THE POTENTIAL OF THE SUPEROXIDE DISMUTASE INHIBITOR, DIETHYLDITHIOCARBAMATE AS AN ADJUVANT TO RADIOTHERAPY

by

Charles Kent

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Department of Radiotherapy

Faculty of Medicine

University of Cape Town

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To Mish, the winner.

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Contents

Abstract

It has long been known that oxygen has the potential to be toxic to biologic systems and that this toxicity is not due to oxygen itself, but due to the production of oxygen radicals. One of these potentially toxic radicals, superoxide (O_2^-) can be generated as a result of ionizing radiation, and if not adequately removed can proceed to cause cell damage. Superoxide dismutase (SOD) is one of the key enzymes involved in the defence against oxygen toxicity. SOD activity can be inhibited by diethyldithiocarbamate (DDC), a powerful copper chelator.

If inhibition of SOD by DDC increases the lifetime and effectiveness of radiation induced O_2^-, it follows that the potential exists for DDC to enhance the effect of radiation. DDC is however also a thiol compound, and thus may act as a radioprotector by modifying tissue oxygenation status or by free radical scavenging.

This study has concerned itself primarily with the inhibition of superoxide dismutase by diethyldithiocarbamate in order to sensitize tumours to ionizing radiation. The use of DDC as an inhibitor of SOD has however meant that any sensitization resulting from SOD inhibition could be masked by a radioprotective effect by DDC.

The inhibition of SOD by DDC was confirmed in a murine rhabdomyosarcoma, and it was shown that this inhibition can be maintained for up to twenty-four hours after DDC administration. It was hypothesised that there was a potential for the radioprotective effect of DDC to be overcome, if the levels of DDC were low enough at the time of irradiation. Indeed, if DDC was removed from the growth medium of B16 mouse melanoma cells in culture prior to irradiation, a significant sensitization was demonstrated.

It was shown that DDC could act as both a radiosensitizer and as a radioprotector in the same experiment. The dominant action of DDC was found to be dependent on the time allowed between DDC administration and irradiation. If this time was approximately 4 hours, it was possible to show a radiosensitizing effect by means of a tumour growth delay assay.

This time modulation effect of DDC was shown in larger tumours, rather than smaller tumours, which could indicate that tumour oxygenation is an important criterion in determining the response to radiation of DDC treated cells.

It was shown that B16 mouse melanoma cells exposed to 43°C after DDC pretreatment were sensitized to thermal damage.

This work suggests that some caution should be exercised when DDC is put forward as either a radiosensitizer or a radioprotector in the clinic, but that DDC may have potential as a thermosensitizer.

Chapter 1
The toxicity of oxygen

This section aims at providing an introduction to oxygen toxicity, the chemistry of oxygen and superoxide radicals. Some historical perspectives on the origins of oxygen toxicity and the evolution of protective mechanisms are included since the enzyme being studied in this thesis, superoxide dismutase, is thought to have arisen early in evolution as a protective mechanism against oxygen toxicity.

Evolution of our solar system

This has been very succinctly described by Dickerson (1978) It is thought that the sun and planets of our solar system were formed from a revolving cloud of gas, dust particles and debris. Due to rotational and gravitational energy, the sun coalesced at the centre of this cloud. Local inhomogeneities at varying distances from the centre became aggregation points for the formation of the planets. The larger planets, Jupiter, Saturn, Uranus and Neptune probably represent a fair approximation of the composition of this original cloud, since their constituent elements are close to that of the universe at large. (They are composed mainly of hydrogen, helium, methane, ammonia and water). The small inner planets Mercury, Venus, Earth and Mars are richer in the heavier elements and poorer in the volatile

gases which could escape the weak gravitational pull of these planets. A combination of low gravity and high temperature led to a loss of most of the earth's volatile constituents soon after the planet coalesced.

The relative abundance of oxygen was greatly increased, because it was locked into the non-volatile minerals. The original surface may have been too hot for water to remain liquid, but as soon as the temperature dropped below the boiling point, water released from the interior by outgassing processes such as volcanism would have condensed to form the original oceans. This would give rise to a secondary atmosphere composed of water vapour, methane, carbon dioxide, ammonia and hydrogen sulphide.

The early atmosphere

This atmosphere - the character of which was reducing rather than oxidising, is probably where life arose. Oparin (1936) and Haldane (1954) first proposed that a reducing atmosphere (with no free oxygen) was a requirement for the evolution of life from non-living organic matter. Urey (1952) and Miller (1953) conducted laboratory experiments where they demonstrated that spark discharges (e.g. lightning) in aqueous mixtures of hydrogen, methane and ammonia gave rise to aldehydes, carboxylic acids, amino acids and strings of amino acids.

From this is postulated the development of primitive protein and nucleic acid chains from the polymerisation

of these organic compounds, which eventually formed droplets and a membrane with an internal environment. The final stage would be the development of some type of reproductive machinery to ensure that daughter cells had all the chemical and metabolic capabilities of the parents.

The first living organisms

Some of the first living organisms, similar to green and purple sulphur bacteria, were able to produce energy rich molecules by photosynthesis using H_2S as a source of hydrogen atoms :

$$12H_2S + 6CO_2 \xrightarrow{\text{light energy}} \text{Glucose } (C_6H_{12}O_6) + 12S + 6H_2O$$

Later the predecessors of blue-green algae and higher plants mastered the technique of photosynthesis using water as a source of hydrogen atoms :

$$12H_2O + 6CO_2 \xrightarrow{\text{light energy}} \text{Glucose } (C_6H_{12}O_6) + 6O_2 + 6H_2O$$

This process released oxygen into the early atmosphere, and transformed it into the one in which all subsequent life developed.

With the evolution of photosynthetic cells that produced oxygen, the earth's atmosphere slowly began to change. As a result of photosynthesis, the concentration of

oxygen in the earth's atmosphere rose to 1 percent about 600 - 1000 million years ago and to about 10% some 100 million years ago. The present concentration of 21% has been created and is maintained by photosynthesis. The accumulation of oxygen in the air made possible the evolution of organisms that produce most of their chemical energy by cellular respiration. As the oxygen content of the atmosphere rose, it also changed the atmosphere from a reducing to an oxidising environment. Living matter was thus exposed to oxygen toxicity, that is , oxidations which were harmful or perhaps even lethal to the cells.

The evolution of protective mechanisms

There was considerable evolutionary pressure to develop protective mechanisms against oxygen toxicity or to retreat to environments that oxygen did not penetrate.

Anaerobic organisms comprise a wide range of organisms with varying oxygen tolerance. For 'strict' anaerobes, for example *Treponema denticola* and several *Clostridia*, oxygen exposure is lethal, whereas moderate anaerobes such as faecal bacteria can survive some oxygen exposure (Halliwell & Gutteridge, 1985).

The damaging effects of oxygen on strict anaerobes appears to be due to the oxidation of essential cellular components. Some enzymes in anaerobes are also inactivated by oxygen, for example the nitrogenase

enzyme, which catalyses the reduction of nitrogen to ammonia in *Clostridium pasteurianum*, is inactivated due to the oxidation of essential components in its active site. This enzyme is essential for the survival of the organism in environments poor in nitrogen compounds (Halliwell & Gutteridge, 1985).

The efficient use of oxygen in aerobic organisms paved the way for the development of more complex multicellular organisms. However oxygen is dangerous and often lethal in high concentrations, and thus a selective pressure existed which favoured the proliferation of cells which had developed mechanisms to cope with the damaging effects of oxygen.

Why oxygen is toxic to living organisms

Before considering these mechanisms, it is pertinent to examine why oxygen is toxic to living organisms.

The history of early biological and medical research into oxygen toxicity has been excellently reviewed by Gilbert (1981).

Soon after the discovery of oxygen by Priestley in the 1770s, it was recognised that oxygen might have therapeutic value, such as for the treatment of "*asphixias by suffocation*", but also that pure oxygen could be toxic :

> "*If the atmospheric air were perfectly pure, the life of animals breathing it would be much more energetic, better and pleasant in many ways, but at the same time it might be proportionally shortened, and being rapidly consumed by such active air, they might only live one quarter of the time that they live in the ordinary air of the atmosphere, impure though it may be.*" (Macquer, 1777)

The first report of oxygen toxicity was given in 1777 by Scheele :

> "*Plants [peas], however will not grow noticeably in pure fire-air [oxygen].*"

Lavoisier (1782/83) autopsied guinea pigs that had died in oxygen, and found that :

> "*all of them appeared to have died of a burning fever and of inflammatory sickness. Upon inspection, the flesh was very red, the heart was livid ... the lung was very flaccid ... Healthy air is therefore composed of a good proportion between vital air [oxygen] and atmospheric moffete [nitrogen] ... when there is an excess of vital air [oxygen], the animal undergoes a severe illness, when it is lacking, death is almost instantaneous.*"

Perhaps the earliest biochemical suggestion made to explain oxygen toxicity was that oxygen inhibits cellular enzymes (*op cit*, Halliwell & Gutteridge, 1985).

A good example of this is photosynthesis. During photosynthesis, illuminated green plants fix CO_2 into glucose in the Calvin cycle, a complex metabolic pathway where ribulose biphosphate carboxylase combines CO_2 with a 5-carbon sugar (ribulose 1,5-biphosphate) to produce 2 molecules of phosphoglyceric acid. Oxygen inhibits this reaction by competing with CO_2, resulting in less CO_2 fixation and less plant growth at elevated oxygen concentrations.

In general however, the rates of enzyme inactivation by oxygen in anaerobic cells are too slow and too limited in extent to account for the rate at which toxic effects develop, and many enzymes are unaffected by oxygen at all. This led Gershman *et al.* (1954) to propose that most of the damaging effects of oxygen could be attributed to the formation of oxygen radicals.

In order to understand why oxygen is toxic to living organisms, we must first examine the basic chemistry of oxygen, and of free radicals.

Notes on the chemistry of oxygen

In covalent bonding, where electrons are shared between atoms, the atomic orbitals are transformed to molecular orbitals.

There are 2 types of molecular orbitals :

* Bonding molecular orbitals e.g. σ1s, which are more stable than the atomic orbitals.

* Anti-bonding molecular orbitals e.g. σ*1s, which are less stable than the atomic orbitals.

The bonding molecular orbitals are more stable than the atomic orbitals. To illustrate this let us examine hydrogen and helium.

The bonding molecular orbitals are the most stable, and of lowest energy. They are even more stable than the atomic orbitals. For example, hydrogen atoms each have a single electron in the 1s atomic orbital. ($1s^1$) When hydrogen atoms combine to form the hydrogen molecule, the two electrons in the atomic 1s orbitals enter the σ1s bonding molecular orbital, each electron having opposite spin (in accordance with Pauli's principle). Hence H_2 is more stable than isolated H atoms.

For the sake of argument, let us examine what would happen should helium atoms combine to form He_2: helium

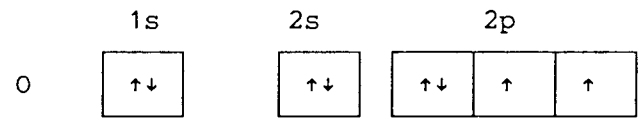
atoms contain 2 electrons in the 1s atomic orbital. ($1s^2$) Should helium atoms combine, 2 electrons would share the σ1s bonding molecular orbital, and the other 2 would share the σ*1s anti-bonding molecular orbital. As mentioned earlier, this state is less stable than the atomic state, thus He_2 does not form.

Combination of p-type atomic orbitals can produce 2 types of molecular orbitals by overlapping in different ways, and are known as σ and π orbitals. Hence for one of the $2p_x$ orbitals combining with another such orbital, there will be two bonding molecular orbitals : $\sigma 2p_x$ and $\pi 2p_x$, and 2 anti-bonding molecular orbitals ($\sigma^* 2p_x$ and $\pi^* 2p_x$). The energy would increase in the order of :

$$\sigma 2p_x < \pi 2p_x < \pi^* 2p_x < \sigma^* 2p_x$$

Forms of molecular oxygen

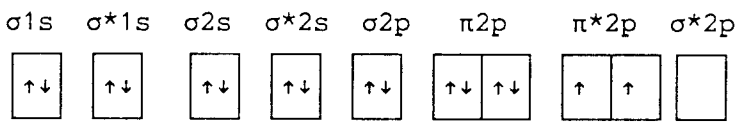
Oxygen has the configuration of $1s^2\ 2s^2\ 2p^4$ or



If the atoms combine to form a diatomic molecule (O_2), the four 1s electrons (2 from each atom) fully occupy both σ1s bonding and σ*1s anti-bonding molecular orbitals, so there is no net bonding. Similarly the four 2s electrons occupy the σ2s bonding and σ*2s anti-bonding molecular orbitals, and again no net bond

results. Of the eight electrons remaining, six fill the $\sigma 2p_x$, $\pi 2p_y$ and $\pi 2p_z$ bonding molecular orbitals, and the remaining two must occupy the next highest molecular orbital in terms of energy. There are 2 such orbitals of equal energy namely the $\pi^* 2p_y$ and $\pi^* 2p_z$ anti-bonding molecular orbitals. By Hund's Rule, each must receive one electron. Since the presence of electrons in anti-bonding orbitals energetically cancels out one of the $\pi 2p$ bonding orbitals, the two oxygen atoms are effectively joined by a double O=O bond.

Ground state O_2

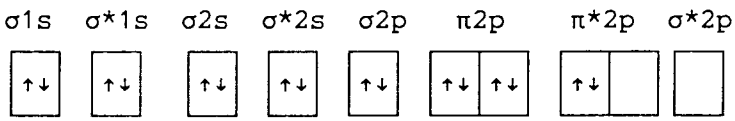


If oxygen attempts to oxidise another atom or molecule by accepting a pair of electrons from it, both of these electrons must be of anti-parallel spin so as to fit in the vacant spaces of the $\pi^* 2p$ orbital. A pair of electrons in an atomic or molecular orbital would not meet this criterion however, as they would both have opposite spin in accordance with Pauli's principle. This imposes a restriction on electron transfer which makes oxygen accept its electrons one at a time and also means that oxygen reacts sluggishly with non-radicals. This spin restriction prevents the spontaneous combustion of many organic compounds in the presence of oxygen.

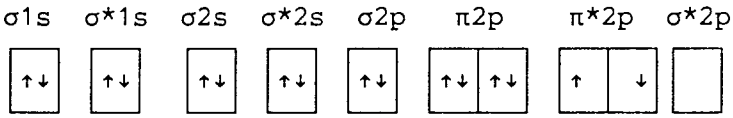
Singlet oxygen

With an input of energy, the O_2 molecule can be converted to singlet oxygen, which is more reactive.

Singlet oxygen can exist as singlet oxygen $^1\Delta g$ O_2, 22.4 kcal above the ground state



or as singlet oxygen $^1\Sigma g^+$ O_2, 37.5 kcal above the ground state.



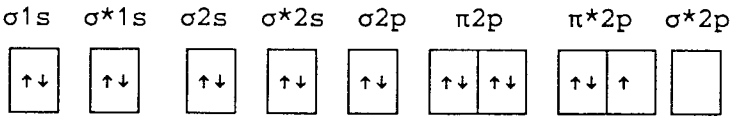
In both forms of singlet oxygen, the spin restriction is removed and the oxidising ability is greatly increased (Halliwell & Gutteridge, 1985).

The superoxide radical

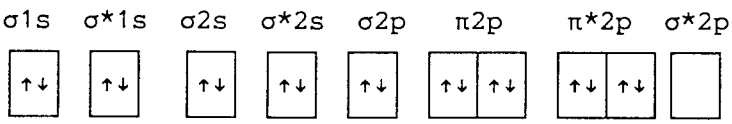
If a single electron is added to ground state O_2, it must enter one of the π*2p anti-bonding orbitals. This weakens the oxygen-oxygen bond, and results in only $1\frac{1}{2}$ covalent bonds between the oxygen atoms in superoxide (O_2^-). Addition of another electron forms the peroxide

ion (O_2^{2-}) which is not a radical and weakens the O-O bond to a single covalent bond.

The superoxide radical, O_2^- can be designated



and the peroxide ion O_2^{2-}



As can be seen, superoxide has only one unpaired electron, and one could argue that it is therefore less of a radical than oxygen in the ground state.

The biology of superoxide radicals

A free radical may be defined as any species capable of independent existence that contains one or more unpaired electrons (Halliwell & Gutteridge, 1985). The presence of unpaired electrons causes the species to be attracted slightly to a magnetic field and sometimes makes the species highly reactive.

Radicals can be formed when a covalent bond is broken, and one electron from the shared electron pair remains with each atom (homolytic fission). The energy required

to break covalent bonds can be obtained from, for example electromagnetic radiation, such as X or gamma rays.

Chemistry of superoxide

One of the reactions of superoxide in aqueous solution is to act as a base, that is a proton acceptor.

When O_2^- accepts a proton, it forms the hydroperoxyl radical ($HO_2^{\bullet}$). $HO_2^{\bullet}$ can dissociate again and release H^+ ions, i.e. act as an acid. Hence when O_2^- and H^+ ions are mixed, an equilibrium is set up :

$$HO_2^{\bullet} \longleftrightarrow H^+ + O_2^-$$

The pH at which the acid ($HO_2^{\bullet}$) and base (O_2^-) are in equal concentrations (i.e.. the pKa) is 4.8. As the pH of most body tissues is in the range 6.4 to 7.5, the ratio $[O_2^-]$ /$[HO_2^{\bullet}]$ will be very large.[1] e.g.. 100:1 at pH 6.8. Hence any O_2^- generated will remain almost entirely in this form, rather than being protonated.

However, superoxide dissolved in aqueous solution disappears very rapidly. The reason for its' disappearance is the dismutation reaction:

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

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[1] Henderson-Hasselbalch equation : $pH = pKa + \log_{10}([base]/[acid])$

The rate constant for this reaction is only 0.3 $M^{-1}.s^{-1}$, whereas the reaction :

$$HO_2^{\bullet} + O_2^- + H^+ \longrightarrow H_2O_2 + O_2$$

has $k_2 = 8 \times 10^7\ M^{-1}.s^{-1}$, and

$$HO_2^{\bullet} + HO_2^{\bullet -} \longrightarrow H_2O_2 + O_2$$

has $k_2 = 8 \times 10^5\ M^{-1}.s^{-1}$.

Hence dismutation is most rapid at acid pHs and will become slower as the pH rises.
O_2^- can also act as a reducing agent (donor of electrons). For example it can reduce cytochrome c, wherein ferric iron is reduced to ferrous iron.

$$cyt_c(Fe^{3+}) + O_2^- \longrightarrow O_2 + cyt_c(Fe^{2+})$$

O_2^- is also a weak oxidising agent (electron acceptor). For example O_2^- oxidises ascorbic acid ($k_2 = 2.7 \times 10^5\ M^{-1}.s^{-1}$)

$$\text{ascorbate} + O_2^- + 2H^+ \longrightarrow H_2O_2 + \text{ascorbate radical}$$

Radicals generate radicals

The reaction of a free radical with a non radical species produces a different free radical, which may or may not be less reactive than the original radical.

The reactions of superoxide will therefore often produce other radicals or species such as H_2O_2, HOCl, O_3 or singlet oxygen which although they are not radicals themselves, can produce free radicals.

Superoxide is produced constantly at low levels in cells by a number of mechanisms.

* It can be produced by normal chemical reactions within the cell.
* It can be produced as a by-product of a leaky electron transfer system.
* Xanthine oxidase produces O_2^- during the oxidation of hypoxanthine.
* O_2^- production plays an essential role in phagocytosis (Baboir, 1978).
* Chemotaxis, lymphocyte regulation, fibrosis and proliferation all involve low levels of O_2^- production (Halliwell, 1990).

Site specific damage

The expression of O_2^- toxicity is through its ability to reduce transition metal compounds (for example copper and

iron compounds), which are subsequently reoxidised by H_2O_2 yielding deleterious entities such as $OH^{\cdot}$ (Czapski & Goldstein, 1987).

This proposed mechanism has given rise to much controversy due to the very high concentrations of $OH^{\cdot}$ scavengers necessary to protect cells from O_2^- mediated toxicity (Czapski, 1984). It has therefore been suggested that the reactions of O_2^- and metal compounds proceed with the metal compound bound to a biological target (Goldstein & Czapski, 1986a). This allows the formation of $OH^{\cdot}$ in the vicinity of the target, making effective $OH^{\cdot}$ scavenging difficult. This results in what is known as the site specific mechanism. (Czapski, 1984).

Radicals produced within the cell may then cause damage resulting in cell death, for example :

* reaction with critical -SH groups on membrane ion pumps.
* DNA fragmentation by strand breakages.
* lipid peroxidation can increase the 'leakiness' of membranes, allowing ions such as Ca^{2+} to cross the membrane. An increased intracellular Ca^{2+} concentration can activate proteases and nucleases and this can lead to the inactivation of many intracellular enzymes and also to DNA damage (Halliwell, 1990).

The role of iron and copper

Superoxide can reduce iron, and together with hydrogen peroxide can form the highly reactive hydroxyl radical. This mechanism is essentially an iron catalysed Haber-Weiss reaction (Haber & Weiss, 1934) as shown below.

$$Fe^{3+} + O_2^- \longleftrightarrow \underset{(Fe^{3+}\!\!-\!\!-O_2^- \longleftrightarrow Fe^{2+}\!\!-\!\!-O_2)}{\text{perferryl}} \longleftrightarrow Fe^{2+} + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow \underset{Fe(IV)=O}{\text{Ferryl}} \longrightarrow OH^- + Fe^{3+} + OH^{\bullet}$$

The Fe(IV) ferryl compound can be either very stable or unstable, depending on what binds to it. The stability of ferryl can therefore limit $OH^{\bullet}$ production in the cell (Halliwell, 1990).

Iron taken up in the gut enters the blood as the plasma protein transferrin, which functions as a carrier molecule. Transferrin binds to iron extremely tightly, making it unavailable to O_2^- attack. Transferrin under normal conditions is only about 30% saturated with iron, so the amount of extracellular iron is extremely low. Transferrin binds to receptors on the cell surface and is internalised, entering the cytoplasm in a vacuole. The contents of this vacuole are then acidified to facilitate the release of iron into the cell. This protein-bound iron can then be used in the synthesis of other iron-

containing proteins. Any iron not required by the cell is stored in the protein ferritin, which appears to prevent an excessive intracellular buildup of non-protein-bound iron. However a small pool of intracellular non-protein-bound iron exists and could be attacked by O_2^- and H_2O_2 to produce $OH^\bullet$.

Indeed, intracellular concentrations of antioxidant enzymes are much higher than extracellular concentrations, where all iron is protein-bound, attached to transferrin and unable to be catalysed by free radical reactions. It is the function of the intracellular antioxidant enzymes to prevent O_2^- coming into contact with the free iron pool (Halliwell, 1990).

Iron overload can lead to, for example, haemachromatosis, many of the consequences of which can be explained in terms of free radical reactions. There is no obvious physiological mechanism for disposing of excess iron. In conditions of excessive intake, for example where acidic beer is brewed or food cooked in iron pots (as in some regions of Africa) or in overdoses of iron tablets, iron overload results, with resultant saturation of transferrin, and an increased amount of free iron in the plasma (McGilvery & Goldstein, 1983).

The reaction of O_2^- with copper ions can also undergo a Haber-Weiss reaction and produce $OH^\bullet$.

$$Cu^{2+} + O_2^- \longrightarrow Cu^+ + O_2$$

$$Cu^+ + H_2O_2 \longrightarrow Cu^{2+} + OH^- + OH^\cdot$$

Copper ions bound to biological targets or chelators can still act as catalysts for the Haber-Weiss reaction.

$$\text{Biol-}Cu^{2+} + O_2^- \longrightarrow \text{Biol-}Cu^+ + O_2$$

$$\text{Biol-}Cu^+ + H_2O_2 \longrightarrow (\text{Biol-}Cu^{2+} \cdots OH^\cdot) + OH^-$$

This modified mechanism describes the 'site specific mechanism' (Goldstein & Czapski, 1986a).

Copper and iron can also catalyse the dismutation of O_2^- by the following reaction.

$$Cu^{2+} \text{ or } Fe^{3+} + O_2^- \longrightarrow Cu^+ \text{ or } Fe^{2+} + O_2$$

$$Cu^+ \text{ or } Fe^{2+} + O_2^- + 2H^+ \longrightarrow Cu^{2+} \text{ or } Fe^{3+} + H_2O_2$$

(Fridovich, 1981).

In this sense copper or iron ions may act either as damaging or protective compounds depending on the relative contribution of the metal catalysed Haber-Weiss reaction and the dismutation reaction respectively.

Chapter 2
A history of superoxide dismutase

As discussed earlier, superoxide, one of the products of the biological reduction of molecular oxygen, can proceed to produce other radicals which can be extremely toxic to the cell. Even though superoxide has only a short lifetime, the production of this free radical has necessitated the evolution of enzymes whose function it is to catalyse the very reaction which occurs rapidly without enzyme catalysis.

The dismutation of superoxide,

$$O_2^- + O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$$

is catalysed by superoxide dismutase (SOD).

SOD was first isolated by Mann & Keilen in 1938, crystalised by Mohammed and Greenberg in 1953 and extensively studied by Markowitz *et al.* (1959) and Carrico & Deutsch (1969) as a copper containing protein of unknown function (*op cit* McCord & Fridovich, 1977). An excellent review of the events that led to the discovery of SOD is presented in McCord & Fridovich's 1977 paper, and is discussed below.

First steps on the road to SOD discovery - sulphite oxidation

In 1941 Handler *et al.* described a sarcosine oxidase activity in rat liver homogenates. They found that sarcosine (N-methyl-glycine) was oxidised to formaldehyde and glycine. (Fig 2.1).

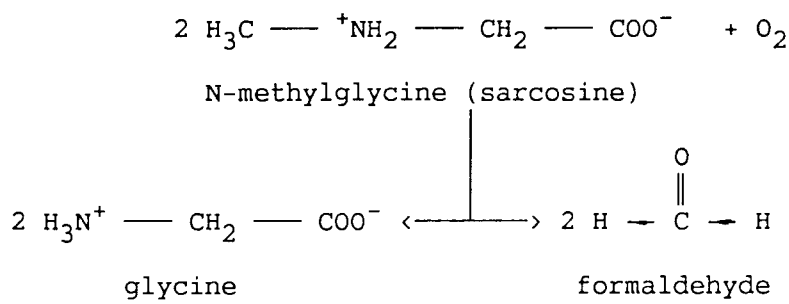


Figure 2.1. Sarcosine is oxidised to formaldehyde and glycine by a compound found in rat liver homogenates.

Heimberg *et al.* (1953), as part of their studies on sarcosine oxidase were examining the use of sulphonic acid analogues of sarcosine as possible competitive inhibitors. The sulphonic acids were themselves oxidised by the liver preparations and one of the products was sulphate. The sulphonic acids were substrates because they dissociated to sulphite which was rapidly oxidised to sulphate in the presence of the liver homogenates.

Thus the existence of an enzyme catalysing the oxidation of inorganic sulphite was demonstrated. The enzyme

activity was found to be localised in liver mitochondria (Fridovich & Handler, 1954).

Hypoxanthine found to facilitate sulphite oxidation

It was found that dialysis of this enzyme caused a loss of activity, which could be restored by addition of a heat stable, dialysable component of the enzyme preparation (Fridovich & Handler, 1955). This substance, which behaved like a classical enzyme cofactor was found to be hypoxanthine. (Fig. 2.2).

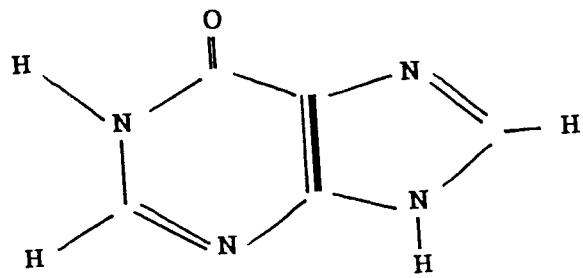


Figure 2.2. Hypoxanthine

The facilitation of sulphite oxidation by hypoxanthine led to the suspicion that the enzyme xanthine oxidase was involved. This enzyme catalysed its substrates hypoxanthine and xanthine to urate. (Fig. 2.3).

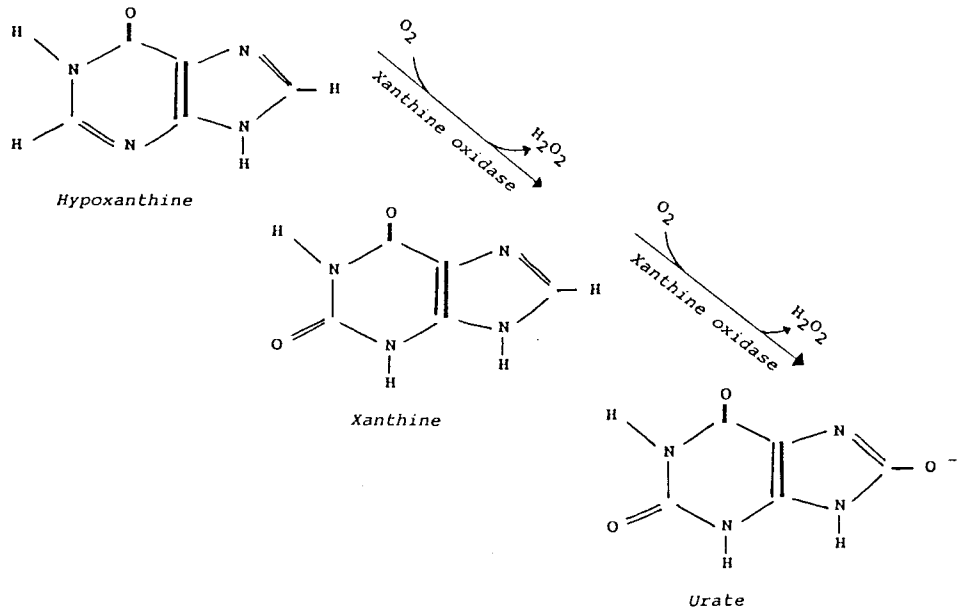


Figure 2.3. Xanthine oxidase catalyses the conversion of hypoxanthine and xanthine to urate.

Free radical chain oxidation of sulphite

The aerobic oxidation of sulphite is complicated by the tendency of sulphite to undergo a free radical chain oxidation. This was shown by an effect of cytochrome c. The sulphite slowly reduced cytochrome c which caused the aerobic oxidation of sulphite; however the relative rates of these reactions were such that thousands of sulphite ions were oxidised per cytochrome c reduced. It was apparent that the oxidation of one sulphite by cytochrome c generated a species, probably a radical, which then caused the aerobic oxidation of many sulphite ions.

Xanthine oxidase initiates the free radical chain oxidation of sulphite

Xanthine oxidase, at very low concentrations was found to catalyse the oxidation of sulphite in the presence of its purine substrates, hypoxanthine and xanthine. In the absence of these substrates, the enzyme had no effect on sulphite oxidation (Fridovich & Handler, 1958a).

Numerous other oxidases were found unable to initiate sulphite oxidation, and thus one had to suppose that the xanthine oxidase reaction produced some special product which was reactive enough to start the sulphite chain reaction. Since uric acid and H_2O_2 were unable to do this, an intermediate of oxygen reduction was suspected (Fridovich & Handler, 1958b).

This was confirmed by Fridovich and Handler in 1961, who demonstrated that O_2^- produced by platinum electrodes in solution initiated sulphite oxidation. They argued that the initiation of sulphite oxidation by xanthine oxidase was due to the generation of oxygen radicals during the course of its catalytic action.

A mystery inhibitor of cytochrome c reduction by xanthine oxidase

At this time, the literature concerning the reduction of cytochrome c by xanthine oxidase was confusing. Cytochrome c was reported to be both a good and a poor electron acceptor. It was found that xanthine oxidase

reduced some samples of cytochrome c, while others were hardly reduced at all. Furthermore, those samples found not reducible by xanthine oxidase were found to contain a potent heat-labile and non-dialysable inhibitor of cytochrome c reduction. This was a very specific inhibitor, since it prevented the reduction of cytochrome c by xanthine oxidase acting on xanthine, but had no effect on the rate of reduction of oxygen (Fridovich & Handler, 1958b).

Was it myoglobin that bound to the active site?

Since the globin of myoglobin was a frequent contaminant of the horse heart cytochrome c being used, myoglobin and the corresponding globin were prepared and were found to be powerful competitive inhibitors of cytochrome c reduction by xanthine oxidase (Fridovich, 1962). Furthermore, enzymatic scavengers of H_2O_2 did not prevent the reduction of cytochrome c by xanthine oxidase.

It appeared most probable that O_2^- generated on the enzyme and bound to it was responsible for the reduction of cytochrome c by xanthine oxidase, and that myoglobin or its globin had inhibited this by binding to this O_2^- site, thereby preventing access by cytochrome c (Fridovich & Handler, 1962).

It was later observed that when methylene blue was added to an aerobic xanthine oxidase - xanthine - cytochrome c reaction, the dye-mediated cytochrome c reduction yielded

different kinetic constants, but still showed classical hyperbolic saturation despite the fact that the enzyme reduced dye was thought to transfer electrons to cytochrome c in free solution. At low cytochrome c concentrations, the reduced dye, unable to find cytochrome c to accept the electrons, could simply autooxidise (McCord & Fridovich, 1968). McCord argued that oxygen could carry electrons in a manner completely analogous to methylene blue, through free solution. At low cytochrome c concentrations, superoxide could largely autooxidise via its dismutation reaction and at high cytochrome c concentrations, the complete scavenging of O_2^- by the cytochrome c would be approached. This led McCord & Fridovich (1968) to predict that superoxide production is first order in xanthine oxidase, while its disappearance via dismutation in free solution is second order in the radical.

Possible presence of a superoxide interceptor

This understanding forced a re-assessment of how the various protein preparations inhibited the phenomenon. No longer could a binding mechanism be invoked, but the inhibitory proteins had to intercept superoxide in free solution, preventing it from reaching cytochrome c. The only explicable means of achieving this was a catalytic decomposition of the radical - a superoxide dismutase.

Superoxide dismutase isolated

McCord then isolated superoxide dismutase (SOD) from bovine erythrocytes on the basis of its enzymic activity. Once it was pure, the identity between this blue-green copper containing SOD and the previously described bovine hemocuprein (Mann & Keilen, 1938) and human erythrocuprein (Markowitz *et al.*, 1959) was apparent (McCord & Fridovich, 1969a).

It was later found that the preparations of myoglobin then used contained a small amount (< 1%) of SOD, which by catalytically scavenging O_2^-, prevented all the reactions dependent on this radical, without inhibiting the enzymatic turnover of xanthine oxidase (McCord & Fridovich, 1977).

