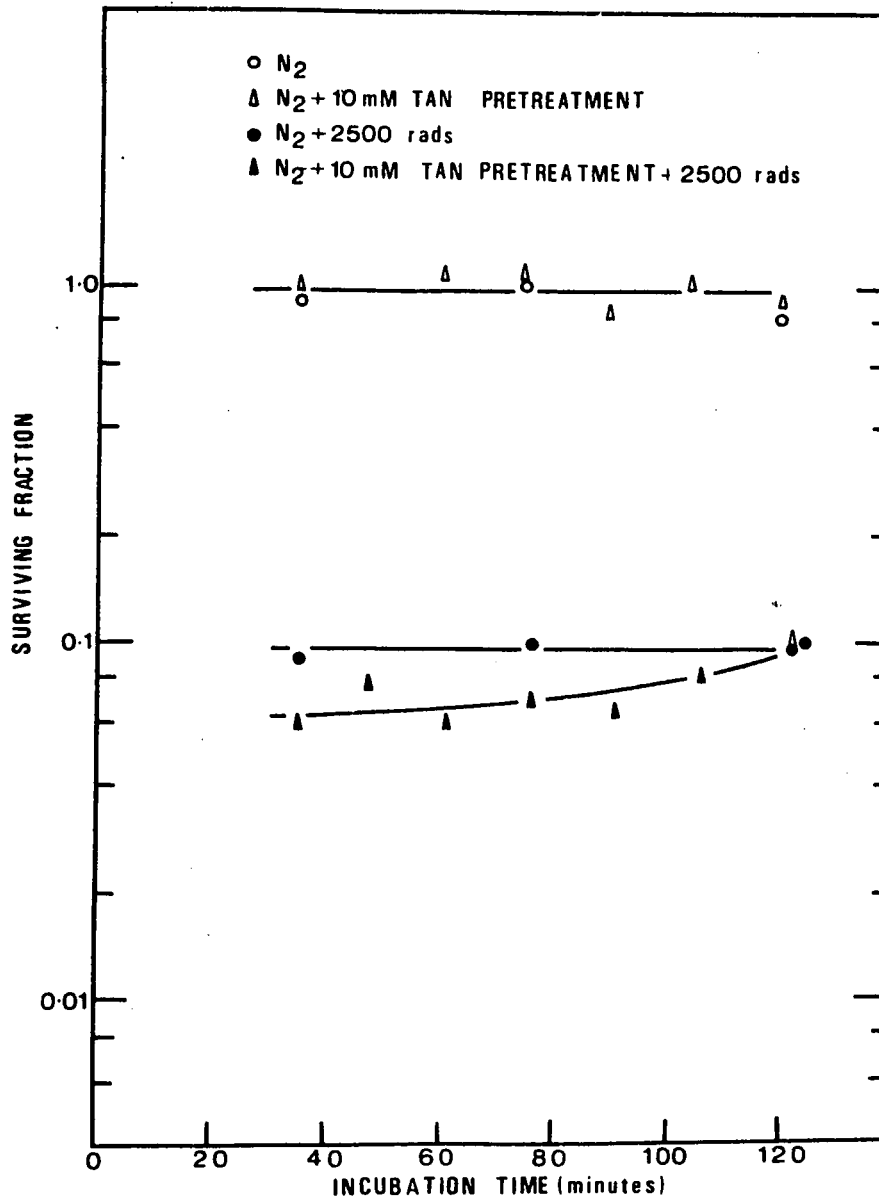


FIGURE 15. Surviving Fraction Following TAN Pretreatment in Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time after the End of Irradiation

Chinese hamster cells were incubated for 1 hour at 20°C in 10 mM TAN. Following a washing with TAN-free-medium, the TAN pretreated cells were centrifuged and the supernatant was removed. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated drug treated (▲) and untreated (●) samples, unirradiated controls were taken from the drug treated (△) and untreated pellets (○). Cells were kept anoxic throughout the experiment.

that some mechanism other than the interaction of TAN with a short-lived reactive species (as occurs for O_2) is involved. A possible explanation of the mode of action of TAN is offered in Section 4.1.3.

It is interesting that the time required for the disappearance of free spins as measured with ESR coincides with the time required for the loss of the pellet pretreatment sensitization. The evidence indicates, however, that the loss of free spins and the loss in sensitization are not causally related.

3.1.11 Cell Pellet Survival Experiment (at 0°C)

Perhaps of greater importance than the gradual loss of a small component of sensitization in cell pellets (Figure 14) is the loss of a large component of sensitization which occurs prior to the earliest possible irradiation times (~ 11 minutes). We showed earlier that the relative number of free spins in the pellet at 11 minutes is $\sim 75\%$ of that in the supernatant (see Figure 13). At this time the sensitization of cells irradiated in pellet is much less than the sensitization observed in either (a) cells irradiated in suspension in the presence of 1 mM TAN (Parker, Skarsgard and Emmerson, 1969) or (b) cells pretreated with 10 mM TAN and irradiated in suspension. The concentration of TAN in (b) has been demonstrated to be less than 10^{-5} Molar (3.1.5).

The loss of the large component of sensitization after pellet formation, therefore, is not due to the removal of TAN free radicals.

To determine if the reduction of sensitization was due to some temperature dependent metabolic process not involving the elimination of free radicals, the cell pellet was repeated keeping the cells at 0°C from the time that TAN was added until the end of centrifugation. The results obtained are shown in Figure 16, where, again, only a small amount of sensitization is present after the pellet is formed. Again, this sensitization disappears with time in much the same way as was illustrated in Figure 15. This argues against the idea that a temperature dependent cellular activity occurring prior to centrifugation could be responsible for the initial reduced effectiveness of TAN observed at high cell concentrations.

If this loss in sensitization at high cell concentrations is a general phenomenon then failure to observe sensitization with TAN in whole animals could be explained by an inherent loss in drug sensitizing ability at the high cell concentrations found in animal tissue.

3.1.12 Cell Concentration Experiment

In order to study TAN sensitization at cell concentrations between those in dilute suspensions (2×10^5

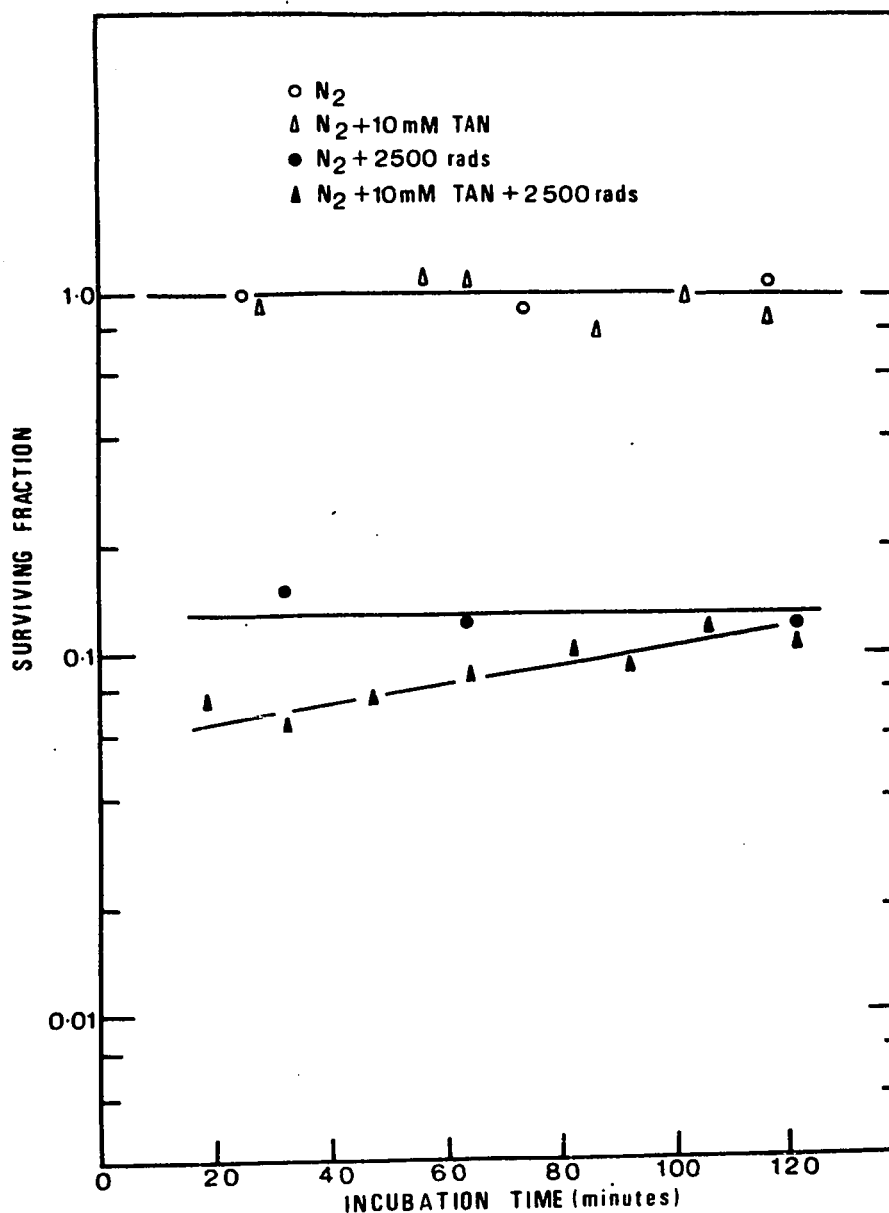


FIGURE 16. Surviving Fraction of TAN Treated Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in growth medium (5×10^6 cells/ml) were incubated under anoxic conditions at 0°C for 1 hour in the presence of 10 mM TAN. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (▲) and untreated (●) samples, unirradiated controls were taken from the drug treated (△) and untreated pellets (○). Cells were kept anoxic throughout the experiment.

cells/ml) and those in cell pellets (7×10^8 cells/ml), the following experiment was performed.

Chinese hamster cells in log phase growth (see 2.1.2) were trypsinized and then resuspended at a concentration of 5×10^6 cells/ml in growth medium (lacking sodium bicarbonate). TAN from a 0.2 Molar solution was then added to the cell suspension to produce a 10 mM TAN solution. Aliquots taken from the cell suspension were placed into 15 ml plastic centrifuge tubes after which the tubes were centrifuged for 6 minutes at 120 g and the supernatant removed. Measured volumes of normal growth medium lacking sodium bicarbonate and containing 10 mM TAN were added to the cells in the tubes to give the desired cell concentrations, and anoxia was obtained by gassing each tube with certified pre-purified nitrogen (0.7 litres/min) for 60 minutes at 23°C prior to irradiation with 2900 rads. Following irradiation the cells were centrifuged (8 min at 40 g), resuspended in normal growth medium, diluted and plated as described in 2.3.3 to determine colony forming ability.

From the results shown in Figure 17, it can be seen that there is a progressive loss in sensitization with increasing cell concentration, and at the highest cell concentration (pellet), the sensitization is greatly reduced. The survival values at the lowest and highest cell concentrations confirm the results obtained in the cell suspension and cell pellet experiments, respectively.

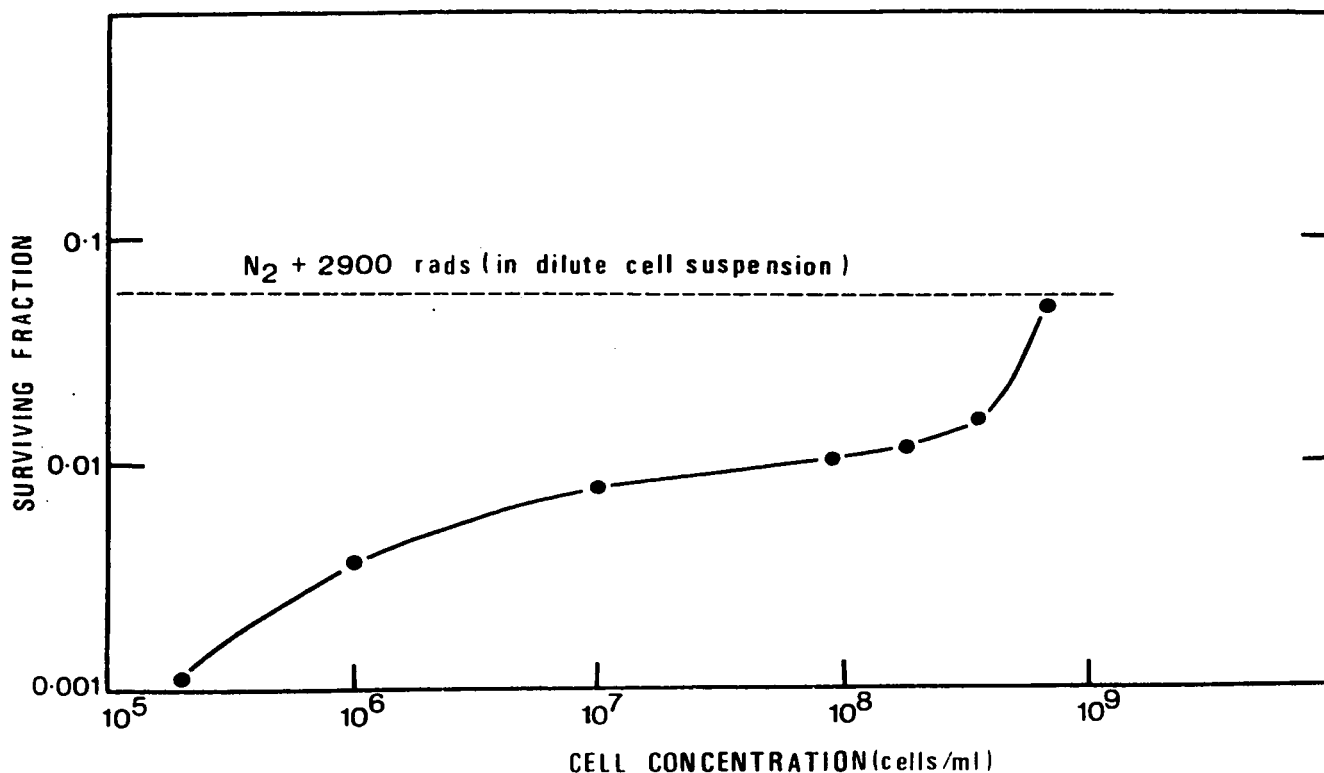


FIGURE 17. Surviving Fraction of Chinese Hamster Cells Subjected to Treatment with 10 mM TAN as a Function of the Cell Concentration at the Time of Irradiation

Chinese hamster cells suspended in growth medium (minus sodium bicarbonate) were placed into plastic centrifuge tubes in the presence of 10 mM TAN and gassed for 60 minutes with nitrogen prior to irradiation with 2900 rads. Following irradiation the cells were centrifuged, resuspended in TAN-free medium and plated to determine colony forming ability.

3.2 p-NITROACETOPHENONE (PNAP) RESULTS

Based on Adams' criterion of electron affinity for selecting radiosensitizers (Adams and Cooke, 1969), PNAP was tested as a chemical sensitizer and was found to preferentially sensitize anoxic cells to ionizing radiation in several biological systems (Asquith, Wilson and Adams, 1970; Adams et al., 1971). The low concentrations of PNAP required (micromolar) to achieve good sensitization in other systems prompted us to test this compound in our mammalian system.

3.2.1 PNAP Sensitization

In these experiments, PNAP was added to a concentration of 500 μM to Chinese hamster cells 60 minutes prior to irradiation, and was removed immediately after irradiation by centrifuging the cells (8 minutes at 40 g) and resuspending them in PNAP-free medium. The effects of 500 μM PNAP on anoxic and aerobic survival are shown in Figure 18. In anoxic cells PNAP, present at the time of irradiation gives a DMF of 1.6, although the extent of sensitization was found to vary between experiments from DMF's of 1.4 to 1.6. This result is similar to that of Chapman, Webb and Borsa (1971), who report DMF's varying between 1.3 and 1.6 in their Chinese hamster lung cell line V79-379-A. The presence of 500 μM

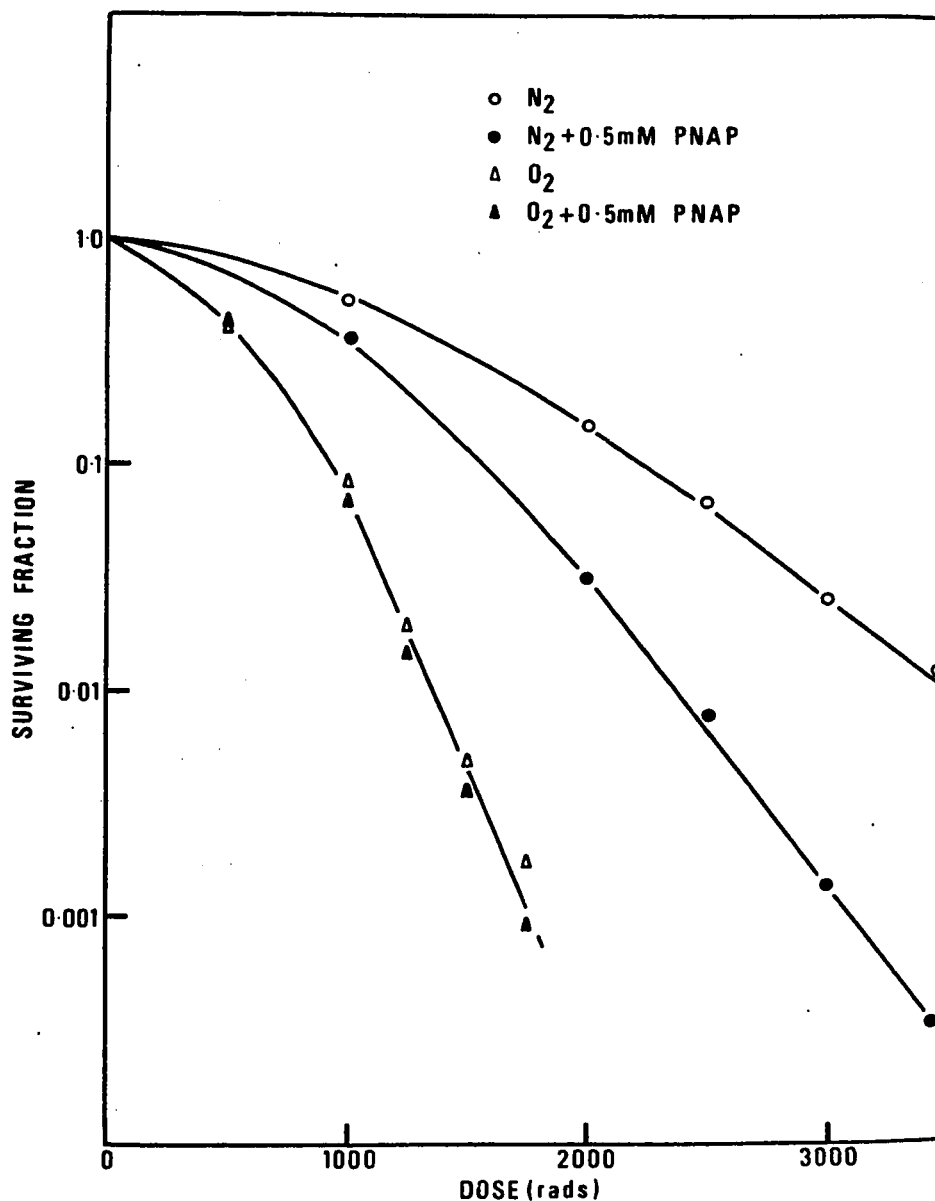


FIGURE 18. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen, Nitrogen Plus PNAP, Oxygen, and Oxygen Plus PNAP

Prior to irradiation, Chinese hamster cells suspended in normal growth medium (minus sodium bicarbonate) at a cell concentration of 2×10^5 cells/ml were gassed with N₂ in the absence (○) or the presence (●) of 500 μM PNAP, or they were gassed with O₂ in the absence (△) or the presence (▲) of 500 μM PNAP. Following irradiation the cells were washed with drug-free medium and plated to determine the colony forming ability. Cells treated with PNAP were exposed to the drug for one hour prior to irradiation.

PNAP does not alter the aerobic survival response.

The 0.5% ethanol present in the PNAP-treated cells was also present in the anoxic and aerobic controls and was found to have no effect on cell survival. The presence of 500 μ M PNAP (\sim solubility limit in aqueous solution) was non-toxic (i.e., did not alter the plating efficiency of unirradiated controls) to the Chinese hamster cells for exposure times at least up to 2 hours.

3.2.2 Pretreatment with PNAP

Pretreatment of Chinese hamster cells with 500 μ M PNAP was performed as described for TAN (Section 3.1.2). Following a 1 hour exposure to the drug at 23°C the cells were washed two times with drug free medium lacking sodium bicarbonate, gassed and irradiated under anoxic conditions. The lack of any pretreatment sensitization (see Figure 19) rules out the binding of cellular non-protein sulfhydryls as a possible mechanism of PNAP sensitization, provided we assume that the cell does not have the capacity to replenish a lost supply of sulfhydryls during periods as short as 45 minutes (Harris, Painter and Hahn, 1969). The results contrast with those of TAN where a substantial pretreatment effect was observed.

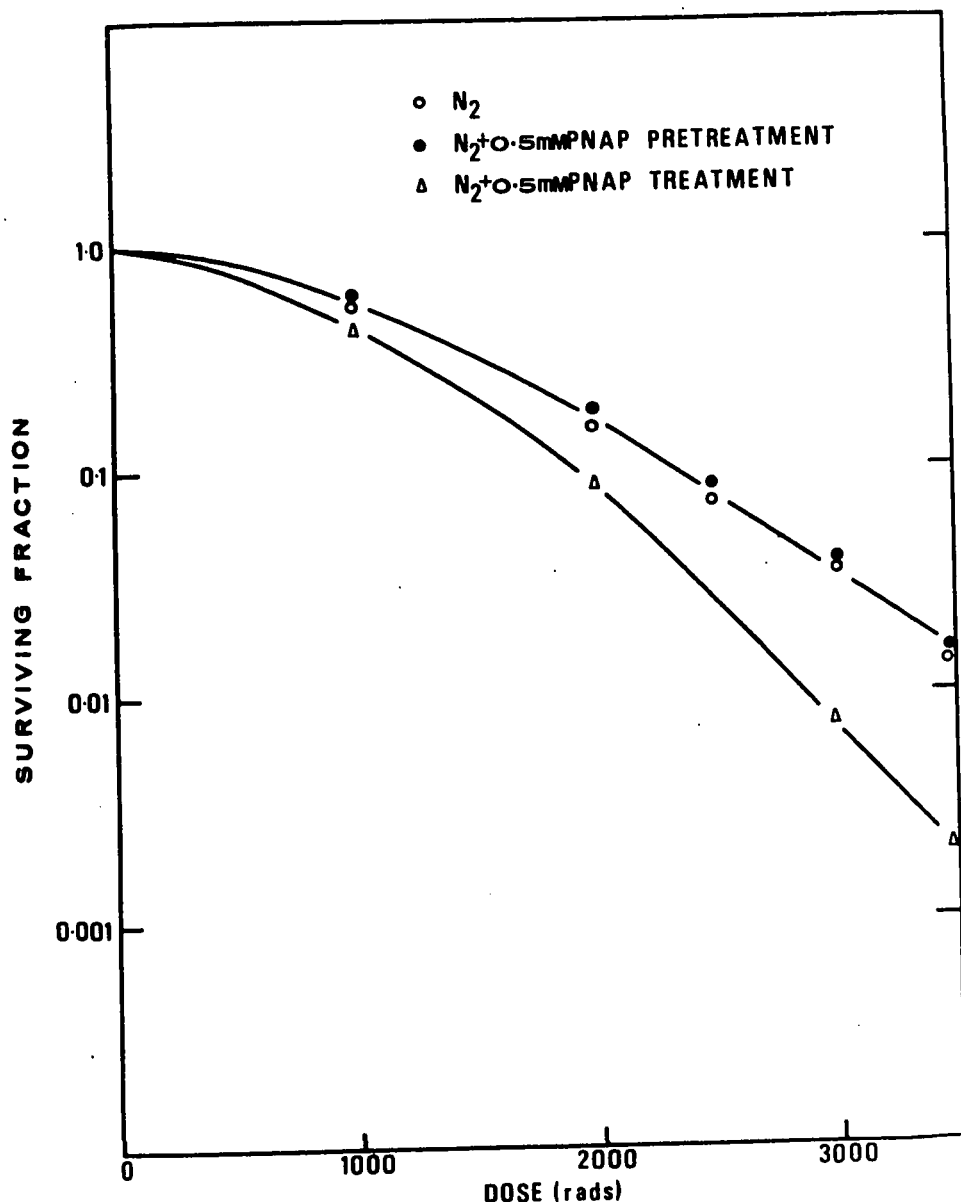


FIGURE 19. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen and Irradiated Following Pretreatment with 500 μ M PNAP

Chinese hamster cells suspended in normal growth medium (minus sodium bicarbonate) were incubated in the absence (O) or the presence (●) of 500 μ M PNAP for 60 minutes at 23°C, after which time they were centrifuged twice and resuspended in drug-free medium (lacking sodium bicarbonate). The cells were then placed in a glass irradiation vessel, gassed with N₂ for 45 minutes at 23°C, irradiated and assayed for colony forming ability (2.3.3). Cells were also irradiated in the presence of 500 μ M PNAP (Δ).

3.2.3 Post-treatment with PNAP

Post-treatment of Chinese hamster cells with 500 μM PNAP was performed as described for TAN (Section 3.1.1). Cells were irradiated and then incubated in the irradiation vessel under anoxic conditions at 23°C. After various incubation times 1 ml aliquots of cell suspension were removed from the vessel and added to 9 ml of aerobic medium containing PNAP. Following a 1 hour exposure to 500 μM PNAP, the cells were centrifuged, resuspended in PNAP-free medium and plated to determine colony forming ability. Two types of controls were obtained by (1) removing cells from the vessel and adding them to 9 ml of normal medium and by (2) irradiating cells in the presence of 500 μM PNAP and diluting in PNAP-free medium after various incubation times.

The results in Figure 20 show that there is no PNAP post-treatment sensitization for even the very short incubation times. Cells irradiated in the presence of 500 μM PNAP and incubated under anoxic conditions show a gradual decrease in survival with longer incubation times, a phenomenon that is not observed in the controls, i.e., cells irradiated in the absence of the drug. It appears that the "PNAP damage" occurring at the time of irradiation increases under prolonged anoxic conditions.

The results of both the pretreatment and post-treatment experiments indicate that PNAP must be present at the time

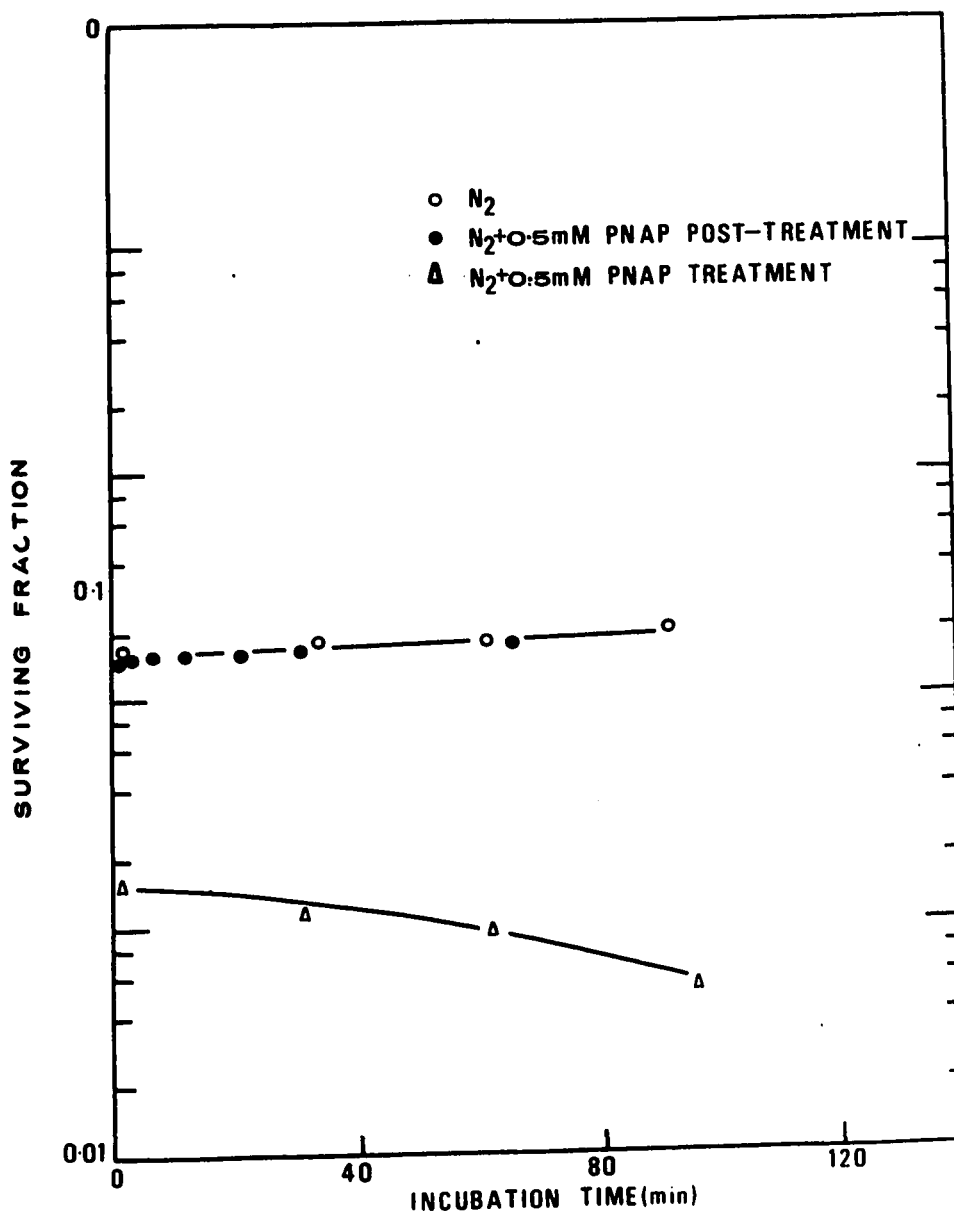


FIGURE 20. Surviving Fraction of Chinese Hamster Cells to PNAP Post-Treatment at 23°C as a Function of Incubation Time after Termination of Irradiation

Chinese hamster cells suspended in growth medium lacking sodium bicarbonate were incubated at 23°C under anoxic conditions prior to, during and following irradiation. At various times after the end of irradiation, aliquots were removed from the anoxic irradiation vessel and added to 500 μ M PNAP (\bullet). Following a 1 hour exposure at 23°C to the aerobic PNAP solution, the cells were centrifuged twice, resuspended in drug-free medium and plated in order to determine colony forming ability. Controls were obtained by irradiating cells in the absence (\circ) and in the presence (Δ) of the drug and then placing samples from the vessels into aerobic medium.

of irradiation in order to effect sensitization, a result that is consistent with the Indirect Action Model of Adams and Cooke (1969).

3.2.4 PNAP Sensitization in Cell Pellets

The sensitization produced by 10 mM TAN was found to be much less in cells irradiated in pellet (Figure 14) than in cells irradiated while in dilute cell suspension (Figure 11). In order to determine if this cell concentration effect is a characteristic observed with other drugs a cell pellet experiment was performed using PNAP. The experiment was carried out as described in Sections 2.4.1 and 2.4.2 with 500 μ M PNAP being added to the cell suspension prior to gassing.

The results are shown in Figure 21. The sensitizing ability of PNAP is greatly reduced when cells at 20°C are irradiated at a high cell concentration, the survival being approximately the same as for TAN-treated cell pellets.

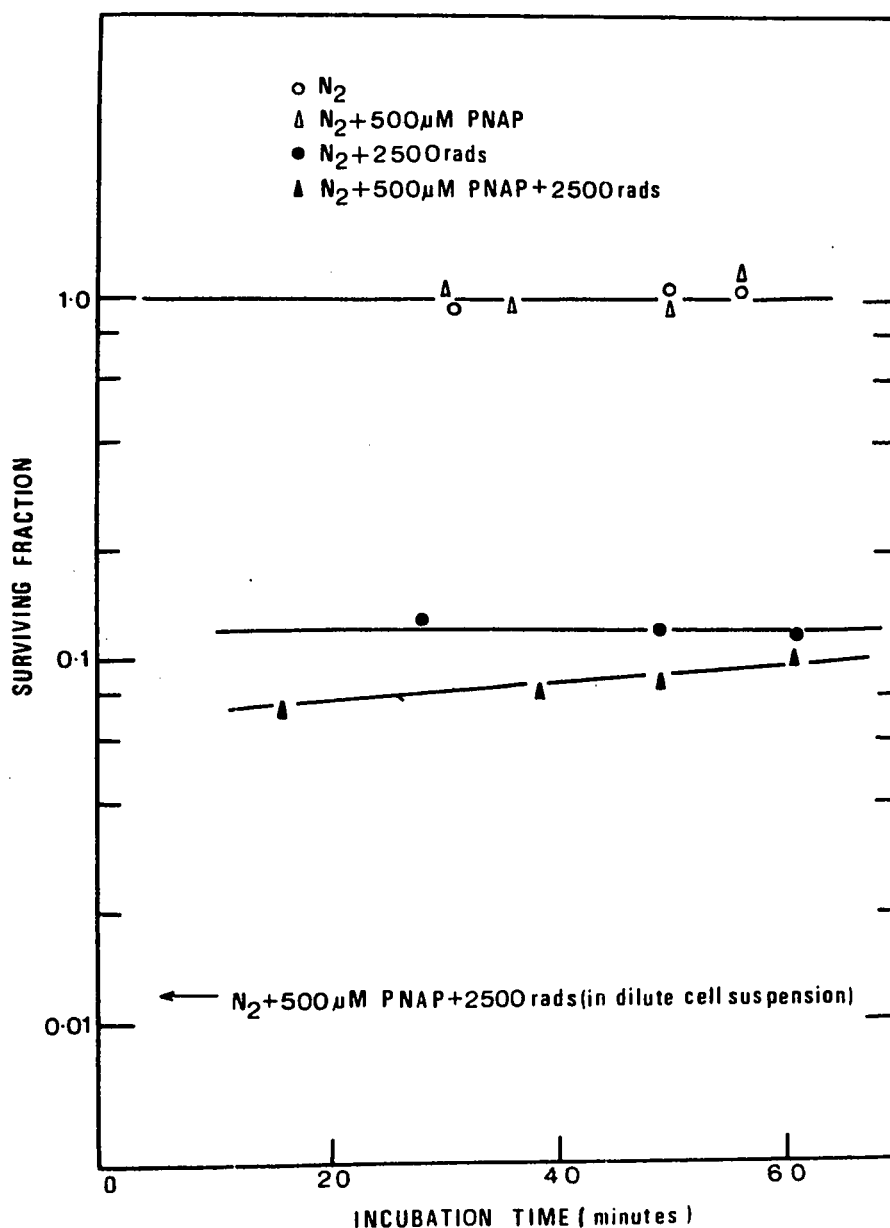


FIGURE 21. Surviving Fraction of PNAP Treated Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in growth medium (5×10^6 cells/ml) were incubated under anoxic conditions at 20°C for 1 hour in the presence of 500 µM PNAP. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (▲) and untreated (●) samples, unirradiated controls were taken from the drug treated (△) and untreated pellets (○). Cells were kept anoxic throughout the experiment.

3.3 DIMETHYL FUMARATE RESULTS

Dimethyl fumarate, an electron affinic compound, was tested in our mammalian cell system because of the large sensitization it produces in anoxic suspensions of the bacterium Serratia marcesens (Adams and Cooke, 1969).

3.3.1 Dimethyl Fumarate Sensitization

Dimethyl fumarate was added to a concentration of 200 μM (the maximum non-toxic concentration for a 2 hour exposure to the drug - see Figure 22) to dilute suspensions of Chinese hamster cells 1 hour prior to irradiation under either anoxic or aerobic conditions. The results in Figure 23 show that 200 μM dimethyl fumarate gives a DMF of 1.8 under anoxic conditions while showing a small effect on the aerobic response. This small effect under aerobic conditions does not rule out this compound as a potential radiotherapeutic agent in view of the large sensitization produced under anoxic conditions.

3.3.2 Pretreatment with Dimethyl Fumarate

A pretreatment experiment was performed by exposing Chinese hamster cells (2×10^5 cells/ml) to 200 μM dimethyl fumarate for 1 hour at 23°C under aerobic conditions, removing the drug by twice centrifuging (40 g for 8 minutes) and

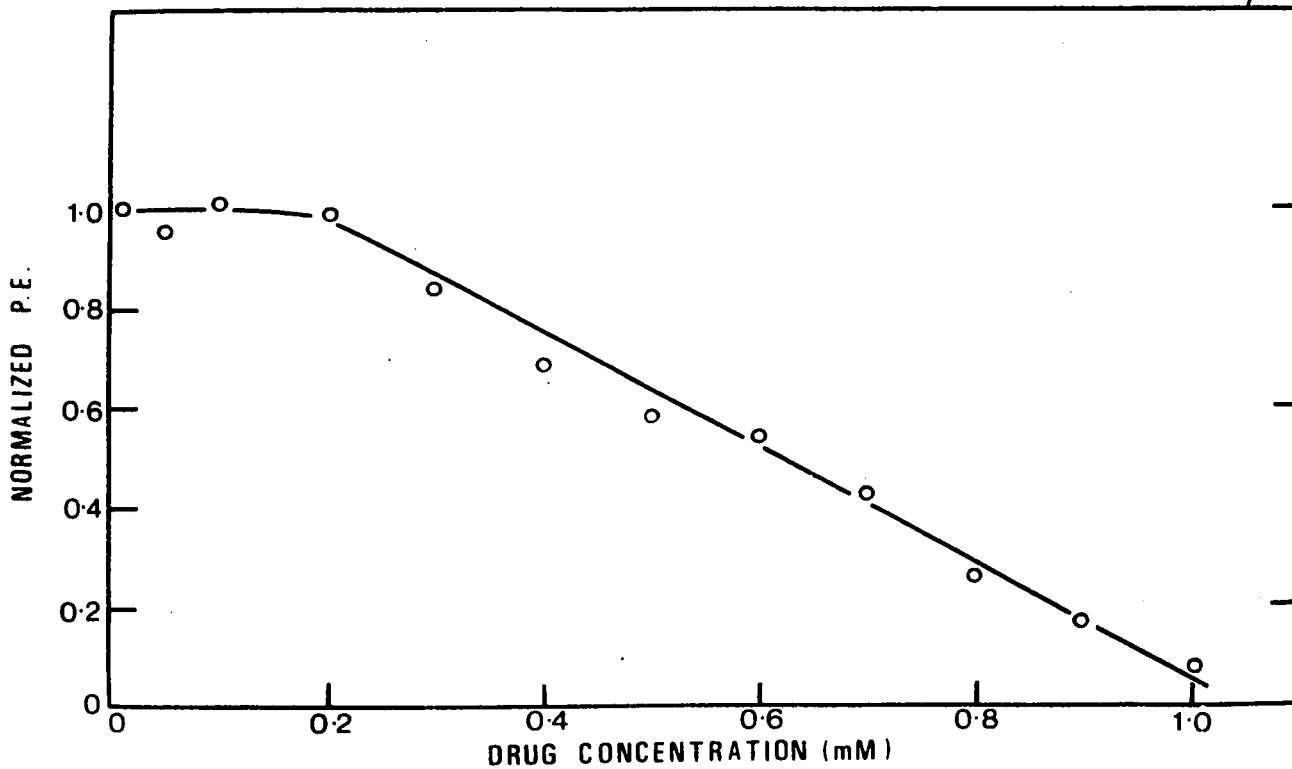


FIGURE 22. The Normalized Plating Efficiency of Chinese Hamster Cells Exposed to Aerobic Solutions of Dimethyl Fumarate for Two Hours as a Function of the Dimethyl Fumarate Concentration

Chinese hamster cells (2×10^5 cells/ml) suspended in aerobic growth medium were exposed to a concentration of dimethyl fumarate for a period of two hours at 23°C . The cells were then centrifuged twice, resuspended in drug-free medium, and plated in order to determine colony forming ability.