Evolution of SOD

Superoxide dismutases are thought to have arisen early in evolution at the time when aerobic life was developing (Steffens *et al.*, 1983), as a defence mechanism against oxygen toxicity. Respiring organisms, including bacteria (Yost & Fridovich, 1973), fungi (Misra & Fridovich, 1972b), algae (Misra & Keele, 1975), plants (Asada *et al.*, 1973), molluscs and arthropods (Tegelstrom, 1975), fish (Bannister *et al.*, 1976), birds (Weisiger & Fridovich, 1973) and mammals (McCord & Fridovich, 1969b), all contain superoxide dismutases.

Oxygen sensitive obligate anaerobes do not contain the enzyme (McCord *et al.*, 1971).

It is thought that superoxide dismutases evolved along two separate lines into three groups with different catalytic ions in their active sites (Fridovich, 1981). There are superoxide dismutases based on iron (FeSOD), manganese (MnSOD) and copper plus zinc (CuZnSOD).

MnSOD and FeSOD appear to be related by extensive amino acid homology, whereas CuZnSOD represents an independently evolved group (Fridovich, 1981). All of these enzymes have comparable catalytic efficiency in catalysing the dismutation of O_2^-.

Copper zinc superoxide dismutase

CuZnSODs are cytoplasmic enzymes, predominantly found in eukaryotes. An exception is the symbiotic bacterium *Photobacterium leiognathi*. Since its host, the pony fish, has evolved a special gland to house this photobacterium, the symbiosis is one of long standing. It is speculated that the bacterium obtained the CuZnSOD by gene transfer from the host fish (Puget & Michelson, 1974).

Each molecule of CuZnSOD is a dimer of identical subunits that contain approximately 153 amino acids, a copper and a zinc ion (Getzoff *et al.*, 1989) The Cu ion, essential in the catalysis of the dismutation reaction is

cyclically oxidised and reduced during successive encounters with O_2^-, as shown below :

$$SOD\text{-}Cu^{2+} + O_2^- \longrightarrow SOD\text{-}Cu^{+} + O_2$$

$$SOD\text{-}Cu^{+} + O_2^- + 2H^+ \longrightarrow SOD\text{-}Cu^{2+} + H_2O_2$$

(Fridovich, 1979).

Both reactions have rate constants of 1.6×10^9 $M^{-1}.s^{-1}$ and both rates are probably limited by the availability of substrate (Klug *et al.*, 1972). This availability of substrate is increased as most of the surface of the molecule is negatively charged except for channels of positive charge that direct O_2^- to the active site (Cudd & Fridovich, 1982).

The structure of bovine CuZnSOD has been elucidated by X-ray crystallography to a resolution of 2Å (Tainer *et al.*, 1982). Each subunit is composed of a set of eight antiparallel ß-strands forming a flattened barrel, plus two external loops forming the active site. (Fig 2.4) The two subunits are joined by noncovalent, predominantly hydrophobic interactions between the ß-barrels such that the active sites are on opposite sides of the dimer.

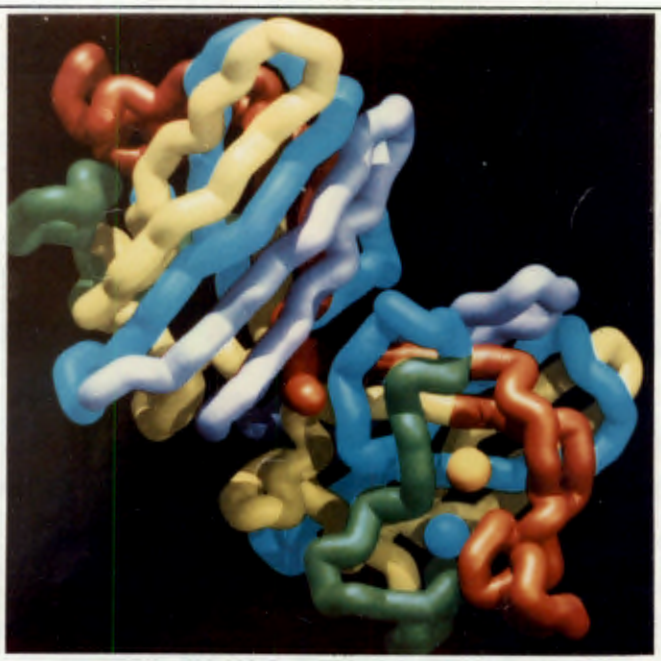


Figure 2.4 A computer graphic analysis of the human SOD dimer showing the ß-strands and the active site (Cu ion - yellow sphere ; Zn ion - blue sphere). Photographed from the cover of Proteins 5 No.4 (1989).

The copper ion is held at the active site by interaction with nitrogens in imidazole ring structures of four histidine residues. It is situated on the surface of the barrel, at the base of a channel formed by the two external loops (Tainer *et al.*, 1982). The one loop contains most of the residues important in the electrostatic guidance of O_2^- to the Cu ion (Getzoff *et al.*, 1983).

The second loop, which binds to Zn^{2+}, contributes to the high stability of the enzyme (Forman & Fridovich, 1973). CuZnSOD is an unusually stable enzyme which can remain active in 8M urea (Malinowski & Fridovich, 1979) and 4% sodium dodecyl sulphate (Forman & Fridovich, 1973) and

has a conformational melting temperature of 96°C (Lepcock *et al.*, 1985 ; Roe *et al.*, 1988).

Iron and manganese superoxide dismutase

FeSOD has been found only in prokaryotes and algae, whereas MnSOD is found in both prokaryotes and eukaryotes. In eukaryotic organisms, MnSOD is localised in the mitochondrial matrix supporting speculations concerning the symbiotic origin of these organelles (Fridovich, 1981). It is thought that mitochondria evolved as a symbiosis between a primitive eukaryote (with CuZnSOD) and a prokaryote (with MnSOD) that eventually became incorporated into the eukaryotic cytoplasm.

Amino acid sequence analysis has confirmed a close evolutionary relationship between MnSOD and FeSOD (Steinman, 1978). As with CuZnSOD, the enzymes operate by a redox cycle in which the active site metal is reduced by one O_2^- and then reoxidised by another O_2^- as shown below where Me^n represents a heavy metal cation.

$$Me^n + O_2^- \longrightarrow Me^{n-1} + O_2$$

$$Me^{n-1} + O_2^- + 2H^+ \longrightarrow Me^n + H_2O_2$$

(Fridovich, 1981). Iron and manganese oscillate between trivalent and divalent states, and copper oscillates between divalent and univalent states during the catalytic cycle.

Biological role of superoxide dismutase and the antioxidant enzymes

It has been argued that SOD is not unique in its function to catalyse O_2^- dismutation (Fee, 1981) and that many copper compounds have similar catalytic efficiencies to that of the native SOD. Indeed, many copper compounds have been shown to have SOD activity *in vitro* (Czapski & Goldstein, 1987).

A possible explanation for the inability of these copper compounds to catalyse O_2^- dismutation *in vitro* is that they can form ternary complexes with biological macromolecules such as DNA. It has been shown that ternary complexes of DNA and some copper complexes react extremely slowly with O_2^- (Goldstein & Czapski, 1986b).

Superoxide generated enzymatically, photochemically or radiochemically has been shown to peroxidise lipids (Petkau & Chelack, 1974), cleave DNA (Lown & Weir, 1978), inactivate enzymes (Lin *et al.*, 1978) and lyse erythrocytes (Stone *et al.*, 1978).

Superoxide and H_2O_2 individually may not be as reactive as other free radicals, but they can 'conspire' to produce $OH^{\bullet}$ by the iron catalysed Haber-Weiss reaction as shown in Chapter 1 and thus cause widespread biological damage (Fridovich, 1981), and this would certainly explain the need for a comprehensive enzymatic defence mechanism against such events.

Figure 2.5 summarises the enzymatic defense mechanisms against radiation induced oxygen toxicity.

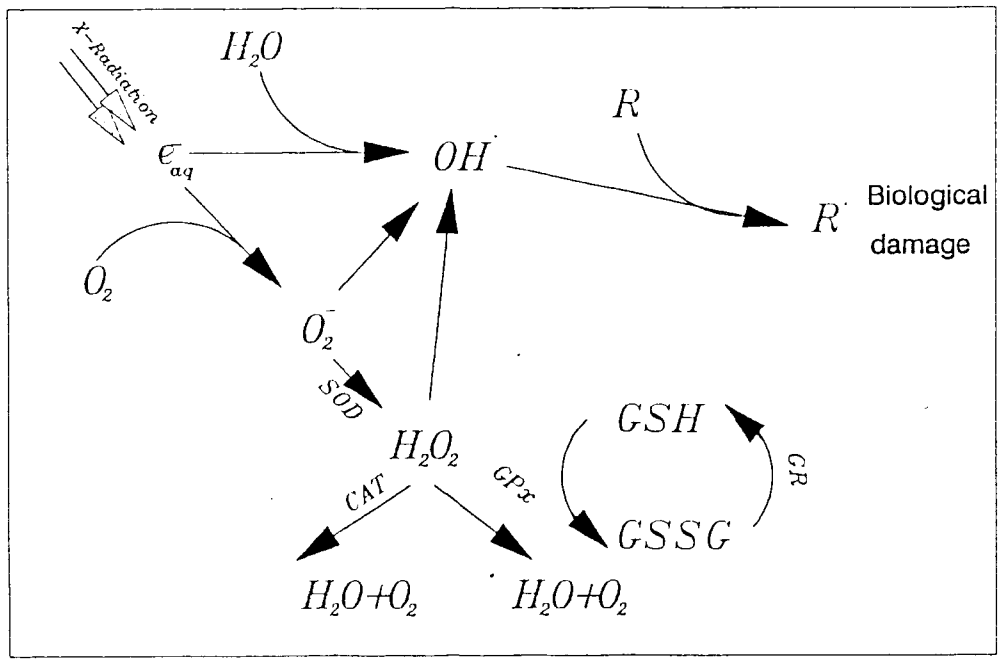


Figure 2.5 The enzymatic defense mechanisms against radiation induced oxygen toxicity. SOD - superoxide dismutase; CAT - catalase; GPx - glutathione peroxidase; GR - glutathione reductase; GSH reduced glutathione; GSSG - oxidised glutathione; R - biologically important molecule; e-aq - hydrated electron.

Klug *et al.* (1972) irradiated a solution of sodium formate, EDTA, and $CuSO_4$ with pulses of 5 MeV electrons of $1\frac{1}{2}$ µseconds duration and then measured the absorbance at 245 nm, and found that in the absence of SOD, O_2^- can remain for about 20 seconds, whereas it is catalysed to oxygen and H_2O_2 within 2 msec in the presence of SOD.

Hydrogen peroxide can be removed by catalysis by either catalase (CAT), which catalyses the reaction :

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

or glutathione peroxidase (GPx), which catalyses the reaction :

$$2SH_2 + 2H_2O_2 \xrightarrow{GPx} 4H_2O + 2S$$

The catalase reaction proceeds as follows :

$$\text{CAT-Fe(III)} + H_2O_2 \xrightarrow{k_1} \text{compound 1}$$

$$\text{compound 1} + H_2O_2 \xrightarrow{k_2} \text{CAT-Fe(III)} + 2H_2O + O_2$$

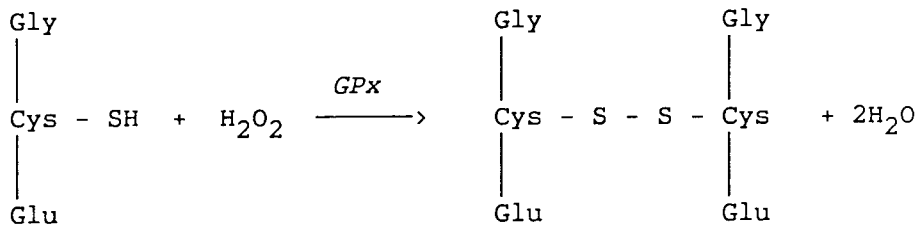
Compound 1 is thought to be either CAT-Fe(III)-HOOH or CAT-Fe(V)=O. The second order rate constants for rat liver catalase are 1.7 X 10^7 $M^{-1}.s^{-1}$ and 2.6 X 10^7 $M^{-1}.s^{-1}$ respectively (Halliwell & Gutteridge, 1985).

Glutathione peroxidase also catalyses the oxidation of GSH to GSSG at the expense of H_2O_2.

$$H_2O_2 + 2GSH \xrightarrow{GPx} GSSG + H_2O$$

Glutathione is a simple tripeptide (glutamic acid - cysteine - glycine) in its reduced form. In the

oxidised form (GSSG), two molecules of GSH join together as the -SH groups of cysteine are oxidised to form a disulphide bridge -S-S-.



2 GSH GSSG

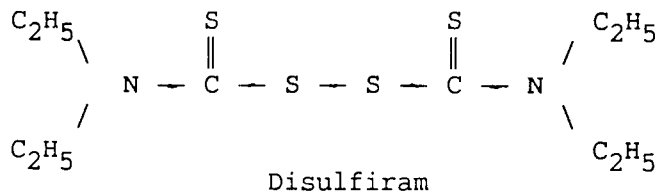
The ratio of [GSH]/[GSSG] in normal cells is kept high, and this is done by glutathione reductase (GR) which catalyses the reduction of GSSG back to GSH in the reaction :

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2\ GSH + NADP^+$$

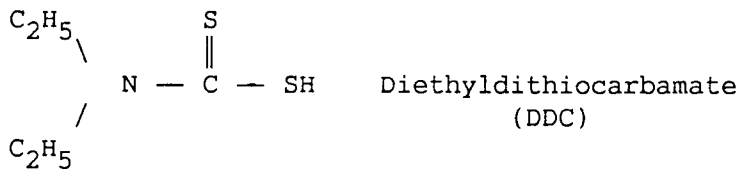
This then is the major enzymatic antioxidant defense, as if these reactive species go unchecked they can proceed to cause lethal damage to the cell as explained in Chapter 1.

Chapter 3
Diethyldithiocarbamate

Diethyldithiocarbamate is the reduced form of disulfiram (tetraethylthiuram disulphide) or Antabuse. Disulfiram has been used for over 30 years in the treatment of chronic alcoholism.



Disulfiram



Diethyldithiocarbamate (DDC)

Diethyldithiocarbamate (DDC) has been used in the treatment of nickel carbonyl (Sunderman, 1979), arsenic and thallium poisoning, (Zemaitis & Greene, 1979) and in the treatment of Wilson's disease (hepatolenticular degeneration), a genetic disturbance of copper metabolism where biliary excretion of copper from the liver, and the formation of ceruloplasmin, the principal means of copper export from the liver, are below normal. This results in a buildup of copper in various organs and can result in development of liver cirrhosis and neurological degeneration. Treatment involves the administration of a chelating agent, usually penicillamine or

diethyldithiocarbamate to remove the excess copper (Sunderman *et al.*, 1963).

DDC has also been suggested as an adjuvant to cis-platinum chemotherapy as it has been shown to limit kidney, gut and bone marrow toxicities in animal studies without limiting the antitumour effect of cis-platinum (Bodenner *et al.*, 1986; Dible *et al.*, 1987). It has also been suggested as a 'rescue' therapy to combined cis-platinum and radiation (Douple & Richmond, 1982).

It has been used in humans at a dose of 600 mg/m^2 (Parades *et al.*, 1988) but did not provide any protection from cis-platinum toxicity.

DDC has also been shown to suppress ejaculation in dogs due to suppression of noradrenaline biosynthesis (Kimura *et al.*, 1979).

SOD inactivation by DDC

As mentioned in Chapter 2, copper ions in CuZnSOD are essential in the catalysis of the dismutation reaction. DDC removes the copper from SOD and thus inactivates it (Heikkila *et al.*, 1976).

Heikkila & Cohen (1977) measured the content of SOD in brain, liver and blood of mice treated with DDC. (Table 3.1) Tissues were removed 3 hours after i.p. injection of 1.5 g/kg DDC and their SOD activity measured.

Table 3.1. Superoxide dismutase activities in brain, liver and blood of mice three hours after treatment with 1.5 g/kg DDC. (From Heikkila & Cohen, 1977).

Tissue	SOD (µg/g) ± SD		
	Control	DDC	% Loss
Brain	100 ± 6	52 ± 10	48%
Liver	697 ± 80	200 ± 32	71%
Blood	88 ± 12	12 ± 12	86%

This dose of DDC, if uniformly distributed throughout the animal represents an average concentration of 9 X 10^{-3}M.

In contrast to the large decreases in SOD activity, liver and blood catalase measured 3 hours after 1.5 g/kg DDC were 85 ± 5% (n=20) and 97 ± 7% (n=11) of their respective control values. It was concluded that the large decreases in SOD activity brought about by DDC *in vivo* was not the result of non-specific tissue damage.

These investigators also measured the loss in SOD activity in mouse liver 3 hours after injection with various doses of DDC. (Heikkila & Cohen, 1977). This is shown in Table 3.2.

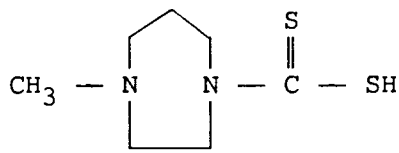
Table 3.2 SOD activity in mouse liver 3 hours after injection with DDC. (From Heikkila & Cohen, 1977).

DDC dose (g/kg)	Liver SOD (µg/g ± SD)	% Loss
0	632 ± 48	-
0.5	368 ± 80	42%
1.0	200 ± 48	68%
1.5	168 ± 32	74%

In another experiment, male Swiss-Webster mice were injected i.p. with a solution of DDC in isotonic saline. Mice were injected with DDC at doses between 0.5 and 1.5 g/kg. At various time periods the animals were sacrificed, tissues removed and their SOD content determined (Heikkila, 1984). It was shown that 1.5 g/kg DDC resulted in losses of SOD ranging from 50 - 90% in various tissues. It was argued that this treatment of mice clearly resulted in a loss of tissue SOD activity. However it was also stated that the mode of action of DDC is complex and most probably does more than just inactivate SOD. DDC is known to inactivate other copper containing enzymes, and furthermore DDC is a modestly good reducing agent as well as a free radical scavenger (Heikkila, 1984). It was argued that any conclusions about SOD actions *in vivo*, based on DDC experiments should be drawn with extreme caution.

Structural analogues of DDC

FLA-57 is a structural analogue of DDC, and like DDC is a potent copper chelator and a commonly used inhibitor of the enzyme dopamine-ß-hydroxylase (Florvall & Carrodi, 1970).



FLA-57

SOD (1 mg/ml) and FLA-57 (10^{-2}M) were incubated alone and together at 37°C in water for 2 hours after which 10 µl aliquots were assayed for SOD activity (Heikkila & Cohen, 1977). The inactivation *in vitro* of SOD by FLA-57 appeared similar to that by DDC. This inactivation was completely reversed by incubation with $CuSO_4$. The results were interpreted by these investigators as indicating that the -NCSSH grouping is an important structure for the inactivation of SOD.

A number of other copper chelating compounds (both cuprous and cupric chelators) such as methimazole, penicillamine, propylthiouracil, phenylthiazolythiourea, pyridyldiphenyltriazine, cuprizone, bathocuprine and diethyldithiophosphate were tested as SOD inhibitors (Heikkila & Cohen, 1977). These compounds had no effect on SOD activity *in vitro*.

Heikkila & Cohen (1977) suggested that these potent copper chelators fail to inhibit SOD *in vitro* as they cannot chelate protein bound copper which is in contrast to DDC and FLA-57.

Interestingly, although exposure to penicillamine had no demonstrable effect *in vitro*, it has been shown to inhibit SOD activity *in vivo* (Albergoni *et al.*, 1975). Penicillamine administration twice daily for 20 days at 150 mg/kg caused relatively modest losses in SOD activity

compared to the large losses caused by a single injection of DDC.

Chapter 4
Radiosensitization by DDC

Increased radiation haemolysis

Stone *et al.* (1978) showed that human erythrocytes were radiosensitized by DDC. They argued that substantial amounts of haemolysis (the loss of integrity of the membrane, leading to the liberation of haemoglobin and other cellular macromolecules) could be produced by O_2^- if the rate of O_2^- production exceeded the rate of O_2^- removal by SOD. In the absence of DDC, there was no haemolysis up to about 2 Gy, however when suspensions were incubated with 2.4 mM DDC for 90 minutes prior to irradiation, complete haemolysis was demonstrated at 1 Gy. This effect was still observed even when the DDC was added immediately or 1 hour after radiation. If DDC was washed out from the cells before irradiation, very little haemolysis was observed. They asserted that this meant that the concentration of DDC entering the erythrocyte was insufficient to inhibit SOD to a critical level or that a large concentration of DDC must be present at all times to maintain the inhibitory effects on SOD.

Modification of slope and shoulder of survival curve

Lin *et al.* (1979a) showed that DDC sensitized DON cells (an established cell line derived from the lung tissue of Chinese hamsters) to 2.5 Gy gamma-irradiation. Cells were incubated with DDC for 1 hour at 25°C prior to radiation. They showed that DDC enhanced the effect of radiation by a factor of 1.7 for cells exposed to 10^{-4}M DDC for 30 minutes prior to irradiation (Table 4.1). The enhancement appeared to be concentration dependent.

Table 4.1 Change in survival of DON cells after various concentrations of DDC or simultaneous treatment with DDC and 2.5 Gy of gamma radiation. (From Lin et al., 1979a).

Radiation Dose	Control	DDC Concentration 10^{-4}M	10^{-5}M	10^{-6}M	10^{-7}M
	% survival relative to corresponding controls				
0 Gy	100%	62.1%	82.4%	87.8%	94.5%
2.5 Gy	100%	37.4%	59.6%	72.7%	82.1%
Enhancement ratio		1.7	1.4	1.2	1.1

In a further set of experiments, Lin *et al.* (1979b) exposed DON and 3T3 cells (an established cell line derived from mouse embryonic fibroblasts) to DDC alone and in combination with gamma irradiation. Cells treated with DDC consistently showed lower levels of survival, which were even further reduced when the cells were exposed to radiation as well. Analysis of the survival curves of these two cell lines showed that DDC modified the slope as well as the shoulder of the curve.

They argued that the lowered survival of the DDC pretreated cells must be, at least in part due to their reduced ability to defend themselves against O_2^- related toxicity.

Mitochondrial alterations

In electron microscopy studies, Lin *et al.* (1979b) noted that DDC treated cells showed marked changes in the mitochondria. These included swelling with clearing of the mitochondrial matrix and the formation of myelin figures of mitochondrial membranes. Some mitochondria showed inhomogenous matrices and modified internal and external membranes which suggested a moderate degree of degeneration. Myelin figures were also present in the cytoplasm of DDC treated 3T3 cells. It was argued that these mitochondrial alterations are essentially similar to the pathology of cells exposed to hyperbaric oxygen, as described by Hughson *et al.* (1977).

The mitochondrial changes noted would be consistent with the cells having lower levels of respiration and ATP production, which could have serious implications in the post irradiation-repair period. Another mitochondrial enzyme, cytochrome oxidase has been reported to be inhibited by DDC *in vivo*, possibly because of the fact that this enzyme is copper containing (Frank *et al.*, 1978). Inhibition of this enzyme could lead to increased O_2^- since mitochondrial electron transfer systems can generate superoxide (Nohl & Henger, 1978).

Cytochrome oxidase can also act as a superoxide scavenger (Markossian *et al.*, 1978) and therefore an inhibition of cytochrome oxidase might further add to the effect of SOD inhibition by DDC.

Biphasic Toxicity of DDC

The literature concerning this subject is summarised in Table 4.2.

Table 4.2 A summary of previous work showing the biphasic toxicity of DDC

Concentration :	Low		--->	High
Author/Experiment	Non-toxic	Toxic	Non-toxic	Toxic
Rigas *et al.*, 1979 lymphocytes	2.5×10^{-6}M	2.5×10^{-5}M	2.5×10^{-5}M	$>2.5 \times 10^{-5}$M
Westman & Midander, 1984 Chinese hamster cells	1×10^{-6}M	1×10^{-4}M	$>1 \times 10^{-4}$M	3×10^{-3}M
Maners *et al.*, 1985 mouse fibroblasts		2.9×10^{-8}M	5.8×10^{-7}M	1.5×10^{-5}M
Lin *et al.*, 1985 V79 cells	1×10^{-6}M	1×10^{-5}M	$>1 \times 10^{-5}$M	1×10^{-3}M
Heikkila *et al.*, 1976			1.5 g/kg	
Cristensen *et al.*, 1974 mouse (in vivo)		0.5 - 0.6 g/kg		

Rigas *et al.* (1979) observed a biphasic toxicity of DDC. These workers measured cell survival and RNA synthesis from [^{3}H]-uridine incorporation in lymphocytes, monocytes and polymorphonuclear granulocytes following 2 hours

incubation with various concentrations of DDC at 37°C. Low concentrations of DDC (1 X 10^{-9}M to 2.5 X 10^{-6}M) appeared to increase cell survival, while higher concentrations (2.5 X 10^{-5}M) were toxic. As the concentration was increased further, toxicity decreased and cell survival reached a maximum around 2.5 X 10^{-4}M. At even higher concentrations, DDC became toxic again, and cell survival decreased again. The incorporation of [^{3}H]-uridine followed a similar course.

Rigas *et al.* (1979) found that the first phase of toxicity (2.5 X 10^{-5}M) could be partially reversed by the addition of 2.5 X 10^{-5}M $ZnCl_2$, but not by the addition of 2.5 X 10^{-5}M or 1 X 10^{-2}M $CuCl_2$. On the other hand, the inhibition of [^{3}H]-uridine incorporation by 2.5 X 10^{-3}M DDC was not reversed by 2.5 X 10^{-5}M or 1 X 10^{-2}M $ZnCl_2$, but was partially reversed by 1 X 10^{-2}M $CuCl_2$.

These investigators argued that the inhibition of zinc metalloenzymes by DDC plays a crucial role in the first toxic phase, and inhibition of copper metalloenzymes in the second. They also argued that DDC may be biphasically toxic *in vivo*, as large doses of DDC (1.5 g/kg) have been given to mice without ill effects (Heikkila *et al.*, 1976), yet lower doses (0.5 - 0.6 g/kg) were found to be toxic (Christensen *et al.*, 1974).

In a later experiment (Rigas *et al.*, 1980) it was noted that radiosensitization of human lymphocytes by DDC was

also biphasic, and that these phases coincided with the biphasic toxicity of DDC (1 X 10^{-5}M and 1 X 10^{-3}M). The group found that SOD was only inhibited in the second phase, and suggested that it may be a contributing cause of this radiosensitization. The mechanism of the first phase is not known.

'Paradoxical' Toxicity

In this regard, Rigas *et al.*, (1980) maintain that DDC appears to differ from the 'paradoxical' toxicity of other radioprotective thiols, which is reversed by increasing their concentration, (Vergroessen *et al.*, 1967; Delrez & Firket, 1968; Sawada & Okada, 1970) and is due to H_2O_2 produced through oxidation of the thiol by molecular O_2 (Tagaki *et al.*, 1974). Furthermore, in contrast to these thiols which are radioprotective at concentrations at which their toxicity has become reversed, DDC at similar concentrations is radiosensitizing and inhibits SOD. Nevertheless, the possibility that the mechanism of the first phase of toxicity and of its reversal is the same as that of the 'paradoxical' toxicity of these thiols cannot be excluded. This first phase could be due to the formation of toxic metal chelates of the type DDC$\cdots\cdot M^{n+}$, and that the reversal of toxicity at higher concentrations could be due to the formation of non-toxic metal chelates of the type DDC$\cdots\cdot M^{n+}\cdots\cdot$DDC (Rigas *et al.*, 1980).

Westman & Midander (1984) showed that exposure to 1 X 10^{-6}M DDC for 90 minutes had no effect on the survival of Chinese hamster cells. As the DDC concentration was increased, the toxicity increased reaching a maximum at 1 X 10^{-4}M. At concentrations above 1 X 10^{-4}M DDC, toxicity decreased until 3 X 10^{-3}M, where it increased again.

Maners *et al.* (1985) demonstrated a bimodal toxicity curve using mouse fibroblasts exposed to DDC concentrations ranging from 2.9 X 10^{-8}M to 1.5 X 10^{-5}M. They showed 97% cell toxicity at 1.5 X 10^{-5}M, 15% toxicity at 5.8 X 10^{-7}M and 94% toxicity at 2.9 X 10^{-8}M.

Lin *et al.* (1985) incubated V79 cells (derived from a Chinese hamster cell line) in medium containing DDC at concentrations ranging from 1 X 10^{-6}M to 1 X 10^{-3}M for 60 minutes. They found that DDC was not toxic at a concentration of 1 X 10^{-6}M, but when the concentration increased to 1 X 10^{-5}M, DDC became toxic. As the concentration was further increased, DDC became less toxic. At concentrations of 1 X 10^{-3}M and greater, DDC again became toxic.

When DDC was combined with other cytotoxic agents such as radiation, adriamycin, bleomycin as well as hyperthermia (exposure to 43°C) it was found that the biphasic nature of the toxicity remained, but was enhanced by the other agents (Lin *et al.*, 1985).

DDC inhibits SOD

Westman & Marklund (1980) showed a 95% inhibition of SOD after Chinese hamster cells were incubated with 3 X 10^{-3}M DDC for 90 minutes. This inhibition remained after DDC was removed by four successive medium changes. The SOD inhibition decreased with time, but was found still to be significant for a few hours after the removal of DDC. They found that DDC pretreated cells had an increased slope, the ratio of $D_{0\text{-CONTROL}}/D_{0\text{-DDC}}$ being 1.23 indicating radiosensitization.

DDC effect not dependent on pO_2

Westman & Marklund (1983) again examined the question of the effect of DDC on radiation haemolysis of erythrocytes. They found that by exposing erythrocytes to 3 X 10^{-3}M DDC for 90 minutes at 37°C produced a 98% inhibition of SOD. After 24 hours, the SOD activity was still only 10% of the control level. The activities of catalase (CAT) and glutathione peroxidase (GPx) were unaffected by this DDC pretreatment.

This pretreatment of cells with DDC followed by 4 washings to remove DDC had very little effect on the glycerol haemolysis time (GLT_{50}). Exposure to ionizing radiation led to a shortening of the GLT_{50}, and this was dose related. Pretreatment with 1 X 10^{-3}M DDC for 90 minutes prior to irradiation in DDC-free medium resulted in a small, but not statistically significant

(ratio=1.04) decrease in slope when compared to the radiation only GLT_{50} curve, however the intercept values on the Y axis (GLT) were found to differ significantly ($p<0.005$). They concluded that the effect of DDC was additive to radiation rather than potentiating. When DDC remained on the cells throughout the experiment, they found that concentrations of 10^{-3}M and lower had no effect additional to that which was seen after DDC pretreatment. However when 1 X 10^{-2}M DDC was present during the whole experiment, an increased sensitivity to radiation was seen.

The addition of exogenous SOD and CAT to these experiments did not protect the cells significantly against radiation haemolysis. It was argued that this suggested that in spite of a marked inhibition of SOD, no increase in susceptibility to glycerol haemolysis was seen, which indicated that O_2^- and H_2O_2 formed in the medium are of negligible importance in the radiation haemolysis of erythrocytes.

Westman & Midander (1984) irradiated V79 cells in argon, oxygen and air following 3 X 10^{-3} M DDC pretreatment for 90 minutes. The clonogenic survival curves after irradiation yielded results as detailed in Table 4.3.

The reduction of D_0 (reciprocal of the regression slope constant of the final straight portion of the survival curve) with DDC pretreatment was found to be

statistically significant in all oxygenation conditions, however there were no statistically significant differences between the dose modifying factors (DMFs) obtained in argon, air and oxygen.

Table 4.3 D_0 values for survival curves after treatment with radiation alone, or pretreatment with 1 X 10^{-3}M DDC for 90 minutes prior to irradiation in DDC-free medium under various conditions of oxygenation.

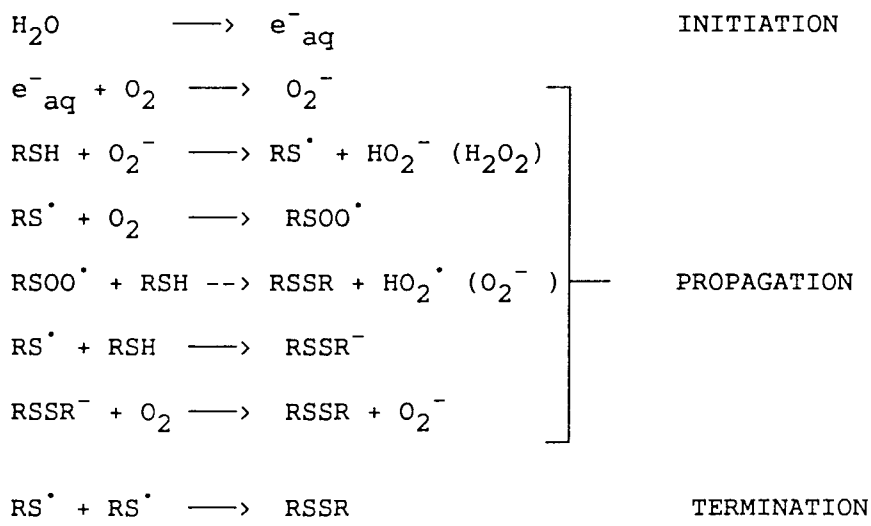
	D_0 (Argon)	D_0 (Air)	D0 (Oxygen)
Control	3.32 Gy	1.39 Gy	1.15 Gy
+ DDC	2.53 Gy	1.10 Gy	0.76 Gy
DMF	1.31	1.26	1.51

The authors argued that this showed that the radiosensitizing effect of DDC was not dependent on the oxygen partial pressure at the time of irradiation as the DMF in argon, where no O_2^- is formed, did not differ from the DMF in air, and moreover an increase in oxygen concentration to 100% was not shown to enhance the DDC sensitization further. As the cells were returned to aerobic conditions immediately after irradiation, they suggested a long term post irradiation increase of radiation damage in DDC treated cells, and not an increase of immediate radiation damage. Indeed, fast kinetic studies (Michael *et al.*, 1973) as well as studies demonstrating the importance of molecular oxygen for fixation of aerobic radiation damage (Samuni *et al.*, 1978) do not favour the participation of the superoxide radical in the immediate radiation effects.

Post irradiation treatment by DDC also sensitizes

Westman & Midander (1984) treated V79 cells with 1 X 10^{-3}M DDC after exposure to radiation. They found that the radiosensitizing effect of DDC could be produced when DDC was added 0.5 or 2 hours, but not 20 hours after radiation. The authors concluded that superoxide dependent processes were involved in the mediation of delayed radiation damage, and that the temporary inhibition of SOD did not influence immediate radiation damage, but that long term delayed radiation damage was modified. In agreement with this conclusion are the results of Petkau & Chelack, (1976) where it was shown that addition of SOD after irradiation protected membranes.