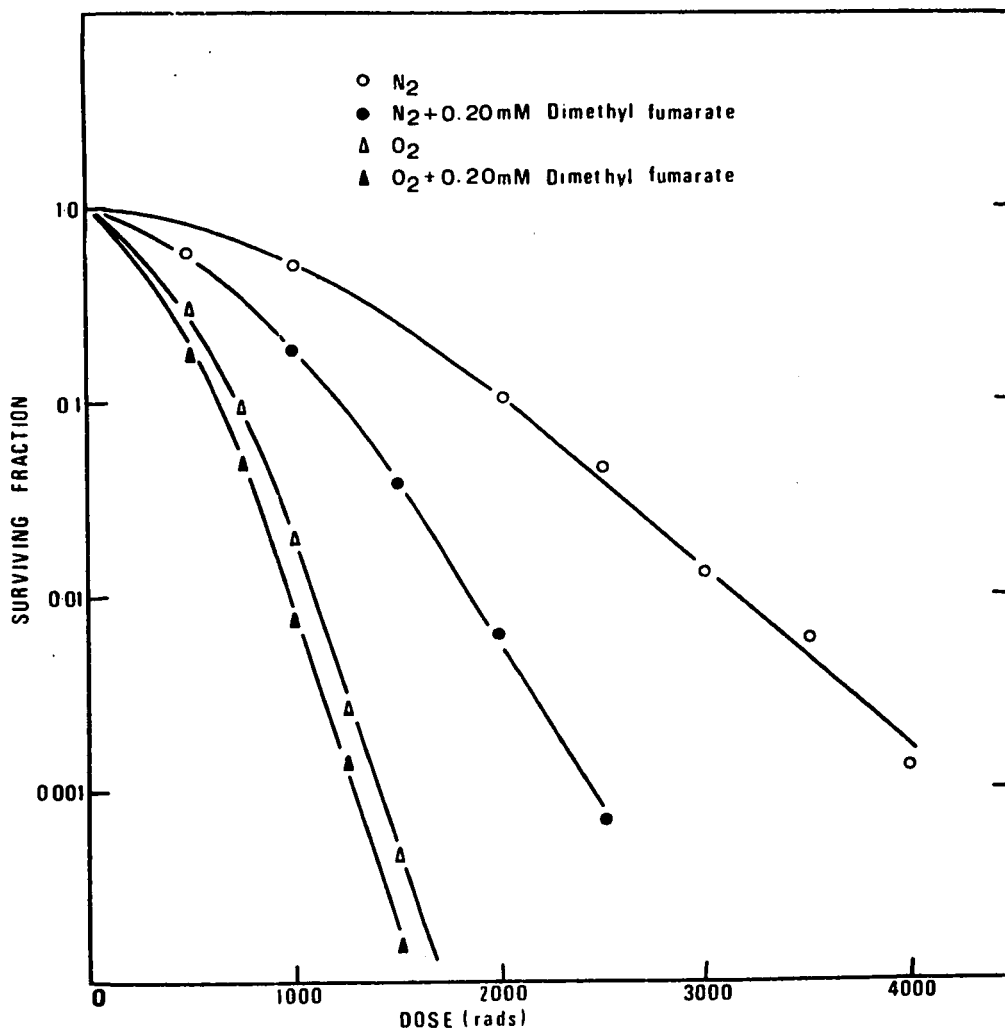


FIGURE 23. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen, Nitrogen Plus Dimethyl Fumarate, Oxygen and Oxygen Plus Dimethyl Fumarate

Prior to irradiation, Chinese hamster cells suspended in normal growth medium (minus sodium bicarbonate) at a cell concentration of 2×10^5 cells/ml were gassed with N₂ in the absence (○) or the presence (●) of dimethyl fumarate, or they were gassed with O₂ in the absence (△) or presence (▲) of dimethyl fumarate. Following irradiation, the cells were washed with drug-free medium and assayed for colony forming ability (see 2.3.3). Cells treated with dimethyl fumarate were exposed to the drug for 1 hour prior to irradiation.

resuspending the cells in drug-free medium (minus sodium bicarbonate). The cells were then gassed with N_2 , irradiated and plated as described in Section 2.3.3. The results shown in Figure 24 demonstrate a large pretreatment effect (DMF = 1.4), exceeding the pretreatment effect obtained with 10 mM TAN (DMF = 1.3).

ESR measurements taken following TAN pretreatment showed the compound to be effectively washed from the cells, but no similar technique is available to determine the extent to which dimethyl fumarate remains in the cell following the washing with drug-free medium. However, there is no evidence to indicate that dimethyl fumarate is not effectively washed from the cells. This compound is very water soluble, and the low concentration required to achieve sensitization suggests that the compound easily traverses cell membranes.

The large pretreatment sensitization obtained with dimethyl fumarate indicates that this compound, as is true of TAN, can sensitize by a mechanism that does not involve the interaction with a radiation produced short-lived reactive species. However, a mechanism of action involving the binding of cellular sulfhydryl groups cannot be ruled out here. It is also possible that this compound is concentrated at crucial sites within the cell in which case the above argument may not be valid.

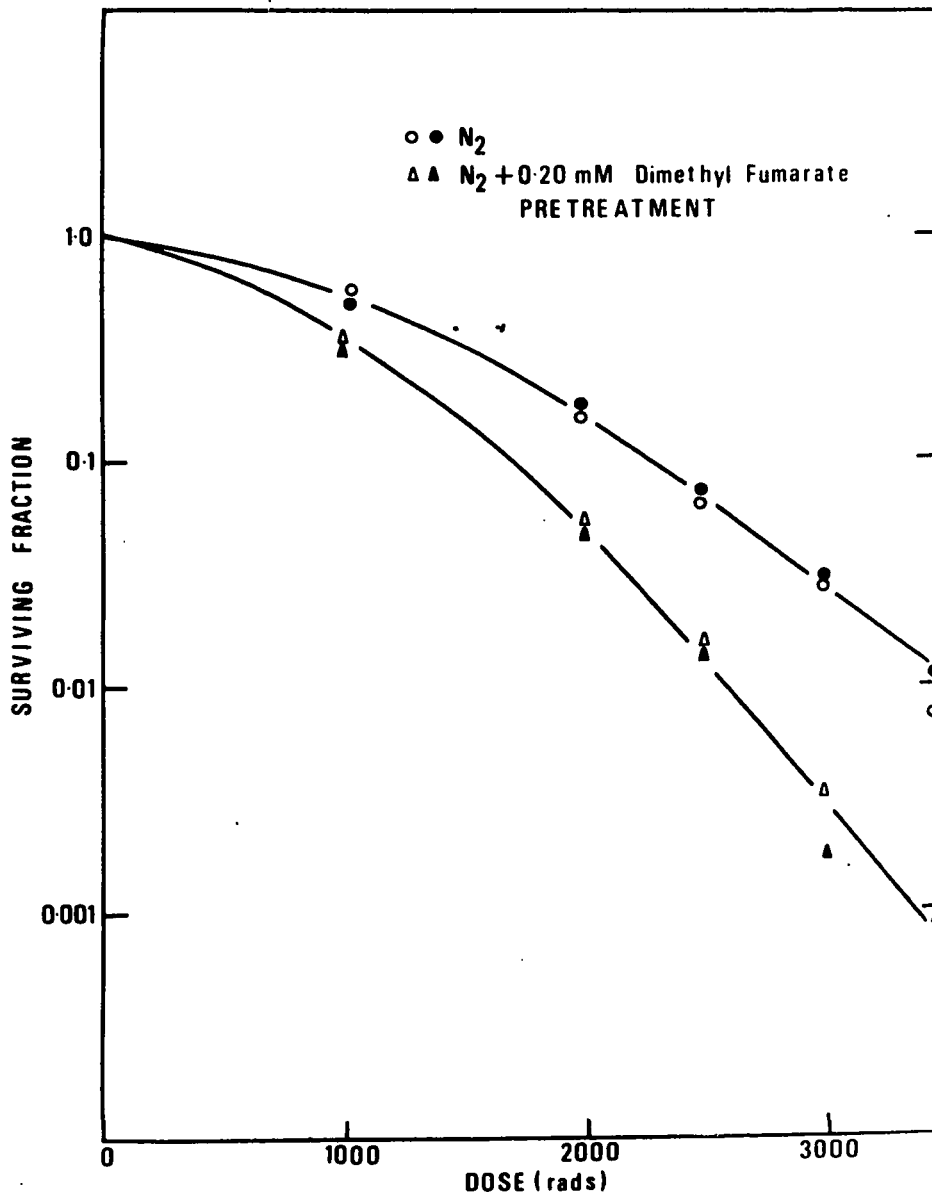


FIGURE 24. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen and Irradiated Following Pretreatment with Dimethyl Fumarate

Chinese hamster cells suspended in growth medium (minus sodium bicarbonate) were incubated in the absence (○●) or the presence (▲▲) of 0.20 mM dimethyl fumarate for 60 minutes at 23°C, after which time they were centrifuged twice and resuspended in TAN-free growth medium lacking sodium bicarbonate. The cells were then placed in a glass irradiation vessel, gassed with N₂ for 45 minutes at 23°C, irradiated and assayed for colony forming ability (see 2.3.3).

3.3.3 Post-treatment with Dimethyl Fumarate

Post-treatment of Chinese hamster cells with 200 μM dimethyl fumarate was carried out as described for TAN (Section 3.1.6). Following irradiation under anoxic conditions the cells were incubated at 23°C under anoxia and at various times after the termination of irradiation 1 ml aliquots were taken from the irradiation vessel and placed into 9 ml aerobic solutions of dimethyl fumarate for a 1 hour period. The cells were then centrifuged, resuspended in drug free medium and plated to determine colony forming ability.

The results shown in Figure 25 reveal a small post-treatment effect that disappears in less than 1 hour. Although the sensitization is less than for TAN post-treatment it does contrast with PNAP where no post-treatment effect was observed. The results also indicate that dimethyl fumarate can achieve a small degree of sensitization by interacting with a long-lived transient state of the cell that exists following irradiation.

3.3.4 Dimethyl Fumarate Sensitization in Cell Pellets

The pellet experiments performed with TAN and PNAP showed a loss in the sensitizing ability of these drugs at high cell concentrations. The pellet experiment was repeated (see Sections 2.4.1 and 2.4.2) to determine if sensitization

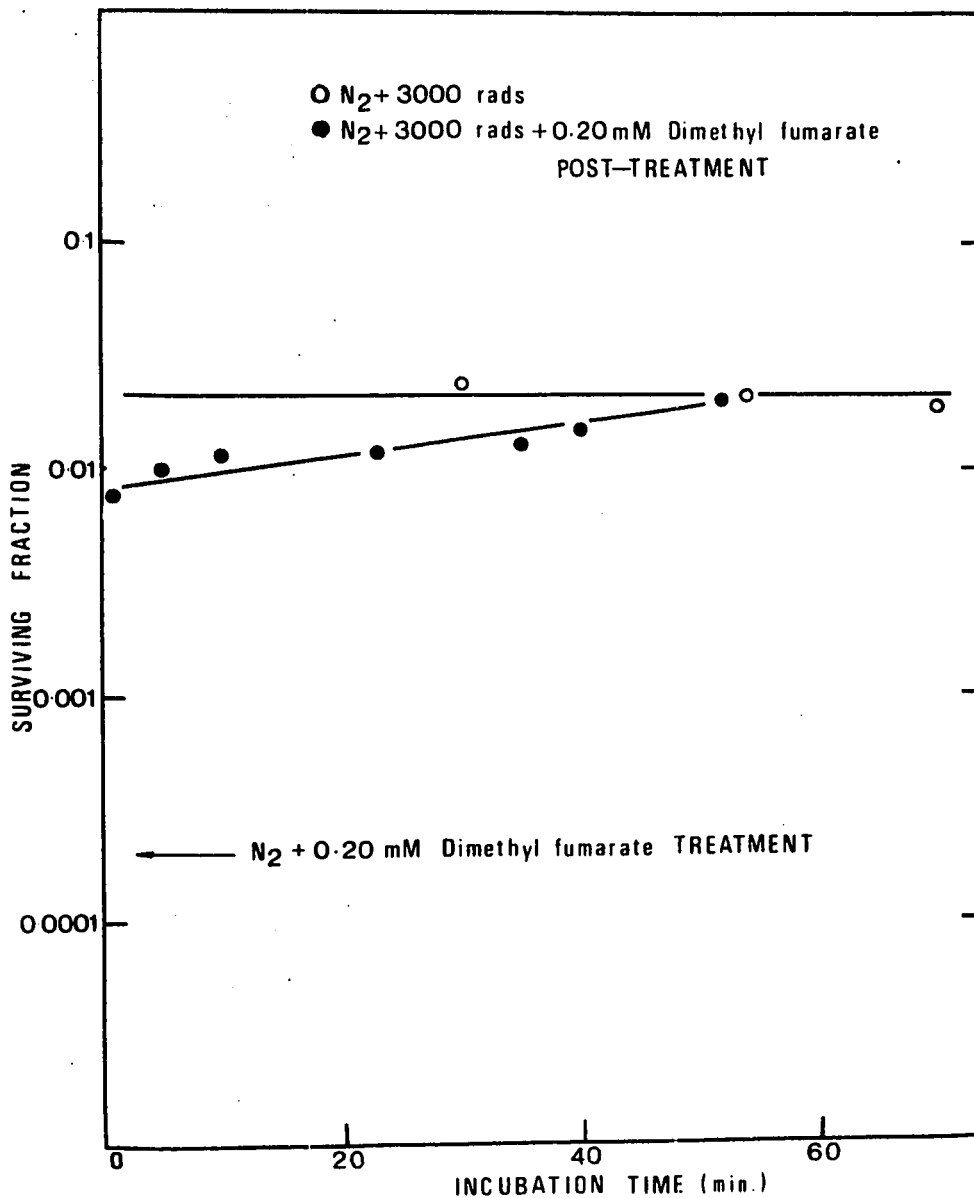


FIGURE 25. Surviving Fraction of Chinese Hamster Cells Subjected to Dimethyl Fumarate Post-Treatment at 23°C as a Function of Incubation Time After the Termination of Irradiation

Chinese hamster cells suspended in growth medium (lacking sodium bicarbonate) were incubated at 23°C under anoxic conditions prior to, during and following irradiation. At various times after the end of irradiation, aliquots were removed from the anoxic irradiation vessel and added to 0.20 mM dimethyl fumarate. After a 1 hour exposure at 23°C to the aerobic dimethyl fumarate solution, the cells were centrifuged twice, resuspended in drug-free medium and assayed for colony forming ability (2.3.3).

with dimethyl fumarate also varies inversely with the cell concentration. The results in Figure 26 show a substantial sensitization, that, although less than for cells irradiated in suspension, (Figure 23) is larger than that obtained in cell pellets with TAN and PNAP.

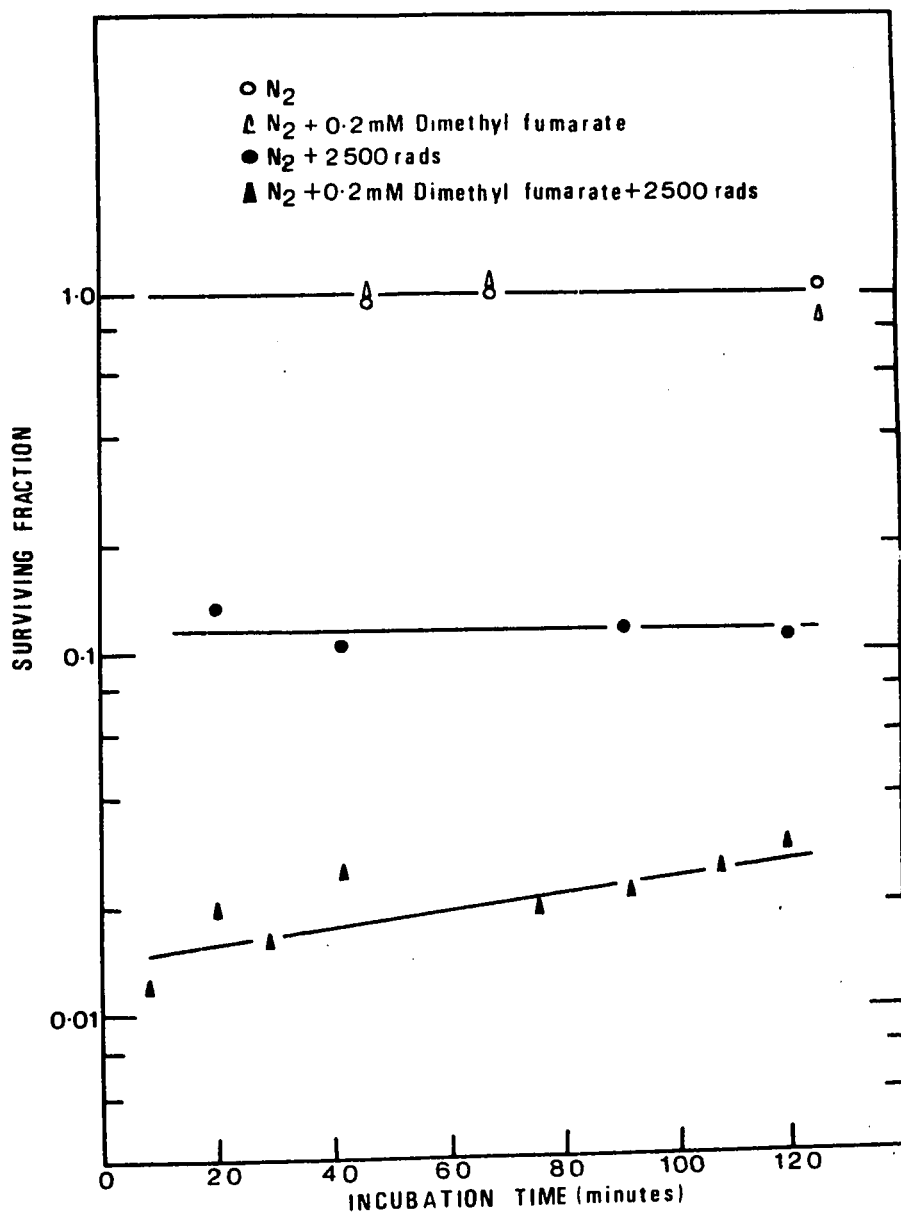


FIGURE 26. Surviving Fraction of Dimethyl Fumarate Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in growth medium (5×10^6 cells/ml) were incubated under anoxic conditions at 20°C for 1 hour in the presence of 0.2 mM dimethyl fumarate. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (\blacktriangle) and untreated (\bullet) samples, unirradiated controls were taken from the drug treated (Δ) and untreated pellets (O). Cells were kept anoxic throughout the experiment.

3.4 NITROFURAZONE RESULTS

Nitrofurazone is a member of the class of compounds known as the nitrofurans, which have been in use for the past three decades in the treatment of human diseases such as genitourinary and gastrointestinal tract infections. The pharmacology of these electron affinic compounds is well understood.

Upon hearing of the large sensitization achieved with some of the nitrofurans in mammalian cell systems by Chapman's group at the National Research Council in Pinawa, Manitoba (personal communication), we obtained a sample of the nitrofurazone from Dr. D. McCalla (McMaster) for testing in our system.

3.4.1 Nitrofurazone Sensitization

Nitrofurazone was added to dilute suspensions of Chinese hamster cells to determine this compound's ability to sensitize when present during irradiation. The sensitizing capacity of three different drug concentrations [all of which yielded plating efficiencies greater than 70% for an exposure of 2 hours 20 minutes in air (see Figure 27)] are shown in Figure 28.

Nitrofurazone concentrations of 44 μM , 175 μM and 260 μM produce dose modifying factors of 1.5, 1.8 and 1.8

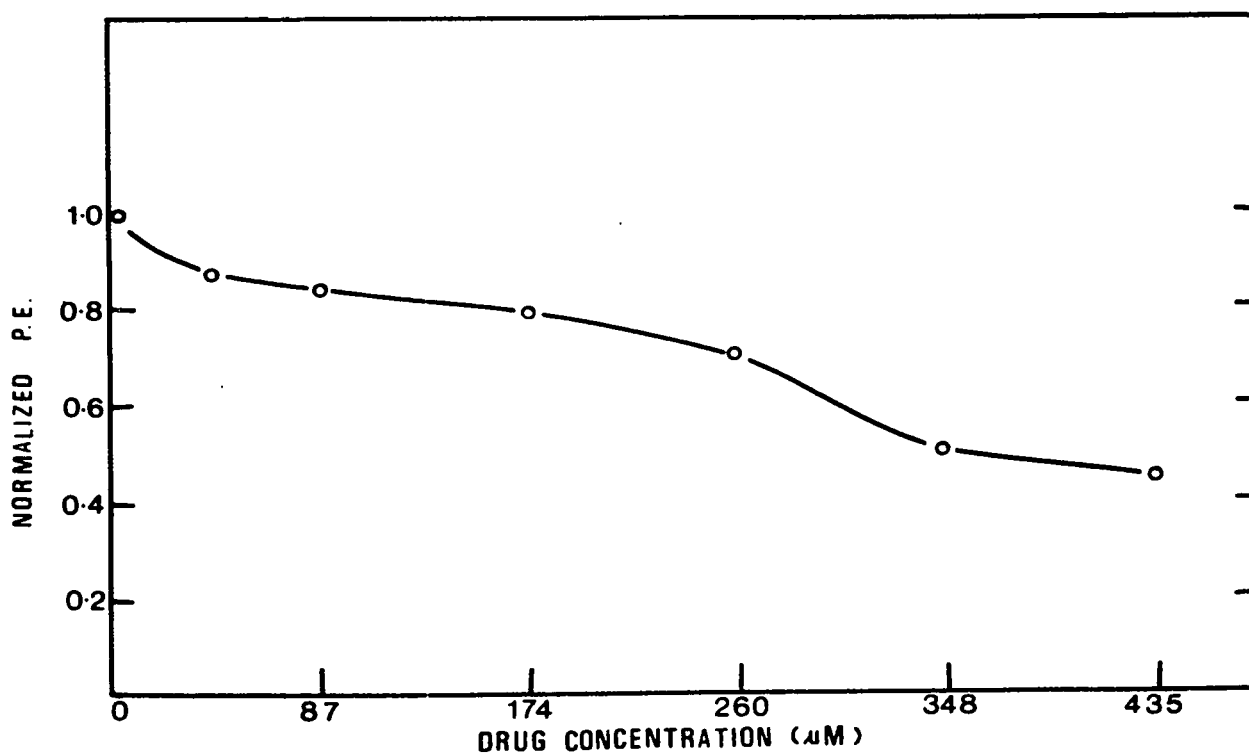


FIGURE 27. The Normalized Plating Efficiency of Chinese Hamster Cells Exposed to Aerobic Solutions of Nitrofurazone for Two Hours and Twenty Minutes as a Function of the Nitrofurazone Concentration

Chinese hamster cells (2×10^5 cells/ml) suspended in aerobic growth medium were exposed to a concentration of nitrofurazone for a period of two hours and twenty minutes at 23°C . The cells were then centrifuged twice, resuspended in drug-free medium, and plated in order to determine colony forming ability.

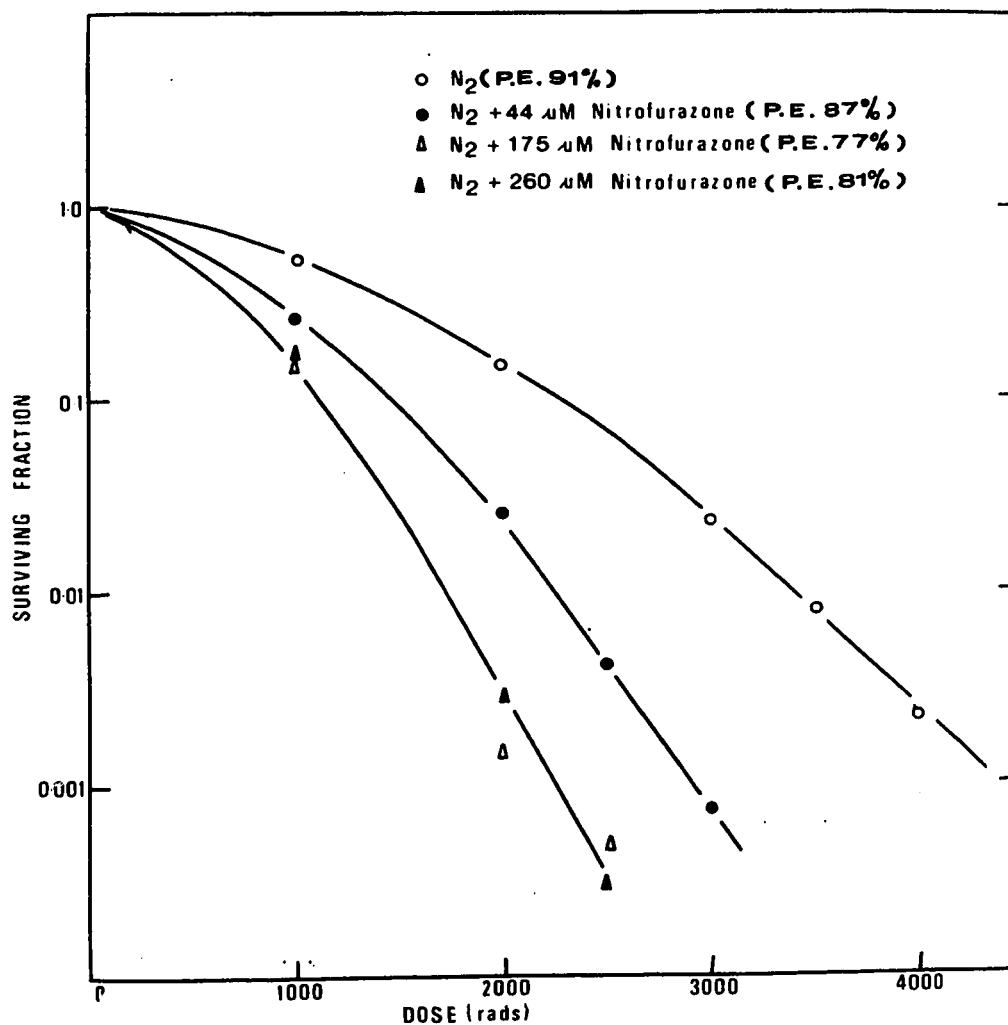


FIGURE 28. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen or Nitrogen plus Nitrofurazone

Chinese hamster cells suspended in normal growth medium (minus sodium bicarbonate) and at a cell concentration of 2×10^5 cells/ml were gassed with N₂ in the presence of 0 (O), 44 μM (●), 175 μM (Δ), or 260 μM (▲) nitrofurazone prior to irradiation. Following irradiation the cells were washed with drug free medium and plated in order to determine colony forming ability. Cells treated with dimethyl fumarate were exposed to the drug for 1 hour prior to irradiation.

respectively under anoxic conditions, while having no effect on aerobic survival (see Figure 29). The toxicity of nitrofurazone is greater when oxygen (or air) is present during drug treatment (see plating efficiencies of Figures 28 and 29). All nitrofurazone solutions were shielded from light to prevent decomposition (Paul and Paul, 1964).

3.4.2 Pretreatment with Nitrofurazone

Chinese hamster cells suspended at 2×10^5 cells/ml in normal growth medium (lacking sodium bicarbonate), were exposed to 44 μM , 170 μM or 260 μM nitrofurazone for 1 hour at 23°C, centrifuged twice and resuspended in drug-free medium lacking sodium bicarbonate. Following a 45 minute gassing with N_2 , the cells were irradiated, diluted and plated to determine colony forming ability (Section 2.3.3.).

The results in Figure 30 show a small pretreatment effect with both 170 μM (DMF = 1.09) and 260 μM (DMF = 1.15) nitrofurazone. Pretreatment with 44 μM nitrofurazone has no effect on cell survival. The very small effect of pretreatment on survival makes it unlikely that nitrofurazone sensitizes by a mechanism that involves SH binding. In view of the reduced plating efficiencies using 170 μM and 260 μM nitrofurazone, an alternative explanation is possible for the reduced survival shown in Figure 30. If the reduced plating efficiencies are the result of a selective toxicity

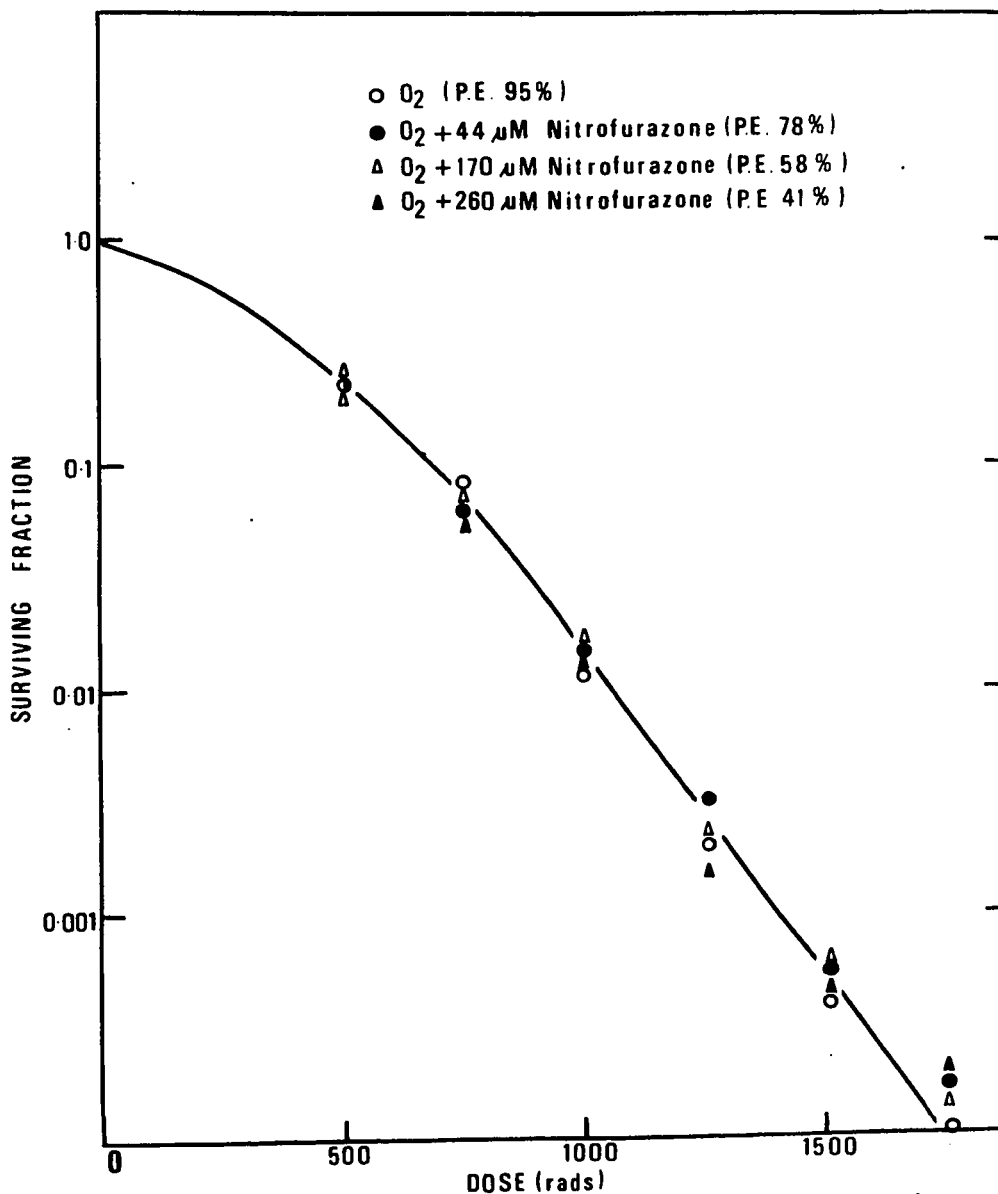


FIGURE 29. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Oxygen, and Oxygen Plus 44 μM, 170 μM, and 260 μM Nitrofurazone

Chinese hamster cells suspended in growth medium (minus sodium bicarbonate) at a cell concentration of 2×10^5 cells/ml were gassed for 45 minutes in a glass irradiation vessel in the presence of oxygen (○), or oxygen plus 44 μM (●), 170 μM (△) or 260 μM nitrofurazone prior to irradiation. Following irradiation the cells were diluted and plated in order to determine the colony forming ability (2.3.3). Cells treated with nitrofurazone were exposed to the drug 60 minutes prior to irradiation.

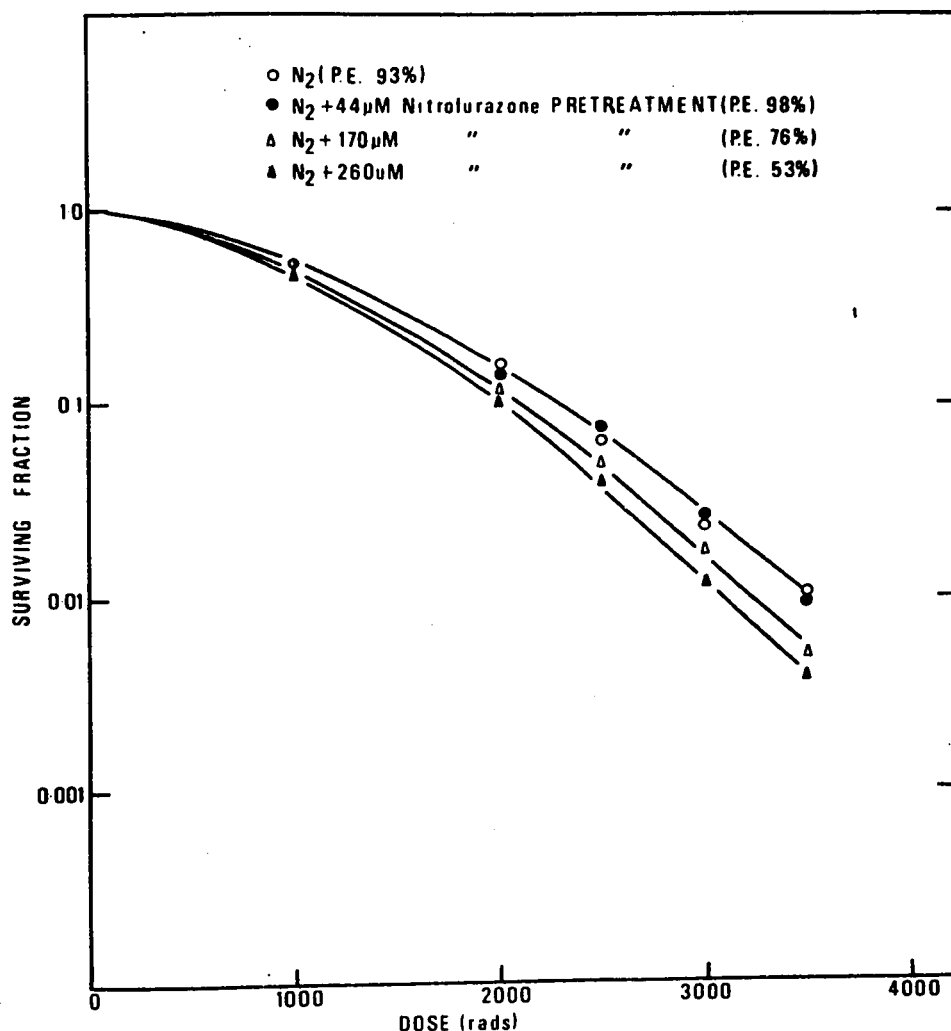


FIGURE 30. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen and Irradiated Following Pretreatment with 44 μM, 170 μM and 260 μM Nitrofurazone

Chinese hamster cells suspended in growth medium (lacking sodium bicarbonate) were incubated in the absence (○) or in the presence of 44 μM (●), 170 μM (Δ) and 260 μM (▲) nitrofurazone for 60 minutes at 23°C, after which time they were centrifuged and resuspended in drug free medium (lacking sodium bicarbonate). The cells were then placed in a glass irradiation vessel, gassed with N₂ for 45 minutes at 23°C, irradiated and assayed for colony forming ability (2.3.3).

to a radioresistant subpopulation of the cell suspension, then the cells surviving the drug pretreatment will have a lower radioresistance and thus a lower survival than the original population. However, the results of Figure 29 suggest that toxic effects resulting in reduced plating efficiencies do not produce subpopulations with altered radioresistance, since the survival curves are indistinguishable even though the control plating efficiencies change substantially.

3.4.3 Post-treatment with Nitrofurazone

Post-treatment of Chinese hamster cells was performed as described in Section 3.1.6 with irradiated cells being exposed to aerobic solutions of 44 μM and 170 μM nitrofurazone for 1 hour following various post-irradiation incubation intervals at 23°C. A small post-treatment effect that disappears in approximately 1 hour is observed for both concentrations of the drug (see Figure 31).

The pre- and post-treatment results demonstrate that nitrofurazone is very ineffective in this system unless it is present at the time of irradiation. Except for a small component of sensitization that does not appear to involve interactions with radiation produced, short-lived species, the results are consistent with the Direct Action Model of Adams and Cooke (1969).