Stone *et al.* (1978) showed that 2.4 X 10^{-3}M DDC added immediately after or 1 hour after irradiation resulted in a significantly greater amount of erythrocyte haemolysis than after radiation alone. This was seen as indicating that O_2^- was still present in sufficient quantities to do damage, or was being generated by other reactions. They offered a mechanism proposed by Packer & Winchester (1970) to substantiate this :



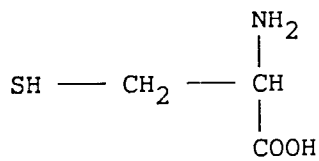
The final product of this chain reaction is disulphide. The finding that gamma-irradiation of erythrocytes led to an increase of disulphide linkages and a decrease in free sulphydryl groups, (Sutherland & Phil, 1968) was consistent with this mechanism. Thus inhibition of SOD by DDC after irradiation could still lead to sensitization, should the reactions proposed above occur.

Chapter 5
Radioprotection by DDC

Many compounds can protect against the lethal effects of radiation not by directly affecting the radiosensitivity of the cells, but rather by causing vasoconstriction, or disturbing the normal metabolism. This may occur to such an extent that the oxygen concentration in critical organs is reduced, thus offering a measure of protection because cells in which the oxygen concentration is low are more resistant to sparsely ionizing radiation (Hall, 1988). However, it must be emphasised that the compounds that act in this way, such as sodium cyanide, CO, adrenaline, histamine and serotonin are not protectors *per se*.

Sulphydryl compounds

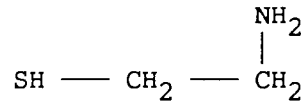
True protectors are generally sulphydryl-containing compounds, the simplest of which is cysteine (Hall, 1988).



Cysteine

In 1949, Patt *et al.* discovered that cysteine could protect mice against lethal effects of X-irradiation when large amounts were injected prior to exposure. At about

the same time, cysteamine was also found to protect animals from total body irradiation (Patt, 1953).



Cysteamine

Although these compounds were too toxic for clinical use, their discovery prompted great interest in finding other non-toxic radioprotective compounds, especially after the explosion of atomic bombs at Hiroshima and Nagasaki during World War II.

Many similar compounds have been tested and found to act as radioprotectors. A common feature of these compounds is a free SH group at one end of the molecule and a strong basic function such as an amine at the other end separated by a chain of two or three carbon atoms.

Competition with oxygen for fixation of damage

It was shown in bacteria that protection by sulphydryl compounds was dependent on oxygen concentration (Alper, 1962), a phenomenon which was later demonstrated in mammalian cells both *in vitro* (Chapman *et al.*, 1973) and *in vivo* (Harris & Phillips, 1971). It is thought that sulphydryl compounds work by scavenging free radicals produced by ionizing radiation. These radicals may combine with oxygen :

$$F^{\bullet} + O_2 \longrightarrow FO_2^{\bullet}$$

forming a highly reactive radical product, which can seriously damage biologically sensitive molecules.

Sulphydryl compounds block this process by reacting with free radicals in competition with oxygen (Koch & Howell, 1981). The protective effect of sulphydryls therefore tends to parallel the oxygen effect, being maximal for sparsely ionizing radiation and minimal for densely ionizing radiation. It might be predicted that with effective scavenging of all free radicals, the largest possible dose reduction factor (DRF) i.e..

$$DRF = \frac{\text{dose of radiation in presence of drug}}{\text{dose of radiation in absence of drug}}$$

(to produce a given level of lethality)

would equal the oxygen enhancement ratio (OER) with a value of 2.5 to 3.0 (Hall, 1988).

A second mechanism of thiol radioprotection has been suggested by Durand (1983), whereby thiols deplete all the oxygen and thereby induce anoxia. Durand & Olive (1989) have recently shown that thiol manipulation markedly influences the oxygenation status of cells (due to thiol oxidation reactions) and that this mechanism is of greater significance at lower oxygen tensions.

WR-2721

In the years that followed World War II, the Walter Reed Army Hospital in Washington D.C. synthesised more than 2000 compounds in an attempt to find the 'perfect radioprotector', one that would protect without debilitating side effects. At an early stage it was found that the toxic side effects of these compounds could be reduced if the sulphydryl group was 'covered' by a phosphate group. One of the most effective of these compounds that were developed in this period was WR-2721 (Hall, 1988).

$$NH_2 \text{ —— } (CH_2)_3 \text{ —— } NHCH_2CH_2SPO_3H_2$$

WR-2721

WR-2721 was found to give good protection to the blood forming organs, with a DRF of 2.7, approaching the theoretical maximum value of 3 (Yuhas *et al.*, 1980).

In radiotherapy a radioprotector, to be effective, has to protect normal tissues to a greater extent than tumour tissue. Successful protectors should also be hydrophilic, since lipophilic compounds would not show any differential uptake between tumour and normal cells (Hall, 1988). WR-2721 has been shown to be actively concentrated by normal tissues, yet only be passively absorbed by solid tumours (Yuhas, 1980).

A differential protection with WR-2721 has been shown in small laboratory animals irradiated with a large single dose of X-rays (Yuhas, 1973). It has not unequivocally been shown that there is a similar differential protection when tumours are irradiated with multiple small radiation doses. However phase I toxicity trials with WR-2721 in humans showed dose limiting toxicities including hypotension, hypertension, emesis, somnolence and allergic reactions (Blumberg *et al.*, 1982; Glick *et al.*, 1982). These side effects tended to limit the amount of drug given to levels lower than necessary to give maximum protection.

Chemical radioprotectors appear to be more efficient in protecting tissues from low LET compared to high LET radiation (Sigdestad *et al.*, 1975; Sigdestad *et al.*, 1976). Sigdestad *et al.* (1986) showed that WR-2721 was a more efficient as a protector against low LET radiation. This could be due to greater influence of free radical scavenging and/or hydrogen ion donation because of :

* LET differences in subcellular dose distribution
* The degree to which radiolysis of water affects cell killing by radiations of different qualities.

The induction of hypoxia by thiol compounds as demonstrated by Durand & Olive (1989) could lead to an increased survival after low LET compared to high LET

radiation because of the greater oxygen effect on low LET radiation injury.

Diethyldithiocarbamate

In 1953 Bacq found that diethyldithiocarbamate (DDC) offers protection to mice against the lethal effects of ionizing radiation. The protective properties of DDC were found to be as good as cysteamine (Alexander *et al.*, 1955), one of the best chemical radioprotectors known at that time. The relationship between the chemical structure of dithiocarbamic acid and its derivatives and their radioprotective action has been extensively studied by Van Bekkum (1956). Ammonium dithiocarbamic acid, sodium dimethyldithiocarbamate and Na-DDC gave maximum protection, whilst further substitution at the N or S position decreased the radioprotective properties. The corresponding disulphide of DDC, tetraethylthiuram disulphide (disulfiram), as well as tetraethylthiuram monosulphide did not have any radioprotective action.

Stromme (1965b) studied the metabolism of DDC and disulfiram in rats. It was found that upon absorption, disulfiram immediately becomes converted to DDC. This DDC (either formed *in vivo* from disulfiram or administered as such) was found to be metabolised at a high rate, the main end products being the S-glucuronide of DDC, inorganic sulphate and carbon disulphide. It was shown that DDC is an obligatory intermediate in the metabolism of disulfiram, and thus it appeared

contradictory that only DDC and not disulfiram possessed radioprotective qualities. Stromme & Eldjarn (1966) used ^{35}S-labelled compounds in mice to study the metabolism of DDC and disulfiram. They found that DDC is metabolised along three main routes :

* conjugation with glucuronic acid
* oxidation to sulphate
* decomposition to diethylamine and volatile carbon disulphide, which is eliminated via the lungs.

A marked difference in organ distribution was found which was in agreement with results of other radioprotective S-containing compounds. The highest amounts were shown in plasma, liver and kidney, whereas the lowest amount was found in the brain. Organs which play an important role in the radiation syndrome such as bone marrow, spleen and intestinal mucosa showed intermediate amounts 10 minutes after 15 mg ^{35}S-labelled DDC was injected i.p. It was shown by Stromme & Eldjarn (1966) that initially (during the first 40 minutes after injection) high concentrations of free unmetabolised thiol were found, and during the following 40 minutes, this concentration decreased rapidly. At the same time, the concentration of the S-glucuronide of DDC, sulphates and protein bound metabolites increased.

The total concentrations of ^{35}S found in tissues after disulfiram administration were found to be only $^{1}/_{10}$ -

$^{1}/_{20}$ of that found with DDC. This may explain why disulfiram does not show any radioprotective qualities.

No free DDC was found in tissues at the time of exposure to irradiation after disulfiram administration. Stromme & Eldjarn (1966) argued that the resorption of water insoluble disulfiram was so slow that the organism was capable of metabolising this compound fast enough to prevent any measurable build up of disulfiram or DDC.

Radioprotection due to non protein-bound DDC

At that time the mechanism of radioprotection by DDC was unclear. *In vitro* studies had failed to demonstrate any mixed disulphide formation of DDC with biological low molecular weight disulphides (Eldjarn & Pihl, 1960) and with S-S groups of proteins (Stromme, 1965a). *In vivo* data however, suggested that some mixed disulphide formation occurred on both the soluble and structural proteins. This was thought probably to be due to an initial oxidation of DDC to disulfiram, which is known to have a great ability for oxidising and forming mixed disulphides with endogenous thiol groups (Stromme, 1965a). However, in contrast with the considerable amounts of protein-bound sulphur recovered from mice treated with other radioprotective sulphur containing compounds, (Eldjarn & Pihl, 1956; Eldjarn & Pihl, 1960) the amounts of sulphur detected after DDC administration represent only a minute fraction of the total sulphur given. This, together with the fact that disulfiram

under conditions where no radioprotection was demonstrated, gave rise to the same amount of protein-bound sulphur as that obtained with DDC made it unlikely that protein-bound metabolites played any important role in its radioprotective action.

Stromme & Eldjarn (1966) argued that, of the metabolites, the free (i.e. non protein-bound) thiol, DDC seems to be the most likely immediate active agent in radioprotection, and this would explain why disulfiram fails to protect as in contrast to the amounts of free thiol after DDC administration, there is a complete absence of free thiol after disulfiram administration.

Duration of DDC action

Craven *et al.* (1976) determined the time sequence of absorption and elimination of DDC and compared this to its duration of action. ^{35}S-labelled DDC was given by stomach tube to Wistar rats at a rate of 25 mg/kg. After 15 minutes the DDC concentration in the plasma was 11.3 µg/ml. Analysis of the plasma at this time showed equal quantities of free DDC and dithio-S-glucuronide together with a small amount of inorganic sulphate.

Following oral administration of ^{35}S-labelled DDC, it was rapidly excreted, 60% of the dose being cleared in 3 hours. By 72 hours, 96% of the administered dose could be accounted for in the excretory products. 50% of this

was via the lungs in the form of carbon disulphide and most of the remainder in the urine.

The duration of DDC action was measured by the ability of DDC to prolong the sleeping time induced by pentabarbitone sodium (45 mg/kg i.p.). DDC was administered orally to mice at various times before the injection of pentabarbitone and the sleeping time was recorded for each animal. Prolongation of sleeping time was maximal when DDC was administered 60 minutes before pentabarbitone sodium, and no prolongation was noted when this time interval was three hours, showing that the duration of action of DDC is not in excess of three hours, and at this time over half of the DDC dose had been eliminated.

Ionizing radiation can induce haemolysis in red blood cells. Bartosz & Leyko (1981) examined the effect of various free radical scavengers on radiation damage to red blood cell membranes. When comparing the effect of these scavengers, the relative haemolysis was expressed by :

$$H_{rel} = \frac{\text{haemolysis of samples irradiated with protector}}{\text{haemolysis of samples irradiated without protector}}$$

Erythrocytes were incubated with DDC for 2 hours at 37°C and then irradiated with a ^{60}Co source to a dose of 5 Gy. They showed no radiosensitization as suggested by Stone

et al. (1978) , but instead radioprotection, as shown in Table 5.1.

Table 5.1 The effect of DDC (present during irradiation) on the radiation induced haemolysis of bovine erythrocytes.

DDC conc [mM]	H_{rel} %
1.0	42.7 ± 6.0
2.5	35.8 ± 1.0
5.0	61.4 ± 1.8
Cysteine 5.0	82.6 ± 0.4

Evans *et al.* (1983a) extended their work to the *in vivo* situation, where they showed that the $LD_{50/30}$ in F1 hybrid (C57/Bl X BALB/c) mice was increased from 7.8 Gy to 14 Gy when non-toxic concentrations of DDC were injected prior to total body irradiation (TBI). Their results are presented in Tables 5.2 and 5.3.

Table 5.2 $LD_{50/30}$ as a function of time of injection of 1000 mg/kg DDC in F1 hybrid (C57/Bl X BALB/c) mice.

Time of injection	$LD_{50/30}$ (Gy)	95% Confidence
1 hr after TBI	8.6	(8.2 - 9.0)
2 hr before TBI	14.0	(13.4 - 16.4)
30 min before TBI	13.4	(12.9 - 13.9)

Table 5.3 $LD_{50/30}$ as a function of DDC dose injected 30 minutes before total body irradiation in F1 hybrid (C57/Bl X BALB/c) mice.

Dose of DDC (mg/kg)	$LD_{50/30}$ (Gy)	95% Confidence
0	7.8	(7.1 - 8.2)
500	7.7	(7.0 - 8.4)
1000	14.9	(14.3 - 15.5)
1400	13.4	(12.9 - 13.9)

An $LD_{50/7}$ of 17.0 (15.6 - 18.9) Gy for death by the gastrointestinal syndrome was obtained with no DDC present and 16.5 (16.2 - 16.8) Gy when 1000 mg/kg DDC was injected 30 minutes prior to irradiation, suggesting that DDC offers no protection against the gastrointestinal syndrome in these mice.

Using ^{35}S-labelled DDC, Evans *et al.*, (1983a) showed that higher levels of DDC were obtained in kidney, lung and bone marrow compared to tumour tissue, a result previously demonstrated by Stromme & Eldjarn (1966). It was argued that this data supports a differential uptake in normal versus tumour tissue.

In an effort to show that bone marrow cells were protected by DDC, Evans *et al.* (1983a) performed a standard spleen colony assay (Till & McCulloch, 1961) where survival fractions are based on injecting bone marrow cells, from mice which have received graded doses of total body irradiation, into ten recipient mice and determining the number of spleen colonies eight days later. Their results showed an absence of a shoulder on the survival curves for spleen colonies in mice which

were treated with 1000 mg/kg DDC, injected 30 minutes prior to irradiation as well as for mice without DDC pretreatment. However, they showed an increase in D_0 from 0.8 Gy with irradiation alone to 1.2 Gy with DDC pretreatment giving a DMF of 1.5.

DDC stimulation of stem cells

Allalunis-Turner & Chapman (1984) also performed a spleen colony assay and observed large increases in spleen colonies in C57/Bl/10J mice after a treatment of various doses of DDC without irradiation (Colony enhancement ratio, CER = 3.9). When spleen colony assays for DDC and irradiation were normalised to account for this observed enhancement of colony forming units with DDC alone, the DMF after a single dose of 30 mg DDC ranged from 0.9 to 1.1 as opposed to 1.4 to 2.9 with non-normalised results.

The same investigators suggested that the difference between the DMF obtained in this study and the large DMFs reported for DDC in other studies could be due to the resolution of DDC effects into a drug dependent stimulation of stem cells and a true radioprotective effect. It was therefore possible to overestimate the protective effect of DDC.

The mechanism of DDC stimulation of haematopoetic cells has yet to be determined. Allalunis-Turner & Chapman (1984) gave, as a possible explanation for the ability of

DDC to protect cells, its ability to trigger cells into cycle, possibly into a more radioresistant phase, an effect that has been documented for endotoxin, a non-thiol radioprotector (Queensberry *et al.*, 1973; Smith *et al.*, 1966).

Multiple doses of DDC (15 mg at 48 hr, 24 hr and 15 minutes prior to irradiation) were not as effective as a single dose, producing little protection and little enhancement of colony forming units. It was suggested that bone marrow could become refractory to the effects of DDC, and while this would not affect the potential usefulness of DDC in single dose, whole body irradiation schemes, the use of DDC in fractionated radiotherapy would be compromised (Allalunis-Turner & Chapman, 1984).

Evans (1985), using a similar spleen colony assay with F1 hybrid (C57/Bl X BALB/c) and C3H/Km mice was unable to demonstrate any stimulation of the bone marrow by DDC alone. He showed that DDC protected bone marrow in F1 hybrid mice with a DMF of 1.5. DDC offered a lesser degree of protection when similar experiments were performed in hypoxic conditions (mice were bathed in 5.5% oxygen in nitrogen for 5 minutes prior to and during irradiation, which without DDC resulted in an oxygen enhancement ratio (OER) of 2.3, indicating that an hypoxic state did in fact occur following this treatment). (DMF = 1.2).

A similar result was obtained with the $LD_{50/30}$ assay after total body irradiation, where a DMF_{AIR} of 1.9 was obtained compared to a $DMF_{HYPOXIA}$ of 1.2 in animals breathing the 5.5% oxygen mixture.

Radioprotection with concomitant tumour radiosensitization

In a further experiment, Evans (1985) exposed C3H/Km mice bearing a RIF-1 fibrosarcoma to total body irradiation in air or hypoxia. Following irradiation, the tumour was excised and an *in vitro* cell survival assay used to determine the radiation response of the tumours. Bone marrow was also removed from these mice and used in a spleen colony assay. It was found that DDC, as in the F1 hybrid mice, protected the bone marrow with a DMF of 2.1 in air breathing animals.

In the tumours however, DDC pretreatment resulted in sensitization by a factor of 2.6 in air breathing mice, and by a factor of 13 in mice breathing the 5.5% oxygen mixture. Evans argued that these results which demonstrated bone marrow radioprotection by DDC (aerobic>hypoxic) with concomitant tumour radiosensitization (hypoxic>aerobic) strongly suggested a large therapeutic gain factor, and had a direct application to the clinic (Evans, 1985).

Chapter 6
Hypothesis

Apparently contradictory findings, namely that DDC can act as a radiosensitizer or as a radioprotector, have been demonstrated by various investigators over the last few years. The major contributions to this debate in the literature have been reviewed in the last two chapters.

It is possible that these two positions can be reconciled. There is evidence that DDC inhibits SOD, and that SOD is an important antioxidant enzyme that can protect cells against the damaging effect of radiation produced oxygen radicals. However, there is also extensive evidence that thiols, such as DDC can protect cells by competing with oxygen for the fixation of oxygen induced damage, by scavenging radiation induced radicals, or by inducing anoxia in the tissues.

In the present work, it is hypothesised that radiosensitization can occur as a result of SOD inhibition by DDC, but can be masked by a radioprotective effect if the tissue levels of DDC are too high at the time of irradiation.

Thus, to resolve the apparent discrepancies, it is postulated that the introduction of time delays between DDC administration and radiation may reveal definitive

information as regards the action of DDC. This thesis describes *in vitro* and *in vivo* investigations along these lines.

The first work done in this regard concerned the measurement of SOD and other antioxidant enzymes at various times after DDC administration.

In the presentation of these investigations, it was decided to present the methods and results of each enzyme study separately, followed by a general discussion at the end of the chapter.

The hypothesis was also tested *in vivo* using the tumour growth delay assay. These investigations form the major part of this thesis, and in the discussion of this section, the hypothesis is developed further.

Many intrinsic problems in the analysis of growth delay assays came to light during these investigations. These problems together with an alternative method of analysing this kind of data are presented in Chapters 8 - 10.

The effect of a time delay between DDC administration and radiation on the $LD_{50/30}$ is examined in Chapter 11.

In vitro studies of the effect of DDC on mouse melanoma cells to both radiation and hyperthermia are reported in Chapters 12 and 13.

Chapter 7
DDC and the enzymatic defence against oxygen toxicity

In this chapter studies which examine the effect of DDC on enzymes involved in the defence against oxygen toxicity are described.

Objectives

* To confirm that DDC inhibits superoxide dismutase (SOD).

* To examine the time course of this inhibition.

* To examine the effect of DDC on other enzymes in the oxygen defence system, viz. catalase and glutathione peroxidase.

Methods

Superoxide dismutase assay

The method of Misra and Fridovich (1972a) was used to measure SOD activity. The assay is based on the autooxidation of adrenaline to an adrenochrome at pH 10.2.

Adrenaline is stable in acidic solutions, but oxidizes to adrenochrome with increasing facility as the pH is raised.

It has been proposed that the autooxidation of adrenaline is initiated by traces of heavy metals present as contaminants in reaction mixtures. Indeed, EDTA has been demonstrated to inhibit this autooxidation by Misra & Fridovich (1972a) who also showed that SOD inhibited this reaction to a maximum of 91% at pH 10.2. They showed that virtually all the oxidation at this pH was produced by an O_2^- dependent pathway, and that the involvement of O_2^- diminished with decreasing pH, becoming undetectable below pH 8.5. They proposed two alternative mechanisms of adrenaline oxidation, each of which assumed greater relative importance depending on the pH.

At high pH, where RH_3^- represents adrenaline, R represents adrenochrome and Me^n represents a heavy metal cation, the oxidation could be initiated by traces of heavy metal cations (reaction a).

$$RH_3^- + Me^n \longrightarrow RH_3^{\cdot} + Me^{n-1} \qquad (a)$$

$$RH_3^{\cdot} + O_2 \longrightarrow RH_2 + O_2^- + H^+ \qquad (b)$$

$$RH_2 + O_2^- + H^+ \longrightarrow RH^{\cdot} + H_2O_2 \qquad (c)$$

$$RH^{\cdot} + O_2 \longrightarrow R + O_2^- + H^+ \qquad (d)$$

Superoxide could also initiate the oxidation and start a chain reaction :

$$RH_3^- + O_2^- + 2H^+ \longrightarrow RH_3^{\cdot} + H_2O \quad (e)$$

Clearly SOD should strongly inhibit the formation of adrenochrome by reacting with O_2^-.

Misra & Fridovich (1972a) argued that the effect of EDTA on this mechanism would be twofold. Firstly it would inhibit the rate of adrenaline oxidation due to chelation of heavy metal cations in reaction a. Secondly it would emphasise the role of O_2^- in adrenaline oxidation (reaction e), with a concomitant increase in sensitivity towards SOD. They argued that this would occur because of the EDTA-metal cation complex reacting to produce O_2^- as discussed by McCord & Fridovich (1969b). EDTA had no effect on the activity of SOD in other assay systems.

At low pH, Misra & Fridovich (1972a) proposed that the organic radical generated by the initiating event could lead to adrenochrome formation by a series of dismutation reactions such as :

$$RH_3^{\cdot} + RH_3^{\cdot} \longrightarrow RH_2 + RH_4 \quad (f)$$

$$RH_3^{\cdot} + RH_2 \longrightarrow RH^{\cdot} + RH_4 \quad (g)$$

$$RH^{\cdot} + RH^{\cdot} \longrightarrow R + RH_2 \quad (h)$$

Here SOD would not inhibit adrenochrome formation. The reduced metal cation generated in reaction (a) alone could be reoxidised by reaction with oxygen :

$$Me^{n-1} + O_2 \longrightarrow Me^{n} + O_2^{-} \qquad (i)$$

Thus an assay for superoxide dismutase was described. The production of adrenochrome in reaction mixtures containing 3 X 10^{-4} M adrenaline, 1 X 10^{-4} M EDTA and 0.05 M sodium carbonate buffer at pH 10.2 at 30°C was followed at 480 nm.

Preparation of tumour samples after intratumoural administration of DDC

Male BALB/c mice were inoculated with a transplantable rhabdomyosarcoma in the right hind gastrocnemius muscle as described in Appendix 2.

DDC solutions for intratumoural administration were prepared as described in Appendix 2.

Five tumour-bearing mice were used in each group, 4 having been injected with 20 µl DDC intratumourally at doses of 50, 100 and 150 mg/kg body weight, the remaining mouse serving as a control. One to twenty four hours after DDC administration, the mice were sacrificed and the tumours excised. Non-necrotic tumour tissue was homogenised in a Potter-Elvehjem homogeniser in 5 ml potassium phosphate/EDTA buffer, which was then clarified

by centrifugation in a Sorvall RC-5B centrifuge at 30 000 X *g* for 30 minutes at 5°C.

Superoxide dismutase extraction

The method of Sykes *et al.* (1978) for SOD extraction was used. Direct assay of SOD was not possible as extracts of homogenised tissue were often too highly coloured by haemoglobin, thus further clarification of the sample was necessary.

Following tissue homogenisation and centrifugation, the supernatant fluid was removed and treated with 0.25 volume ethanol and 0.15 volume chloroform, both chilled to 5°C. This precipitated the haemoglobin complexes, which were removed by centrifugation for 30 minutes at 30 000 X *g* at 4°C in a Sorvall RC-5B centrifuge.

The supernatant was removed and K_2HPO_4 was added to a final concentration of 30% (w/v) and stirred using a vortex mixer until all the salt had dissolved. The mixture was centrifuged for 20 minutes at 17 210 X *g* at 4°C to bring about a clean separation of the phases. The lighter phase, essentially water and ethanol, containing the bulk of the enzyme was carefully removed. The enzyme was precipitated by the addition of 0.75 volume cold acetone (-20°C). This mixture was centrifuged for 30 minutes at 17 210 X *g* at 4°C, and the acetone supernatant discarded, leaving the SOD containing precipitate which was resuspended in 0.75 ml distilled

water. This was allowed to stand at 5°C overnight to ensure complete solubilisation of the enzyme whereafter it was centrifuged for 30 minutes at 17 210 X *g* at 5°C to remove any traces of flocculated material. The water-clear supernatant constituted the tumour extract and was assayed immediately.

Measurement of superoxide dismutase activity

The reagents used are detailed in Appendix 1.

A standard solution of superoxide dismutase (SOD) was prepared in water using bovine erythrocyte SOD (Sigma, St. Louis, Mo.), the activity of which was 28 000 units/ml. The Na_2CO_3 / EDTA buffer was oxygenated by bubbling O_2 gas through the solution for 5 minutes.

The reaction was started by adding a 0.0125 ml stable, acidic aliquot of adrenaline to 1.1 ml buffer in a quartz glass cuvette. The rate of adrenochrome formation was then measured spectrophotometrically at 480 nm at 30°C with a Pye-Unicam SP8-400 double beam spectrophotometer.

A standard curve for SOD activity using a 0.28 units/µl SOD solution was generated by plotting the percentage of inhibition of the rate of change of the absorption of adrenochrome at 480 nm against SOD concentration (Fig 7.1).

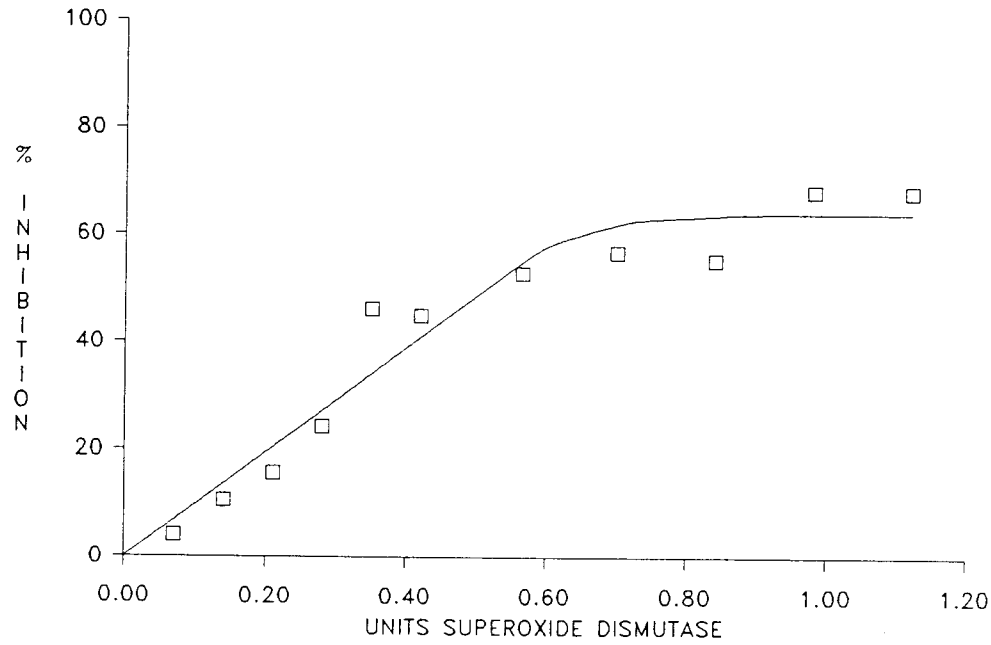


Figure 7.1 The standard curve for superoxide dismutase concentration vs the inhibition of the rate of change of absorption of adrenochrome at 480 nm.

Tumour SOD activity was measured by adding 0.02 ml tumour extract to 1.08 ml buffer prior to the addition of the adrenaline aliquot. The subsequent reduction in the rate of formation of adrenochrome was then used to calculate SOD activity.

Figure 7.1 shows that the graph of percentage inhibition of the rate of adrenochrome formation as determined by absorption changes at 480 nm against SOD concentration is not linear, but reaches a plateau at about 0.6 units SOD

(or 50% inhibition of the rate of adrenochrome formation). Three or four replicates of SOD activity for each tissue extract were made, and volumes of buffer and sample were adjusted to give readings in the linear part of the standard curve, that is, such that there was an inhibition of from 0 to 50% in the rate of adrenochrome formation.

Protein determination

Protein content of each sample was measured fluorometrically with a Perkin-Elmer fluorescence spectrophotometer using the method of Bohlen *et al.* (1973) as this method allows protein determinations in the nanogram range. The method is described in Appendix 1. Calculation of SOD content of tumour samples was performed in terms of units of enzyme per mg protein.

Results

Tumours used in this experiment ranged in size from 0.201 to 1.327 cm^3 with a mean volume of 0.471 cm^3 ± 0.196 cm^3 (mean ± SD). Care was taken to exclude necrotic tissue from the samples. The size of the tumours had no demonstrable effect on SOD activity in otherwise untreated (control) tumours. Linear regression analysis was performed on the tumour size against SOD activity, and a correlation co-efficient of r=0.1323 obtained.

Tumour superoxide dismutase activity

Untreated (control) mice had mean SOD activities ranging from 46.6 to 101.4 units/mg protein with a mean activity of 77.6 ± 16.2 units/mg protein (mean ± SEM).

Analysis of SOD activity up to 24 hours after doses of 50, 100, and 150 mg/kg DDC showed that DDC reduced the activity of SOD (Table 7.1; Figs. 7.2 - 7.4).

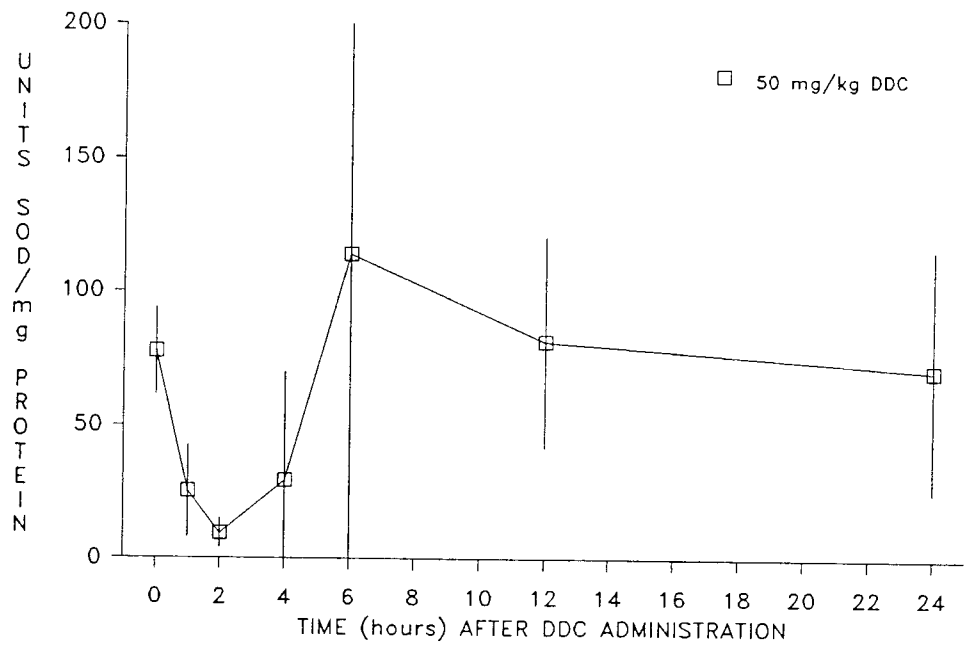


Figure 7.2 Tumour SOD activity after intratumoural administration of 50 mg/kg DDC. Maximum inhibition of SOD activity (87.7%) was achieved two hours after DDC administration. Bars represent standard deviation.

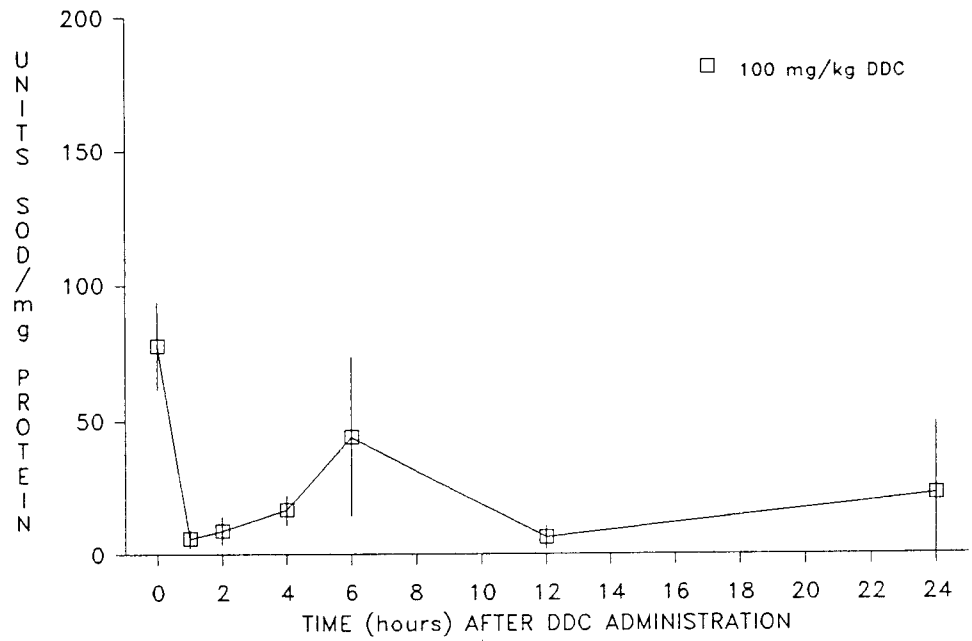


Figure 7.3 Tumour SOD activity after intratumoural administration of 100 mg/kg DDC. Maximum inhibition of SOD activity (92.5) was achieved one hour after DDC administration, and the SOD activity remained low up to 24 hours after DDC administration. Bars represent standard deviation.