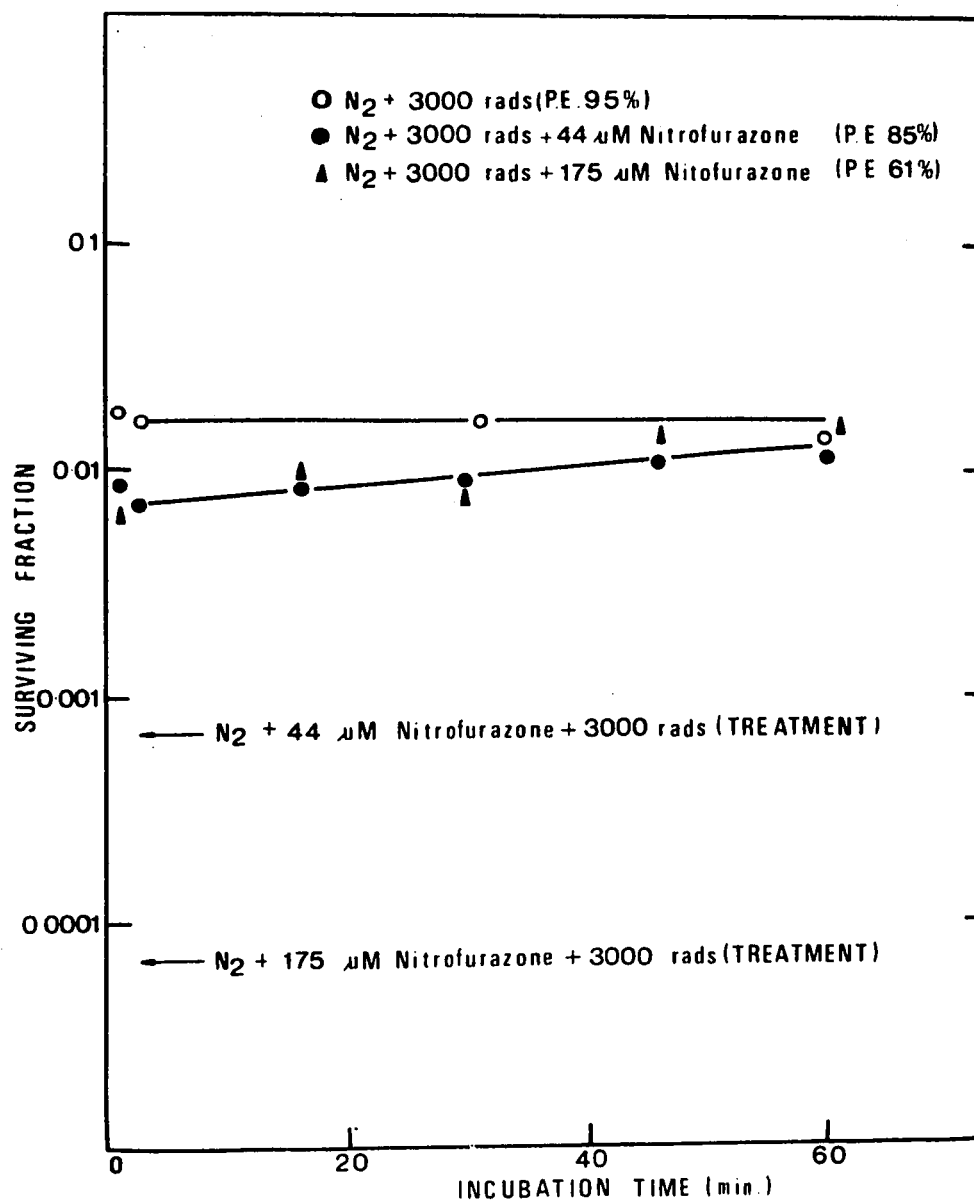


FIGURE 31. Surviving Fraction of Chinese Hamster Cells Subjected to Nitrofurazone Post-Treatment at 23°C as a Function of Incubation Time after the Termination of Irradiation

Chinese hamster cells suspended in growth medium (lacking sodium bicarbonate) were incubated at 23°C under anoxic conditions prior to, during, and following irradiation. At various times after the end of irradiation, aliquots were removed from the anoxic irradiation vessel and added to 44 μM , and 175 μM nitrofurazone. After a 1 hour exposure at 23°C to the aerobic nitrofurazone solution, the cells were centrifuged twice, resuspended in drug free medium and assayed for colony forming ability.

3.4.4 Nitrofurazone Sensitization in Cell Pellets

Pellet experiments as described in Sections 2.4.1 and 2.4.2 were performed using 175 μM and 360 μM nitrofurazone in order to determine if this compound also loses effectiveness as a sensitizer at high cell concentrations. The results of Figure 32 show a moderate sensitization with both concentrations of the drug. A higher drug concentration (350 μM) was used as the toxicity of the drug in nitrogen was found to be less than that measured in air in the toxicity experiment (Figure 27). The fact that the same sensitization is obtained with both 175 μM and 350 μM nitrofurazone is not surprising in view of the fact that a 175 μM drug concentration in suspension produces a maximum dose modifying effect (Figure 28).

It has been reported that the nitrofurans bind to serum proteins (Paul et al., 1960), a result that could be relevant to the radiotherapeutic use of these compounds if they are to reach the tumor via the circulatory system. In order to determine if high concentrations of serum protein would reduce the effectiveness of the drug, the pellet experiment was repeated, with 175 μM nitrofurazone being added to cells suspended in 100% fetal calf serum. The results in Figure 33 show the sensitization to be the same as for cells suspended in normal growth medium prior to pellet formation.

Thus, the radiosensitizing ability of nitrofurazone is not affected by the presence of high concentrations of

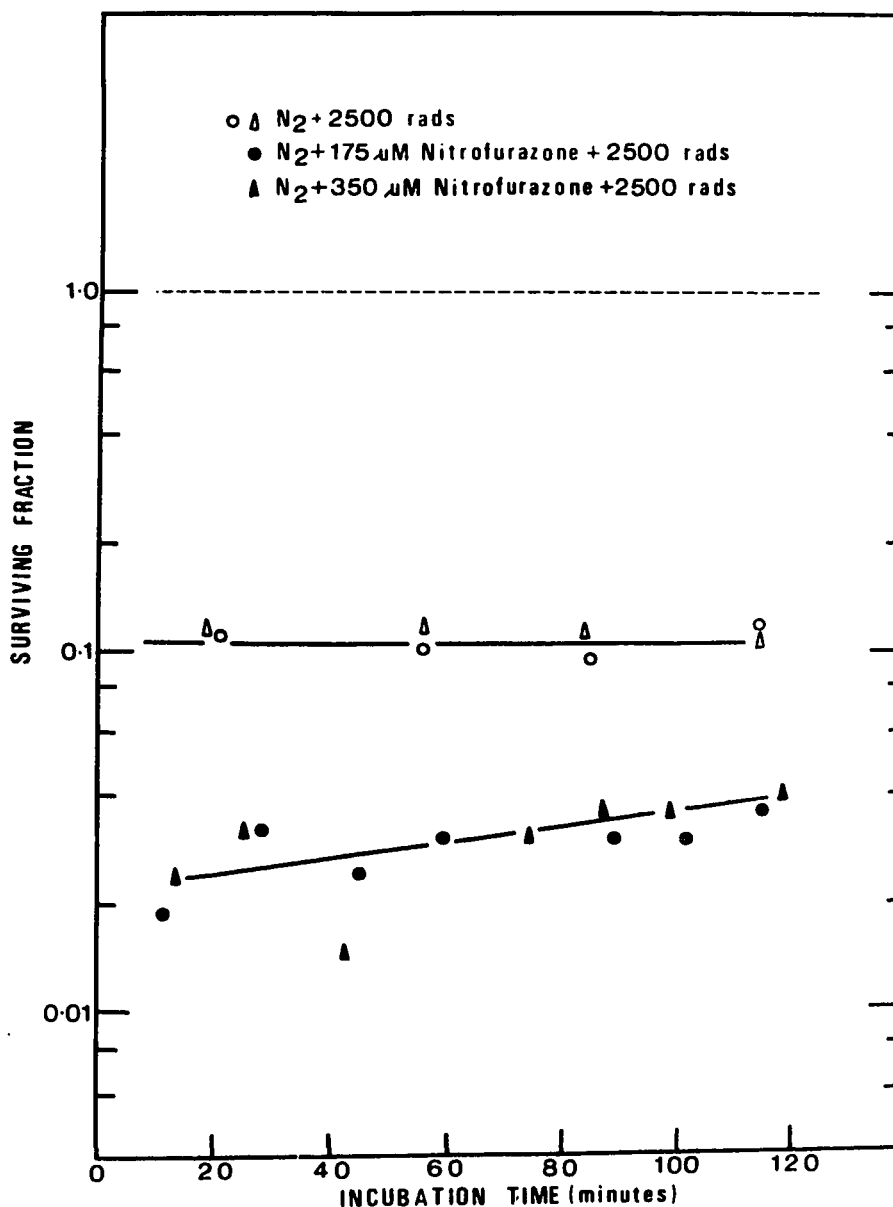


FIGURE 32. Surviving Fraction of Nitrofurazone Treated Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in growth medium (5×10^6 cells/ml) were incubated under anoxic conditions at 20°C for 1 hour in the presence of 175 μM and 360 μM nitrofurazone. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (●, ▲) and untreated (○, Δ) samples, unirradiated controls were taken from the drug treated and untreated pellets (----). Cells were kept anoxic throughout the experiment. The expected surviving fraction for $N_2 + 175 \mu M$ nitrofurazone + 2500 rads (in dilute cell suspension) is $\sim 10^{-3}$.

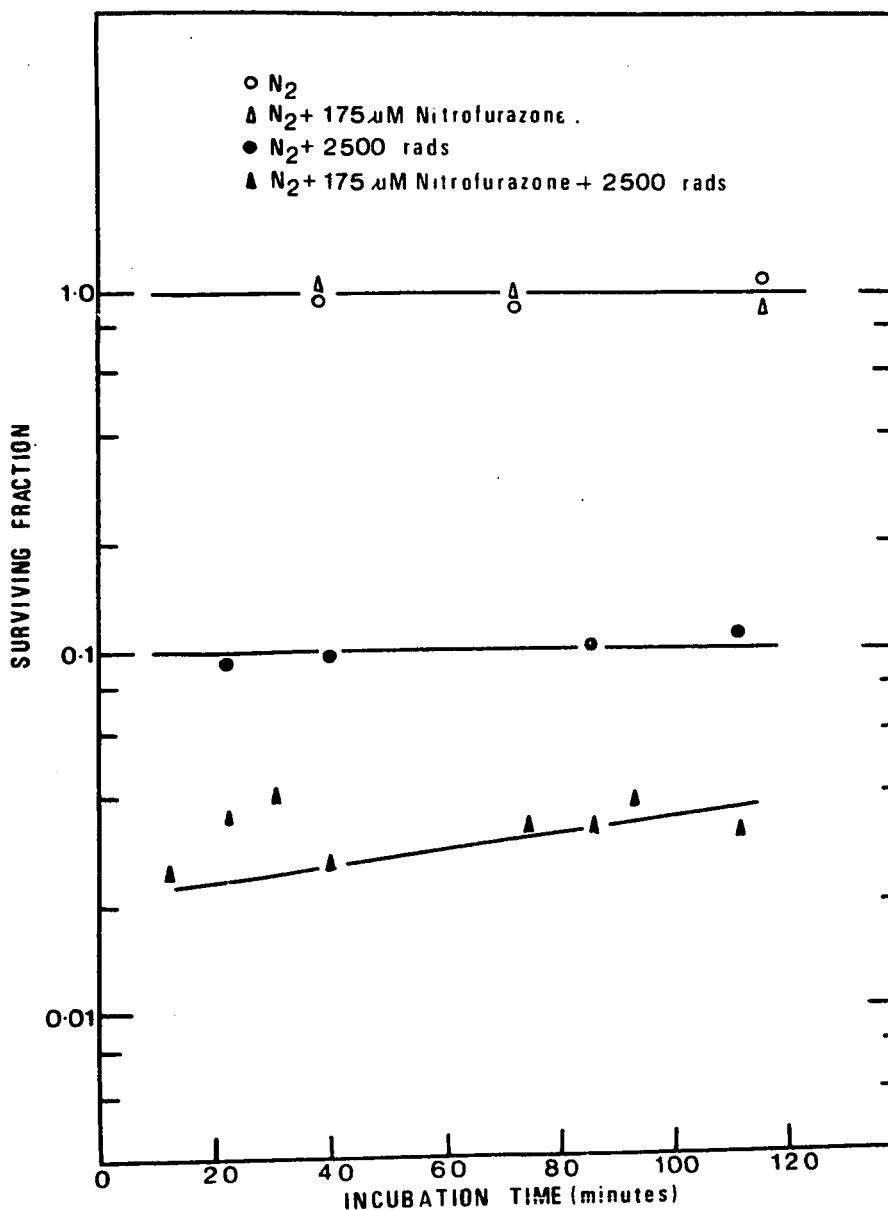


FIGURE 33. Surviving Fraction of Nitrofurazone Treated Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in 100% fetal calf serum (5×10^6 cells/ml) were incubated under anoxic conditions at 20°C for 1 hour in the presence of 175 μM nitrofurazone. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (\blacktriangle) and untreated (\bullet) samples, unirradiated controls were taken from the drug treated (\triangle) and untreated pellets (\circ). Cells were kept anoxic throughout the experiment.

serum proteins but as with the other drugs tested, it does vary inversely with the cell concentration.

3.5 PNAP-M RESULTS

The experiments with PNAP (Section 3.2) have shown this compound to exhibit good anoxic sensitization in cell suspension at a drug concentration of 500 μM . With the purpose of increasing the solubility and the hope of maintaining good sensitizing properties G. E. Adams ordered the synthesis of a Mannich derivative of PNAP, referred to as PNAP-M. We obtained a small sample of PNAP-M from Dr. G. F. Whitmore upon hearing of its exceptional sensitizing properties, and prepared a 2 mM stock solution which was stored frozen.

3.5.1 PNAP-M Sensitization in Cell Pellets

The growing interest in the loss of drug sensitization at high cell concentrations prompted us to first try this compound as a sensitizer in Chinese hamster cell pellets. Because of the small quantity of PNAP-M available, we used the toxicity data of G. F. Whitmore (unpublished data) which show the drug to have a low toxicity at a 100 μM concentration for short exposure times. The experiment was performed as described in Sections 2.4.1 and 2.4.2 with PNAP-M from a 2 mM stock solution being added to the cell suspension to a concentration of 100 μM . The results of Figure 34 show a large sensitization which exceeds that for all the other drugs even in cell suspension.

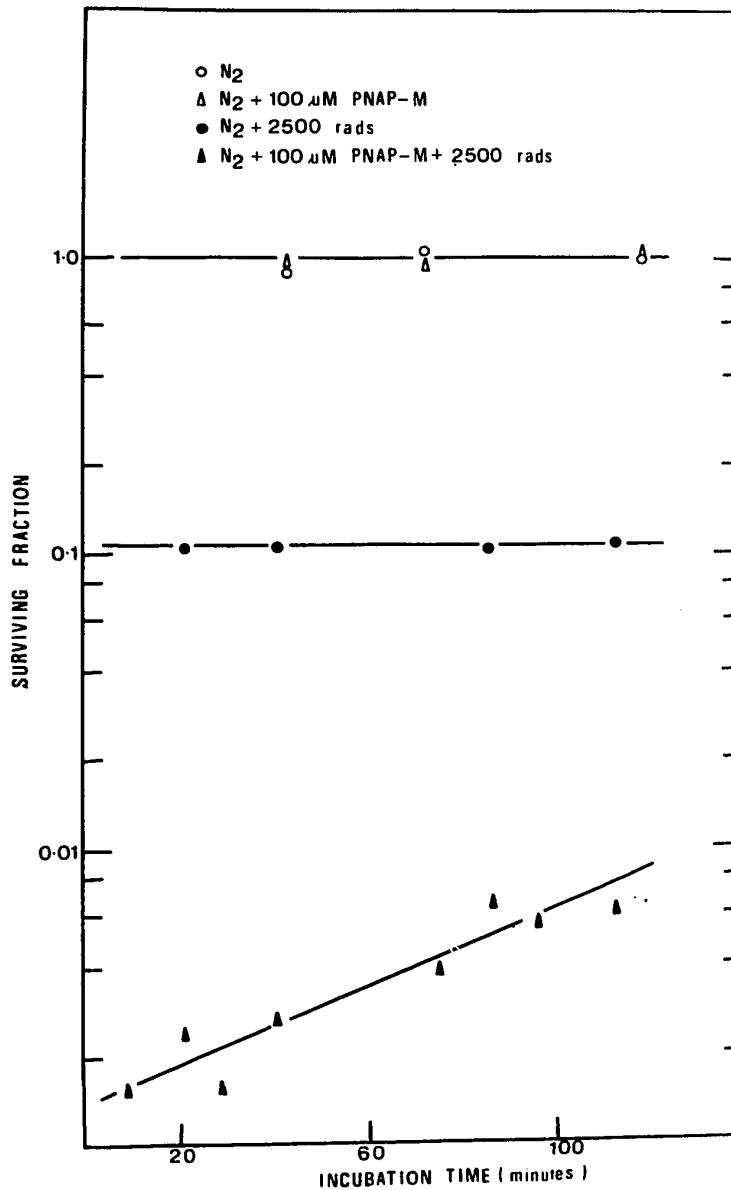


FIGURE 34. Surviving Fraction of PNAP-M Treated Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in growth medium (5×10^6 cells/ml) were incubated under anoxic conditions at 20°C for 1 hour in the presence of 100 μ M PNAP-M. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (\blacktriangle) and untreated (\bullet) samples, unirradiated controls were taken from the drug treated (Δ) and untreated pellets (\circ). Cells were kept anoxic throughout the experiment. The PE = 77% in unirradiated controls.

The toxicity studies of Whitmore show PNAP-M to be less toxic to mammalian cells when they are suspended in 100% fetal calf serum than when suspended in normal growth medium containing only 10% serum. To determine if the smaller toxicity is accompanied by a smaller sensitizing ability, the pellet experiment was repeated (2 days later) by adding PNAP-M from the stock solution to cells suspended in 100% fetal calf serum. The results shown in Figure 35 reveal a smaller sensitizing effect than was obtained for cells suspended in normal growth medium prior to pellet formation (see Figure 34). The loss of toxicity and sensitizing ability of the drug in fetal calf serum could reflect the binding of the compound to serum components, making it unavailable to the cells.