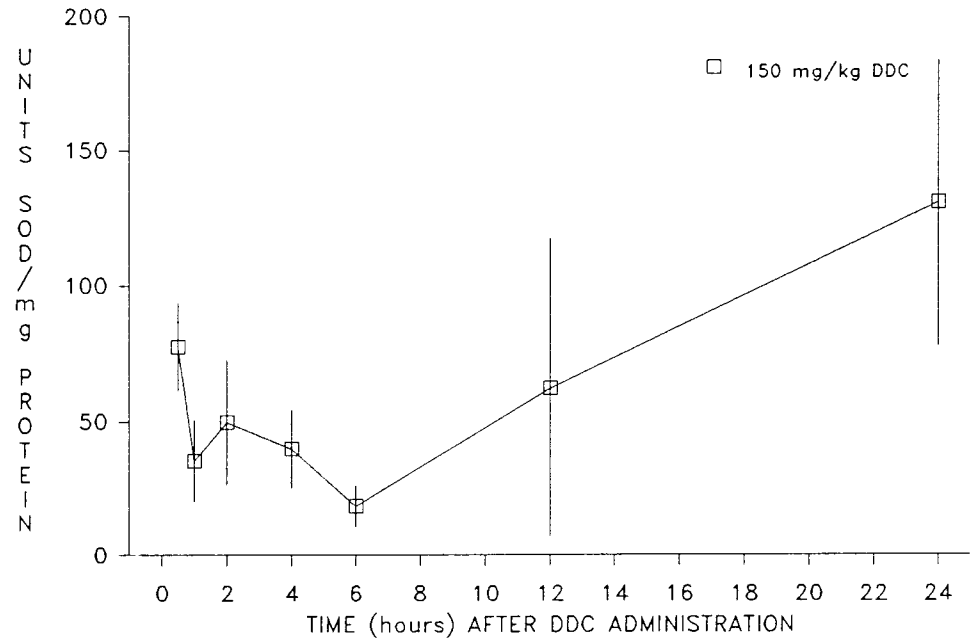


Figure 7.4 Tumour SOD activity after intratumoural administration of 150 mg/kg DDC. The inhibition of SOD activity after this dose of DDC was not as great as seen with lower doses. Bars represent standard deviation.

Table 7.1 shows the inhibition of SOD activity as a percentage of control SOD activity where SOD was inhibited by DDC treatment. Where a significant difference at or close to the 5% level was noted, the *t* values and probability are tabulated.

Table 7.1 SOD activity as a percentage of control SOD activity in DDC treated tumours. (N.S. = not significant), t-values read at 29 degrees of freedom.

DDC dose	Time between DDC and assay	% inhibition of control SOD activity	significance
50 mg/kg	1 hr	67.3	N.S.
50 mg/kg	2 hr	87.7	t=2.132, p<0.05
50 mg/kg	4 hr	61.9	N.S.
100 mg/kg	1 hr	92.5	t=2.253, p<0.05
100 mg/kg	2 hr	88.7	t=2.158, p<0.05
100 mg/kg	4 hr	78.5	t=1.908, N.S.
100 mg/kg	6 hr	43.4	N.S.
100 mg/kg	12 hr	91.7	t=2.232, p<0.05
100 mg/kg	24 hr	70.6	N.S.
150 mg/kg	1 hr	54.5	N.S.
150 mg/kg	2 hr	36.2	N.S.
150 mg/kg	4 hr	48.8	N.S.
150 mg/kg	6 hr	76.2	N.S.

Exposure to 50 mg/kg DDC caused a decrease in SOD activity at 1, 2 and 4 hours after DDC administration, the 87.7% decrease at 2 hours being significant at the 5% level (Fig. 7.2 ; Table 7.1). Six hours after DDC administration, SOD activity had returned to control levels.

When a higher DDC dose (100 mg/kg) was administered, the inhibitory effect of DDC was even greater, and a 92% inhibition of SOD activity was noted 1 hour after DDC administration. SOD activity remained low up to 24 hours after DDC administration, where it was still only 29.4% of control activities (Fig. 7.3 ; Table 7.1). The activities of SOD at 1, 2 and 12 hours after treatment were found to be significantly different from control activities at the 5% level.

When the dose of DDC was further increased to 150 mg/kg, DDC also caused a reduction in SOD activities, but this was not as great as when a lower dose of DDC was administered (Fig. 7.4 ; Table 7.1). The maximum inhibition of SOD activity noted at this DDC dose was 76.2% at 6 hours after DDC administration.

The data obtained in the present work shows that inhibition of SOD does not appear to be linearly dependent on DDC concentration.

Methods

Glutathione peroxidase assay

Glutathione peroxidase was first discovered in erythrocyte lysates as a consequence of its ability to protect haemoglobin from oxidative breakdown (Mills,

1957). It is made up of 4 protein subunits, each of which contains 1 atom of selenium at its active site. Glutathione is a substrate for the enzyme.

Glutathione peroxidase (GPx) has been shown to catalyse with high specificity the detoxification of hydrogen peroxide by the oxidation of reduced glutathione according to the reaction :

$$2\ GSH + H_2O_2 \xrightarrow{GPx} GSSG + 2H_2O$$

The assay method of Paglia & Valentine (1967) measures the rate of GSH oxidation by H_2O_2 as catalysed by GPx present within the tissue sample. Rather than measure the progressive loss of GSH, however, this substrate is maintained at a constant concentration by the addition of exogenous glutathione reductase (GSSG-r) and NADPH, so that any GSSG formed is immediately converted to the reduced form.

$$2\ GSH + H_2O_2 \xrightarrow{GPx} 2H_2O + GSSG \xrightarrow[NADPH \rightarrow NADP]{GSSG\text{-}r} 2GSH$$

To prevent decomposition of H_2O_2 by catalase in the tissue samples, azide, a catalase inhibitor is added to the reaction mixture.

The rate of GSSG formation is then measured by following the change in absorbance of the reaction mixture at 340 nm as NADPH is converted to NADP. The enzymatic activity of GPx in this reaction mixture was found to be maximal at pH 8.0 with negligible activities below pH 6.0 and with a rapid increase in the non-enzymatic oxidation of GSH above pH 7.0 (Paglia & Valentine, 1967).

The method used in this work was a modification of Paglia & Valentine (1967) as made by Lawrence & Burk (1976) which was more sensitive to the enzymatic oxidation of GSH with H_2O_2 as substrate.

Preparation of tissue samples after intratumoural administration of DDC

Male BALB/c mice were inoculated with a transplantable rhabdomyosarcoma in the right hind gastrocnemius muscle as described in Appendix 2.

DDC solutions for intratumoural administration were prepared as described in Appendix 2.

Four tumour-bearing mice were used in each group. They were injected with 20 µl DDC intratumourally at a rate of 0.5, 50 and 250 mg/kg body weight. The control mouse was injected with 20 µl 0.9% saline. One or four hours after DDC administration, the mice were sacrificed and the tumour, liver and normal gastrocnemius (left hind leg) muscle were excised. These tissues were

homogenised in a Potter-Elvehjem homogeniser in 2 ml 10 mM potassium phosphate buffer at pH 8.0 which was 30 mM in KCl. The supernatant, after centrifugation for 20 minutes at 4°C at 17 210 X *g* in a Sorvall RC-5B centrifuge was used as the enzyme source in this assay.

Measurement of glutathione peroxidase activity

The reagents used are detailed in Appendix 1.

The total reaction volume was 2 ml and consisted of 0.5 ml potassium phosphate buffer (pH 7.0); 0.2 ml EDTA; 0.2 ml NaN_3; 0.2 ml NADPH; 1 µl GSSG-reductase; 0.2 ml GSH; 0.489 ml H_2O and 0.2 ml H_2O_2 in a 3 cm^3 quartz glass spectrophotometer cuvette.

All reagents except the enzyme source and the hydrogen peroxide were added prior to each experiment. The enzyme source (0.01 ml) was added to the sample cuvette and allowed to incubate for 2 minutes at room temperature, before initiation of the reaction by the addition of 0.2 ml H_2O_2. The reference cuvette contained all reagents present in the sample cuvette, but the enzyme source was replaced by an equal volume of distilled water.

The absorbance at 340 nm (measured using a Pye Unicam SP8-400 spectrophotometer) was recorded for 3 minutes and the activity of the enzyme was calculated from the absorbance change per minute. Enzyme units were defined

as the number of µmoles of NADPH oxidised per mg protein, and were calculated on the basis of a molar absorptivity for NADPH at 340 nm of 6.22 X 10^{-6} (Horecker & Kornberg, 1948) as follows :

$$\text{GPx Activity} = \frac{\Delta \text{ O.D.}_{340}/1 \text{ min}}{6.22} \times \frac{2 \text{ ml}}{\text{sample vol.}} \times \frac{1}{\text{mg protein/ml of sample}}$$

Protein determination

Protein was measured spectrophotometrically using a Pye-Unicam SP8-400 double beam spectrophotometer by the method of Lowry *et al.*, (1951) (Appendix 1).

Results

Tumours used in this experiment ranged in size from 0.271 to 1.416 cm^3 with a mean volume of 0.663 cm^3 ± 0.250 cm^3 (mean ± SD). Linear regression analysis was performed on the tumour size against GPx activity in saline treated (control) tumours, and a correlation coefficient of $r=0.6299$ ($p>0.1$, 3 DF) obtained. It was therefore concluded that no relationship between tumour size and tumour GPx activity could be demonstrated.

Tissue glutathione peroxidase activity

Table 7.2 shows that in saline treated (control) mice, GPx activity in the liver was about six fold greater than

in the tumour and twenty fold greater than in the leg muscle.

Table 7.2 GPx activity in tumour, leg muscle and liver of saline treated (control) mice.

	GPx activity units/mg protein (mean ± SD)	95% confidence
Tumour	0.041 ± 0.008	0.026 - 0.056
Muscle	0.013 ± 0.006	0.001 - 0.025
Liver	0.240 ± 0.096	0.052 - 0.427

Analysis of glutathione peroxidase activity 1 hour after intratumoural administration of DDC showed a slight decrease of GPx activity at a dose of 0.5 mg/kg and 50 mg/kg DDC, but not at a dose of 250 mg/kg in the tumour, leg muscle and liver (Fig. 7.5, 7.6, 7.7). When 4 hours were allowed between DDC administration and assay, a slight increase in GPx activity was observed in the tumour samples (Fig. 7.5) at all dose levels of DDC tested. This increase was not observed in the liver or the leg muscle.

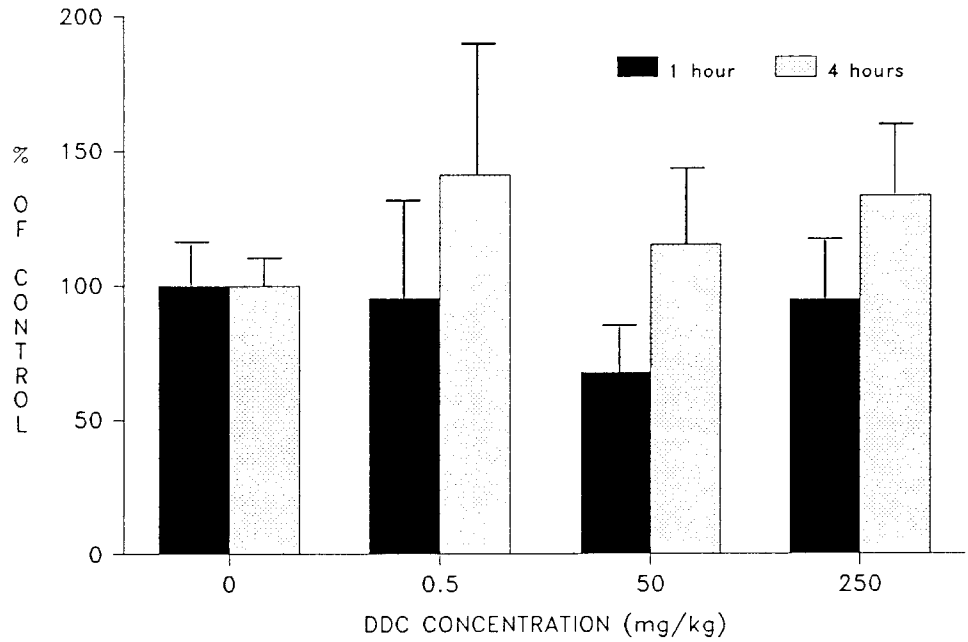


Figure 7.5 Tumour glutathione peroxidase activity after DDC administration in BALB/c mice. Assays were performed 1 and 4 hours after DDC administration. Bars represent one standard deviation.

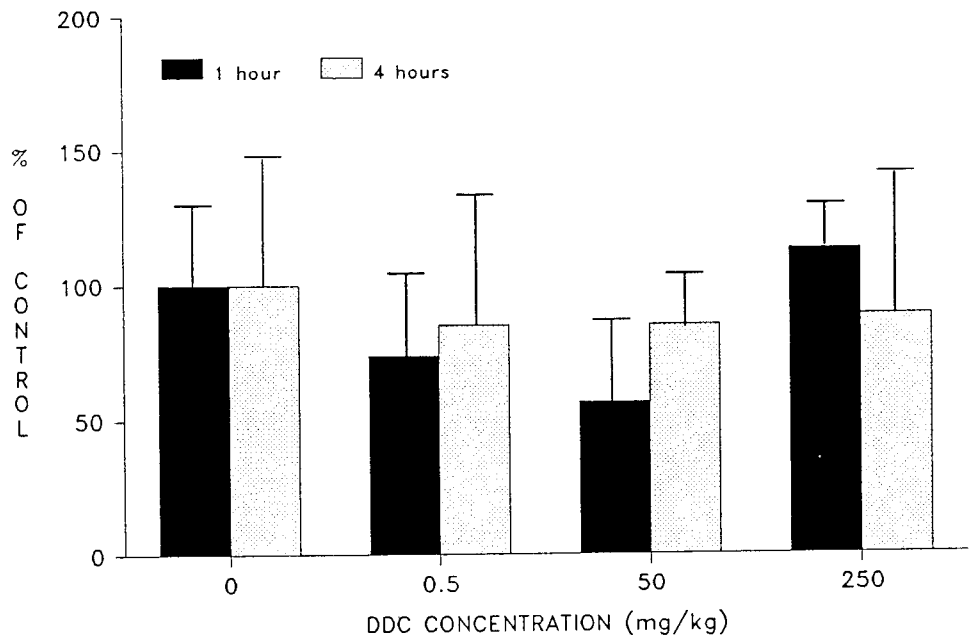


Figure 7.6 Muscle glutathione peroxidase activity after DDC administration in BALB/c mice. Assays were performed 1 and 4 hours after DDC administration. Bars represent one standard deviation.

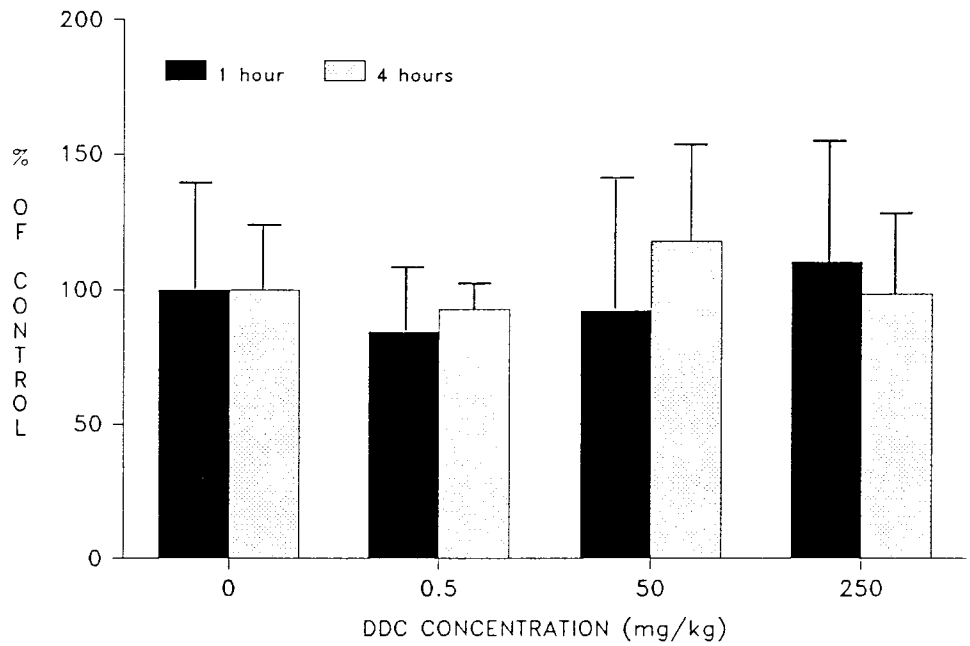


Figure 7.7 Liver glutathione peroxidase activity after DDC administration in BALB/c mice. Assays were performed 1 and 4 hours after DDC administration. Bars represent one standard deviation.

The changes in GPx activity after DDC administration observed here were found to be no different at the 5% significance level from saline treated mice using the Student's *t*-test.

Methods

Catalase assay

Most aerobic cells contain catalase (CAT). In mammals, catalase is present in all major organs, especially the liver and erythrocytes. Catalase was first isolated and obtained in crystalline form from ox liver by Sumner in 1941. Most purified catalases have been shown to consist of 4 protein subunits, each of which contains a haem (Fe(III)-protoporphyrin) group bound to its active site.

Catalase is responsible for the decomposition of hydrogen peroxide in cells by the following reaction.

$$2H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$$

This is a 2 stage reaction :

$$CAT\text{-}Fe(III) + H_2O_2 \longrightarrow \text{compound 1}$$

$$\text{compound 1} + H_2O_2 \longrightarrow CAT\text{-}Fe(III) + 2H_2O + O_2$$

The exact structure of compound 1 is uncertain, however the equations show that the complete removal of H_2O_2 requires the impact of 2 molecules upon a single active site (Halliwell & Gutteridge, 1985).

The catalase activity may be measured by following either the decomposition of H_2O_2 or the liberation of O_2. The decomposition of H_2O_2 can be followed directly by the change in absorbance at 240 nm per unit time, which is a measure of catalase activity.

The method of Holmes & Masters (1970), a modification of the original method of Beers & Sizer (1952), was used for catalase activity determination in this work.

Preparation of tissue samples after intratumoural administration of DDC

Male BALB/c mice were inoculated with a transplantable rhabdomyosarcoma in the right hind gastrocnemius muscle as described in Appendix 2.

DDC solutions for intratumoural administration were prepared as described in Appendix 2.

Four tumour bearing mice were used in each group. They were injected with 20 µl DDC intratumourally at a rate of 0.5, 50 and 250 mg/kg body weight. The control mouse was injected with 20 µl 0.9% saline. One or four hours after DDC administration, the mice were sacrificed and the tumour, liver and leg muscle were excised. These tissues were homogenised in a Potter-Elvehjem homogeniser in 2 ml 10 mM potassium phosphate buffer at pH 8.0 which was 30 mM in KCl. The supernatant, after centrifugation

for 20 minutes at 4°C at 17 210 X *g* in a Sorvall RC-5B centrifuge was used as the enzyme source in this assay.

Measurement of catalase activity

The reagents used are detailed in Appendix 1.

The H_2O_2 concentration was kept low (10 mM) as the enzyme is known to be inactivated by high concentrations of its substrate (Thomson, 1963). High concentrations of H_2O_2 also lead to the formation of oxygen bubbles in the cuvette.

The total reaction volume was 3 ml and consisted of 1.5 ml tris hydroxymethylmethylamine, 1.1 ml distilled water, 0.3 ml H_2O_2 and 0.1 ml tissue sample in a 3 cm^3 quartz glass spectrophotometer cuvette. Distilled water was substituted for the tissue sample in the reference cuvettes.

Absorbance change at 240 nm was recorded using a Pye Unicam SP8-400 double beam spectrophotometer. Enzyme units were defined as the number of µmoles of H_2O_2 converted per minute per mg protein, and were calculated on the basis of a molar absorption coefficient for H_2O_2 at 240 nm of 43.6 $M^{-1}.s^{-1}$ (Holmes & Masters, 1970) as follows :

$$\text{CAT Activity} = \frac{\Delta \text{ O.D.}_{240}/\text{min}}{0.0436} \times \frac{3 \text{ ml}}{\text{sample volume}} \times \frac{1}{\text{mg protein/ml of sample}}$$

Protein determination

Protein was measured spectrophotometrically using a Pye-Unicam double beam spectrophotometer by the method of Lowry *et al.* (1951)

Results

Tumours used in this experiment ranged in size from 0.271 to 1.416 cm^3 with a mean volume of 0.663 cm^3 ± 0.250 cm^3 (mean ± SD). Linear regression analysis was performed on the tumour size against CAT activity in saline treated (control) tumours, and a correlation co-efficient of $r=0.6915$ ($p>0.1$, 3 DF) obtained. No relationship between tumour size and tumour CAT activity could therefore be demonstrated.

Tissue catalase activity

Table 7.3 shows that in saline treated (control) mice, CAT activity in the liver was about eight fold greater than in the tumour and twenty fold greater than in the leg muscle.

Table 7.3 CAT activity in tumour, adjacent muscle and liver of saline treated (control) mice.

	CAT activity units/mg protein (mean ± SD)	95% confidence
Tumour	0.825 ± 0.209	0.416 - 1.235
Muscle	0.363 ± 0.162	0.045 - 0.680
Liver	6.291 ± 1.351	3.644 - 8.938

There did not appear to be any significant effect of DDC administration on the catalase activity in the tumour, leg muscle or liver (Fig. 7.8, 7.9, 7.10). Muscle catalase activity was slightly decreased after 0.5 and 250 mg/kg DDC was injected 1 hour prior to assay, but the decrease was within the 95% confidence limits of control values (Fig. 7.9). The higher CAT activity observed at 50 mg/kg DDC is probably artifactual since the standard deviation at this point is large.

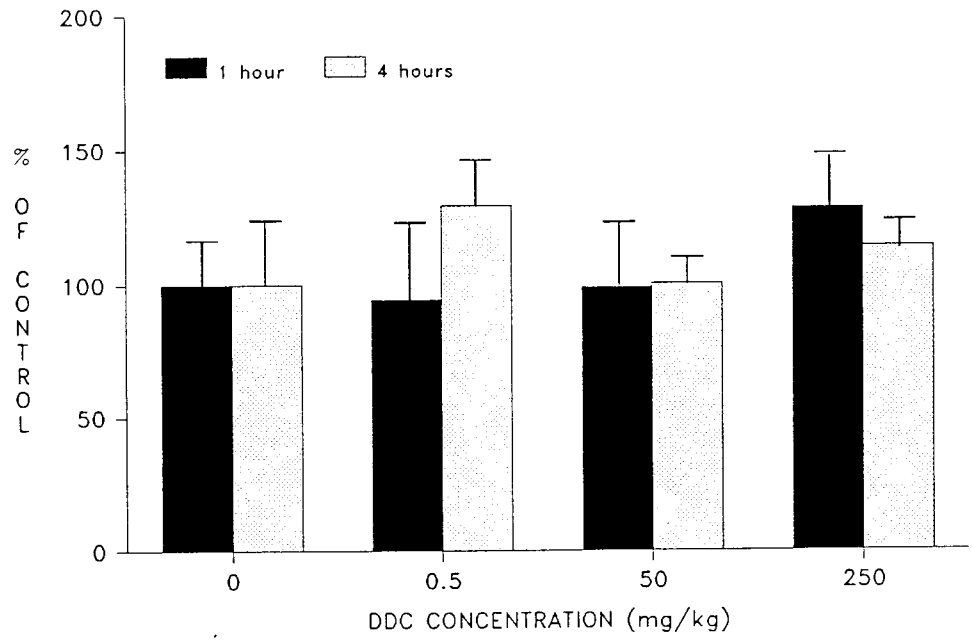


Figure 7.8 Tumour catalase activity after DDC administration in BALB/c mice. Assays were performed 1 and 4 hours after DDC administration. Bars represent one standard deviation.

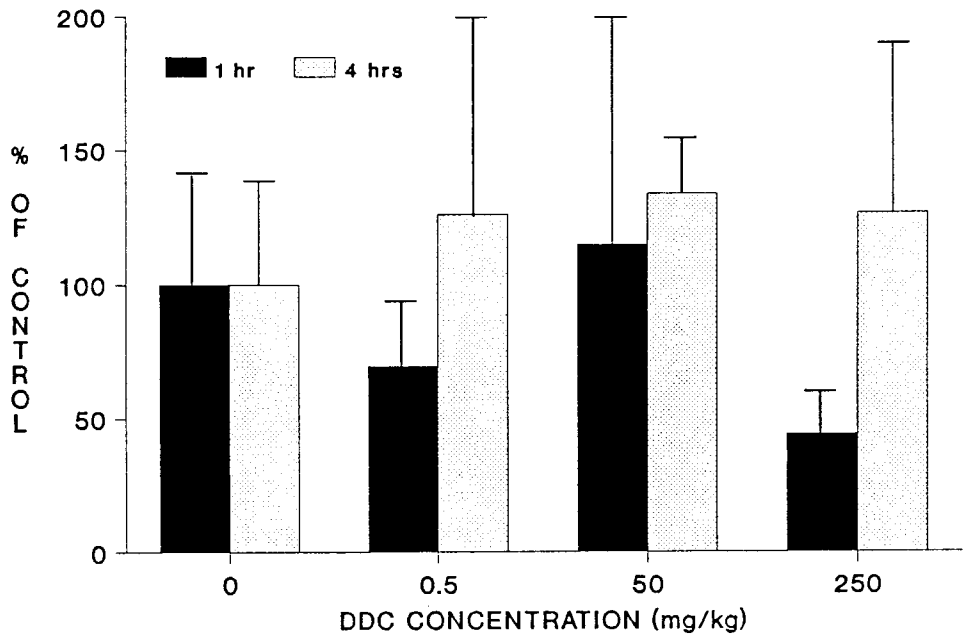


Figure 7.9 Muscle catalase activity after DDC administration in BALB/c mice. Assays were performed 1 and 4 hours after DDC administration. Bars represent one standard deviation.

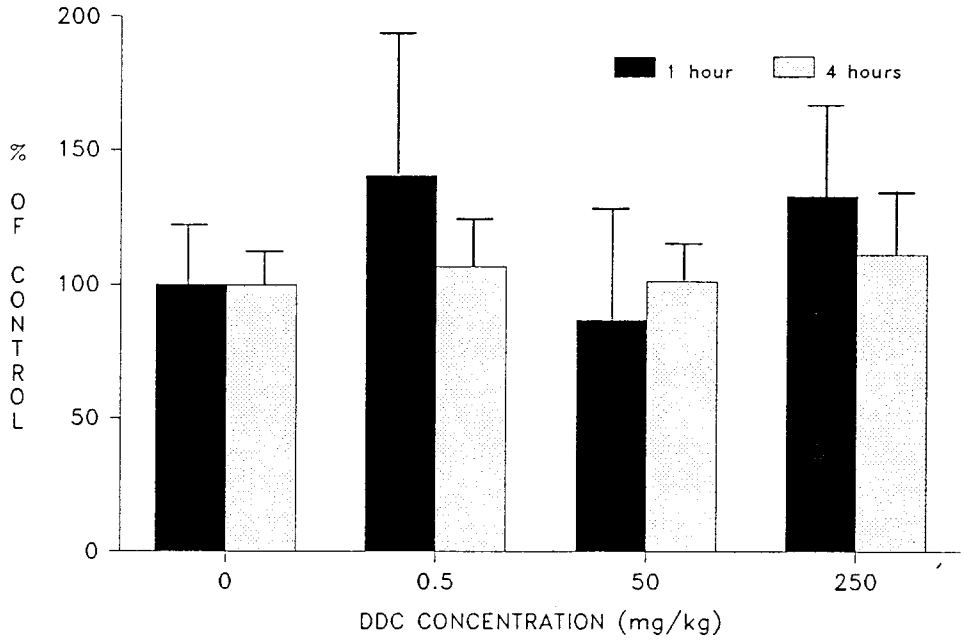


Figure 7.10 Liver catalase activity after DDC administration in BALB/c mice. Assays were performed 1 and 4 hours after DDC administration. Bars represent one standard deviation.

The changes in CAT activity after DDC administration observed here were found to be no different at the 5% significance level from saline treated mice using the Student's *t*-test.

Discussion

In 1978 Stone *et al.* showed *in vitro* that if DDC was removed from erythrocytes prior to irradiation, no effect

on sensitivity was seen. The authors concluded that this was due to insufficient SOD inhibition.

The present work has shown that this is not necessarily so, as SOD inhibition can be maintained up to 24 hours after DDC treatment. This period is probably long enough for DDC in the tumour to have been completely metabolised. Indeed, 60% of DDC dose can be accounted for in excretory products after 3 hours following oral administration of DDC (Craven *et al.*, 1976).

Westman & Marklund (1980) demonstrated *in vitro* that SOD inhibition could be maintained in Chinese hamster cells after DDC was washed off the cells. These authors found that DDC caused a reduction in SOD activity to about 2% of control (no DDC) activities when SOD activity was assayed immediately after DDC incubation. Thirty minutes after this treatment, SOD activity was still only 5% of the control, and after 24 hours was 10% of control activities.

The work reported in this thesis on *in vivo* SOD activity in tumours after intratumoural DDC administration has shown similar results. DDC caused a marked inhibition of SOD soon after exposure (8% of control, 1 hour after 100 mg/kg DDC) which was sustained for up to 24 hours (Fig 7.3).

It is also of interest to note that when the DDC concentration was increased (150 mg/kg), a concomitant increase in SOD inhibition was not noted. In fact the higher concentration of DDC was less effective in inhibiting SOD. This 'biphasic concentration effect' has been shown for human lymphocytes, (where SOD was inhibited at only one of two toxic concentrations) (Rigas *et al.*, 1979 ; Rigas *et al.*, 1980), survival of Chinese hamster cells (Westman & Midander, 1984), survival of mouse fibroblasts (Maners *et al.*, 1985) and enhancement of heat, radiation, bleomycin and adriamycin toxicity (Lin *et al.*, 1985).

In view of the fact that DDC has been shown to have both radiosensitizing (due to SOD inhibition) and radioprotective properties, it is of interest to note that SOD inhibition can be maintained for some time after DDC administration both *in vitro* (Westman & Marklund, 1980) and *in vivo* (Fig. 7.3) This is complicated by the concentration effects of DDC, but in principle, a situation could be reached where SOD activity is lowered in the cell, at the same time as free DDC levels are too low to be of any radioprotective consequence.

There was no significant effect of DDC on catalase or glutathione peroxidase (Figs 7.5 - 7.10). This is in agreement with the findings of Marklund & Westman (1980) who found no or only insignificant effects of DDC on manganese-SOD, catalase, cytochrome oxidase and protein

sulphydryl levels as well as glutathione peroxidase (Westman & Marklund, 1983).

There have been conflicting results in the literature as to the effect of DDC on glutathione peroxidase activities. A dose of 400 mg/kg DDC has been shown to cause an increase in mouse liver GPx after 2 hours (Weiss *et al.*, 1985). DDC induced resistance to hyperoxia has been argued to be associated with an increased GPx activity (Deneke & Fanburg, 1980). A decreased GPx activity was reported in mouse lung and liver following a 1.2 g/kg dose of DDC (Goldstein *et al.*, 1979). However, it has been shown that DDC exhibits 'GPx-like' activity and could be substituted for GPx in the assay of Lawrence & Burk, (1976) When GPx was replaced by DDC, a concentration dependent enzyme-like activity was seen (Kumar *et al.*, 1986). This may be a significant factor in *in vivo* assays of GPx activity following DDC treatment.

Conclusions

There is a 90% decrease in SOD activity in DDC treated tumours. This decrease is maximal shortly after DDC administration, but SOD activity remains lowered up to 24 hours after DDC administration.

The implications of this are that a situation could arise where the DDC concentration in the tumours is not high enough to cause radioprotection (due to metabolism of

DDC), yet the SOD activity is low enough to allow radiosensitization.

The inhibition of SOD is not necessarily linearly dependent on dose of DDC administered to the tumours.

The observation that a higher dose of DDC resulted in a more modest decrease in SOD activity was not significant at the 5% level, however in view of the work of other investigators concerning a 'biphasic' concentration effect of DDC, this point deserves to be raised.

No significant effect of DDC on catalase or glutathione peroxidase activity could be demonstrated.

Chapter 8
Tumour growth delay

The principles of the tumour growth delay assay, the tumour bed effect and the results of initial studies on *in vivo* DDC pretreatment are described in this chapter.

Principles of the tumour growth delay assay

The delay in tumour growth by treatment can be used as a measure of the effectiveness of the treatment (Thomlinson, 1980). The pattern of regression (if any) and subsequent regrowth is followed by making regular measurements of tumour size. The time taken for the treated tumours to reach a certain endpoint size is recorded and from this is subtracted the time taken for untreated tumours to reach that same size.

One of the main problems in the interpretation of this data stems from the fact that the pattern of regrowth is usually different in treated and untreated tumours, making the choice of endpoint size at which to assess the delay critical in determining the magnitude of that delay.

Mechanisms of damage that result in growth delay

The growth retardation of tumours after radiation can result from either :

* damage to the stroma or vasculature (Tumour Bed Effect, TBE).
* damage to tumour cells.

It is perhaps pertinent to discuss the tumour bed effect at this point.

The tumour bed effect

It has long been known that tumour cells transplanted into pre-irradiated subcutaneous tissue take longer to establish tumours than cells transplanted into non-irradiated tissues, and that further growth of tumours in the irradiated tissue is reduced, for example (Vermund *et al.*, 1956 ; Hewitt & Blake, 1968 ; Summers *et al*, 1964 ; Urano *et al*, 1980). This phenomenon is known as the Tumour Bed Effect or TBE and is thought to be due to the reduced ability of irradiated tissue to provide blood vessels to tumours.

Normal tissue stroma that surrounds or infiltrates tumours plays a significant role in the growth and response of tumours to therapy. Tumour vasculature is formed by the stroma of the tumour bed in response to angiogenic factors released by the tumour cells (Folkman, 1974). The formation of tumour blood vessels is usually inadequate to sustain tumour growth and leads to necrosis and hypoxia, which can result in resistance to ionizing

radiation. The inadequate blood supply to tumours may also compromise drug delivery (Steel & Adams, 1975).

Pattern of growth retardation

If the growth retardation results from stromal and not tumour cell damage, then it is feasible that some modifying agents may modify stromal damage to a greater degree than tumour cell damage. Begg (1980) has discussed this point, which is illustrated in Figure 8.1.

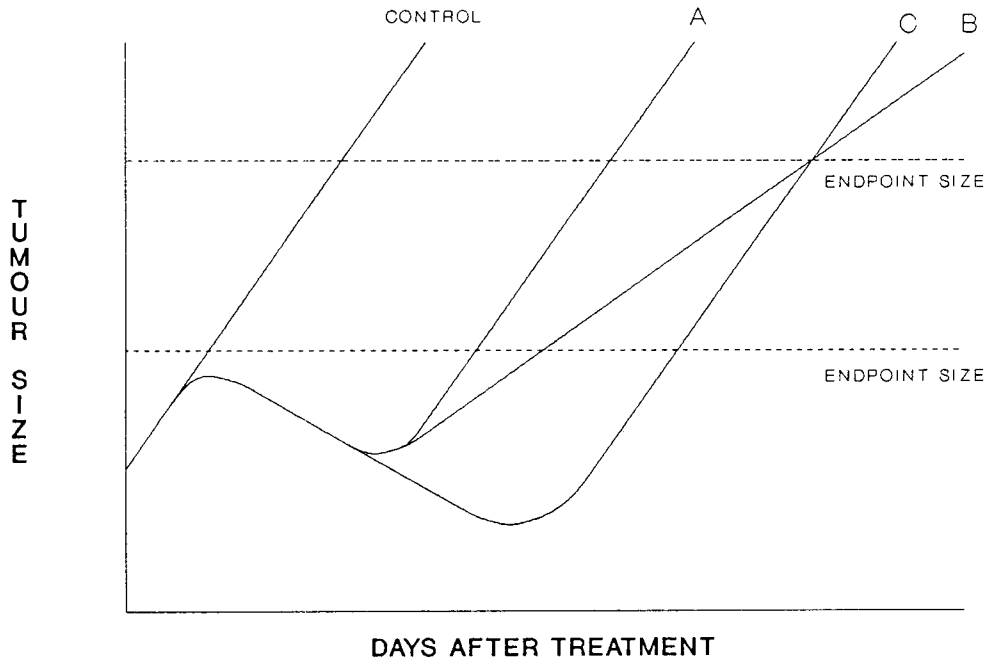


Figure 8.1. Schematic growth curves for three different types of treatment A, B & C. A and B both produce equal cell killing, but B produces more stromal damage, hence treatment B results in slower tumour regrowth. C produces a greater amount of cell kill than A or B, but causes no stromal damage and results in the same growth delay as treatment B. Redrawn from Begg (1980).

Treatment A produces no stromal damage, thus after the initial tumour shrinkage due to the treatment, the tumour regrows at the same rate as the control. Treatment B however results in an equal amount of cell kill as treatment A, but in addition, causes some stromal damage, thus after the initial shrinkage, the tumour regrows at a slower rate than the control. Treatment C on the other hand results in more cell kill than treatment A or treatment B, but causes no stromal damage and regrows at the same rate as the control.

The growth delay estimate would show treatment B as being more effective than treatment A and equally effective as treatment C. If slower regrowth occurs as a result of stromal damage (B), the estimates of tumour growth delay should be based on the smallest possible endpoint size, for example marginally larger than the size at treatment, in order to avoid artificially large delays resulting from growth retardation.

Choice of endpoint size

The choice of endpoint size for this assay depends largely on the pattern of growth retardation. In many tumours the growth rate is initially slowed after treatment - which may be the result of slow clearance of cellular material (Begg, 1987), whereafter the growth rate again approaches that of untreated controls.

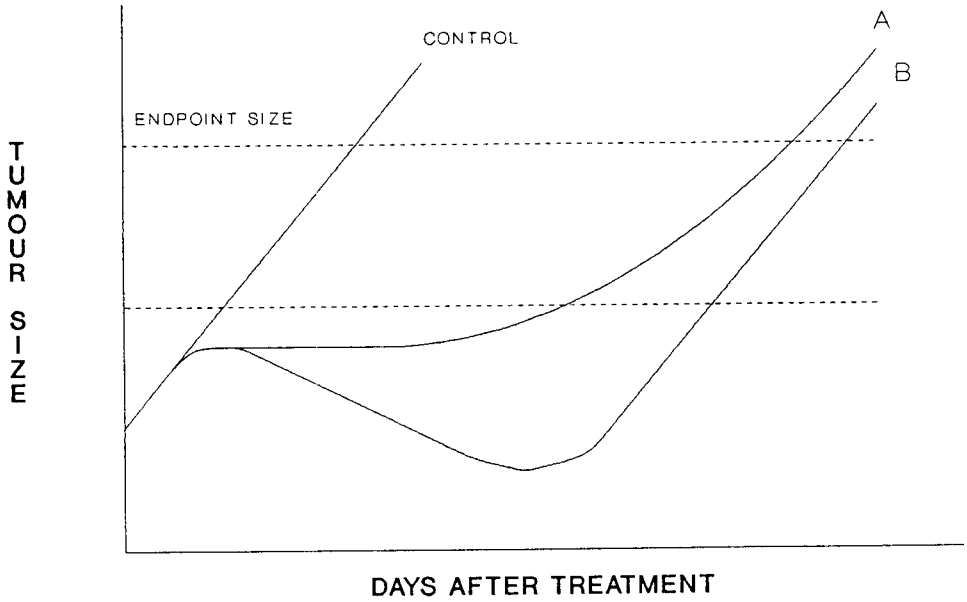


Fig 8.2. Representation of the effect of shrinkage on the shape of the regrowth curve. When no shrinkage occurs (A), the initial regrowth rate appears slower even if the proliferation rate of surviving cells remains unchanged. (Redrawn from Begg, 1980).

If no tumour shrinkage occurs after treatment, the regrowth rate will be slow initially, giving the appearance of a tumour bed effect (Curve A in Fig 8.2). This is because the influence of a large non-growing mass may considerably slow the observed tumour growth rate. In this case, the choice of a small endpoint size will lead to an incorrect low estimate of growth delay. In this case an endpoint size should be chosen large enough not to cause the growth delay estimate to occur in this artificially slow growth period. If shrinkage occurs, (Curve B in Fig 8.2), the influence of this non-growing

mass diminishes, and the tumour as a whole rapidly attains the growth rate of the surviving cells. Here an endpoint size should be as low as possible so as not to allow the estimate to be influenced by changes in growth rate due to the TBE as well as increasing necrosis and hypoxia with increasing size (Hill, 1980).

A third possibility is shown in Figure 8.3, where the data can only be fitted assuming a changed proliferation rate of surviving cells due to the tumour bed effect. Thus growth delay estimates will increase as endpoint size increases.

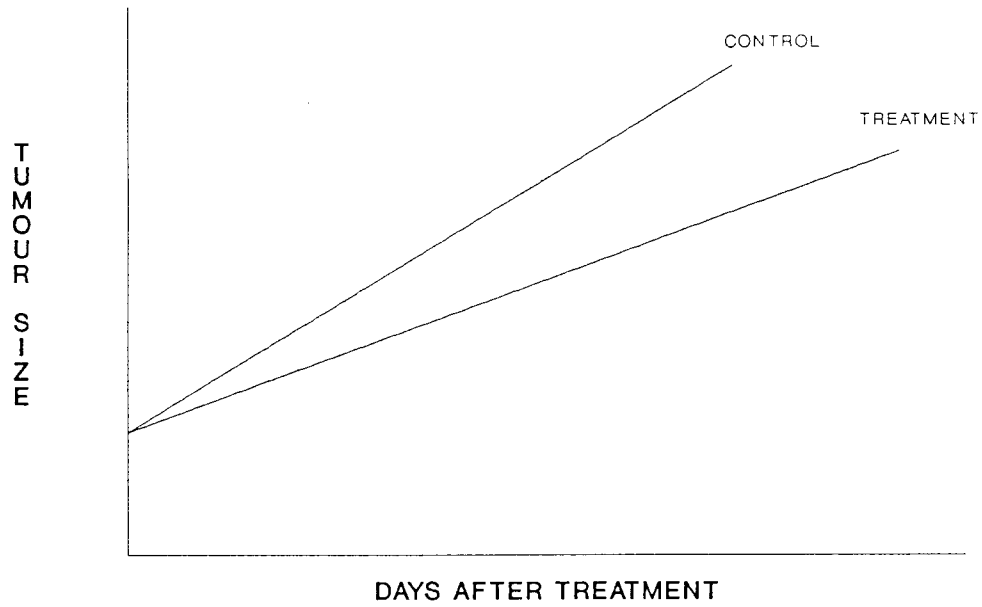


Fig. 8.3. In this growth curve, the lack of shrinkage cannot account for growth retardation, and a changed proliferation rate of surviving cells is indicated.

Thus, because tumours show different growth patterns of regrowth, it is important to measure tumour size regularly.

Tumour size at treatment

Tumour size at treatment is important as blood supply deteriorates and resistance to radiation and drugs increases with increasing tumour size (Stanley *et al.*, 1979). As a consequence, for an equal amount of cell kill; large, more slowly growing tumours will produce a bigger estimate of growth delay than small, faster growing tumours. (Begg, 1980).

It is therefore important to treat tumours that fall within a narrow size range in order to minimise the variability of the results.

Initial studies on tumour growth delay

The results presented here are those of exploratory studies used in the formulation of the hypothesis outlined in chapter 6. At the stage that these experiments were performed, it was believed that DDC pretreatment could result in a significant decrease of SOD (as shown in chapter 7) and that this could result in sensitization to ionizing radiation, but it was not known to what extent the radioprotective properties of DDC would influence this sensitization.

Objectives

* To examine the effect of a time delay of 1 - 24 hours between DDC pretreatment and irradiation on tumour growth in a murine rhabdomyosarcoma
* To calculate tumour growth delay times in these mice.

Methods

Male BALB/c mice bearing a 3-methylcholanthrene induced rhabdomyosarcoma were used. The mice and tumours were maintained as described in Appendix 2.

X-irradiation

Tumour bearing mice were restrained on an acrylic plastic jig and immobilised with elastic bands as shown in Fig. 8.4. The mice were briefly anaesthetised in ether prior to immobilisation. A Philips RT 100 medium voltage X-ray unit was used for the X-irradiations, operating at 100 kVp and 8 mA, with an HVL of 3 mm Al giving a dose rate of from 7.93 to 8.04 Gy/min at the centre of the field. A 2.5 cm diameter circular applicator with an SSD of 10 cm was used and placed directly above the tumour.

Figure 8.4 The irradiation setup for X-irradiations. A 2.5 cm diameter circular applicator with an SSD of 10 cm was used and placed directly above the tumour.

The X-ray unit was calibrated with a flat (small volume - 0.3 cm^3) ionization chamber connected to a Baldwin Farmer secondary standard dosimeter, and placed at the end of the 2.5 cm diameter circular applicator with an SSD of 10 cm. The dose rate at this distance in the centre of the field was measured to be 7.36 Gy/min when the output of the machine was 65 Roentgens/min, measured at 30 cm with a standard 5 cm diameter circular applicator. The output of the machine was measured weekly, and corrections were made for changes in output in the calculation of dose rate. The X-ray dose to the tumours was 11 Gy - 18 Gy.

^{60} Cobalt gamma irradiation

Tumour bearing mice were restrained in acrylic plastic jigs as shown in Fig 8.5. The mice, apart from the tumour-bearing legs were shielded with 10 cm thick lead blocks. 0.5 cm thick acrylic plastic strips placed over the tumours were used as build-up. The mice were briefly anaesthetised in ether prior to immobilisation. Irradiation was performed using an Eldorado 6 ^{60}Co source, with a fixed cast lead collimator.

Figure 8.5 The irradiation setup for irradiation using the Eldorado 6 Cobalt Unit.

The dose rate was measured with a 0.5 cm^3 ionization chamber connected to a Baldwin Farmer secondary standard dosimeter, at 80 cm from the source in a 40 X 40 cm field

with full secondary electron build-up and backscatter provided by 40 cm of wood. The dose rate was 0.516 Gy/min in June 1987, which increased to 0.821 Gy/min in October 1987 as a result of a source change, and was 0.579 Gy/min in June 1990.

In order to calibrate the Eldorado 6 for irradiation of the mouse tumours using the geometric arrangement described above, the 0.5 cm^3 ionization chamber was placed in the position of the tumour and the dose measured.

Neutron irradiation

BALB/c mice were inoculated with a 3-methylcholanthrene induced rhabdomyosarcoma in the same way as for the X-ray experiments. They were briefly anaesthetised in ether before being gently restrained in an acrylic plastic jig with elastic bands as shown in Fig. 8.6. Neutron irradiations were performed using the p(66)/Be(40) neutron therapy isocentric unit of the National Accelerator centre in Faure, South Africa.

Figure 8.6 The irradiation setup for irradiation using the Faure neutron therapy beam.

A 20 X 20 cm field was used, and the mice were positioned such that only the tumour bearing leg was within the field. The jig was placed on 9 cm of acrylic plastic which provided backscatter, and a 20 X 20 X 2 cm closed plastic box containing a tissue equivalent solution (containing 65.6% H_2O, 26.8% glycerol and 7.6% urea (Goodman, 1969)) placed above the tumours served as buildup. An SSD of 150 cm was used. Total dose (neutron + gamma) at the position of the tumours was kindly determined by Dr D.T.L. Jones, using the international protocol for neutron dosimetry (Mijnheer *et al.*, 1987). The dose to the tumours was 7 Gy and 9 - 10

mice were used in each treatment group. Assessment of tumour growth delay was done in the same manner as for the X-ray experiments.

Assessment of response

Tumour growth delay was assessed by measuring the 3 orthogonal diameters, using vernier calipers, of each tumour 3 times per week. The volume of the tumour was calculated assuming a spherical shape by the relationship :

$$V = 4/3\ \pi r^3 \qquad \text{where } r = \frac{1}{3} \times \frac{(a + b + c)}{2}$$

and a, b and c are the orthogonal tumour diameters, until a volume of 0.6 cm^3 was obtained, at which point the mice were sacrificed.

Tumour volume at the time of treatment ranged from 0.155 to 0.333 cm^3 with a mean of 0.219 cm^3 ± 0.038 cm^3 (mean ± SD) Some tumours did not grow in the normal oblate spheroid shape, but in a multilobed or sausage shape. Such irregular shaped tumours were not used in the estimation.

Validation of tumour volume measurement

In order to validate the method used to assess tumour response, certain tumours were measured more than once

during a measurement session. Measures of central tendency were then calculated.

Table 8.1 below shows that during repeated measurements of the same tumour, the standard deviation was usually around 10% of the mean volume.

Table 8.1 Validation of tumour measurement.

Mouse number	mean tumour volume (cm^3)	SD	coefficient of variation	number of measurements
B4	0.453	0.043	9.5%	14
C2	0.359	0.043	11.9%	4
F3	0.352	0.033	9.4%	4
A1	0.270	0.021	7.7%	4
D4	0.300	0.025	8.4%	4
H3	0.360	0.037	10.4%	4

Results and discussion

Growth delay times after DDC pretreatment

Tables 8.2 to 8.4 show the median growth times*, tumour volume doubling times and tumour starting sizes after pretreatment with DDC prior to X-irradiation.

* median of the times taken for a tumour to reach 0.6 cm^3.

Table 8.2 Median growth time, volume doubling time and tumour starting sizes for mice treated with 50 mg/kg DDC intratumourally, followed by a time delay of 1 to 24 hours prior to an 11 Gy X-ray dose.

Treatment	Time between DDC and radiation	number of mice	Growth Time (days)	Doubling Time (days)	Cures	Initial Tumour Volume (cm^3) mean ± SD
None	-	15	6.2	4.0	0	0.255 ± 0.044
11 Gy only	-	8	12.4	9.3	0	0.208 ± 0.016
DDC + 11 Gy	1 hour	4	8.8	3.8	0	0.190 ± 0.036
DDC + 11 Gy	2 hours	4	16.7	8.5	0	0.198 ± 0.010
DDC + 11 Gy	4 hours	6	15.7	9.0	0	0.219 ± 0.034
DDC + 11 Gy	6 hours	4	10.7	7.8	0	0.198 ± 0.028
DDC + 11 Gy	12 hours	4	11.1	6.5	0	0.200 ± 0.020
DDC + 11 Gy	24 hours	4	14.1	11.0	0	0.214 ± 0.035

Table 8.3 Median growth time, volume doubling time and tumour starting sizes for mice treated with 100 mg/kg DDC intratumourally, followed by a time delay of 1 to 24 hours prior to an 11 Gy X-ray dose.

Treatment	Time between DDC and radiation	number of mice	Growth Time (days)	Doubling Time (days)	Cures	Initial Tumour Volume (cm^3) mean ± SD
None	-	15	6.2	4.0	0	0.255 ± 0.044
11 Gy only	-	8	12.4	9.3	0	0.208 ± 0.016
DDC only	-	5	4.0	3.5	0	0.191 ± 0.015
DDC + 11 Gy	1 hour	4	11.8	10.0	0	0.231 ± 0.020
DDC + 11 Gy	2 hours	8	12.8	9.5	0	0.232 ± 0.033
DDC + 11 Gy	4 hours	8	26.3	19.5	3	0.219 ± 0.026
DDC + 11 Gy	6 hours	4	23.5	19.6	1	0.209 ± 0.037
DDC + 11 Gy	12 hours	8	6.2	4.9	0	0.229 ± 0.038
DDC + 11 Gy	24 hours	4	21.3	8.5	0	0.190 ± 0.030

Table 8.4 Median growth time, volume doubling time and tumour starting sizes for mice treated with 150 mg/kg DDC intratumourally, followed by a time delay of 1 to 24 hours prior to an 11 Gy X-ray dose.

Treatment	Time between DDC and radiation	number of mice	Growth Time (days)	Doubling Time (days)	Cures	Initial Tumour Volume (cm^3) mean ± SD
None	-	15	6.2	4.0	0	0.255 ± 0.044
11 Gy only	-	8	12.4	9.3	0	0.208 ± 0.016
DDC + 11 Gy	1 hour	4	15.9	10.4	1	0.191 ± 0.027
DDC + 11 Gy	2 hours	4	10.4	7.1	1	0.188 ± 0.025
DDC + 11 Gy	4 hours	12	12.6	11.6	3	0.249 ± 0.055
DDC + 11 Gy	6 hours	4	13.8	9.5	1	0.235 ± 0.039
DDC + 11 Gy	12 hours	4	13.4	10.3	1	0.235 ± 0.040
DDC + 11 Gy	24 hours	4	6.6	4.0	0	0.231 ± 0.036

When these results were first reported (Kent & Blekkenhorst, 1988), the mean growth times and growth delays were calculated; however in some of the treatment groups, tumour cures were noted and no estimate of growth delay could be calculated. This led to the unfortunate situation where for treatments within the range of curability, growth delay estimates could only be calculated for those tumours which recurred ; i.e. the subgroup least affected by the treatment. The subgroup that was most affected by the treatment (cured tumours) did not recur, and therefore provided no data for calculation of growth delay. This kind of problem and the need for a statistical solution have been discussed previously (Wheldon & Brunton, 1982).

In Tables 8.2 to 8.4, the median growth time is used rather than the mean as the median growth time can

accommodate cured tumours (as long as they do not comprise more than 50% of the group) and thus allow comparisons to be made between treatment groups. Where no cures were noted mean growth times could be compared for significant differences using the Student's *t*-test.

The growth times of tumours receiving 50 mg/kg DDC pretreatment (Table 8.2) at different times prior to irradiation were compared to the growth times of tumours receiving radiation only using a Student's *t*-test, however these differences were not significant at the 5% probability level. When the DDC dose was increased, (Tables 8.3 ; 8.4) some tumour cures are noted making comparisons of mean tumour growth time or volume doubling time impossible.

Although it is not possible to base any conclusions on these results, as no significant differences could be detected between treatment groups, it is clear that DDC has the ability to help cure some of these tumours in certain circumstances, thus some discussion is warranted.

When the time delay between DDC administration and X-irradiation was short, for example 1 hour, pretreatment with 50 or 100 mg/kg DDC resulted in a slight shortening of the tumour growth time when compared to those tumours treated with radiation only. When this time delay was increased to 4 hours, the growth times increased and

cures were noted at 100 mg/kg indicating that tumour sensitization took place.

Treatment with 100 mg/kg DDC 4 or 6 hours prior to irradiation resulted in 4 out of 12 tumours regressing completely. The tumour doubling times also followed a similar pattern. The doubling time was reduced when a 50 mg/kg DDC dose was administered 1 hour prior to irradiation, but was not affected by a higher 100 mg/kg DDC dose when comparison was made with radiation only.

The increase in tumour growth time after a 4 to 6 hour delay between 50 mg/kg DDC and irradiation was not reflected in the volume doubling time, but was shown when the DDC dose was increased to 100 mg/kg.

The starting tumour volumes in these groups were comparable, and should not have affected their response.

The result of increasing the DDC dose to 150 mg/kg was that about 25% of the tumours were cured when the delay between DDC administration and irradiation was 1 to 12 hours (Table 8.4).

Systemic administration of DDC

It was felt that it would be important to determine if systemic administration of DDC could also affect the tumour response.

The tumours used in this later experiment were found to be more radioresistant than those used in the earlier experiments with intratumoural administration of DDC. For this reason a higher dose of radiation, 18 Gy was chosen.

Table 8.5 shows that when DDC was injected intraperitoneally 1 hour prior to irradiation, tumours grew to 0.6 cm^3 4.5 days faster than tumours treated with X-rays alone. When a longer delay was allowed between DDC administration and irradiation, a tumour growth delay of 2.9 days was seen when 2 hours was allowed between DDC pretreatment and irradiation. It is probable that a dose of 100 mg/kg DDC injected systemically resulted in lower DDC concentrations than when DDC was injected intratumourally. For this reason a dose of 1000 mg/kg DDC was given which resulted in 1 out of 5 tumours regressing.

None of these differences were significant at the 5% level (Comparison of mean growth times - Student's *t*-test).

Table 8.5 Tumour growth time and cures in BALB/c mice after systemic DDC pretreatment and radiation.

Treatment	Days to 0.6 cm^3 (mean ± SD)	Cures
18 Gy only	16.9 ± 3.2	0/9
100 mg/kg DDC 1 hr before 18 Gy	12.3 ± 0.5	0/5
100 mg/kg DDC 2 hr before 18 Gy	19.8 ± 1.8	0/5
100 mg/kg DDC 4 hr before 18 Gy	15.8 ± 1.3	0/5
1000 mg/kg DDC 4 hr before 18 Gy	——	1/5

Neutron irradiation

Table 8.6 shows that tumours treated with DDC and neutrons grew faster than those treated with neutrons alone. The mean growth time of tumours treated with 100 mg/kg DDC 1 hour prior to 7 Gy neutrons was found to be significantly different from the mean growth time of tumours treated with neutrons alone ($p<0.05$). There was no difference in the growth time of tumours treated with DDC 1 or 4 hours prior to irradiation.

Table 8.6 Tumour growth times for BALB/c mice treated with neutrons with and without prior treatment with DDC. t - values, read at 17 degrees of freedom are for comparisons of DDC treatments with neutron only treatments. N.S. = not significant.

Treatment	Days to reach 0.6 cm^3 mean ± SD (n=9)	significance
7 Gy neutrons only	12.8 ± 0.9	
100 mg/kg DDC 1 hr before 7 Gy neutrons	10.2 ± 0.8	t = 2.147 ; p<0.05
100 mg/kg DDC 4 hr before 7 Gy neutrons	10.1 ± 0.9	t = 2.009 ; N.S.

The protection factor (PF = 1.3) shown by DDC pretreatment 1 hour prior to neutron irradiation is interesting as it has been previously demonstrated that chemical radioprotectors are more efficient at protecting tissues from low LET radiation than high LET radiation (Sigdestad *et al.*, 1975 ; Sigdestad *et al.*, 1976).

Sigdestad *et al.* (1986) gave as possible reasons for the difference the following points :

a) The influence of free radical scavenging and/or hydrogen ion donation would be greater in low LET because of (i) the degree to which radiolysis of water affects cell killing by radiations of different qualities, and (ii) LET differences in the subcellular dose distribution.

b) The induction of hypoxia by the radioprotector (see chapter 5) would be expected to increase survival to a greater extent after low LET, because of the greater oxygen effect on low LET radiation injury.

It is important to note that the Faure neutron beam has a small gamma component which is highest at the edge of the field (Yudelev & Jones, 1988), where the tumours were situated for the irradiations.

Problems of tumour growth delay studies

Already at this point in the study, some of the limitations of the growth delay assay became apparent. Some of the growth delay times were 'censored'. i.e. the mice never reached the endpoint in question (0.6 cm^3 tumour volume in our case), because their tumours were 'cured'. This kind of experiment where tumours are measured after subcurative therapy is also prone to loss of data from other sources such as premature death (death before the endpoint is reached) due to metastasis or intercurrent disease.

It must be pointed out at this stage that growth delay assays are very frequently used to quantify tumour response to radiation or some other cytotoxic treatment modality, but that volume changes do not necessarily mirror the effect of the treatment on tumour cell survival (Jung *et al.*, 1981). Indeed, the volume changes may depend on not only the surviving tumour cells, but also on factors such as the rate at which the tumours are repopulated by the surviving cells, the rate of resorption of radiation-sterilised cells, the necrotic portion of the tumour, and also on the numbers of host cells migrating into the tumour.

Nevertheless, the tumour growth delay is a very useful method of assessing the response of experimental tumours to radiation and cytotoxic treatment. The assay

requires fewer animals than 50% tumour control dose studies (TCD_{50}) and is therefore more economical.

Denekamp (1979, 1980) reviewed the different assays available for measuring the response of tumours *in situ* to therapy, namely animal survival time, regression rate of tumours, regrowth delay, local tumour control, and loss of incorporated radioactivity. It was argued that any single assay was adequate, provided a dose response relationship could be demonstrated. Survival time and regression rate studies probably yielded the least valuable information, but the tumour growth delay assay appeared to be more closely dependent on cell survival.

The tumour growth delay assay has been shown to yield well defined dose response curves after localised treatment, (for example Thomlinson & Cradock (1967), Field *et al.*, (1968), Denekamp & Harris (1975)).

In dealing with data which contained censored growth delay times, various authors have taken different approaches. Williams *et al.*, (1984) either excluded animals from his analysis, or time of death was taken as the minimum regrowth time, but only if it was longer than the mean of that dose group.

Suit *et al.*, (1978) on the other hand argued that the allotment of some arbitrary value to a cured tumour had no sound theoretical basis.

Robinson *et al.*, (1974) used the slope of the regrowth curve as a measure of effectiveness of treatment, rather than the delay to reach a certain size.

Sheldon & Smith (1975) plotted the mean reciprocals of the growth delay time, regarding controlled tumours to take 'infinite time' to grow to the particular endpoint.

Nowak *et al.*, (1978) used the median doubling time of each treatment group to assess the response of treatment. Some tumours that failed to regrow and therefore had infinitely large doubling times were not a problem, because unless more than half the tumours in a particular group fail to regrow, the median is a parameter that allows these animals to remain part of the analysis.

Fowler *et al.*, (1980) have analysed tumour local control data in tumour growth delay experiments in a number of ways :

* by regarding the tumour growth delay as infinite, and using reciprocals of growth time to obtain an average.
* by allocating some arbitrary growth time - 150, 300 or 1000 days.
* by using the lifespan of the mouse.
* by using the latest time at which a recurrence has ever been observed.

Fowler *et al.*, (1980) examined experiments where both a tumour growth delay and a tumour control (TCD_{50}) assay had been performed to determine enhancement ratios for some adjuvant treatments. They found that agreement between enhancement ratio estimates derived from the regrowth results and tumour cure (TCD_{50}) results was good when these methods were used to handle cured tumours in several experimental situations. When cured tumours were merely ignored, this agreement between tumour growth delay assays and TCD_{50} assays was poor.

Assessment of tumour response using the Kaplan-Meier method

It was realised in the early stages of the investigations reported in this thesis that the use of any of the methods for growth delay described above was not adequate. It was thus concluded that the best way of dealing with censored growth delay data was to make use of the method proposed by Kaplan & Meier (1958). This method allowed the inclusion of growth delay data for tumours both from animals in which tumours were cured, and for tumours lost due to death from intercurrent disease before the endpoint was reached.

This method has been used for some time in clinical trials to estimate survival after different treatments. The prognosis of patients with cancer is often described by a single figure such as 5 year survival, but only the

survival curve shows the complete time course of the disease. The curve shows the probability at different times of survival. The methods used in constructing a survival curve can be used to analyse the time taken to reach any event. Indeed the term 'survival curve' used in the context of the Kaplan-Meier model is merely a less clumsy way of saying 'the probability at successive times that an event has not occurred (Peto, 1984).

The calculation of a survival curve requires only two parameters, namely :

1) a **response variable** : This is essentially a logical yes / no variable. - (Did the patient die ?).
2) a **time variable** : This is the time taken to reach the particular response in question. For example, if the patient died - how long after the start of treatment did he/she survive ? If the patient is still alive / lost to follow up - How long has that patient survived ?

There was thus no reason why a survival curve could not be constructed for the data presented in this thesis, using the time for the tumour to grow to a certain endpoint size as the time variable, and the achievement of the endpoint size or not as the response variable.

This method has been called many names in clinical studies, 'actuarial curve', 'product-limit curve',

'Kaplan-Meier curve', or simply the survival curve. A modified, but essentially identical method, where data is grouped into weeks, months or years is known as the 'Life Table' method (Sylvester *et al.*, 1978).

Comparison of survival curves

In a review of tests for comparing survival curves, Breslow (1984) argues that the standard test for comparison of survival curves is the Mantel-Haenzel or logrank test (Mantel & Haenzel, 1959), (Mantel, 1966).

That the Kaplan-Meier method used in the analysis of tumour growth delay data in this thesis was a suitable method, was underscored by the recent publication of a paper by Stuschke *et al.*, (1990). The authors used computer simulations to test the appropriateness of the method in situations where censored data was present.

They argued that omission of censored growth delay data often leads to biased estimates. They found that the power of the logrank test and the conventional *t*-tests were about the same when comparing uncensored data from two therapy groups. The tests applicable to censored data (logrank) had a markedly higher power than tests applied to a reduced set of complete observed growth delay (Student's *t*-test, Mann-Whitney *u*-test). They asserted that the Kaplan-Meier method is suitable for quantitative and qualitative analysis of growth delay data up to a censoring rate of 30%.

In view of the fact that this approach to growth delay is somewhat novel, details of the method of calculating a survival curve are presented in Appendix 5.

Conclusions

A time dependent modulation effect of DDC was observed, after both intratumoural and systemic administration of DDC. It appeared that a greater degree of sensitization could be achieved if a time lapse was allowed between DDC administration and radiation. This sensitization could be masked by a radioprotective effect if the time lapse was short.

The evidence provided here is not yet adequate to characterise the fine balance between the radioprotective and radiosensitizing action of DDC, nor to determine the concentration and time dependence of these effects.

The presence of 'censored' data made these effects of DDC difficult to describe statistically.

The methods used for analysis of tumour growth delay data were inadequate. The Kaplan-Meier method is suggested as an appropriate alternative.

Chapter 9
A prospective randomised trial in tumour-bearing mice of irradiation with and without prior treatment with DDC

In the previous chapter it was suggested that a 4 hour time delay between DDC administration and irradiation could possibly result in tumour sensitization, and that if this time delay was short, for example 1 hour, the sensitization could be masked by a radioprotective effect.

In order to confirm the results presented in the previous chapter, it was decided to repeat the experiments; but with a change in the methods to limit the possibility of bias in the measurement of tumours and interpretation of results. This was achieved by randomisation and in the way in which data was collected.

Two prospective randomised trials are described where tumour bearing mice were allocated to either :

* DDC pretreatment 1 or 4 hours prior to irradiation.
* Saline placebo pretreatment 1 or 4 hours prior to irradiation.

Methods

Experimental animals and tumours

Female BALB/c mice bearing a 3-methylcholanthrene induced rhabdomyosarcoma were used.

The tumours were maintained by serial passage of tumour from an original group that was inoculated with 3-methylcholanthrene (Appendix 2). As the tumours used in this experiment were derived from different original stock, it was found that they were faster growing and more radioresistant than those used in the initial experiments described in chapter 8. A brief description of the protocol is given below.

Eligibility and exclusion criteria

8 to 12 week old female BALB/c mice bearing a 3-methylcholanthrene induced rhabdomyosarcoma in the right hind gastrocnemius muscle with a volume of between 0.170 and 0.300 cm^3 were considered eligible for the trial.

Mice were excluded from the trial if a) the tumour was not approximately spherical in shape, and b) the tumour was situated on the upper part of the thigh such that it was not possible to position the entire observable tumour within the radiation field.

Stratification and randomisation

A stratified randomisation was performed. The volume of each tumour was calculated as described in chapter 8, and the mice divided into two strata, those bearing small tumours (0.17 - 0.23 cm^3) and those bearing large tumours (0.231 - 0.3 cm^3). The strata were randomised separately. This ensured that the same number of mice bearing small and large tumours fell into the different treatment arms.

Randomisation was performed so that a similar number of mice fell into each treatment group. This was done by generating the numbers 1 to 12 in random order by means of a random number generator*. These numbers were then sequentially drawn, and the treatment allocated in the following way :

No's 1 - 3 : Treatment A	:	DDC pretreatment 1 hour prior to radiation
No's 4 - 6 : Treatment B	:	DDC pretreatment 4 hours prior to radiation
No's 7 - 9 : Treatment C	:	saline pretreatment 1 hour prior to radiation
No's 10 - 12 : Treatment D	:	saline pretreatment 4 hours prior to radiation

This meant that for every twelve mice entered into a stratum, 3 were allocated to each treatment group.

Treatment

A solution of DDC was prepared in 0.9% saline such that 20 µl could be injected at a dose of 100 mg/kg body

* Turbo Pascal Ver.4.0 (Borland)

weight. In the placebo arms, 20 µl saline was injected into the tumours.

The mice were immobilised and the tumours irradiated with gamma rays from an Eldorado 6 ^{60}Co unit as described in Chapter 8. The dose to the tumours was 11 Gy in a single dose. The trial was later repeated with a tumour dose of 25 Gy.