Two weeks after the 2 mM stock solution of PNAP-M was prepared, the pellet experiment was repeated to confirm the large sensitization exhibited in Figure 34. However, no results were obtained due to the high toxicity (<10% plating efficiency) of the compound. A fresh stock solution of PNAP-M was prepared and the cell pellet experiment repeated once more, this time within one hour of preparing the stock solution. This time, the toxicity was much less (~ 70% plating efficiency) but the sensitization as well was greatly reduced. Experiments done by Gulyas and Whitmore (1972) and by Adams (1972) may offer an explanation of these anomalous

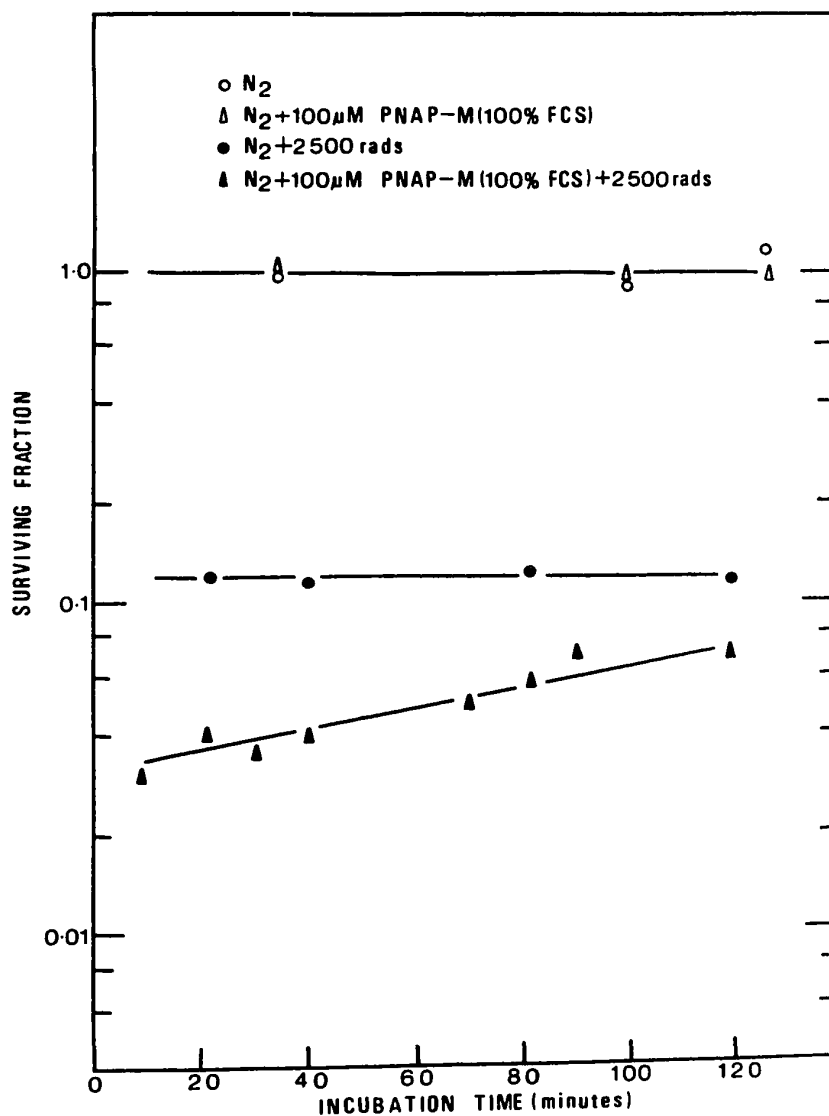


FIGURE 35. Surviving Fraction of PNAP-M Treated Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 20°C After the End of Centrifugation

Chinese hamster cells suspended in 100% fetal calf serum (5×10^6 cells/ml) were incubated under anoxic conditions at 20°C for 1 hour in the presence of 100 µM PNAP-M. The cells were then centrifuged, and the supernatant was withdrawn. After various incubation times at 20°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming ability. In addition to the irradiated, drug treated (▲) and untreated (●) samples, unirradiated controls were taken from the drug treated (△) and untreated pellets (○). Cells were kept anoxic throughout the experiment. The PE = 84% in unirradiated controls.

results. There is evidence that PNAP-M itself is not the sensitizing compound. Instead radiosensitization is produced by some altered form of the molecule that appears within a few hours during incubation of the PNAP-M solution. Following further incubation, the molecule possessing radiosensitizing properties is altered to form a toxic product, with little or no sensitizing ability. This could explain our inability to reproduce the results of Figure 34.

The results with PNAP-M are exciting in that they demonstrate that a large degree of sensitization can be obtained even at high cell concentrations using chemical radiosensitizers. However, the instability and toxicity of this compound in its present form pose problems in its use.

3.5.2 PNAP-M Sensitization in Dilute Cell Suspension

Before we were aware of the activation and toxicity problems associated with PNAP-M, an experiment was performed to determine the sensitizing ability of 100 μ M PNAP-M in cell suspension with both normal growth medium (lacking sodium bicarbonate) and 100% fetal calf serum. Thirty minutes prior to irradiation, freshly prepared PNAP-M was added to suspensions of Chinese hamster cells. The cells were irradiated under anoxic conditions, centrifuged (40 g for 8 minutes), resuspended in drug-free medium and plated to determine colony forming ability.

The results of Figure 36 reveal a very large sensitization in normal growth medium (DMF = 5), and moderate sensitization in 100% fetal calf serum (DMF = 1.9). The reduced sensitization in 100% fetal calf serum shown here is consistent with the reduced sensitization in pellet for cells suspended in 100% fetal calf serum. Thus, perhaps not all of the sensitization lost in the second pellet experiment (with 100% FCS) was due to an inactivation of the frozen stock solution of PNAP-M but, perhaps as well, some binding of the drug to serum was taking place in the pellet.

The extremely large sensitization obtained in suspension using normal growth medium must be viewed with some skepticism in view of the low plating efficiency of the unirradiated controls (39%). The surviving subpopulation could have a mean radioresistance less than that of the whole cell population (see Section 3.4.2). As well, the drug may be producing damage to the cell which acts synergistically with ionizing radiation damage.

The survival of anoxic cells suspended in 100% fetal calf serum is seen to be slightly higher than for cells suspended in growth medium (lacking sodium bicarbonate), a result which has been confirmed in several experiments.

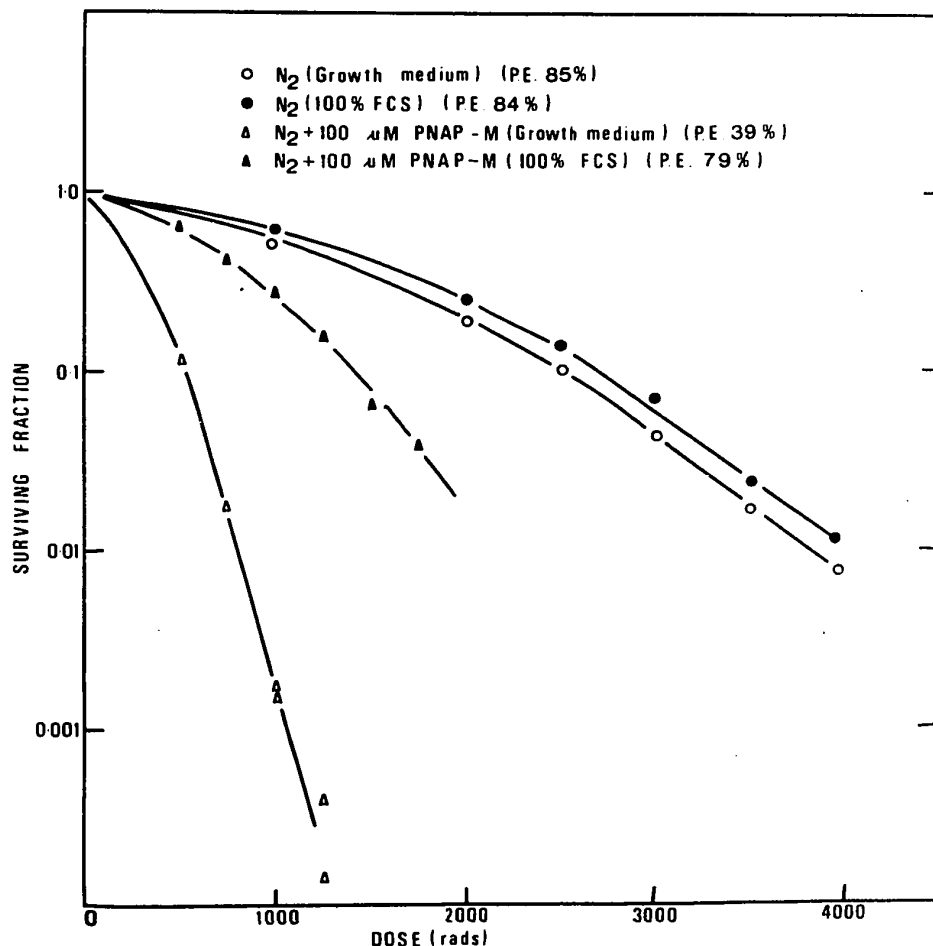


FIGURE 36. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen and Nitrogen Plus PNAP-M

Chinese hamster cells were suspended in either growth medium with (Δ) or without (O) 100 μ M PNAP-M, or in 100% fetal calf serum (FCS) with (\blacktriangle) or without (\bullet) 100 μ M PNAP-M. The cells were gassed with N₂ for 30 minutes prior to irradiation. Immediately following irradiation the drug was removed by centrifuging and resuspending the cells in drug-free medium. Cells treated with PNAP-M were exposed to the drug for 30 minutes prior to irradiation.

3.6 OXYGEN RESULTS

The importance of oxygen as a radiosensitizer in biological systems, and its involvement in the problems of radiotherapy have already been discussed. The motivation for studying chemical radiosensitization is the desire to increase the radiosensitivity of anoxic tumour cells to a level close to that for the healthy oxygenated cells. Since the pellet experiments showed the chemical radiosensitizers to be less effective in cell pellets than in cell suspensions, it was of interest to know if the same phenomenon occurs with oxygen at high cell concentrations.

3.6.1 Oxygen Sensitization in Pellets

We have already seen the sensitizing effect of oxygen when it is present during irradiation. In Chinese hamster cells oxygen produces a dose modifying factor of 2.8 (see Figure 23).

A pellet experiment as described in Section 2.4.3 was performed by gassing the control cells with N_2 and the oxygenated cells with O_2 for the duration of the experiment. Both controls and oxygenated cells were kept at $0^\circ C$ from the time that gassing started until the completion of irradiation. The results are shown in Figure 37. The surviving fraction for oxygenated cells (2×10^{-4}) is

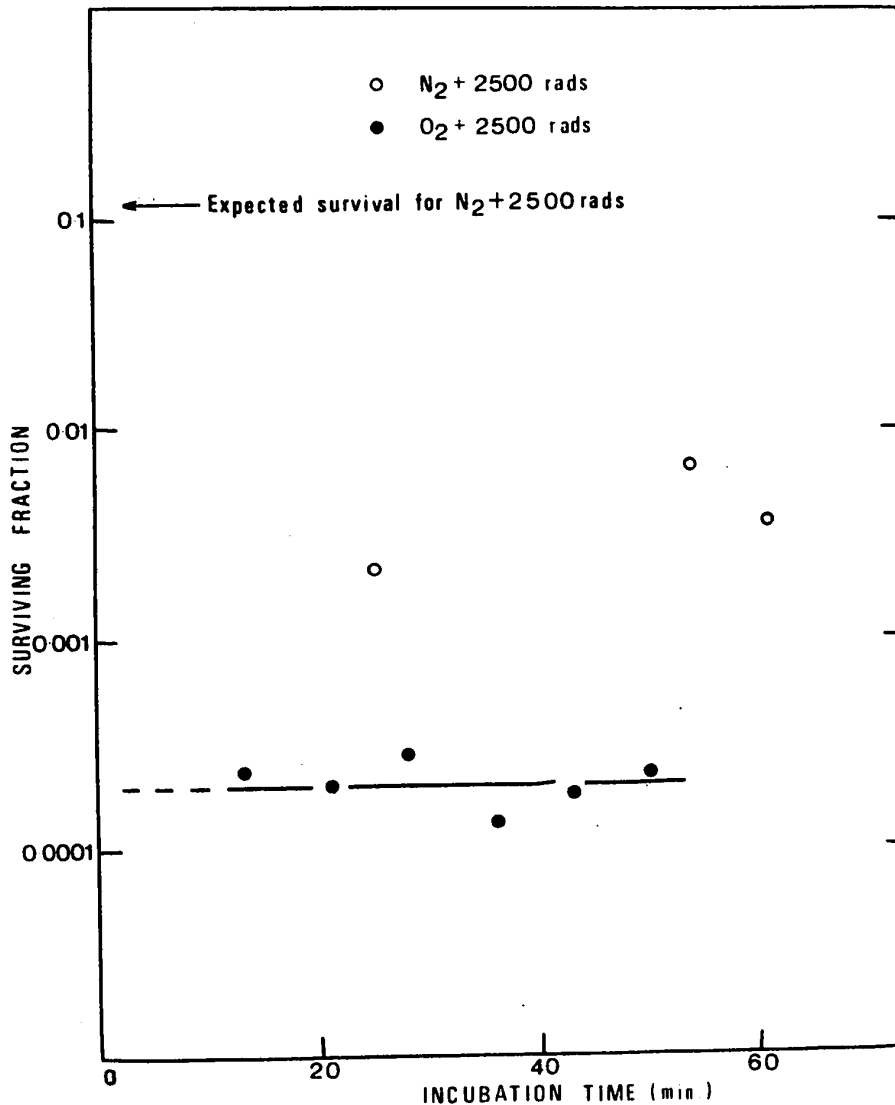


FIGURE 37. Surviving Fraction of Aerobic Chinese Hamster Cells Irradiated in Pellet Form as a Function of the Incubation Time at 0°C After the End of Irradiation

Chinese hamster cells suspended in growth medium (5×10^6 cells/ml) were incubated at 0°C in the presence of O₂. The cells were then centrifuged and the supernatant was withdrawn. After various incubation times at 0°C, samples of the cell pellet were irradiated, diluted in growth medium and plated to determine colony forming. The arrow indicates the surviving fraction obtained for the N₂ + 2500 rad controls in the pellet experiments performed at 23°C.

larger than the surviving fraction expected (3×10^{-5}) from cell suspension experiments.

It appears then, that oxygen also shows a reduced effectiveness at high cell concentrations. The constant value of the surviving fraction for increasing incubation time seen for the oxygenated cells suggests that the reduced oxygen effect in pellet is not due to reduced oxygen concentrations in the cell as a result of metabolic activity at 0°C.

The failure to obtain reproducible controls ($N_2 + 2500$ rads) in this experiment is characteristic of all experiments that were performed at a temperature of 0°C throughout the irradiation period. It is for this reason that this type of 0°C pellet experiment was not performed using the other sensitizers. The survival of 0°C irradiated anoxic controls is generally one to two logarithms lower than the survival of controls obtained in the pellet experiments performed at 20°C. This reduced cell survival at 0°C probably results from oxygen contamination of the pellet samples. This contamination most likely occurs between the time that the cell sample is removed from the pellet and the end of irradiation. During this time the sample is in contact with a plastic pipet which probably leaks small amounts of oxygen (Chapman, Sturrock, Boag and Crockall, 1970). The resulting oxygen contamination could possibly

sensitize some of the cells in the pellet sample. In addition some O_2 contamination may occur when the pellet is removed from the 50 ml tube containing the pellet and placed into a 12 ml tube prior to irradiation (see Section 2.4.2).

The drop in survival which occurs when anoxic cells are irradiated in pellet at $0^\circ C$ is not seen when the pellets are irradiated at $20^\circ C$. This presumably indicates that utilization of oxygen in these dense populations is rapid enough to compensate for small oxygen contaminations in the $20^\circ C$ pellets. The large fluctuation in control cell survival in pellet at $0^\circ C$ is not characteristic of the uniform survival usually observed in the anoxic pellet experiments performed at $20^\circ C$. It is not likely, therefore, that the reduced control survival at $0^\circ C$ in pellet is a result of a uniform decrease in the inherent cellular radiosensitivity at $0^\circ C$. In addition the radiosensitivity of cells in suspension is unchanged at $0^\circ C$ (see Figure 9).

3.7 OTHER DRUGS

Four of the electron-affinic compounds tested in our cell system failed to preferentially sensitize anoxic cells to the lethal effects of ionizing radiation.

3.7.1 Phenyl Pyruvate Results

Chinese hamster cells were suspended in a 10 mM phenyl pyruvate solution (2.7.6) 1 hour prior to irradiation under either anoxic or aerobic conditions. The exposure to the drug under the conditions of the experiment did not alter the plating efficiency. The results in Figure 38 show that the drug has no effect upon either the anoxic or aerobic survival response.

The low water solubility of this compound suggests that it may be unable to penetrate the cell membrane, and reach the target site in the cell.

3.7.2 2-Methyl-1,4-Naphthoquinone Results

Suspensions of Chinese hamster cells were irradiated under anoxic or aerobic conditions in the presence of 1 μ M 2-methyl-1,4-naphthoquinone (the maximum non-toxic concentration for exposure times up to 2 hours). The results shown in Figure 39 show that this compound also fails to sensitize anoxic or aerobic cells.

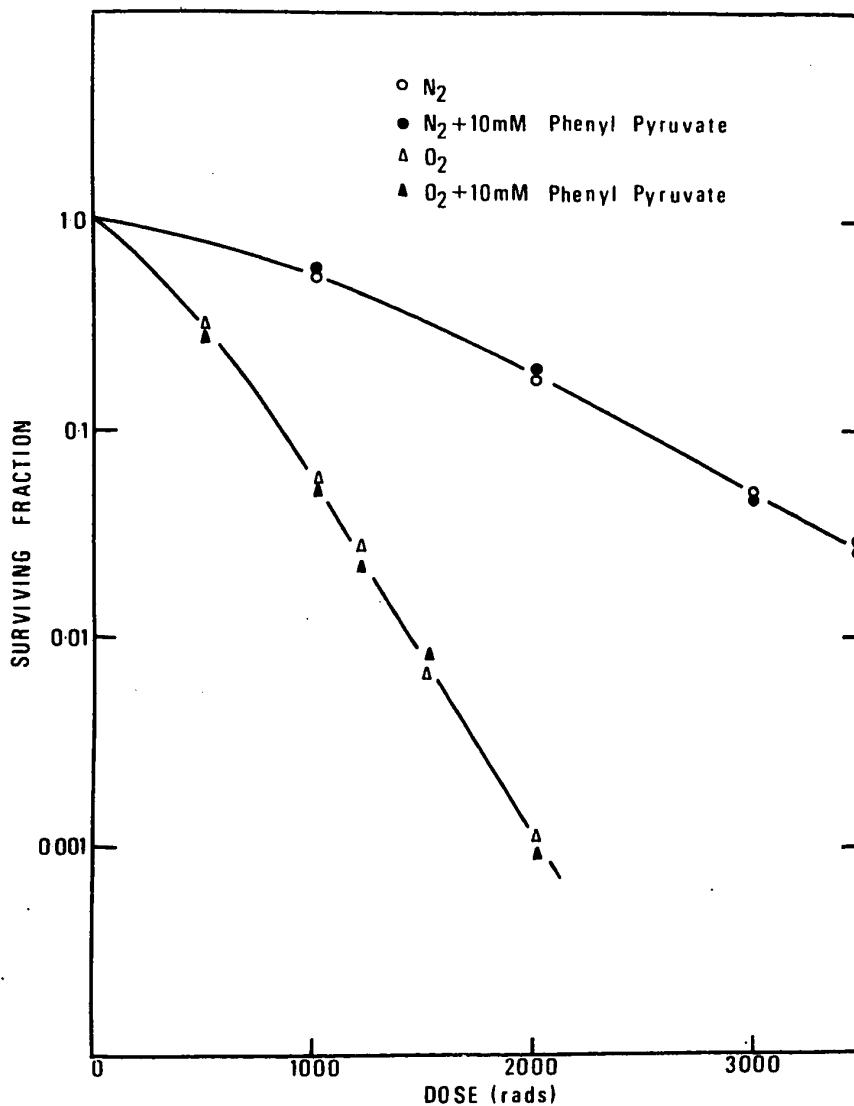


FIGURE 38. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen, Nitrogen Plus Phenyl Pyruvate, Oxygen, and Oxygen Plus Phenyl Pyruvate

Chinese hamster cells suspended in growth medium (minus sodium bicarbonate) at a cell concentration of 2×10^5 cells/ml were exposed to N₂ (○), N₂ + 10 mM phenyl pyruvate (●), O₂ (Δ) and O₂ + 10 mM phenyl pyruvate (▲) prior to irradiation. Following irradiation, the cells were washed with drug-free medium and assayed for colony forming ability (2.3.3). Cells treated with phenyl pyruvate were exposed to the drug for 1 hour prior to irradiation.