Data collection and analysis

Once a mouse was randomised, it was allocated an identity number, which was marked on the tail. A record of each mouse's treatment allocation was kept with this identity number.

No record of the mouse's treatment group was ever kept with the data retrieval forms, or on the cages. Mice from different treatment groups also shared the same cage. This meant that during the routine measurement of the mouse tumours, the treatment group was unknown to the investigator. This was done to make the assessment of tumour response as 'blind' as possible.

Tumours were measured three times per week and the volume calculated as described in Chapter 8. The tumours were measured until a volume of 0.6 cm^3 was reached, whereupon the mice were sacrificed by prolonged exposure to ether. The mean tumour volume at each day was plotted against

the number of days after treatment. This data is presented in Fig 9.1.

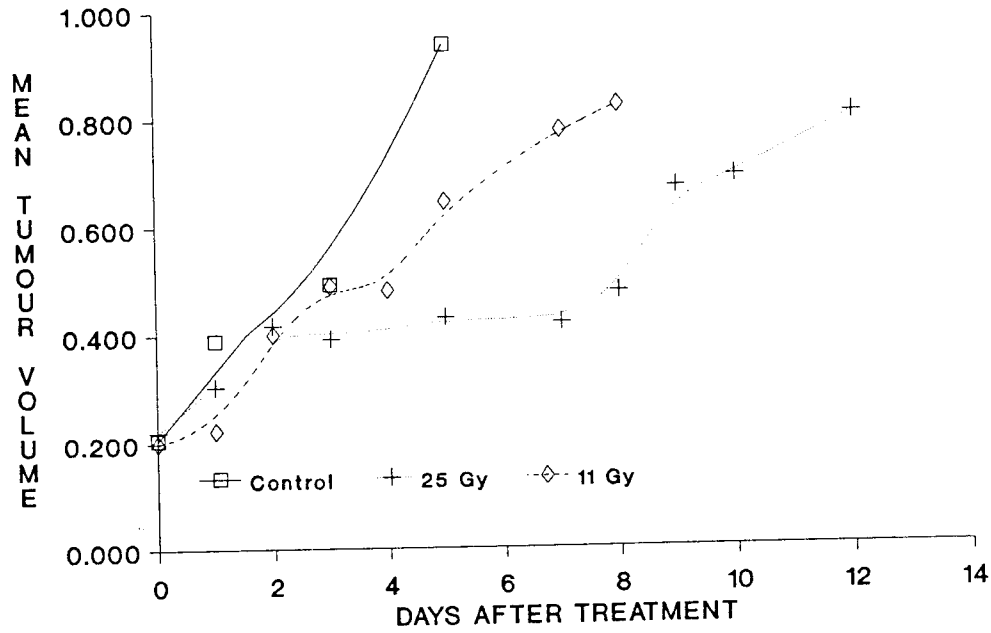


Figure 9.1 Mean tumour volume following no treatment (solid line), 11 Gy (dashed line) and 25 Gy (dotted line). Each point is the mean of between 10 and 30 determinations. Uncertainty bars have been omitted to preserve clarity.

Fig. 9.1 shows that no shrinkage was observed following treatment. After 25 Gy, the tumour growth rate was initially slowed, before regrowth at a slower rate than the control, suggesting the possibility of a stromal damage in addition to tumour cell kill (see chapter 8). The response after 11 Gy was similar to that after 25 Gy, except that the initial slower growth rate was of

considerably shorter duration. Based on this graph, it was felt that an endpoint size of 0.6 cm^3 was justified.

Tumour regression or 'cure' was defined as the absence of any palpable tumour for a period of at least 30 days, whereupon the mouse was sacrificed.

Results

Comparison of placebo arms

On comparison of the two placebo arms where 20 µl of 0.9% saline was injected either 1 or 4 hours intratumourally prior to irradiation with 11 Gy gamma rays, no difference in the response of these 2 groups was discernable in either the large tumour stratum (Fig 9.2) or the small tumour stratum.

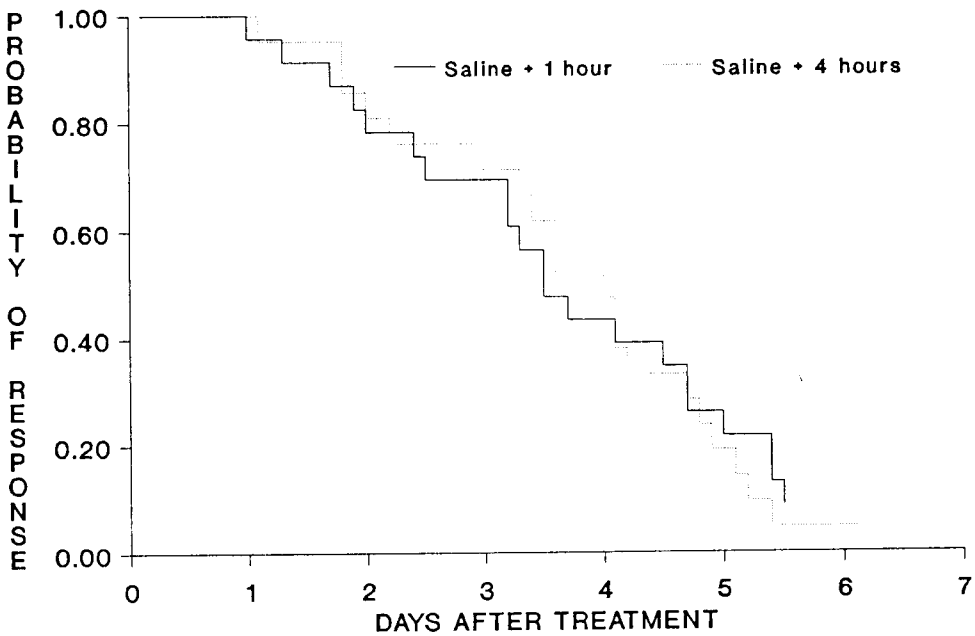


Figure 9.2. Response curves of the two placebo arms.
(solid line) n=23, large stratum Rx : 20 µl saline 1 hr before 11 Gy.
(dotted line) n=21, large stratum Rx : 20 µl saline 4 hr before 11 Gy.
A response was defined as the tumour reaching a volume of 0.6 cm^3.

The Kaplan-Meier median growth times (see Appendix 5) of the placebo arms are presented in Table 9.1.

Table 9.1 Kaplan-Meier median growth times after placebo treatment and 11 Gy.

Treatment arm	LARGE STRATUM		SMALL STRATUM	
	number	growth time	number	growth time
20 µl saline 1 hr before 11 Gy	23	3.3 days	16	4.0 days
20 µl saline 4 hr before 11 Gy	21	3.6 days	17	4.1 days

As the Kaplan-Meier curves were so similar for the two placebo groups it was decided to analyse their mean growth times. As there were no 'censored' data points, this kind of analysis was possible. A Student's *t*-test was performed, and a *t* value of 0.176 was obtained. This indicated that there was no significant difference between these two groups as expected, thus these 2 treatment arms were combined for any further comparisons.

Comparison of DDC arms

Fig. 9.3 shows that there was a significant difference between the DDC groups in the large tumour stratum where the growth time after a DDC pretreatment 4 hours prior to irradiation was increased significantly (Chi square= 4.93, $0.05>p>0.01$, logrank test) when compared to a DDC pretreatment 1 hour prior to 11 Gy.

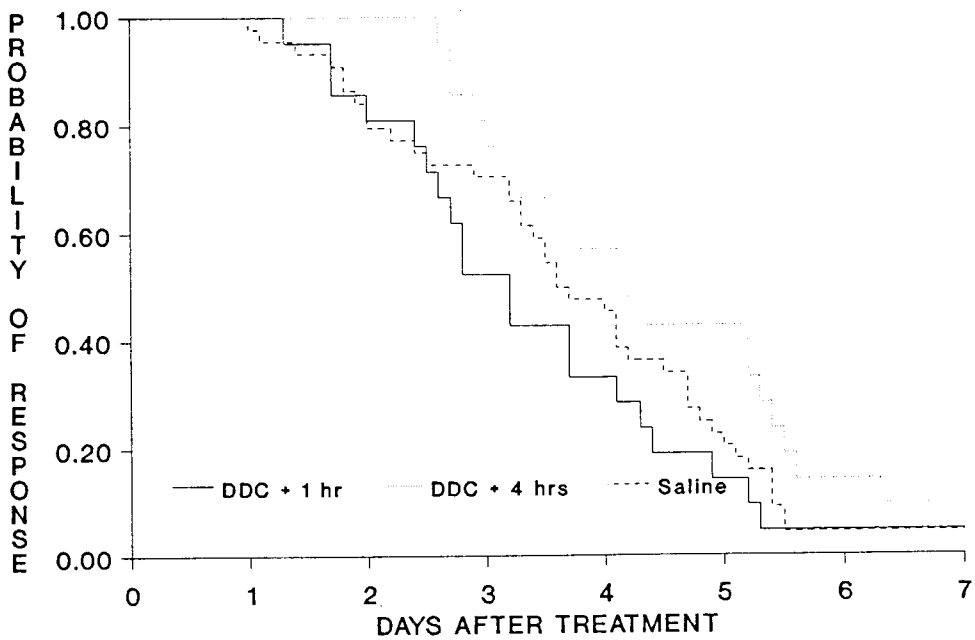


Figure 9.3 Response curves of the two DDC arms of the large tumour strata.
(solid line) n=21, large stratum Rx : 100 mg/kg DDC 1 hr before 11 Gy.
(dotted line) n=21, large stratum Rx : 100 mg/kg DDC 4 hr before 11 Gy.
(dashed line) n=44, large stratum Rx : 20 µl saline before 11 Gy.
A response was defined as the tumour reaching a volume of 0.6 cm^3.

Table 9.2 Shows the Kaplan Meier median growth times of these 3 groups.

In addition, on comparison of the mean growth times of the two DDC arms, a significant difference was found using the Student's *t*-test ($t=2.519$, 40 DF, $p<0.02$). No tumour cures were noted.

Table 9.2 Kaplan-Meier median growth times after DDC pretreatment and 11 Gy for the large tumour and small tumour strata.

Treatment arm	LARGE STRATUM		SMALL STRATUM	
	number	median growth time	number	median growth time
20 μl saline before 11 Gy	44	3.6 days	33	4.6 days
100 mg/kg DDC 1 hr before 11 Gy	21	3.2 days	18	5.0 days
100 mg/kg DDC 4 hr before 11 Gy	21	4.2 days	16	4.9 days

There was no significant difference between the mean growth times, using a Student's *t*-test, of the two large tumour DDC strata and the saline controls. The difference in mean growth time between DDC pretreatment 4 hours prior to irradiation and the saline control was however, close to the 5% significance level (t=1.956, 63 DF $0.1 > p > 0.05$).

There was no measurable difference in the response curves of the DDC groups and saline controls in the small tumour strata (Fig. 9.4).

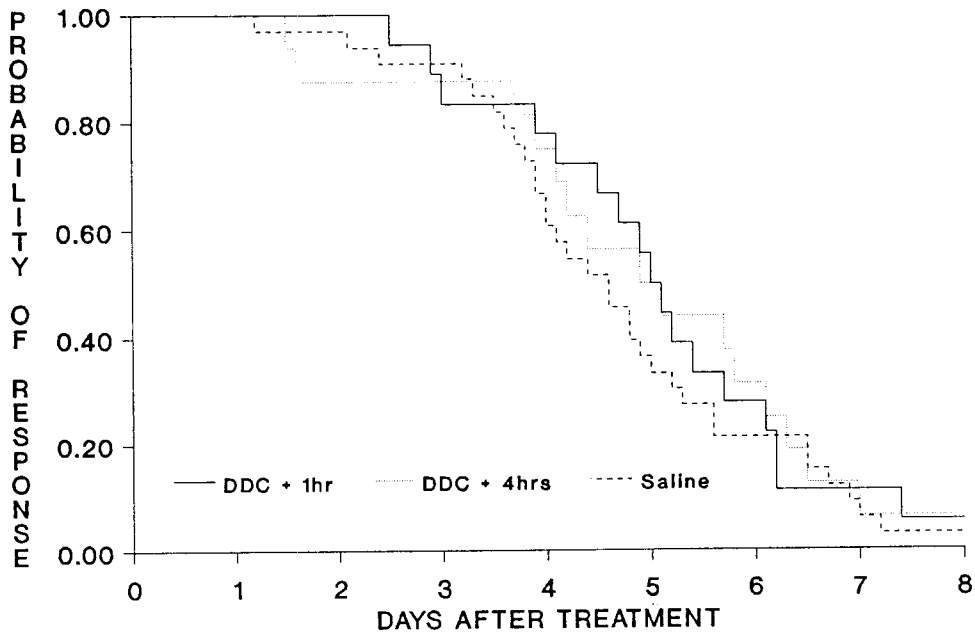


Figure 9.4 Response curves of the two DDC arms of the small tumour strata.
(solid line) n=18, small stratum Rx : 100 mg/kg DDC 1 hr before 11 Gy.
(dotted line) n=16, small stratum Rx : 100 mg/kg DDC 4 hours before 11 Gy.
(dashed line) n=33, small stratum Rx : 20 µl saline before 11 Gy.
A response was defined as the tumour reaching a volume of 0.6 cm^3.

The observation that DDC administration affected the radiation response of larger, but not smaller tumours was unexpected. Therefore the trial was repeated with a higher radiation dose to check if this effect could be replicated.

As was seen with the 11 Gy trial, no difference between the DDC arms was observed in the small tumour stratum

(Fig. 9.5). In the large tumour stratum however, a significant difference (Chi-square = 4.21 0.05› p ›0.01, logrank test) was again seen between the two DDC arms (Fig. 9.6).

The Kaplan-Meier median growth times for the large and small tumour strata after 25 Gy are presented in Table 9.3.

Table 9.3 Kaplan-Meier median growth times after DDC pretreatment and 25 Gy for the large tumour and small tumour strata.

Treatment arm	LARGE STRATUM number	LARGE STRATUM median growth time	SMALL STRATUM number	SMALL STRATUM median growth time
20 µl saline before 25 Gy	10	11.5 days	16	12.0 days
100 mg/kg DDC 1 hr before 25 Gy	10	9.9 days	16	12.4 days
100 mg/kg DDC 4 hr before 25 Gy	10	13.5 days	15	14.0 days

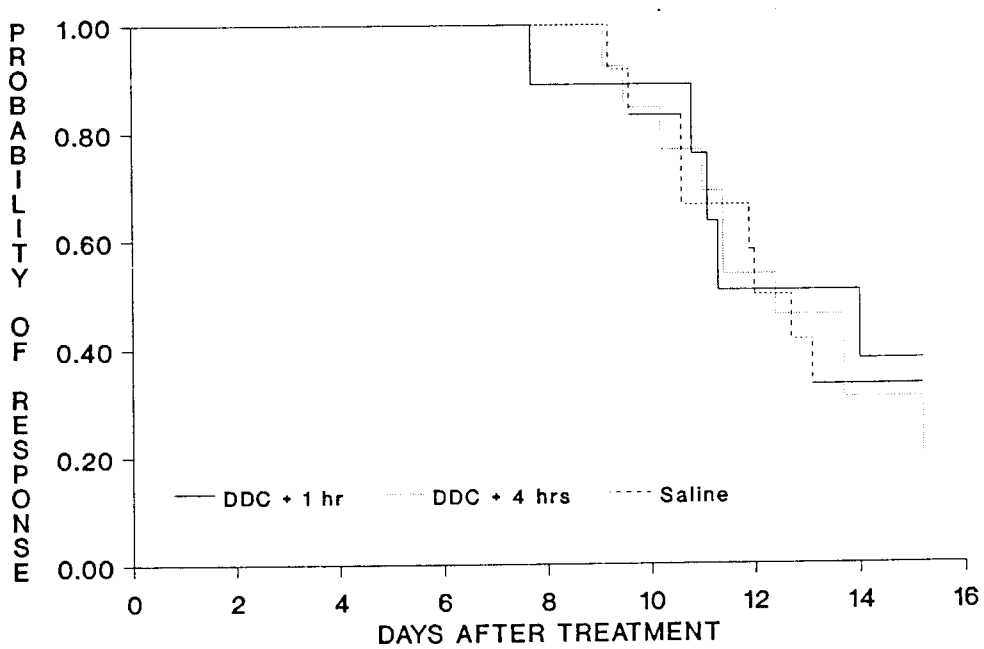


Figure 9.5 Response curves of the two DDC arms of the small tumour strata after DDC pretreatment and 25 Gy.
(solid line) n=16, small stratum Rx : 100 mg/kg DDC 1 hr before 25 Gy.
(dotted line) n=15, small stratum Rx : 100 mg/kg DDC 4 hr before 25 Gy.
(dashed line) n=16, small stratum Rx : 20 µl saline before 25 Gy.
A response was defined as the tumour reaching a volume of 0.6 cm^3.

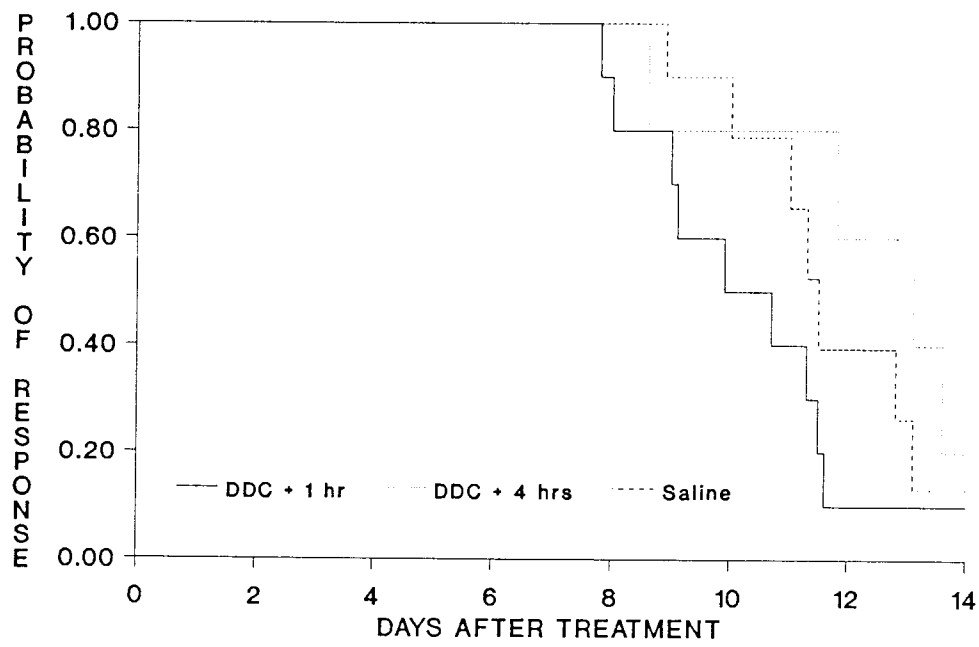


Figure 9.6 Response curves of the two DDC arms of the large tumour strata after DDC pretreatment and 25 Gy.
(solid line) n=10, large stratum Rx : 100 mg/kg DDC 1 hr before 25 Gy.
(dotted line) n=10, large stratum Rx : 100 mg/kg DDC 4 hr before 25 Gy.
(dashed line) n=10, large stratum Rx : 20 µl saline before 25 Gy.
A response was defined as the tumour reaching a volume of 0.6 cm^3.

Discussion

The results presented here confirm the hypothesis presented in chapter 6 that DDC could modulate the effects of radiation injury depending on the time lapse between DDC administration and irradiation. It appeared that in larger tumours (0.230 - 0.300 cm^3) DDC sensitized the tumours to radiation when the time between DDC

administration and radiation was four hours, whereas when this time delay was only one hour, the sensitization appeared to be masked by a radioprotective effect.

The radioprotective effect was most likely a reflection of higher tumour DDC concentrations at the time of irradiation when the time delay was only 1 hour, even though SOD activity was greatly inhibited. When the time delay was increased to 4 hours, the tumour DDC concentration was probably less than at 1 hour, and this allowed the lowered SOD activity to take the dominant role in modifying the response to radiation.

The fact that this time modulation effect of DDC was not observed in the small tumour stratum is interesting. The reasons for this are not clear, but it could be postulated that the clearance time of DDC is faster in small tumours, as the poorly developed tumour vasculature, which could 'trap' DDC in larger tumours, probably does not yet exist in smaller tumours.

It is also possible that the chance of penetrating a major draining blood vessel when administering the DDC, and thus 'losing' the entire dose is greater in smaller tumours. In either of these events, there would be little opportunity for DDC to have any effect either as a radiosensitizer or as a radioprotector.

It is also possible that the hypoxic fraction of the tumour increases with increasing tumour size due to poorly developed tumour vasculature (Stanley *et al.*, 1979). If this is so, then the work of Evans (1985) is very relevant. He showed, using an excision assay that, in mice breathing 5.5% O_2 in nitrogen, there was a ten fold difference in survival of cells after radiation between DDC treated and untreated mice. This could be contrasted with a two fold difference in survival when air-breathing mice were used. The inference here was that DDC was more effective as a sensitizer under hypoxic conditions.

There is also extensive evidence that radioprotection by thiols is related to the level of oxygenation at the time of irradiation (Denekamp *et al.*, 1981a ; Lunec, 1981 ; Parkins *et al.*, 1983). Indeed, Durand (1983) and Durand & Olive (1989) have argued that thiols deplete the cell of oxygen, and that this is the mechanism of radioprotection by thiol compounds.

If the larger tumours did indeed have a greater proportion of hypoxic cells, then a DDC pretreatment may have contributed to further hypoxia and therefore radioresistance in those tumours.

It has been shown that the duration of action of DDC did not exceed 3 hours, and that following oral administration, 60% of the dose was cleared in 3 hours

and was excreted via the lungs and urine (Craven *et al.*, 1976).

It is possible therefore that if the time between DDC administration and irradiation is 4 hours, the effect of DDC on oxygen status in the tumour would have normalised, allowing SOD inhibition to take the dominant place in determining the final response to the radiation.

Conclusions

A statistically significant time modulation effect of DDC has been demonstrated where DDC was protective if administered 1 hour prior to irradiation, and sensitized when administered 4 hours prior to irradiation.

This effect was dependent on the size of the tumour. This could be a reflection of the lower level of oxygenation of larger tumours.

Chapter 10
The effect of DDC pretreatment on the tumour bed effect

In Chapter 8 the effect of the tumour bed effect (TBE) on the growth delay assay was discussed. It is known that the tumour response to modifying agents (sensitizers, protectors, hyperthermia, induced hypoxia etc) may modify stromal damage to a different degree compared with damage to tumour cells. This has been shown for WR 2721 (Begg & Terry, 1983 ; Williams *et al.*, 1984). For this reason, it was decided to examine tumour growth delay following DDC pretreatment and radiation in a situation where either the tumour bed effect was absent, or where only the tumour bed effect was present - i.e. where the tumour cells were not exposed to radiation or DDC. These studies are discussed in this chapter.

Methods

BALB/c mice bearing the 3-methylcholanthrene induced rhabdomyosarcoma on the right hind leg were used in all experiments. Procedures concerning the maintenance of animals and tumours as well as the irradiation of mice and preparation of DDC for intratumoural administration have been described in Appendix 2.

In vivo excision assay

The aim of this experiment was to examine tumour growth delay following treatment where the tumour bed effect was absent.

Tumour-bearing mice were treated with DDC and irradiation, following which the mice were sacrificed and the tumours excised and cell suspensions prepared. These cell suspensions were then inoculated into the legs of untreated recipient mice. Tumour growth was then recorded as described previously.

Assay procedure

The tumour bearing mice were divided into treatment groups as follows :

* No treatment.
* 20 µl saline injected intratumourally prior to 15, 25 or 35 Gy total body irradiation.
* 100 mg/kg DDC injected intratumourally 1 hour prior to 15, 25 or 35 Gy total body irradiation.
* 100 mg/kg DDC injected intratumourally 4 hours prior to 15, 25 or 35 Gy total body irradiation.

Six mice were used for each radiation dose in each treatment group. For the irradiations, mice were placed in an acrylic plastic box with dimensions 15 X 15 X 2.5 cm. The box was closed with a 0.5 cm thick acrylic lid

which served as build up. Many small holes were drilled into the box to provide ventilation during irradiation. An air pump was also used to blow air into the box during irradiation. Total body irradiation (TBI) was performed with an Eldorado 6 ^{60}Co source at an SSD of 80 cm.

Mice were sacrificed by cervical dislocation and the tumours excised within 30 minutes of radiation. A tumour cell inoculum containing approximately 1.6 X 10^6 cells (Appendix 2) was injected into the right hind gastrocnemius muscle of 6 untreated recipient mice per treatment group. Tumour volume was measured three times per week until a volume of 0.6 cm^3 was reached.

Results

The mean growth times following DDC and radiation treatment to the tumour prior to excision and re-inoculation into an untreated mouse are given in Table 10.1.

Table 10.1. The mean growth times following treatment to the tumour prior to excision and re-inoculation into an untreated mouse. *t*-values are given where a significant difference between the DDC treated tumours and saline treated controls exits.

Treatment prior to inoculation	Mean growth time (days)	Significance
Control : no treatment	10.5 ± 0.6	
20 µl saline before 15 Gy	13.9 ± 0.5	
100 mg/kg DDC 1 hr before 15 Gy	15.6 ± 0.5	t = 5.356 p<0.001
100 mg/kg DDC 4 hr before 15 Gy	15.5 ± 1.3	t = 2.653 p<0.05
20 µl saline before 25 Gy	17.9 ± 0.8	
100 mg/kg DDC 1 hr before 25 Gy	18.0 ± 1.7	N.S.
100 mg/kg DDC 4 hr before 25 Gy	17.7 ± 0.8	N.S.
20 µl saline before 35 Gy	23.7 ± 2.7	
100 mg/kg DDC 1 hr before 35 Gy	3/6 tumours 'cured'	
100 mg/kg DDC 4 hr before 35 Gy	4/6 tumours 'cured'	

A significant difference between growth times of DDC and saline treated mice was noted when the pre-inoculation radiation dose was 15 Gy. This difference was not seen when the pre-inoculation dose was 25 Gy. At even higher pre-inoculation doses, some tumours failed to grow within 30 days. It was not possible to compare these groups with a Kaplan-Meier analysis as the censoring rate was above 30%.

Fig. 10.1 and Fig 10.2 show the pattern of tumour growth following pre-inoculation treatment with DDC or saline and 15 or 25 Gy respectively.

The difference between saline and DDC pre-treatment prior to 15 Gy is illustrated in both Figure 10.1 and Figure 10.2. It is important to note that the slopes of the curves of all of the treated groups are the same as the untreated controls. This indicated that the tumour cells were proliferating at the same rate as the untreated controls, a characteristic of the absence of a tumour bed effect.

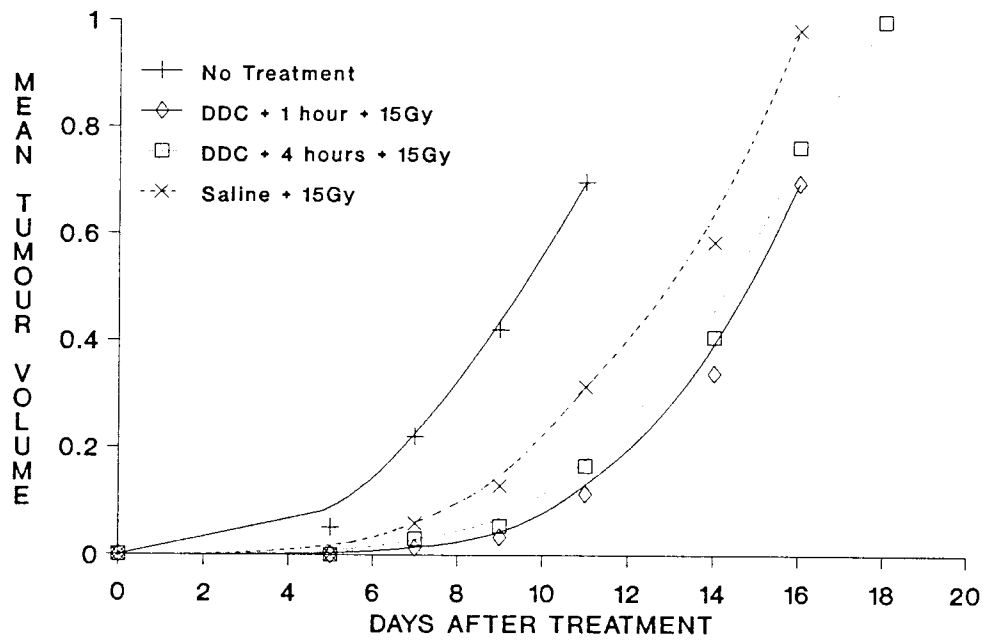


Figure 10.1. The pattern of tumour growth following DDC or saline administration prior to 15 Gy gamma irradiation. The tumour was excised and re-inoculated into an untreated mouse prior to estimation of growth time. Each point is the mean of six determinations. Uncertainty bars have been omitted to preserve clarity.

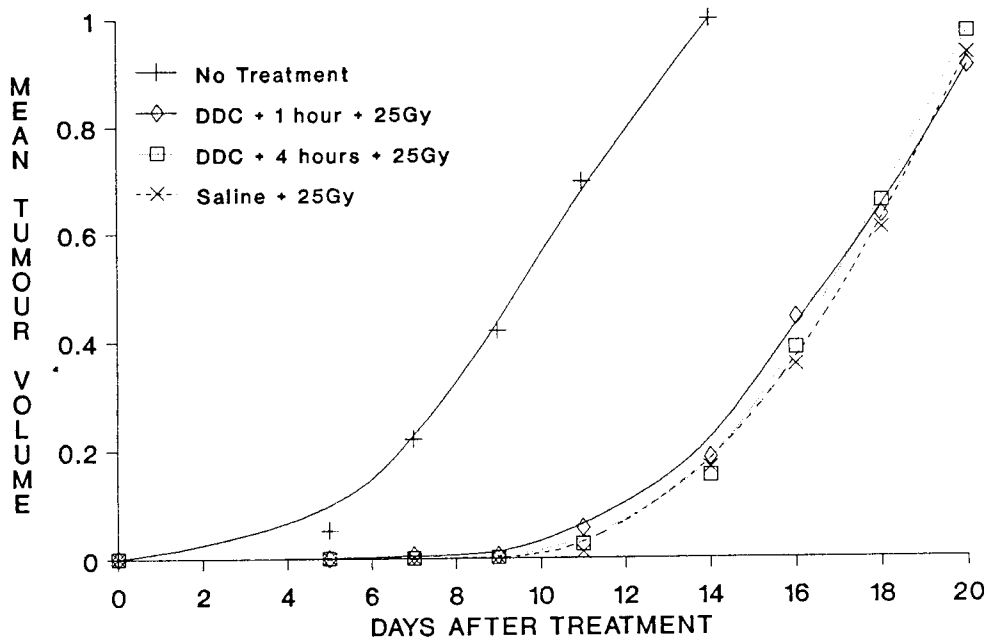


Figure 10.2. The pattern of tumour growth following DDC or saline administration prior to 25 Gy gamma irradiation. The tumour was excised and re-inoculated into an untreated mouse prior to estimation of growth time. Each point is the mean of six determinations. Uncertainty bars have been omitted to preserve clarity.

Methods

The tumour bed effect assay

The principles of the tumour bed effect have been described in chapter 8. In order to examine tumour growth delay after DDC pretreatment and irradiation to

the tumour bed only, the following treatment protocol was applied.

A dose of 25 Gy was chosen as it was felt that this would definitely result in a visible TBE. The aim of this experiment was to examine whether DDC affected the TBE, and for this reason it was important that a clear TBE be demonstrated. Milas *et al.* (1986), using an MCA-4 tumour which exhibits a strong TBE, showed that the TBE started to appear at 5 Gy, increased sharply with increasing radiation dose and approached a plateau at approximately 15 - 20 Gy. For this reason a dose of 25 Gy was chosen.
A dose of 100 mg/kg or 20 µl 0.9% saline was injected directly into the right hind gastrocnemius muscle either 1 or 4 hours prior to irradiation of the leg with 25 Gy gamma rays.

The mice were restrained in an acrylic plastic jig for leg irradiations in the same way as for leg tumour irradiations as described in Chapter 9.

Twenty-four hours after irradiation, a tumour cell suspension containing approximately 1.6×10^6 cells (Appendix 2) was injected into the gastrocnemius muscle of the irradiated leg.

Tumour volume was then measured three times per week as described in Chapter 8, until a volume of 0.6 cm^3 was reached.

Four groups of 10 mice each were used in this experiment, and were placed into the following treatment groups :

* No treatment.
* 20 µl saline injected prior to 25 Gy irradiation to the right hind leg.
* 100 mg/kg DDC injected 1 hour prior 25 Gy irradiation to the right hind leg.
* 100 mg/kg DDC injected 4 hours prior to 25 Gy irradiation to the right hind leg.

Results

Table 10.2 shows the mean growth times of tumours following DDC or saline and 25 Gy irradiation of the tumour bed prior to tumour cell inoculation. No difference was detectable between DDC pretreatment 1 or 4 hours prior to irradiation, or between these treatments and the saline controls.

Table 10.2. The mean growth times of tumours following treatment to the tumour bed or stroma prior to tumour inoculation.

Treatment prior to inoculation	**Mean growth time (days)**
Control : no treatment	19.2 ± 0.6
20 µl saline before 25 Gy	22.3 ± 2.8
100 mg/kg DDC 1 hr before 25 Gy	21.6 ± 1.6
100 mg/kg DDC 4 hr before 25 Gy	22.5 ± 2.2

Figure 10.3 shows the pattern of tumour growth following inoculation. It can be seen that tumour cells inoculated into a pre-irradiated leg took longer to establish tumours than the controls, and further that their growth rate was reduced. This was especially evident when the tumours grew larger.

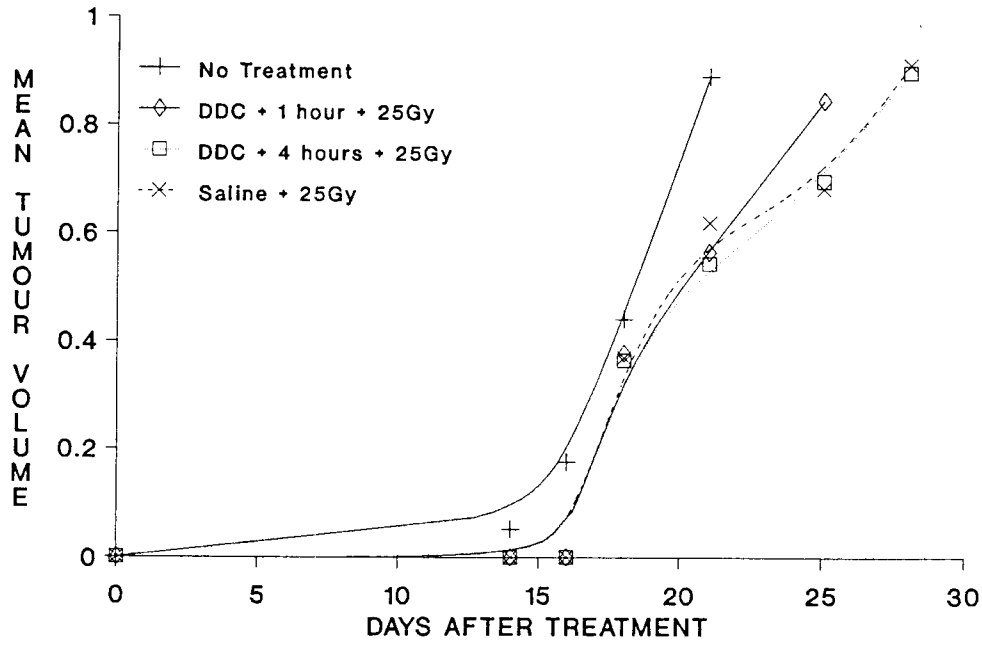


Figure 10.3 The pattern of tumour growth following DDC or saline administration prior to 15 Gy gamma irradiation to the stroma or tumour bed. Each point is the mean of ten determinations. Uncertainty bars have been omitted to preserve clarity.

Discussion

The experiments in this chapter illustrate a more detailed analysis of tumour growth delay which enables the separate analyses of actual growth delay representing tumour cell kill and changes in growth rate representing stromal injury. For any clinical application of modifiers of the effects of radiation, it may be

important to distinguish between these two modes of action (Williams *et al.*, 1984).

A comparison of the growth curves from the excision assay, where TBE is absent and the TBE curve, where there is no direct injury to the tumour cells clearly shows these two modes of action. Figs. 10.1 and 10.2 show growth curves with similar slopes and no change of growth rate, whereas Fig. 10.3 shows a reduced slope following irradiation of the tumour bed. Clearly, the results presented in the previous chapter represent a combination of these effects (see Fig 9.1).

It was not possible to show that DDC made tumour cells sensitive to radiation in all repetitions of the excision assay (Table 10.1), nor were any significant differences between the two DDC pretreatment groups (DDC administration 1 or 4 hours prior to radiation) detected.

Fig 10.1 shows that DDC sensitized the *tumour cells* to 15 Gy irradiation, tumours taking significantly longer to reach an endpoint volume of 0.6 cm^3 after DDC pretreatment.

An important feature of the excision assay described here is that the tumours were all greater than 0.3 cm^3 at the time of irradiation and probably had a fair proportion of hypoxic cells. In chapter 9, tumour sensitization was only noted in the 'larger' tumour stratum (volume = 0.230

- 0.300 cm^3), and it was argued that DDC may be more effective as a sensitizer under hypoxic conditions.

The fact that no effect of DDC on the stroma could be demonstrated could also be a reflection that the response of cells to DDC and radiation is dependent on the level of oxygenation of the tissue, as it is most likely that the irradiated muscle in this experiment was well oxygenated.

Conclusion

The action of DDC in modifying the radiation response of tumours is due to a change in sensitivity of the tumour cells rather than the tumour bed or stroma.

Chapter 11
Survival after DDC administration

The protective effect of DDC against total body irradiation (TBI) has been recognised for almost 35 years. One of the earliest reports is that of Van Bekkum (1956), who showed that DDC protected mice significantly against the lethal effects of total body irradiation. Recent reports corroborate this finding, for example Evans *et al.* (1983a) showed that 100 mg/kg DDC injected 30 minutes before TBI increased the $LD_{50/30}$ from 7.8 Gy to 14.9 Gy. The $LD_{50/30}$ is the dose of radiation required to kill 50% of the mice within 30 days.

In view of the results of the previous chapters, showing a time-modulation effect of DDC, it was decided to investigate whether a time delay of 4 hours prior to irradiation would reverse this protective effect.

Objectives

* To examine the effect on mouse survival of DDC administered 1 or 4 hours prior to total body irradiation as measured by the $LD_{50/30}$ assay.

Methods

Experimental animals and DDC pretreatment

C57/Bl mice, bred and maintained as described in Appendix 2 were used in the survival experiments.

DDC solutions for intraperitoneal administration were prepared as described in Appendix 2. The DDC was administered intraperitoneally in a total volume of 100 µl.

Total body irradiation

Seven to eight mice were placed in an acrylic plastic jig at a time for total body irradiation as described in Chapter 10. Fifteen mice were used for each dose point. Mouse mortality was checked daily for 30 days. Radiation dose response curves for lethality were constructed and fitted by probit analysis as described by Finney (1971) These were used to calculate the values of $LD_{50/30}$. Linear regression analysis was performed on the probits of response versus the natural logarithm of the dose, and the correlation coefficient calculated (Finney, 1971).

Results and Discussion

Table 11.1 shows the results of an experiment where various doses of DDC were injected i.p. into mice 4 hours prior to 7.5 Gy gamma irradiation.

Table 11.1. Survival after treatment with various doses of DDC, followed by 7.5 Gy total body irradiation 4 hours later.

DDC dose (mg/kg)	Survival
0	3/15
0.5	6/7
5	6/7
50	5/7
250	3/7
500	5/15
1000	7/7

In most cases DDC offered some protection against the effects of total body irradiation, however this protection was minimal at a dose of 500 mg/kg where 10 out of 15 mice succumbed to the treatment. When the dose was increased to 1000 mg/kg, DDC again strongly protected the mice. For this reason, 500 mg/kg was the dose of DDC chosen for the $LD_{50/30}$ determinations.

The results of probit analysis for determination of the $LD_{50/30}$ are given in Table 11.2 and Fig. 11.1.

Table 11.2. $LD_{50/30}$ values for C57/Bl mice exposed to total body irradiation with and without prior treatment with DDC.

Treatment	$LD_{50/30} \pm$ SD	correlation coefficient of probit line
Radiation (RT) only	7.50 ± 0.08 Gy	0.870
500 mg/kg DDC 1 hr before RT	8.96 ± 0.20 Gy	0.671
500 mg/kg DDC 4 hr before RT	7.93 ± 0.19 Gy	0.523
500 mg/kg DDC immediately after RT	7.80 ± 0.21 Gy	0.744

When DDC was administered 1 hour prior to total body irradiation, a moderate degree of protection was obtained (DMF = 1.2 at LD_{50}).

No significant differences between the $LD_{50/30}$ values were detected between mice treated with DDC 4 hours prior to irradiation and mice treated with radiation only.

DDC given immediately after irradiation was not found to effect the $LD_{50/30}$ value significantly.

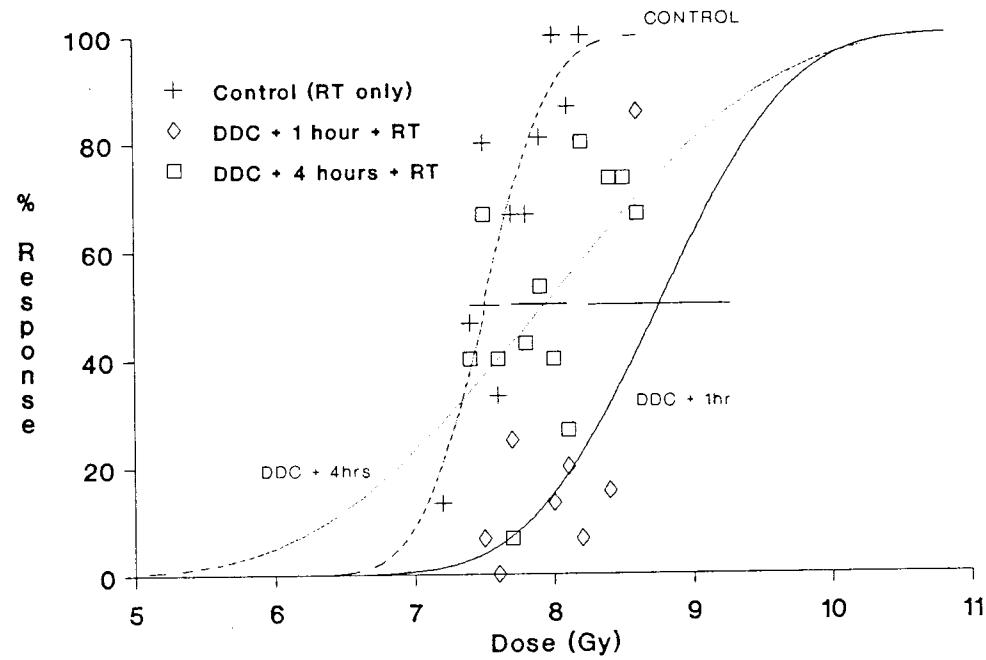


Figure 11.1 Isoeffect curves for lethality in C57/Bl mice after 500 mg/kg DDC administered 1 or 4 hours prior to radiation or radiation only. Bars represent $LD_{50/30}$ ± S.D.

These results are in agreement with those of Evans *et al.* (1983a) who were unable to show any significant radioprotection at a dose of 500 mg/kg DDC 30 minutes before TBI, but maximum protection at a dose of 1000 mg/kg DDC.

The data did not show any sensitization to the effects of TBI by DDC, and in all cases DDC pretreatment resulted in some degree of protection.

The lower $LD_{50/30}$ for DDC pretreatment 4 hours prior to irradiation compared to DDC pretreatment 1 hour prior to irradiation is probably an indication that most of the DDC was metabolised by the time of irradiation. No time modulation effect of DDC is suggested by these results.

Chapter 12
In vitro responses to DDC and irradiation

In Chapter 6 it was hypothesised that radiosensitization could occur as a result of SOD inhibition by DDC, but could be masked by a radioprotective effect if the tissue levels of DDC were too high at the time of irradiation.

This section sets out to examine whether cells in culture can be sensitized to radiation by DDC pretreatment if DDC is removed prior to irradiation.

Objectives

* To determine the survival of B16 mouse melanoma cells after irradiation when the cells have been pretreated with DDC and the DDC washed out of the medium prior to irradiation.
* To examine the effect of a time interval of either one or four hours between DDC administration and irradiation.

Methods

Procedures used for the routine maintenance and treatment of cells are described in Appendix 3.

Briefly, these investigations concerned the response of B16 mouse melanoma cells after having been exposed to various concentrations of DDC in serum-free McCoys 5A

medium for 45 minutes at 37°C. The response of the cells to radiation was assessed after DDC was removed by 3 successive washings in Hanks Balanced Salt Solution (HBSS) and the cells re-incubated in DDC-free, serum containing medium for either 1 or 4 hours.

Results and Discussion

Figure 12.1 shows the survival of B16 mouse melanoma cells following exposure to concentrations of DDC ranging from 10^{-9}M to 10^{-3}M for 45 minutes at 37°C. It was noted that low concentrations of DDC (10^{-9}M ; 10^{-8}M) actually enhanced the survival of cells to above that of control levels. At higher concentrations, DDC toxicity increased.

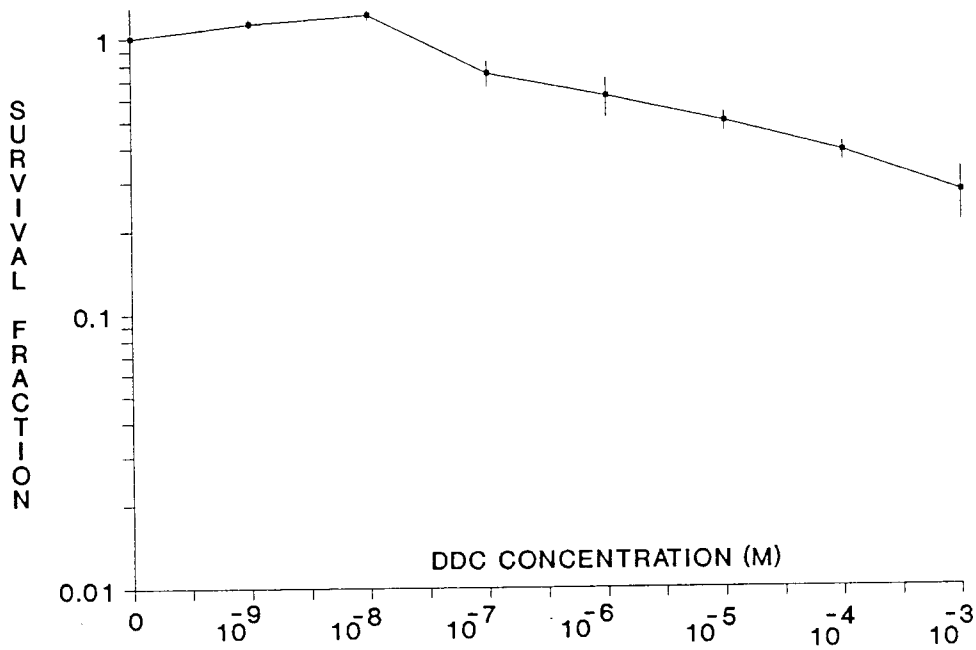


Figure 12.1 Survival of B16 cells after incubation with various concentrations of DDC for 45 minutes. Bars represent the mean ± S.D of 3 replicates.

Figure 12.2 shows the response of B16 cells to 10^{-6}M DDC pretreatment for 45 minutes prior to gamma irradiation. In the case of the DDC pre-treated cells, the survival fractions were calculated as ratios of survival after DDC plus radiation to survival after DDC only. This normalisation of the DDC pre-treatment curve was done to allow assessment of the radiation effect once the effects of DDC had been accounted for.

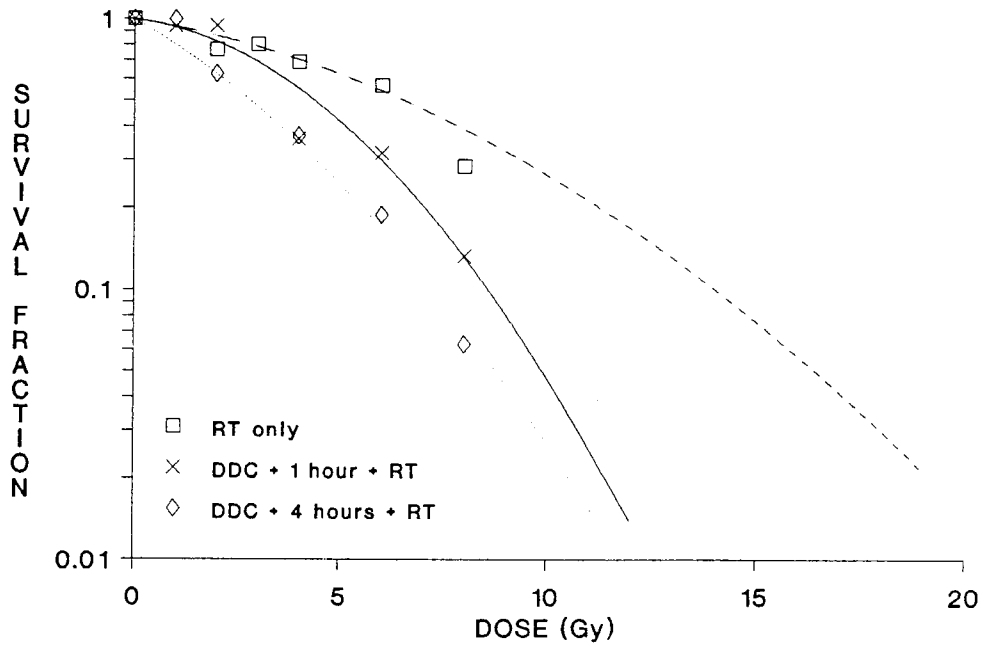


Figure 12.2 Survival curves of B16 cells after pretreatment with 10^{-6}M DDC for 45 minutes followed by a 1 hour (solid line) or 4 hour (dotted line) delay before irradiation (RT) (dashed line) Points represent the mean of 3 replicates.

Stone *et al.* (1978) asserted that a large concentration of DDC must be present at all times to maintain the inhibitory effects of SOD in order to cause radiosensitization. These investigators were unable to show sensitization of erythrocytes to radiation haemolysis when DDC was washed off the erythrocytes prior to radiation, whereas a substantial haemolysis was noted when DDC was present during irradiation.

The results observed in erythrocytes may not be applicable to other cell types, as was found in the work described here. Fig. 12.2 shows that even when DDC was washed off the cells by 3 successive washes in HBSS, a substantial enhancement of radiation induced lethality was noted. The DDC pretreatment sensitized the cells with a DMF (dose modifying factor) of 1.4 when the time between DDC exposure and radiation was 1 hour (see Appendix 3 for the calculation of DMF). When the cells are incubated in DDC-free medium for four hours prior to irradiation, this sensitization was even greater (DMF = 2.5).

Analysis of the curves in Fig 12.2 show that DDC modified both the slope and shoulder of the curve. This result is consistent with that of Lin *et al.* (1979b) who studied cells that were irradiated in the presence of DDC. Lin *et al.* (1979b) argued that the ultrastructural changes in the mitochondria induced by DDC (see Chapter 4) were similar to those of cells following exposure to hyperbaric oxygen. Westman & Marklund (1983) found that by exposing erythrocytes to 3 X 10^{-3}M DDC for 90 minutes produced a 98% inhibition of SOD, and SOD activity was only 10% of control activity 24 hours later. This suggests that the increased sensitivity to irradiation, even 4 hours after DDC pretreatment, must be at least in part due to O_2^- mediated toxicity.

The observation that low concentrations of DDC appear to increase cell survival (Fig. 12.1) is consistent with the work of Rigas *et al.* (1979) and Allalunis-Turner & Chapman (1984).

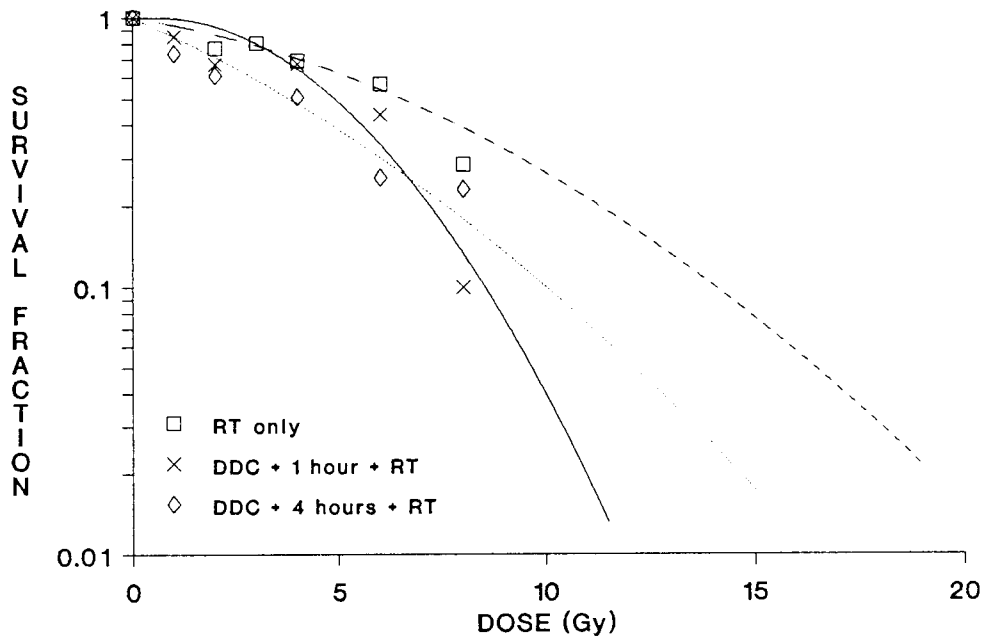


Figure 12.3 Survival curves of B16 cells after pretreatment with 10^{-7}M DDC for 45 minutes followed by a 1 hour (solid line) or 4 hour (dotted line) delay before irradiation (RT) (dashed line) Points represent the mean of 3 replicates.

When a lower concentration (10^{-7}M) was used for DDC pre-treatment, (Fig. 12.3) the effect was much the same, except that the addition of a 4 hour gap between DDC and radiation did not result in as great a sensitization as that seen after 10^{-6}M DDC. The slope of the curve was

also not as greatly reduced as with the higher concentration of DDC, yet the decrease in the shoulder was still evident.

Conclusion

It appears therefore, that DDC pretreatment can sensitize cells to radiation even when DDC has been washed away, and that this sensitization is still seen when a time interval is allowed between DDC pretreatment and irradiation. It is suggested that this is probably due to a lowered SOD activity.

Chapter 13
DDC and Hyperthermia

There is currently great interest in hyperthermia as a treatment modality in cancer. It is clear that the mode of action or the 'hyperthermia target' resulting in cell death may be different to that for ionizing radiation. However there are some indications that heat damage may be modulated or influenced by superoxide (references below). In view of the effect of DDC on SOD, it was considered of great interest to further investigate the effect of DDC on the heat response of cells in culture. In this chapter, these studies are detailed.

Rationale for DDC as a heat sensitizer

McCord *et al.* (1973) showed that cells with lowered SOD activity may be more susceptible to thermal damage than other cells, and postulated that inhibition of SOD by DDC might act as a heat sensitizer. A number of investigators have examined this.

Hahn *et al.* (1975) showed that hyperthermia altered the penetration of bleomycin and adriamycin into mammalian cells. Lin (1979a) extended this reasoning and postulated that if more DDC enters the cell as a consequence of heat treatment, more SOD would be inactivated. This seemed to be consistent with the results of (Ciborowski *et al.*, 1978) which showed a

greater inhibitory effect on SOD activity in cells exposed to increased temperatures.

Evans *et al.* (1983b) showed that if DDC was present in the medium for 1 hour before, but removed prior to heat treatment, the same survival curve as after treatment with heat alone was obtained, indicating that DDC had to be present on the cells during heat exposure to be of any effect. They argued that this rendered a mechanism involving lowered levels of SOD to explain heat sensitization by DDC very unlikely. Instead, they argued for a mechanism where DDC had the ability to affect the repair of potentially lethal damage in cells following heat exposure.

Kapp & Hahn (1979) showed that various thiol compounds including cysteamine, 2-aminoethylisothiourium bromide and cysteine could enhance hyperthermia-induced cell killing. They showed that the drugs alone were not toxic at 37°C, however a marked potentiation of heat killing by these compounds was seen at temperatures above 42°C.

Mitchell & Russo (1983) asserted that exogenous thiols potentiate thermal killing via a peroxide producing reaction involving the formation of a thiol-Cu complex. Issels *et al.* (1984) suggested that superoxide and hydrogen peroxide, generated by the autooxidation of

thiols in the presence of oxygen are involved in the potentiation of heat killing.

As far as DDC itself is concerned, Lin *et al.* (1979a) showed that when DON cells were exposed to 43°C in the presence of DDC, a marked increase in cell killing was noted when compared with either exposure to DDC or 43°C alone.

Lin *et al.* (1985) showed a biphasic toxicity of DDC (see chapter 4), where DDC was toxic at concentrations of 1 X 10^{-5}M and greater than 1 X 10^{-3}M, but not at concentrations of 1 X 10^{-6}M and 1 X 10^{-4}M. The combination of DDC and other cytotoxic agents such as radiation, adriamycin, bleomycin, as well as hyperthermia enhanced cell kill in the two cytotoxic ranges of concentrations (1 X 10^{-5}M ; > 1 X 10^{-3}M). It was argued that this may represent an important mechanism of DDC toxicity that is independent of the toxic effects caused by DDC-induced SOD inhibition or the action of DDC as a thiol compound.

Objectives

* To examine the effect of DDC on cells following exposure to heat, where DDC was washed off the cells prior to heat exposure.

Methods

Procedures used for the routine maintenance and treatment of cells are described in Appendix 3.

Briefly, these investigations concerned the response of B16 mouse melanoma cells after DDC and heat treatment. The cells were exposed to various concentrations of DDC in serum-free McCoys 5A medium for 45 minutes at 37°C. The response of the cells to immersion in a water bath at 43°C for 0 - 60 minutes was assessed after DDC had been washed off and the cells reincubated in DDC-free, serum containing medium for either 1 or 4 hours.

Results and Discussion

Fig 13.1 shows the response of cells to either 43°C alone or pretreatment with 10^{-4}M DDC for 45 minutes 1 or 4 hours prior to exposure to 43°C.

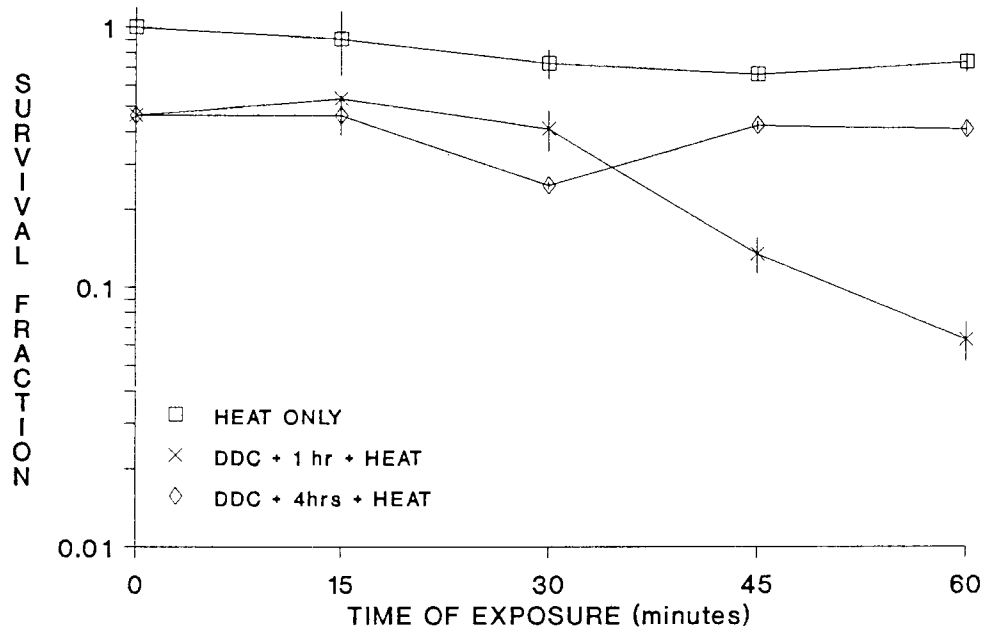


Figure 13.1 Survival of B16 cells after pretreatment with 10^{-4}M DDC for 45 minutes at 37°C followed by a 1 or 4 hour delay before hyperthermia (43°C). Bars represent the mean ± S.D. of 3 replicates.

Survival fractions of the 'heat only' curve were calculated after 'sham-DDC pretreatment' (i.e. cells were exposed to serum-free medium for 45 minutes, followed by 3 successive washings in HBSS). When a time delay of only 1 hour was allowed between DDC incubation and hyperthermia, an enhanced cell kill was seen at heat exposures for 45 or 60 minutes. However when the time between DDC incubation and hyperthermia was 4 hours, this enhancement of cell kill was not seen.

Fig. 13.2 shows the survival of cells after incubation with 10^{-6}M to 10^{-3}M DDC for 45 minutes followed by a 1 hour delay in DDC-free medium at 37°C before exposure to 43°C for 0 - 60 minutes.

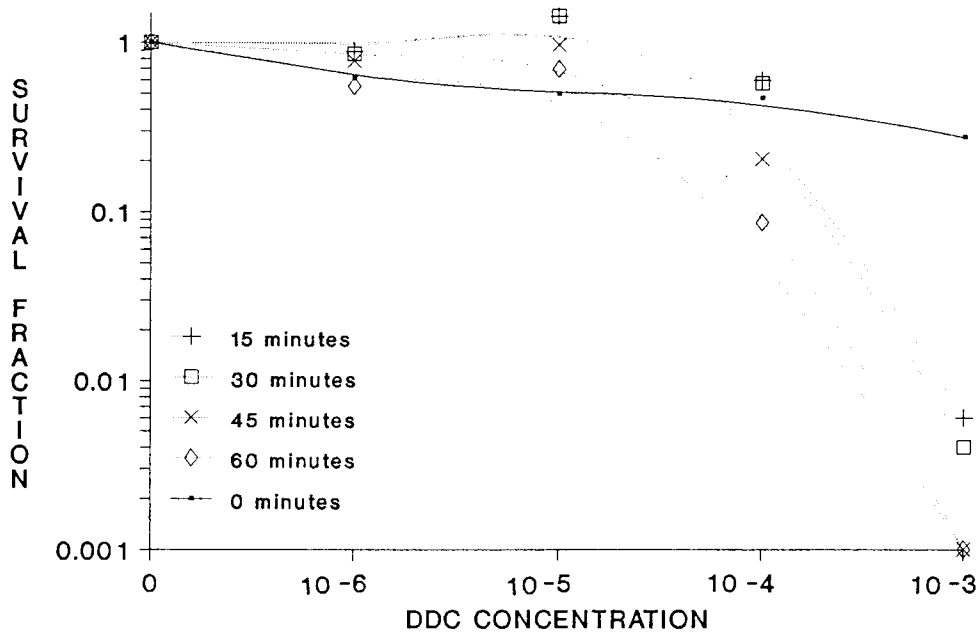


Figure 13.2 Survival of B16 cells after pretreatment with various concentrations of DDC for 45 minutes at 37°C followed by a 1 hour delay before hyperthermia (43°C) of varying durations. Points represent the mean of 3 replicates.

Low concentrations of DDC (1 X 10^{-6}M and 1 X 10^{-5}M) enhanced the survival of heat treated cells. This has been demonstrated before, where DDC was shown to stimulate cell growth, possibly due to an ability to trigger cells into cycle (Allalunis-Turner & Chapman,

1984). It is probably not an expression of thermoprotection. Incubation with higher concentrations of DDC (1 X 10^{-4}M and 1 X 10^{-3}M) on the other hand resulted in an increased cell kill in conjunction with hyperthermia.

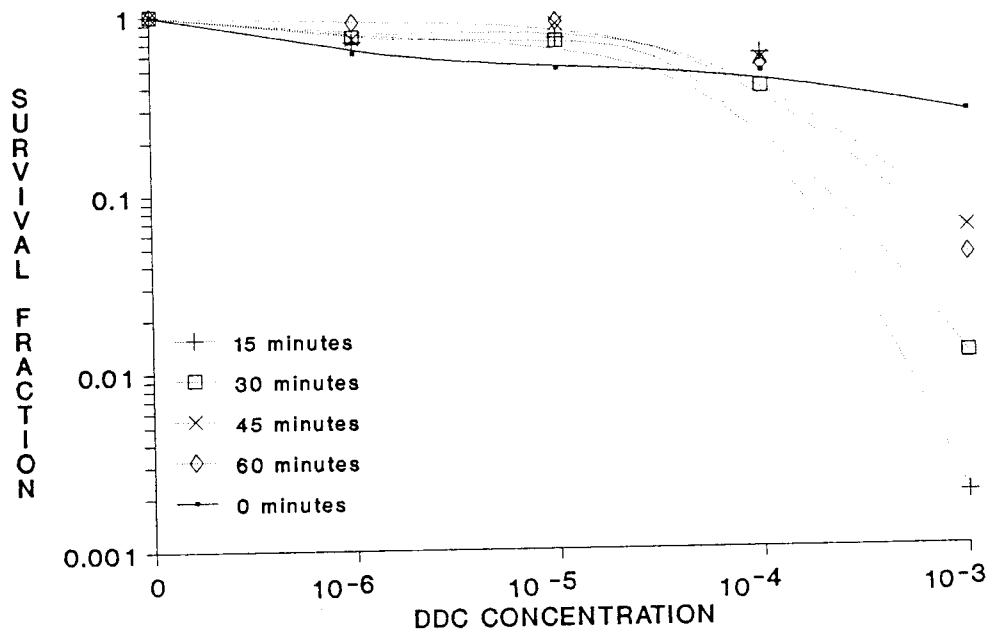


Figure 13.3 Survival of B16 cells after pretreatment with various concentrations of DDC for 45 minutes at 37°C followed by a 4 hour delay before hyperthermia (43°C) of varying durations. Points represent the mean of 3 replicates.

When a 4 hour delay was allowed between DDC pretreatment and hyperthermia, the enhanced survival seen at low concentrations of DDC was not as great as when the delay was 1 hour. Incubation with 1 X 10^{-4}M DDC 4 hours prior

to hyperthermia no longer enhanced the heat toxicity, however at higher concentrations (1 X 10^{-3}M), DDC again enhanced the toxic effects of hyperthermia.

These results show that DDC can sensitize cells to hyperthermia treatment, and this is evident even when DDC is washed off the cells prior to hyperthermia. This is in contrast to the work of Evans *et al.* (1983b) who exposed Chinese hamster cells in Eagle's MEM (minimum essential medium) or HBSS to DDC and temperatures of 43°C. They showed that DDC had to be present during heat exposure in order for it to have any effect.

It could be argued however that a thiol-Cu complex of the type suggested by Mitchell & Russo (1983) involving DDC might have formed during exposure to DDC. Indeed, McCoys 5A medium, used in this work, contains more copper than HBSS or MEM (Biaglow *et al.*, 1984), the medium used by Evans *et al.* (1983). This DDC-copper complex could have become protein-bound, and thus remained after washing.

Activated oxygen species such as superoxide and hydrogen peroxide are also produced during the autooxidation of thiols in the presence of oxygen (Issels *et al.*, 1984 ; Biaglow *et al.*, 1984). This is relevant as a situation may arise where a DDC-Cu complex is involved in producing reactive oxygen species, whose toxicity may be exacerbated by the fact that SOD is inhibited.

It has been shown that hyperthermia treatment can cause the production of superoxide in CHO cells treated with 45°C for 15 minutes (Loven *et al.*, 1988). The addition of exogenous SOD was found to increase survival approximately 2 fold following heat exposure (Downes & Loven, 1988).

Conclusion

DDC is a potential heat sensitizer. It is not clear at this stage whether this is due to an inhibition of SOD, or due to potentiation of heat killing by thiol oxidation or both. Further studies in this direction are warranted.

Chapter 14
Overview and implications of this work

This study has concerned itself primarily with the inhibition of superoxide dismutase by diethyldithiocarbamate in order to sensitize tumours to ionizing radiation.

The use of DDC as an inhibitor of SOD has however meant that any sensitization resulting from SOD inhibition could be masked by a radioprotective effect by DDC.

This contradiction has been the cause of a debate over the last few years on the potential use of DDC as a radiosensitizer or as a radioprotector in the clinic.