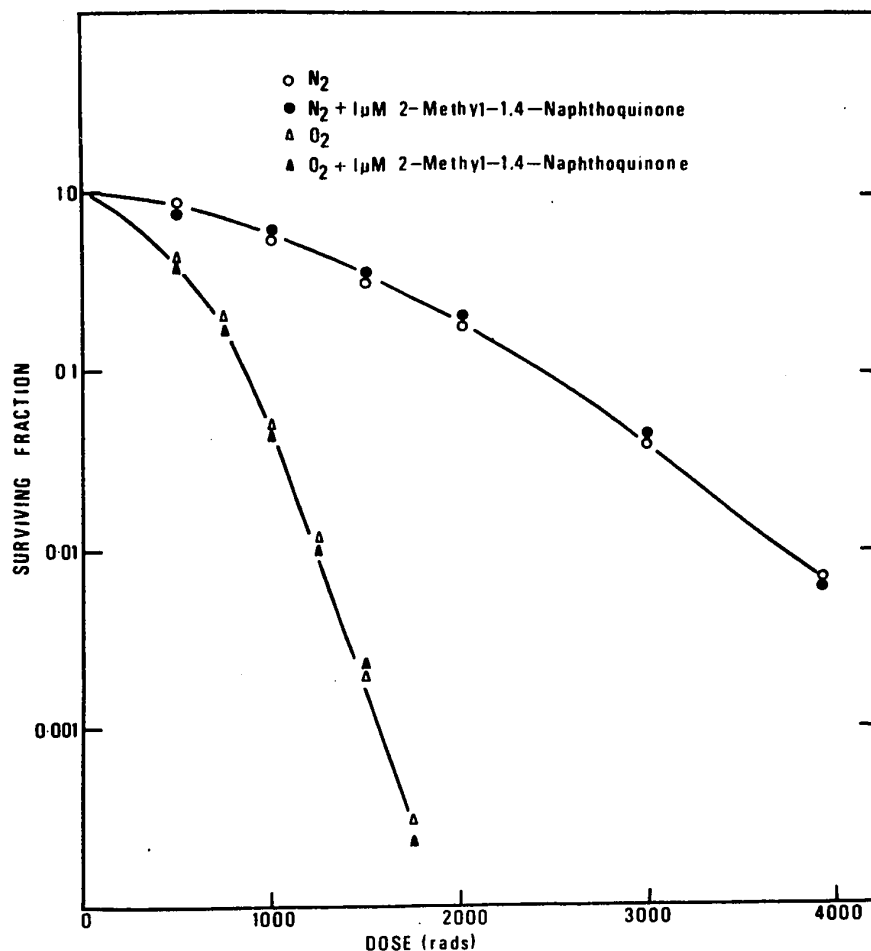


FIGURE 39. Survival Curves for Chinese Hamster Cells Irradiated in the Presence of Nitrogen, Nitrogen Plus 2-methyl-1,4-naphthoquinone, Oxygen and Oxygen Plus 2-methyl-1,4-naphthoquinone

Chinese hamster cells suspended in normal growth medium (minus sodium bicarbonate) at a cell concentration of 2×10^5 cells/ml were exposed to N₂ (○), N₂ + 1 μM 2-methyl-1,4-naphthoquinone (●), O₂ (△) or O₂ + 1 μM 2-methyl-1,4-naphthoquinone prior to irradiation. Following irradiation the cells were washed with drug-free medium and assayed for colony forming ability (2.3.3). Cells treated with 2-methyl-1,4-naphthoquinone were exposed to the drug for 1 hour prior to irradiation.

The failure of this compound to accumulate to a sufficiently high concentration to achieve a sensitizing effect is suggested by the fact that Adams and Cooke (1969) used a 50 μ M concentration of the drug to achieve a sensitizing effect in Serratia marcescens.

3.7.3 Ethyl Pyruvate and Phenyl Glyoxal Results

Chinese hamster cells were suspended in either 10 mM ethyl pyruvate or 100 μ M phenyl glyoxal 1 hour prior to irradiation under either anoxic or aerobic conditions. The concentration of drug used, for both ethyl pyruvate and phenyl glyoxal, was the maximum, non-toxic concentration for exposure times up to 2 hours.

The results of Figure 40 show ethyl pyruvate and phenyl glyoxal to produce DMF's of 1.4 and 1.7 respectively, when present during anoxic irradiation. The presence of ethyl pyruvate or phenyl glyoxal during aerobic irradiation also results in a reduced survival (Figure 41). The relatively large sensitization of aerobic cells observed in Figure 41 is not a desirable characteristic of a radiosensitizer if it is to be used in radiotherapy (see 1.1).

The sensitization of aerobic suspensions of cells may result from the production of toxic products as has been suggested for iodoacetamide (Dean and Alexander, 1962), and hydroxymercuribenzoate (Moroson and Tenney, 1968). Other

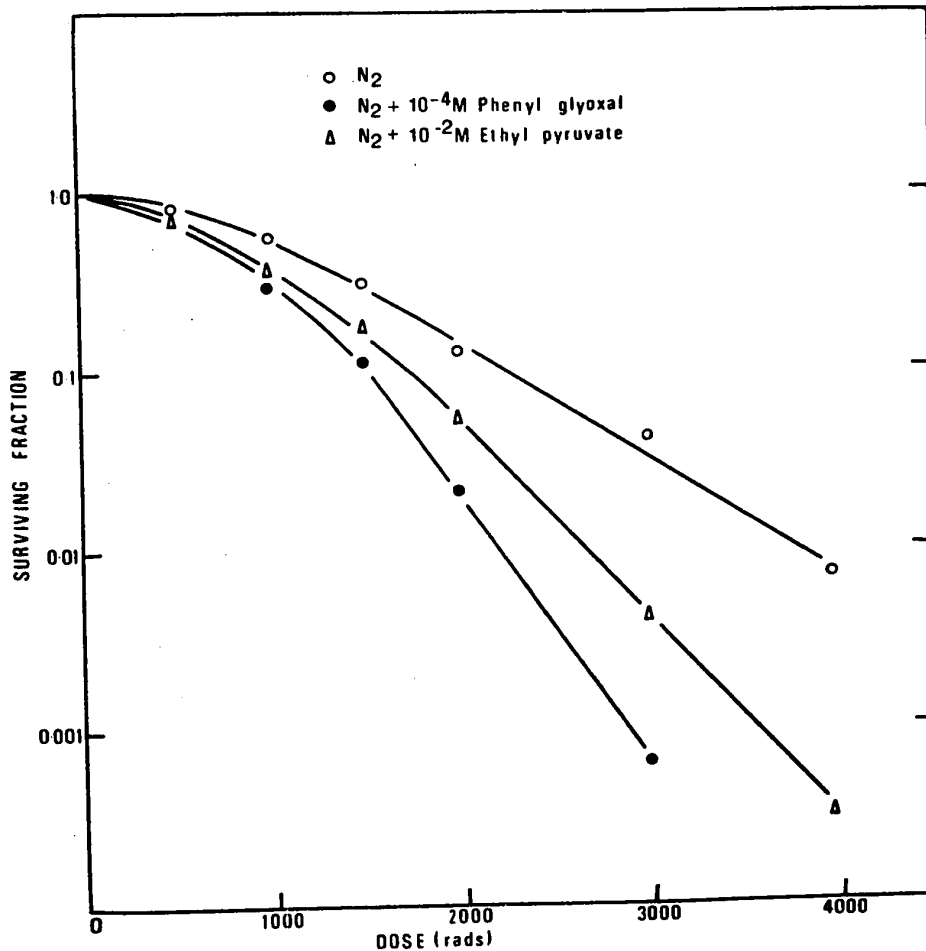


FIGURE 40. Survival Curves of Chinese Hamster Cells Irradiated in the Presence of Nitrogen, Nitrogen Plus Phenyl Glyoxal and Nitrogen Plus Ethyl Pyruvate

Chinese hamster cells suspended in normal growth medium (minus sodium bicarbonate) at a cell concentration of 2×10^5 cells/ml were gassed for 45 minutes in the presence of N_2 (O), $N_2 + 10^{-4}$ M phenyl glyoxal (●), or $N_2 + 10^{-2}$ M ethyl pyruvate (Δ), prior to irradiation. Following irradiation the cells were washed with drug-free medium and assayed for colony forming ability (2.3.3). Cells treated with phenyl glyoxal or ethyl pyruvate were exposed to the drug 60 minutes prior to irradiation.

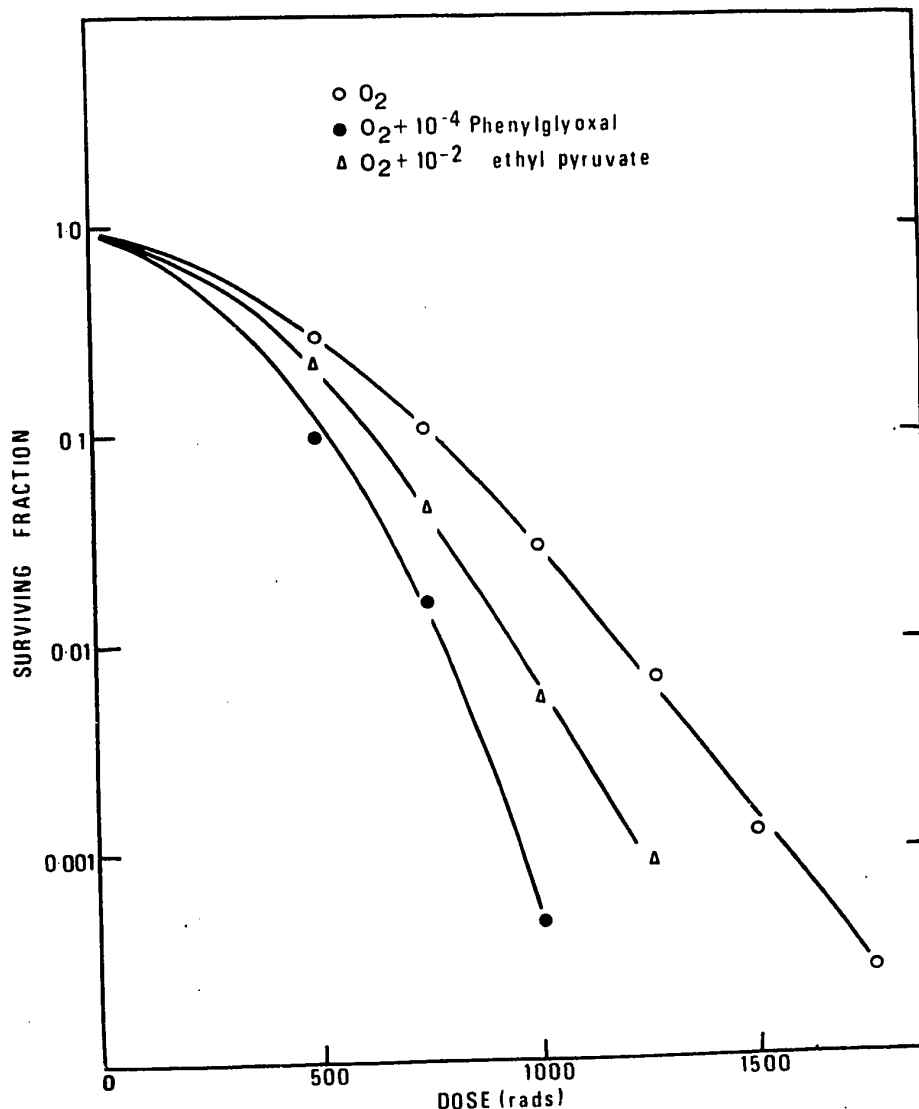


FIGURE 41. Survival Curves of Chinese Hamster Cells Irradiated in the Presence of Oxygen, Oxygen Plus Phenyl Glyoxal and Oxygen Plus Ethyl Pyruvate

Chinese hamster cells suspended in normal growth medium (minus sodium bicarbonate) at a cell concentration of 2×10^5 cells/ml were gassed for 45 minutes in the presence of O₂ (O), O₂ plus 10^{-4} M phenyl glyoxal (●) and O₂ plus ethyl pyruvate (Δ) prior to irradiation. Following irradiation the cells were washed with drug-free medium and assayed for colony forming ability (2.3.3). Cells treated with phenyl glyoxal or ethyl pyruvate were exposed to the drug 60 minutes prior to irradiation.

mechanisms such as the interference with the repair of aerobic damage could be acting to suppress the survival of these cells.

3.8 SEDIMENTATION STUDIES

In this experiment, mouse L-60 cells were exposed to 10 mM TAN 60 minutes prior to irradiation in an anoxic or aerobic environment. The temperature of the cells was maintained at 0°C from 15 minutes prior to irradiation until the time of lysis (2.6).

The weight average molecular weight was determined (Palcic and Skarsgard, 1972) for each irradiation exposure, and the results plotted as the reciprocal of the weight average molecular weight versus dose. The results are shown in Figure 42.

The oxygen enhancement ratio (OER or DMF for oxygen) for single strand breaks is 5, a value greater than that reported by Palcic and Skarsgard (1972). However, the variation in molecular weights between separate experiments indicates that two experiments are not sufficient to establish an accurate value for the DMF.

It is fairly clear, however, that the presence of TAN has not increased the number of DNA single strand breaks in either the aerobic or anoxic cultures. In fact, in these experiments, the presence of TAN seems to have led to increased molecular weights in both cases. The significance, if any, of this result will be examined in greater detail.

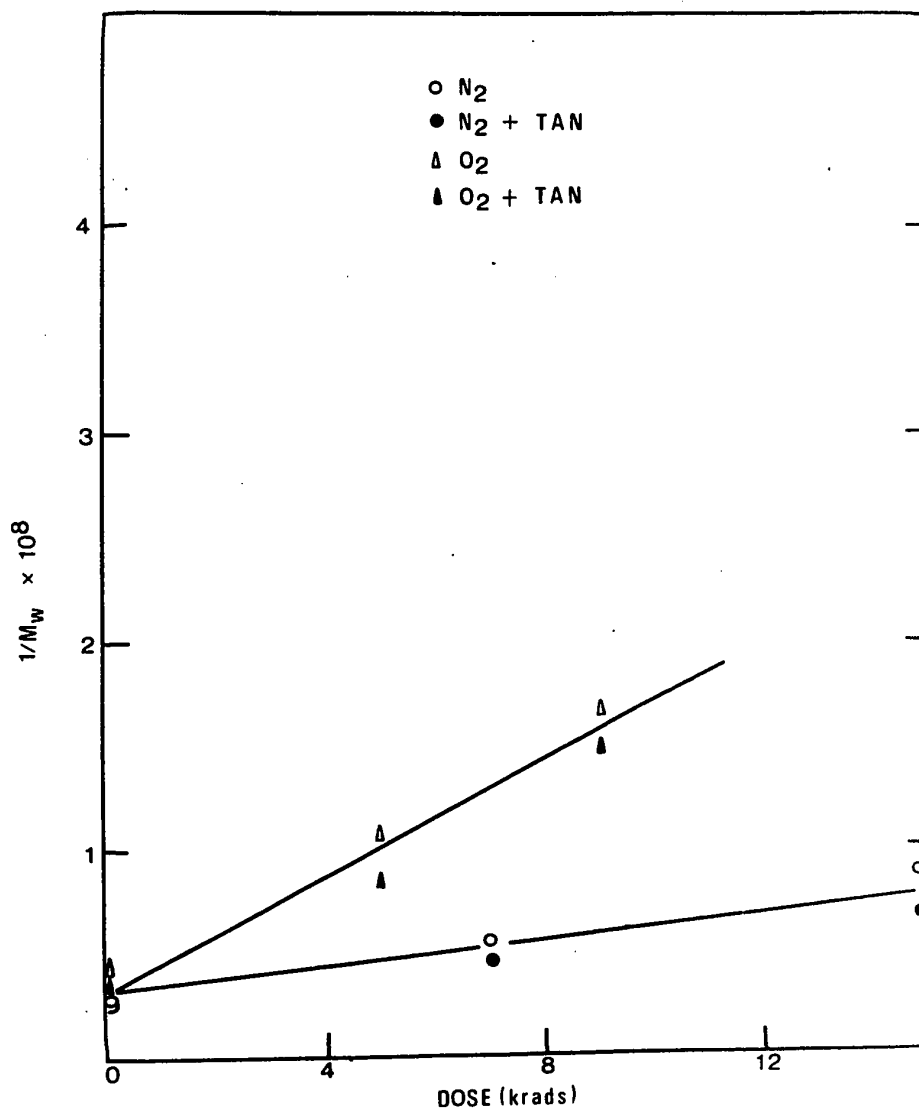


FIGURE 42. The Production of Single Strand Breaks in Mouse L-60 DNA Following Irradiation in the Presence of Oxygen, Oxygen Plus TAN, Nitrogen and Nitrogen Plus TAN

L-60 cells were irradiated at 0°C in the presence of O₂ (△), O₂ plus 10 mM TAN (▲), nitrogen (○) and nitrogen plus 10 mM TAN (●) and then lysed for 12 hours on an alkaline sucrose gradient (2.6.3). The gradients were centrifuged at 16 k rpm for 6.75 hours at 20°C in an SW27 rotor. The weight average molecular weight (Mw) of the DNA was calculated (Palcic and Skarsgard, 1972) and was plotted as 1/Mw versus dose.

4. DISCUSSION

4.1 MECHANISMS OF TAN SENSITIZATION

4.1.1 Inhibition of Repair

The results of the post-treatment experiments in our mammalian cell system (3.1.6) implicate a relatively long-lived transient state of the cell in the mechanism of TAN sensitization. The strong temperature dependence of the post-treatment effect (Figure 10) and the return to the control (N_2 + 3000 rads) survival value of the curves for 35°C and 37°C incubation, suggest that post-irradiation sensitization by TAN involves the interference by the drug with the repair of radiation-induced cellular damage. The completion of the repair process in the absence of TAN allows no subsequent TAN sensitization. Post-treatment after a 1 minute incubation at 33°C produces almost full sensitization while at 35°C and 37°C smaller effects are seen. An increase in the rate of repair at the higher temperatures during the 9 minute irradiation period would result in greater damage repair prior to the addition of TAN, and a subsequent reduction in the post-treatment effect. It appears that at 37°C all of the repair

with which TAN can interfere is complete within 1 hour, if this model is correct.

The results of the post-treatment experiment are not consistent with modes of sensitization such as the interaction of the sensitizer with short-lived reducing species (Adams and Cooke, 1969), the production of a toxic product by the interaction of TAN with a short-lived radiolytic species, or the binding of cellular SH groups by TAN (Howard-Flanders, 1960). In all of the above mechanisms, the lifetimes of the species involved are many orders of magnitude smaller than the 1 minute incubation time referred to in the post-treatment experiments.

The post-treatment results obtained in our mammalian cell system contrast with those performed in Serratia marcescens (Adams, 1970). In that experiment, the addition of TAN only 4 msec after irradiation produced no sensitization. Similar experiments have been performed by Brustad (1968) using E. coli with the same results. It appears that in the bacteria studied, TAN interacts with very short-lived reactive species. The mechanisms of TAN sensitization involved in bacterial and mammalian cells appear to be different, and may reflect differences such as the ability of the drug to diffuse to target sites in the cell and the types of repair mechanisms available to the cell.

Our observation that TAN is able to interfere with recovery between fractionated doses (see 3.1.7) supports the hypothesis that TAN interferes with cellular recovery. However, the magnitude of the recovery suppressed in the fractionated dose experiments should be insufficient to account for the degree of radiosensitization observed with TAN post-treatment (1 minute at 33°C).

It has been observed that TAN treatment does not alter the aerobic response, i.e., neither the slope nor the extrapolation number of the single dose curve is affected by the presence of TAN (Parker, Skarsgard and Emmerson, 1969; and Figure 11, this work). However, the recovery normally seen in aerobic fractionated dose experiments is suppressed in the presence of TAN (see Figure 12). This result is somewhat anomalous since the shoulder or extrapolation number of the survival curve is normally associated with recovery. However, one of the current concepts of the system by which sublethal damage is repaired allows an explanation of this result. The following hypothesis attempts to do this:

In the Repair Model of Survival (Haynes, 1966) the initial damage to the cell is proportional to the radiation dose. The cell has, however, a finite capacity for repair which can be saturated at a suitably high dose. Let us look at the fractionated dose experiment in terms of Haynes' model.

A substantial first dose of irradiation in the presence of oxygen saturates the repair system by creating more damage than the system can begin to repair at once. If a second dose is delivered immediately, the repair system remains saturated and the additional damage leads to exponential killing.

If, however, the two doses are separated by a suitable time interval, then the repair system can "catch up" to the damage from the first dose. The repair system is then unsaturated and a second dose will yield a survival curve which has regained the shoulder, indicating that the repair system is able to begin repair of the new damage. The result is that more of the damage is repaired and the survival is greater than if the two doses were delivered without interruption. If the effect of TAN in the aerobic fractionated dose experiment is to interact at the site of repair (perhaps at the nuclear membrane) and to interrupt the repair process during the interval between doses, then the repair system might remain saturated and no increase in survival would be seen. Thus, the presence of TAN could inhibit recovery in the aerobic fractionated dose experiment.

How, then, can one justify the presence of a shoulder in the single dose survival curve (oxygen) if, as we have just postulated, TAN inhibits recovery? To explain this, it seems necessary to assume that the binding or

association between TAN and the DNA (or the site of DNA repair) is reversible and that repair of the damage registered in oxygen can proceed as soon as the TAN dissociates. It should be mentioned here that the interaction of TAN at the repair site could possibly involve the formation of a TAN lesion, which, in this model would be repairable. During the one hour interval between fractionated doses we would assume that the TAN remains associated or bound with the DNA repair system so that the second dose yields exponential killing. In the single dose experiment, however, one could visualize that even if the TAN dissociated from the repair system several hours after irradiation, repair of unfixed damage might proceed. There is some evidence which supports the notion that free radicals like TAN form loose associations with membranes (Griffith and Waggoner, 1969; Hubbell and McConnell, 1968).

While the above model is based on the idea that for damage registered in the presence of oxygen repair can be deferred for several hours, this is apparently not so for damage produced under anoxic conditions, otherwise we could not expect to see any sensitization in the anoxic TAN treatment experiments where the TAN is normally diluted away within 2 to 3 minutes after irradiation. This fact could either mean that the above model is not valid or that the fixation and/or repair processes are different for

damage produced in the presence or absence of oxygen.

Fractionated dose experiments have been reported by Révész and Littbrand (1970) using a substrain of the Chinese hamster line V79-379-A. In their system they found that low concentrations (0.25 mM) of TAN sensitize and higher TAN concentrations (20 mM) protect. These results are similar to those reported by Sutherland, Duran and Jones (1971), who also used a V79 subline. The sensitization produced at such a low TAN concentration (0.25 mM) may result from an accumulation of the drug in the cells. Rupp et al. (1970) have reported that mouse leukaemic cells accumulate TAN at concentrations 10 to 100 times greater than would be expected by passive diffusion. It is also possible that in the V79 lines, TAN can more easily diffuse to the sites where it is most effective. The reduction of indirect action (see 1.2.3) as a result of the efficient scavenging of free radicals might be a part of the protective effect of high concentrations of TAN.

In the fractionated dose experiments of Révész and Littbrand, cells attached to glass were irradiated under anoxic or aerobic conditions, with or without TAN present. During the 18 hour interval between the fractionated doses, the cells were incubated under aerobic conditions in the absence of TAN. The results of these workers indicated that 0.25 mM TAN did not suppress recovery in cells irradiated

under aerobic conditions. In addition, the anoxic fractionated dose experiments revealed no recovery in the absence of 0.25 mM TAN and no recovery or sensitization in the presence of TAN.

Their results for the aerobic fractionated dose experiment can be explained by the model developed for our system, if we assume that during the 18 hour interval between doses, TAN is released from its binding site and the damage is subsequently repaired. The failure to observe recovery when cells are irradiated under anoxia without TAN, and incubated aerobically is unexpected. Koch and Kruuv (1971), using a V79 subline, have demonstrated that repair does occur following anoxic irradiation when the cells are exposed to oxygen between the fractionated doses. The reason for the failure of Révész and Littbrand to observe anoxic fractionated dose recovery is not known.

The absence of repair in the anoxic fractionated dose experiments (without TAN) of Révész and Littbrand may explain the lack of significant sensitization produced when TAN is present in these experiments. These results are consistent, therefore, with the idea that TAN sensitizes anoxic cells by interfering with cellular repair following ionizing radiation.

4.1.2 TAN Binding

The sensitization produced in Chinese hamster cells following pretreatment with 10 mM TAN (DMF = 1.3) is equivalent to that produced by 1 mM TAN treatment (Parker, Skarsgard and Emmerson, 1969). ESR experiments have revealed, however, that TAN is present as a free radical at a concentration of less than 10^{-5} Molar following 10 mM TAN pretreatment. The degree of sensitization produced in these two cases, therefore, does not correlate well with the average concentration of TAN in the cell at the time of irradiation, and mechanisms such as the interaction of TAN with radiation produced, short-lived, reactive species or the production of toxic products appear to offer unlikely explanations. In both cases the increase in the TAN concentration should correlate with an increase in the production of damaging species and a corresponding greater sensitization. If, however, the cell is able to concentrate the drug at relevant sites within the cell, the above argument might not be valid.

The results of the pretreatment (0°C during TAN treatment, see 3.1.3) experiment suggest that TAN does not sensitize by a mechanism that involves a binding of cellular SH groups.

One mechanism proposed to explain the pretreatment effect is one in which TAN interacts at crucial sites

within the cell, resulting in the formation of TAN adducts and an increased cellular radiosensitivity. The fact that the pretreatment sensitization is independent of the time between removal of TAN and the beginning of irradiation for periods at least up to 4 hours, implies that these adducts must be relatively long-lived. In addition, the 0°C pretreatment experiment (see 3.1.3) suggests that cell metabolism is not involved in the formation of these adducts.

Another model which might explain the pretreatment result is one which supposes that TAN produces in the cell sublethal lesions which are synergistic with radiation produced lesions.

The sensitizing ability of TAN pretreatment has been confirmed by Sutherland and Durand (Conference on Chemical Sensitization of Anoxic Tumour Cells to Radiation, Toronto, April 17, 1972) who have reported that pretreatment with 0.5 mM TAN produces a substantial anoxic sensitizing in their Chinese hamster V79 line. Attempts to sensitize their cell line with 0.5 mM TAN post-treatment, however, were unsuccessful, possibly as a result of the high efficiency of the cellular repair systems during irradiation at 37°C. Results from our post-treatment experiments (see 3.1.6) demonstrate a greatly reduced post-treatment effect at 37°C even at short incubation times.

4.1.3 TAN Sensitization: A Model

Studies with TAN present at the time of irradiation (treatment) were performed by adding TAN to the cells at least 1 hour prior to irradiation and the TAN was removed (i.e., diluted) after irradiation. Thus pretreatment and post-treatment components of sensitization are inherently a part of sensitization which results when TAN is present during irradiation.

A single mechanism which could explain most of the sensitization observed by all three types of treatment is one in which TAN adducts, which form whenever TAN is added to the cells, and which remain in the cell following washing, effectively interfere with the repair of radiation-induced cellular damage. In addition to the effect of TAN adducts, free TAN must also account for a component of sensitization since treatment sensitization (DMF = 1.5) exceeds that for pretreatment (DMF = 1.3).

The possibility that more than one mode of sensitization may be involved in the experiments reported here is not excluded. Under any given treatment condition then, separate mechanisms might be favoured: with TAN present (10 mM) at the time of irradiation, conventional interactions with radiation produced, short-lived species might be favoured, while interference with recovery might predominate in the post-treatment experiment. Pretreatment

sensitization could be the result of interference with recovery and/or synergism between TAN produced and radiation produced lesions. However, if the experimental results are to be explained by these separate mechanisms, one would have to describe as fortuitous the observation that essentially the same sensitization is obtained whether TAN is present during or only after (33°C and 1 min incubation) irradiation.

The equivalent sensitization produced by both treatment and post-treatment (33°C and 1 minute incubation) may indicate that the bulk of TAN present in these two cases is involved in the suppression of repair processes.

Brustad and Nakken (1970) have reported that covalent binding of TAN to DNA may result from the interaction of TAN with long-lived (a few seconds), radiation induced transients of DNA. Barrie Jones (personal communication, 1972) has evidence from preliminary data that some of the free-radicals produced in calf thymus DNA (irradiated in a saline solution) are still present 2 hours after irradiation.

These results suggest that a partial sensitization of our mammalian cells in both TAN treatment and post-treatment experiments could conceivably result from an interaction of TAN with such a long-lived species.

The extreme temperature dependence of the post-treatment experiment, however, deems it unlikely that a

significant amount of the post-treatment sensitization results from such an interaction. The mechanisms involved in eliminating the free radicals would not be expected to have such a strong temperature dependence as is observed with TAN post-treatment. In addition, if the formation of extremely long-lived radicals, as reported by Jones, is characteristic of DNA, then the combined results of Brustaad and Nakken, with those of Jones would suggest that these very long-lived DNA radicals may not interact with TAN.

The results of the TAN sedimentation experiments (Figure 42) show TAN to have no significant effect on the production of single strand DNA breaks. The fact that Emmerson (1970) observed that TAN increased the number of nicks in λ phage DNA suggests that the DNA in our mammalian cells is less vulnerable to direct attack by the TAN molecules than is λ phage DNA in superinfected E. coli. A reduced accessibility of TAN to mammalian cell DNA which these experiments suggest, could offer a possible explanation for the different mechanisms of TAN sensitization suggested for bacteria and mammalian cell systems.

4.2 ELECTRON AFFINIC SENSITIZATION

Three of the electron affinic compounds (PNAP, dimethyl fumarate and nitrofurazone) were examined with respect to their pretreatment and post-treatment capabilities. The pretreatment effectiveness of these compounds ranged from nil (PNAP) to very large (dimethyl fumarate). None of the drugs produced the large post-treatment effect that was observed with TAN.

The sensitization obtained with 500 μ M PNAP (DMF = 1.6) was the same as reported by Chapman, Webb and Borsa (1971). The ineffectiveness of both pretreatment and post-treatment with PNAP is consistent with the direct action model of Adams and Cooke (1969) which requires that the sensitizer be present during irradiation. The absence of a pretreatment effect also suggests that an SH binding mechanism is probably not involved. PNAP is unique in that it is the only drug we have tested that must be present during irradiation to produce a sensitizing effect.

Dimethyl fumarate and nitrofurazone each appear to exhibit pre- and post-treatment sensitizing abilities. The pretreatment effects vary, however, being large for dimethyl fumarate (DMF = 1.4) and small for nitrofurazone (DMF \leq 1.1). In both cases we cannot rule out SH binding as a possible mechanism involved in the pretreatment effect nor can we

rule out the possibility that these compounds are not effectively washed from the cells prior to irradiation. The post-treatment effect of these compounds is small, and in both cases disappears in approximately 1 hour.

The possibility exists that pretreatment sensitization with dimethyl fumarate and nitrofurazone involves the formation of a drug-cell adduct or lesion, as has been suggested for TAN (4.1.2). Similarly, an interference with repair following irradiation could be involved in the post-treatment sensitization of these compounds. However, a large component of sensitization obtained with these compounds may result from an interaction of the drug with a radiation induced, short-lived reactive species, in view of the larger effect obtained when the drug is present at the time of irradiation.

4.3 SENSITIZATION AT HIGH CELL CONCENTRATIONS

The presence of a radiosensitizer during the anoxic irradiation of cells at high cell concentrations resulted in a two-stage loss of the sensitization normally seen with the compound in dilute cell suspension. A large component of sensitization is lost immediately upon formation of the cell pellet and this is followed by the slow disappearance of a smaller component of sensitization. In cells irradiated in the absence of a sensitizing agent no effect of cell concentration was observed, indicating that the loss of a large component of sensitization upon formation of the pellet was not accompanied by a loss in the inherent radiosensitivity of the cell. The slowly disappearing component of sensitization in the pellet could be explained by a metabolic alteration of the sensitizing molecule. For example, nitrofurazone, a compound with a well established pharmacology and one of the sensitizers tested in our pellet system, is known to be metabolized by a great variety of body tissues (Bender and Paul, 1951).

The metabolism of sensitizers by body tissues is a problem in the application of these compounds to radiotherapy. Techniques which will maintain a sufficiently high concentration of these sensitizers in the tissue will be necessary if these sensitizers are to be of practical use.

Perhaps of greater significance is the rapid loss in sensitization which occurs in the cell pellet prior to the time of the first irradiation. TAN pellet experiments performed at 0°C (3.1.11) have shown that a temperature dependent metabolic process is probably not involved in this large initial loss in sensitization. The O₂ pellet experiment (3.6), which was performed at 0°C, confirms this result.

ESR experiments with TAN pellets have suggested that the failure to achieve full sensitization at high cell concentrations is not due to a substantial reduction of the drug concentration in the cells. The concentration of TAN free radicals in the pellets is approximately 80% of that in an equal mass of supernatant, a concentration of TAN which should produce a degree of sensitization much larger than is observed in the cell pellet.

In order to compare the sensitizing effects of these compounds in suspension and in pellet, assume for the moment that the sensitizing effect of these drugs in pellet is strictly a dose modifying one. This assumption seems a reasonable one since the sensitizing effect of these compounds in suspension is nearly completely a dose modifying one. The DMF's obtained directly from the suspension experiments and estimated for the pellets using the single dose survival are recorded in Table 1.

Table 1

Sensitizer	DMF in pellet	DMF in suspension	DMF pellet DMF suspension
TAN	1.1	1.5	0.73
PNAP	1.1	1.5	0.73
Dimethyl fumarate	1.4	1.8	0.78
Nitrofurazone	1.3	1.8	0.72
PNAP-M (in growth medium)	2.0	5	0.40
PNAP-M (in 100% FCS)	1.3	1.8	0.72
O ₂	2.3	2.8	0.82

The ratio (DMF in pellet)/(DMF in suspension) is also recorded. The result is that with the exception of PNAP-M (in growth medium) all of the drugs have a similar ratio. The low ratio for PNAP-M (in growth medium) may reflect an over estimation (due to the high toxicity of the compound, PE = 39%) of the sensitization produced in suspension (3.5.2). The DMF for O₂ was calculated assuming a pellet control survival value at 0°C (N₂ + 2500 rads) equal to the control survival observed in pellets at 20°C.

The equation for the exponential region of a

survival curve is:

$$S = ne^{-kD}$$

where the slope of the curve is $-k$. Since the anoxic survival (in the absence of a sensitizer) is the same for cells irradiated in pellet or suspension, the ratio given in Table 1 is equal to:

$$\frac{k \text{ (for survival curve irradiated in pellet with drug)}}{k \text{ (for survival curve irradiated in suspension with drug)}}$$

Since the slope of the survival curve, $-k$, is just a measure of the sensitivity of the cell, it would appear that (except for PNAP-M in normal medium) the formation of the cell pellet results in a decrease in radiosensitization that is approximately independent of the sensitizer involved.

The cause of this reduced sensitization at high cell concentrations is unknown. It appears that it is not due to a temperature dependent metabolic activity, or a reduced concentration of the sensitizer in the cell pellet. If this cell concentration effect is real, it is difficult to explain in view of the different mechanisms of sensitization probably involved with various sensitizers.

Duran and Sutherland (1971) have used multicellular spheroids, to demonstrate that cell-to-cell contact during irradiation results in an increase in sublethal repair capacity. The importance of this finding to our system is questionable, however, since the cells in our pellet are

not grown together and probably lack the intimate cell-to-cell contact evident in their system. The enhanced anoxic survival observed with cells irradiated in spheroids, a phenomenon that is not observed for our anoxic pellet cells, may simply mean that cell-to-cell interactions in spheroids are different than in our cell pellets.

The importance of the pellet-type experiment as an experimental system intermediate between the in vitro and in vivo systems will not be known until more data from sensitization in in vivo systems is available. If the results in vivo are consistent with the results of our experiments at high cell concentrations, then cell pellets will offer an excellent system for studying mechanisms of sensitization in vivo as well will provide a rapid selection technique for potential in vivo radiosensitizers.

If the decreased sensitizing ability of chemical radiosensitizers at high cell concentrations is a general phenomenon, then, sensitizers chosen for testing in in vivo systems should be those with the largest, in vitro, radiosensitizing abilities at low cell concentrations since we have found that the relative effectiveness in pellet and in suspension is about the same for all drugs tested.

CONCLUSIONS

The purpose of the work discussed in this thesis was to (a) better understand mechanisms of chemical radiosensitization so that the most effective compounds could be selected for therapeutic use and (b) to possibly explain the failure of early attempts to sensitize mammalian cells in vivo.

The sensitizers used in these studies were triacetoneamine N-oxyl (TAN) and several electron affinic compounds.

In this work we have shown:

- (1) The pretreatment of cells with TAN has a substantial sensitizing effect.
- (2) Adding TAN to cells following irradiation (post-treatment) produces a sensitization that depends on (i) the temperature and (ii) the time interval between end of irradiation and the addition of TAN.
- (3) TAN inhibits the recovery of sublethal damage as seen in anoxic and aerobic fractionated dose experiments.
- (4) The presence of TAN during irradiation under anoxic or aerobic conditions does not effect the production of single strand breaks.

- (5) A substantial component of TAN sensitization appears to involve the suppression of damage repair following ionizing radiation.
- (6) Most of the electron affinic compounds tested would appear to be capable of sensitizing by more than one mechanism of action.
- (7) All radiosensitizers studied appear to have reduced sensitizing abilities at very high cell concentrations (centrifugation pellets).
- (8) The loss in sensitization at high cell concentrations appears to be approximately independent of the sensitizer used (except for PNAP-M in normal growth medium).

APPENDIX

List of Publications

1. L. D. Skarsgard, B. Palcic and D. A. Agnew. 1969. Sensitization of anoxic mammalian cells to radiation by organic free radicals: ESR studies (Abstract). Radiat. Res. 39: 455.
2. D. A. Agnew and L. D. Skarsgard. 1970. Anoxic sensitization by triacetoneamine N-oxyl and its effect on cellular recovery and rejoining of single strand breaks. Abstracts of the Fourth International Congress of Radiation Research, p. 6.
3. D. A. Agnew and L. D. Skarsgard. 1972. Sensitization of anoxic mammalian cells to radiation by triacetoneamine N-oxyl. Effect of pre- and post-treatment. Radiat. Res. 51: 97-109.

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