The inhibition of SOD by DDC was confirmed in a murine tumour, and it was shown that this inhibition can be maintained for up to twenty-four hours after DDC administration. This indicated that there was a potential for the radioprotective effect of DDC to be overcome, if the levels of DDC were low enough at the time of irradiation.

Indeed, if DDC was removed from the growth medium of cells in culture prior to irradiation, a significant sensitization was demonstrated.

Perhaps the most important contribution of this thesis to the debate on the role of DDC is that it was shown that DDC could act as both a radiosensitizer and as a radioprotector in the same experiment. The dominant action of DDC was found to be dependent on the time allowed between DDC administration and irradiation. If this time was approximately 4 hours, it was possible to show a radiosensitizing effect by means of a tumour growth delay assay.

In addition, DDC was found to modify the radiation response of tumour cells, rather than the stroma or tumour bed. The time modulation effect of DDC was shown in larger tumours, rather than smaller tumours, which could mean that tumour oxygenation is an important criterion in determining the response to radiation of DDC treated cells.

This work suggests that some caution should be exercised when DDC is put forward as either a radiosensitizer or a radioprotector in the clinic. The work on hyperthermia however, suggests that DDC may have potential as a thermosensitizer, and further investigations in this regard are warranted.

The work on tumour growth delay also demonstrated the inadequacy of the current methods of analysis of tumour growth delay data. It is hoped the use of the Kaplan-Meier method to analyse tumour growth delay data will

become one of the standard methods of analysing growth delay assays with censored data.

Appendix 1
Enzyme measurement

Superoxide dismutase

Reagents

Homogenising buffer: 0.05 M K_2HPO_4 / 1 X 10^{-4} M EDTA :
8.709 g K_2HPO_4, 0.0372 g EDTA were dissolved in water and made up to 1 litre. pH was adjusted to 7.8 with KOH

0.5 M Na_2CO_3 / 1 X 10^{-5} M EDTA buffer :
4.2 g Na_2CO_3, 0.00372 g EDTA were dissolved in water and made up to 1 litre. pH was adjusted to 10.2 with NaOH

0.55% (w/v or 3 X 10^{-4} M) adrenalin solution :
5.5 mg adrenalin (Sigma, St.Louis, Mo.) was added to a solution of water acidified to pH 2.0 with perchloric acid (41 µl perchloric acid + 5 ml water) to prevent autooxidation. Fresh solutions were prepared for each assay.

Glutathione peroxidase

Reagents

Homogenising buffer : 10 mM KH_2PO_4 / 30 mM KCl :

1.36 g KH_2PO_4, 2.25 g KCl were dissolved in water and made up to 1 litre. pH was adjusted to 8.0 with concentrated KOH

50 mM potassium phosphate buffer :
27.21 g KH_2PO_4 was dissolved in water and made up to 1 litre. pH was adjusted to pH 7.0 with concentrated KOH. This resulted in a 0.2 M stock solution, but was used as 0.5 ml/2 ml.

1 mM EDTA :
37.2 mg EDTA dissolved in 10 ml water.

1 mM NaN_3 :
6.5 mg NaN_3 dissolved in 10 ml water.

0.2 mM NADPH :
16.7 mg NADPH dissolved in 10 ml water.

1 mM glutathione
30.7 mg GSH dissolved in 10 ml water.

1 unit/ml Glutathione reductase :
1 µl of a GSSG-r solution with an activity of 2000 units/ml added to a 2 ml sample gave this concentration.

0.25 mM H_2O_2 :
0.267 ml (30% w/v) H_2O_2 was made up to 1 litre with water.

Catalase

Reagents

Homogenising buffer : 10 mM KH_2PO_4 / 30 mM KCl :
1.36 g KH_2PO_4, 2.25 g KCl were dissolved in water and made up to 1 litre. pH was adjusted to 8.0 with concentrated KOH

50 mM tris hydroxymethyl methylamine :
0.606 g Tris was dissolved in 100 ml water and the pH adjusted to 7.4 with concentrated HCl.

10 mM H_2O_2 :
1.05 ml (30% w/v) H_2O_2 was made up to 1 litre with water.

Fluorometric protein assay

The basis of this assay is the reaction of fluorescamine (4-phenylspiro[furan-2(3H),1'-phthalan]-3,3'-dione) with substances containing primary amino groups to yield highly fluorescent products as described by Bohlen (1973). The relative fluorescence during this reaction is highest at pH 8.

Reagents

Standard protein solution :
1 mg bovine serum albumin dissolved in 1 ml water.

0.05 M sodium phosphate buffer :
7.8 g $NaH_2PO_4 \cdot 10H_2O$ (or 5.99 g anhydrous NaH_2PO_4) was dissolved in water and made up to 1 litre. pH was adjusted to 8.0 with concentrated NaOH.

Fluorescamine
Fluorescamine, being insoluble in water was dissolved in dioxane, a water-miscible organic solvent. 30 mg fluorescamine was dissolved in 100 ml dioxane, whereafter it was stored in a glass beaker blacked out with aluminium foil as the solution is light sensitive.

Assay Procedure

10-250 µl aliquots of the sample or a standard protein solution were transferred to glass test-tubes and the volume adjusted to 3 ml with NaH_2PO_4 buffer. 1 ml fluorescamine was added to the buffered protein solution while being stirred with a vortex mixer to ensure rapid mixing. The fluorescence was measured using a Perkin-Elmer Fluorescence Spectrophotometer at an excitation wavelength of 390 nm and an emission wavelength of 475 nm. Sample protein concentrations were estimated by comparison to the standard curve.

Spectrophotometric protein assay

The basis of this assay is the reduction of Folin phenol reagent by copper-protein complexes at pH 10. This causes a colour reaction which can be measured

spectrophotometrically as described by Lowry *et al.* (1951). As the reduction of Folin reagent liberates acid, the solution must be well buffered to maintain colour changes.

Reagents

Standard protein solution :
1 mg bovine serum albumin dissolved in 1 ml water.

Solution A : 2% Na_2CO_3 in 0.1 M NaOH.
4 g Na_2CO_3, 0.8 g NaOH were dissolved in water and made up to 200 ml. To this was added 2 ml 1% $CuSO_4$ and 2 ml 2% NaK tartrate.

Solution B : 1:1 Folin reagent.
10 ml Folin reagent was added to 10 ml water and vortex mixed.

Assay procedure

Protein samples are added to 5 ml of solution A, and the volume adjusted to 6 ml with water in glass test tubes. This was allowed to stand at room temperature to allow the

$$\text{protein} + \text{copper} \longleftrightarrow \text{protein-copper}$$

reaction to occur. After 12 minutes 0.5 ml of solution B is added and vigorously agitated. After 45 minutes

the absorbance was measured spectrophotometrically at 750 nm. All protein determinations were made so that the amounts ranged from 0.05 mg to 0.25 mg, thus falling within the linear range of absorbance at 750 nm.

Appendix 2
Experimental animals and tumours

All procedures on animals described in this thesis were reviewed and approved by the University of Cape Town Ethics Committee.

Experimental animals

BALB/c mice were used in all tumour growth delay experiments. All mice were bred in a specified pathogen-free mouse unit and maintained in the Radiobiology Laboratories of the University of Cape Town Radiotherapy Department. Mice were fed with Epol Mouse cubes and water was allowed *ad libitum*. The temperature in the mouse unit was maintained at 22°C. Mice used in these studies were 6 weeks old with a weight range of 18-22 g before any procedure took place.

3 methylcholanthrene induced rhabdomyosarcoma

A transplantable rhabdomyosarcoma was induced by injecting 0.1 ml of a 1 mg/ml solution of 3-methylcholanthrene in arachis oil into the flanks of male BALB/c mice. It was found that a rhabdomyosarcoma developed in all mice in approximately 3-4 months. The tumour was maintained by serial passage as described below.

Tumour bearing mice were sacrificed by prolonged exposure

to ether, after which tumours were removed by surgical excision. Any obviously necrotic tissue was discarded. Tumours from 3 animals were placed in petri dishes (Falcon Plastics) and finely chopped with crossed scalpel blades in approximately 5 ml of McCoys 5A medium. (pH 7.2 - 7.4). After chopping, the total preparation was aspirated through a 1.2 X 38 mm needle into a sterile syringe, and transferred to a sterile test tube where the aspirate was allowed to settle for a few minutes.

25 µl of the upper layer of this aspirate (1.6 X 10^6 cells) was inoculated into the right hind gastrocnemius muscle. Tumours appeared 10 - 20 days after inoculation. This site was convenient as it was very easy to measure the tumours and it was not necessary to anaesthetise the animal during treatment. It also appeared to cause little distress to the animal.

Preparation of DDC solutions for intratumoural administration

Solutions of DDC were made up at concentrations of 50, 100 and 150 mg/ml in 0.9% saline. A 20 µl intratumoural injection resulted in a dose of 50, 100 and 150 mg/kg respectively for a mouse weighing 20 g.

Appendix 3
Cell Culture Techniques

Cell line and tissue culture techniques

The B16 mouse melanoma cell line is an established line and can be cultured *in vitro* using standard cell culture methods as outlined by Freshney (1987).

The cells were maintained routinely in 50 ml plastic tissue culture flasks (Falcon) in McCoys 5A medium (Gibco) with L-glutamine made up in 2.2 g/l sodium bicarbonate buffer, supplemented with 20 ml/l antibiotics (100 U/l penicillin, 100 µg/l streptomycin and 100 µg/l neomycin) and 10% foetal calf serum (Gibco). The cells were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C.

Harvesting of Cell cultures

Confluent stock cultures were detached from their flasks and harvested using the following procedure. Medium was poured off, and the cells rinsed twice with 2 ml trypsin/EDTA solution. The cells were then incubated for 5 minutes at 37°C. This resulted in the cells becoming detached from the bottom of the flasks. The cells were resuspended in 10 ml McCoys 5A medium with antibiotics and 10% FCS. A sample of the cell suspension was taken and counted in a haemocytometer.

After appropriate dilutions were made, 5 ml aliquots of cell suspension in McCoys 5A medium which contained 500 cells were pipetted into 50 ml plastic tissue culture flasks. These flasks were incubated for 24 hours to allow cell plating to occur before any procedure was performed.

Treatment

Preparation of DDC Medium

A 10^{-2} M DDC solution was made up in distilled water and sterilised by ultrafiltration. This stock solution was further diluted with serum-free medium as required.

DDC pretreatment

After 24 hours the medium was poured off the flasks and replaced with either DDC medium in the case of experimental flasks, or serum-free medium alone in the case of controls. They were then incubated for 45 minutes at 37°C in 95% air and 5% CO_2 whereafter the cells were rinsed three times in 2 ml Hanks balanced salt solution (HBSS), before 5 ml fresh serum containing medium was added.

Irradiation

At one or four hours after DDC pretreatment, the flasks were irradiated with 0 - 8 Gy using a Siemmens Gammatron

Cobalt source with a dose rate of 0.4 Gy/min. The cells were then incubated for 12 days at 37°C in 95% air and 5% CO_2 before being stained and counted.

After 7 days the medium was replaced with fresh medium.

Hyperthermia

A Labotec water bath with a temperature regulator (± 0.1°C) was used for hyperthermia treatments. The screw tops of the culture flasks were tightly closed, and placed in the water bath so that the level of water was just above the level of the medium. Flasks were kept in this situation for 15 - 60 minutes.

Staining and counting

Medium was poured off and the cells rinsed with 3 ml 2% phosphate buffered saline before being fixed in methanol and acetic acid for 15 minutes. They were then stained with an amido black solution for 30 minutes, after which they were rinsed again in methanol and acetic acid. They were then allowed to dry, whereafter the number of colonies in the flask, representing the number of cells surviving in the plated suspension after treatment (Freshney, 1984), was counted.

Plating efficiency was defined as :

$$\frac{\text{No. of colonies counted after no Rx}}{\text{No. of cells originally plated.}}$$

The survival data was then fitted by non-linear least squares regression to the linear quadratic model of Chadwick and Leenhouts (1981) :

$$\ln(SF) = -(\alpha D+\beta D^2)$$

Dose response analysis

The dose modifying factor (DMF) could be calculated at specified biological endpoints (i.e. survival levels) from the ratio of the doses following treatment with radiation only to the doses following pretreatment with DDC. In these investigations, for each pair of survival curves, the DMF was determined at 15 survival levels (0.9 to 0.001), and the mean DMF then calculated.

Appendix 4
Statistical considerations

This appendix describes the rules followed when calculating any descriptive statistics in the discussion of data used in this thesis.

Measures of central tendency

In all discussion of central tendency, the mean, median and standard deviation was used. These are defined as follows.

Mean

Mean : $\bar{x} = (\Sigma x)/n$

where x is each value, and n is the number of observations.

Median

The median is defined as the $({}^{n}/_{2} + 0.5)^{th}$ observation when the observations are ordered from smallest to largest. When $({}^{n}/_{2} + 0.5)$ is not equal to an integer, the mean of the values lying in positions $({}^{n}/_{2})$ and $({}^{n}/_{2} + 1)$ is used as the median value.

Standard deviation

$$SD = \sqrt{\frac{\Sigma(x-\bar{x})^2}{(n-1)}}$$

where $\bar{x}$, n and x are defined as the mean, total number of observations and each observation respectively.

Statements of probability

When the set of observations follow a normal distribution, certain multiples of the standard deviation (e.g. 1.96 X SD = 95%) mark certain limits in the scatter of observations.

When comparing an individual observation against a sample mean, the 95% confidence interval is used. This is defined as :

$$95\% \text{ confidence} = \bar{x} \pm (1.96\ SD)$$

When comparing means of different sets of observations, the following parameters are used.

Standard error of the mean : $SEM = SD / \sqrt{n}$

The 95% confidence limits of the mean when n>30, is :

$$95\% \text{ confidence (mean)} = \bar{x} \pm (1.96\ SEM)$$

When small sample sizes are used, the t distribution is used to calculate the multiples of SEM needed for 95% confidence limits.

Difference between means

For large samples (n>60)

In order to determine whether two sets of observations originate from different populations, the difference between the means is calculated :

$$\bar{x}_{diff} = \bar{x}_1 - \bar{x}_2$$

and the standard error of the difference between the means. :

$$SE_{diff} = \sqrt{\left[\frac{(SD_1)^2}{n_1} + \frac{(SD_2)^2}{n_2} \right]}$$

where the SD_1 and SD_2 are the standard deviations of the two samples, and n_1 and n_2 are the number of observations in each sample.

The number of multiples of the SE_{diff} this difference represents is calculated. If it is greater than 1.96, it is said to be significant at the 5% level. (2.576 : p = 0.01; 3.291 : p = 0.001)

For small samples (n<60) : the t-test

For smaller samples, where more chance variation must be allowed for, the multiples of standard deviation used before are not entirely accurate and the t-distribution is used when statements of probability are made.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{SE_{diff}}$$

The table of t distributions is entered at $(n_1-1)+(n_2-1)$ degrees of freedom.

The correlation coefficient

The calculation of the correlation coefficient is as follows :

$$r = \frac{\Sigma(x-\bar{x})(y-\bar{y})}{\Sigma(x-\bar{x})^2 \ \Sigma(y-\bar{y})^2}$$

with x representing the values of the independent variable, and y representing the values of the dependent variable.

$\bar{x}$ and $\bar{y}$ represent the mean x and mean y respectively.

Appendix 5
Kaplan Meier analysis of survival data

In this discussion of the Kaplan-Meier method, for the sake of clarity, patient survival after a course of treatment is discussed, however 'patient survival' may be substituted for 'mouse not having attained a response (tumour size ≥ 0.6 cm^3)' in the growth delay experiments.

The method of Peto (1984) was used to calculate the Kaplan Meier survival curve. The method follows from the observation that in order to survive one year, a patient has to survive the first day, and the second and the third and so on to the 365^{th} day.

The separate probabilities of surviving each day are multiplied together giving :

$$p(365) = e(1) \times e(2) \times e(3) \times \ldots\ldots\ldots \times e(365)$$

where p(365) is the probability of surviving for a full year, and e(*n*) is the estimated probability of surviving on day *n*.

On day 1, we assume that all patients are alive at the start of treatment. Therefore the probability of surviving day 1 is :

$$e(1) = \frac{\text{(no. of patients)} - \text{(no. of deaths on day 1)}}{\text{(no. of patients)}}$$

On subsequent days, the number of patients under observation decreases. For example, on day n :

* Some patients may have died on or before day n (responses).
* Some patients may have started treatment less than n days ago (withdrawals).
* Some patients may be lost to follow up, and the last information concerning their status was less than n days after treatment (withdrawal).

For each day we calculate a probability of surviving, we therefore have to know how many patients are 'at risk'.

The number of patients 'at risk' on day n is the number who were still alive and under observation at the beginning of the n^{th} day.

Patients who die or are withdrawn on the n^{th} day are therefore at risk on day n, but not on day $(n+1)$.

i.e. $R_n = R_{(n-1)} - D_{(n-1)} - W_{(n-1)}$

where R = no. 'at risk'
D = no. of deaths
W = no. of withdrawals.

The probability of surviving on day n is therefore :

$$e(n) = \frac{R(n) - D(n)}{D(n)}$$

If there are no deaths on day n, then $e(n) = 1$, and the value of $p(n)$ remains unchanged as :

$$p(n) = e(1) \times e(2) \times \ldots \times e(n)$$

$$\text{or } p(n) = \frac{R(1) - D(1)}{R(1)} \times \frac{R(2) - D(2)}{R(2)} \times \ldots \times \frac{R(n) - D(n)}{R(n)} \quad \text{EQN. A}$$

It is therefore necessary to calculate the estimated probability of survival on day n only when $D(n) > 0$.

An example using tumour growth delay data

In this example, a response is defined as the attainment of a tumour volume of 0.600 cm^3. In a group of 25 tumour bearing mice, the first 3 mice to reach this volume do so 5, 7 and 11 days after treatment respectively.

In order to demonstrate how censored (tumour cure / mouse escaped / intercurrent death) data is dealt with, let us assume that one mouse escaped on day 9.

The probability curve is calculated in the following way

Time	Equation A	Probability Curve p(n)
day 0	25/25	(100 %)
day 5	25/25 X 24/25	= 24/25 (96%)
day 7	25/25 X 24/25 X 23/24	= 23/25 (92%)
day 9	25/25 X 24/25 X 23/24 X 23/23	= 23/25 (92%)
day 11	25/25 X 24/25 X 23/24 X 23/23 X 21/22	= 21/25 (84%)

The probability of response is plotted stepwise on a graph against time.

The Kaplan-Meier median is defined as the first day (n) where $p(n) \leq 0.5$.

References

ALBERGONI V., CASSINI A., FAVERO A. and ROCCO G.P. (1975). The effect of penicillamine on some metals and metalloproteins in the rat. *Biochem.Pharmacol.* **24** 1131-1133.

ALEXANDER A., BACQ Z.M., COUSENS S.F., FOX M., HERVE A. and LAZAR J. (1955). Mode of action of some substances which protect against the lethal effects of X-Rays. *Radiat.Res.* **2**, 392-415.

ALLALUNIS-TURNER M.J. and CHAPMAN J.D. (1984). Evaluation of diethyldithiocarbamate as a radioprotector of bone marrow. *Int.J.Radiat.Oncol.Biol.Phys.* **10**, 1569-1573.

ALPER T. (1962). The dependence of chemical protective action on oxygen as studied with bacteria. *Br.J.Radiol.* **35**, 361.

ASADA K., URANO M. and TAKAHASHI M. (1973). The subcellular location of superoxide dismutase in spinach leaves and preparation and properties of crystalline spinach superoxide dismutase. *Eur.J.Biochem.* **36**, 257-266.

BABOIR B.M. (1978). Oxygen dependent microbial killing by phagocytes. *N.Engl.J.Med.* **298**, 659-668.

BACQ Z.M. (1953). Rayons X et agents de chelation. *Bull.Acad.Med.Belg.* **18-19** 226-235.

BANNISTER J.V., ANASTASI A. and BANNISTER W.H. (1976). Cytosol superoxide dismutase from swordfish (*Xiphias gladius*) liver. *Comp.Biochem.Physiol.* **53B**, 235-238.

BARTOSZ G. and LEYKO W. (1981). Radioprotection of bovine erythrocytes to haemolysis. *Int.J.Radiat.Biol.* **39**(1), 39-46.

BEERS R.F. and SIZER I.W. (1952). A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *J.Biol.Chem.* **195**, 133-141.

BEGG A.C. (1980). Analysis of growth delay data : Potential pitfalls. *Br.J.Cancer.* **41**, Suppl IV 93-97.

BEGG A.C. (1987). Principles and practices of the tumour growth delay assay. In *Rodent tumour models in experimental cancer therapy.* ed Kallman R.F. **Pergamon Press**, Oxford U.K. 114-121.

BEGG A.C. and TERRY N.H.A. (1983). Modification of stromal radiosensitivity by misonidazole and WR-2721. *Brit.J.Radiol.* **56**, 565-570.

BIAGLOW J.E., ISSELS R.D., GERWECK L.E., VARNES M.E., JACOBSON B., MITCHELL J.B. and RUSSO A. (1984). Factors influencing the oxidation of cysteamine and other thiols : implications for hyperthermic sensitization and radiation protection. *Radiat.Res.* **100**, 298-312.

BLUMBERG A., NELSON D., GRAMKOWSKI M., GLOVER D.J., GLICK J.H., YUHAS J.M. and KLIGERMAN M. (1982). Clinical trials of WR-2721 with radiation therapy. *Int.J.Radiat.Oncol.Biol.Phys.* **8**, 561-563.

BODENNER D.L., DEDON P.C., KENG P.C., KATZ J.C. and BORCH R.F. (1986). Selective protection against cis-diamminedichloroplatinum(II) induced toxicity in kidney, gut and bone marrow by diethyldithiocarbamate. *Cancer Res.* **46**, 2751-2755

BOHLEN, P., STEIN, S., DAIRMAN W. and UDENFRIEND, S. (1973). Fluorometric assay of proteins in the nanogram range. *Arch.Biochem.Biophys.* **155**, 213-220.

BRESLOW N. (1984). Comparison of survival curves. in *Cancer Clinical Trials : Methods and Practice.* **Oxford University Press**, Oxford U.K. 381-406.

CARRICO R.J. and DEUTSCH H.F. (1969). Isolation of human hepatocuprein and cerebrocuprein. - their identity with erythrocuprein. *J.Biol.Chem.* **244**, 6087-6093.

CHADWICK K.H. and LEENHOUTS H.P. (1981). The molecular model for cell survival following radiation. in *The molecular theory of radiation biology.* edited by R. Frankel. **Springer-Verlag**, Berlin 25-50.

CHAPMAN J.D., REUVERS A.P., BORSA J. and GREENSTOCK C.L. (1973). Chemical radioprotection and radiosensitization of mammalian cells growing *in vitro.* *Radiat.Res.* **56**, 291-306.

CHRISTENSEN H.E., LUGINBYHL T.T. and CARROL B.S. (1974). The toxic substances list. **US Dept HEW, Rockville Md.**, USA. 175-176.

CIBOROWSKI L., KWOCK L. and LIN P.S. (1978). Effect of irradiation, hyperthermia and in vitro conditions on superoxide dismutase activity. *Radiat.Res.* **74**, 545.

CRAVEN M.R., LUSCOMBE D.K. and NICHOLLS P.J. (1976). Absorption, elimination and duration of action of diethyldithiocarbamate in animals. *J.Phar.Pharmacol.* **28**, suppl 38P.

CUDD A. and FRIDOVICH I. (1982). Electrostatic interactions in the reaction mechanism of bovine erythrocyte superoxide dismutase. *J.Biol.Chem.* **257**, 11443-11447.

CZAPSKI G. (1984). On the use of $OH^{\bullet}$ scavengers in biological systems. *Israel.J.Chem* **24**, 29-32.

CZAPSKI G. and GOLDSTEIN S. (1987). The uniqueness of superoxide dismutase (SOD). - Why cannot most copper compounds substitute SOD *in vivo* ? *Free.Rad.Res.Comm* **4**, 225-229.

DELREZ M. and FIRKET H. (1968). The paradoxical *in vitro* effect of a radioprotective agent on mitosis and chromosomes. *Biochem.Pharmacol.* **17**, 1893.

DENEKAMP J. (1979). Experimental tumour systems : Standardization of endpoints. *Int.J.Radiat.Biol.Phys* **5**, 1175-1184.

DENEKAMP J. (1980). Is any single in situ assay of tumour response adequate ? *Br.J.Cancer.* **41**, Suppl IV. 56-63.

DENEKAMP J. and HARRIS S.R. (1975). Tests of two electron affinic radiosensitizers in vivo, using regrowth of an experimental carcinoma. *Radiat.Res.* **61**, 191-203.

DENEKAMP J., MICHAEL B.D., ROJAS A. and STEWART F.A. (1981a). Radioprotection of mouse skin by WR-2721 : The critical influence of oxygen tension. *Int.J.Radiat.Oncol.Biol.Phys.* **8**, 531-534.

DENEKAMP J., MICHAEL B.D., ROJAS A. and STEWART F.A. (1981b). Thiol radioprotection in vivo : The critical role of tissue oxygen concentration. *Br.J.Radiol.* **54**, 1112-1113.

DENEKE S.M. & FANBURG B.L. (1980). Involvement of glutathione enzymes in O_2 tolerance development by diethyldithiocarbamate. *Biochem.Pharmacol.* 29 1367-1373.

DIBLE S.E., SIDDIK Z.H., BOXALL F.E. and HARRAP K.R. (1987). The effect of diethyldithiocarbamate on the haematological toxicity and antitumour activity of carboplatin. *Eur.J.Cancer.Clin.Oncol.* **23**, 813-818.

DICKERSON R.E. (1978). Chemical evolution and the origin of life. *Sci.Amer.* **78**(9), 70-86.

DOUPLE E.B. and RICHMOND R.C. (1982). Enhancement of the potentiation of radiotherapy by platinum drugs in a mouse tumour. *Int.J.Radiat.Biol.Oncol.Phys.* **8**, 501-503.

DOWNES D.L. and LOVEN D.P. (1988). Exogenous superoxide dismutase and catalase protect CHO cells from 45°C heat shock. (Abstract) 36th Conference of the American Radiation Research Society and the 8th Annual Meeting of the North American Hyperthermia Group. April 16-21 1988 p47.

DURAND R.E. (1983). Radioprotection by WR-2721 *in vitro* at low oxygen tensions. Implications for its mechanism of action. *Br.J.Cancer.* **47**, 387-392.

DURAND R.E. and OLIVE P.L. (1989). Radiosensitization and radioprotection by BSO and WR-2721 : the role of oxygenation. *Br.J.Cancer.* **60**, 517-522.

ELDJARN L. and PIHL A. (1956). On the mode of action of X-Ray protective agents. 1. The fixation *in vivo* of cystamine and cysteamine to proteins. *J.Biol.Chem.* **223**, 341-351.

ELDJARN L. and PIHL A. (1960). in *Mechanisms in radiobiology.* Vol 2. edited by ERRERA M. and FORSSBERG **Academic Press**, New York, USA. 231.

ELKIND M.M. (1988). The initial part of the survival curve : Does it predict the outcome of fractionated radiotherapy ? *Radiat.Res.* **114**, 425-436.

EVANS R.G. (1985). Tumour radiosensitization with concomitant bone marrow radioprotection : A study in mice using diethyldithiocarbamate (DDC) under oxygenated and hypoxic conditions. *Int.J.Radiat.Oncol.Biol.Phys.* **11**, 1163-1169.

EVANS R.G., ENGEL C., WHEATLEY C., NIELSEN J. and CIBOROWSKI L.J. (1983a). An *in vivo* study of the radioprotective effect of diethyldithiocarbamate (DDC). *Int.J.Radiat.Oncol.Biol.Phys.* **9**, 1635-1640.

EVANS R.G., NIELSEN J., ENGEL C. and WHEATLEY C. (1983b). Enhancement of heat sensitivity and modification of repair of potentially lethal heat damage in plateau phase cultures of mammalian cells by diethyldithiocarbamate. *Radiat.Res.* **93**, 319-325.

FEE A. in *Oxygen and oxy-radicals in chemistry and biochemistry.* edited by M.A. Rodgers and E.L. Powers. **Academic Press**, New York, USA. p 197.

FIELD S.B., JONES T. and THOMLINSON R.H. (1968). The relative effects of fast neutrons and X-Rays on tumours and normal tissues in the rat. II. Fractionation, Recovery and reoxygenation. *Br.J.Radiol.* **41**, 597.

FINKELSTEIN E. and GLATSTEIN E. (1988). Seduced by oxygen. *Int.J.Radiat.Oncol.Biol.Phys.* **14**, 205-207.

FINNEY D.J. (1971). *Probit Analysis.* **Cambridge University Press**, Cambridge, U.K.

FLORVALL L. and CARRODI H. (1970). Dopamine beta hydrogenase inhibitors - preparation and dopamine beta-hydroxylase inhibitory activity of some compounds related to dithiocarbamic acid and thiuram disulphide. *Acta.Pharm.Suedica.* **7**, 7-22.

FOLKMAN J. (1974). Tumor angiogenesis. *Adv.Cancer.Res.* 19, 331-358.

FOWLER J.F., HILL S.A. and SHELDON P.W. (1980). Comparison of tumour cure (local control) with regrowth delay in mice. *Br.J.Cancer.* **41**, Suppl IV, 102-103.

FOWLER J.F., MORGAN R.L., and WOOD C.A.P. (1963). The biological and physical advantages and problems of neutron therapy. *Br.J.Radiol.* **36**, 77-80.

FRANK L., WOOD D.L. and ROBERTS R.J. (1978). Effect of diethyldithiocarbamate on oxygen toxicity and lung enzyme activity in immature and adult rats. *Biochem.Pharmacol.* **27**, 251-254.

FRIDOVICH I. (1962). Competitive inhibition by myoglobin of the reduction of cytochrome c by xanthine oxidase. *J.Biol.Chem.* **237**, 584-586.

FRIDOVICH I. (1979). Superoxide and superoxide dismutases. *Adv.Inorg.Biochem.* **1**, 67-90.

FRIDOVICH I. (1981). Superoxide radical and superoxide dismutases. in *Oxygen and living processes.* Ed. Gilbert D.L. 250-272. **Springer-Verlag**, New York, USA.

FRIDOVICH I. and HANDLER P. (1954). Further studies of sulphite oxidase. *Federation.Proc.* **13**, 212-213.

FRIDOVICH I. and HANDLER P. (1955). Hypoxanthine : Coenzyme of sulfite oxidase. *Federation.Proc.* **14**, 214.

FRIDOVICH I. and HANDLER P. (1958a). Xanthine oxidase : III Sulfite oxidation as an ultrasensitive assay. *J.Biol.Chem.* **233**, 1578-1580.

FRIDOVICH I. and HANDLER P. (1958b). Xanthine oxidase : IV Participation of iron in internal electron transport. *J.Biol.Chem.* **233**, 1581-1585.

FRIDOVICH I. and HANDLER P. (1961). Detection of free radicals generated during enzymatic oxidations by the initiation of sulfite oxidation. *J.Biol.Chem.* **236**, 1836-1840.

FRIDOVICH I. and HANDLER P. (1962). Xanthine oxidase : V Differential of the reduction of various electron acceptors. *J.Biol.Chem.* **237**, 916-921.

FRESHNEY R.I. (1987). Culture of animal cells. A manual of basic techniques. **Alan R. Liss Inc.**, New York, USA.

FORMAN H.J. and FRIDOVICH I. (1973). On the stability of bovine superoxide dismutase. The effects of metals. *J.Biol.Chem.* **248**, 2645-2649.

GERSCHMAN R., GILBERT D.L., NYE S.W. and FENN W.O. (1954). Oxygen poisoning and X-irradiation : A mechanism in common. *Science* **119**, 623.

GETZOFF E.D., TAINER J.A., STEMPIEN M.M., BELL G.I. and HALLEWELL R.A. (1989). Evolution of CuZn superoxide dismutase and the Greek key ß-barrel structural motif. *Proteins* **5**, 322-336.

GETZOFF E.D., TAINER J.A., WEINER P.K., KOLLMAN P.A., RICHARDSON J.S. and RICHARDSON D.C. (1983). Electrostatic interactions between superoxide and copper, zinc superoxide dismutase. *Nature* **268**, 287-290.

GILBERT D.L. (1981). Perspective on the history of oxygen and life. in *Oxygen and living processes.* Ed. Gilbert D.L. 1-44. **Springer-Verlag**, New York, USA.

GLICK J.H., GLOVER D.J., WEILER C., BLUMBERG A., NELSON D., YUHAS J.M. and KLIGERMAN M. (1982). Phase I clinical trials of WR-2721 with alkylating agent chemotherapy. *Int.J.Radiat.Oncol.Biol.Phys.* **8**, 575-580.

GOLDSTEIN B.D., ROSEN M.G., QUINTAVALLA J.C. and AMORUSO M.A. (1979). Decrease in mouse lung and liver glutathione peroxidase activity and potentiation of the lethal effects of ozone and paraquat by the superoxide dismutase inhibitor, diethyldithiocarbamate. *Biochem.Pharmacol.* **28**, 27-30.

GOLDSTEIN S. and CZAPSKI G. (1986a). The role and mechanism of metal ions and their complexes in enhancing damage in biological systems or in protecting these systems from the toxicity of O_2^-. *J.Free.Rad.Biol.Med* **2**, 3-11.

GOLDSTEIN S. and CZAPSKI G. (1986b). Mechanisms of the reactions of some copper complexes with O_2^-, H_2O_2 and molecular oxygen in the presence of DNA. *J.Am.Chem.Soc* **108**, 2224-2250.

GOODMAN L.J. (1969). A modified tissue equivalent liquid. *Health Physics* **16**, 763.

HABER F. and WEISS J. (1934). Catalytic decomposition of hydrogen peroxide by iron salts. *Proc.Roy.Soc.Lond.* **147**, 332-351.

HAHN G.M., BRAUN J. and HAR-KEDAR I. (1975). Thermochemotherapy : Synergism between hyperthermia (42 - 43°C) and adriamycin (or bleomycin) in mammalian cell inactivation. *Proc.Natl.Acad.Sci.USA.* **72**, 937-940.

HALDANE J.B.S. (1954). The origins of life. in *Adventures in earth history.* P. Cloud (ed) **W.H. Freeman & Co.** 1970. San Francisco, USA.

HALL E.J. (1988). "Radiobiology for the radiologist." **Lippincot**, Philadelphia, USA.

HALLIWELL B. (1990). The role of antioxidants in human disease : new methodology. Plenary Lecture - **First National Conference on free radicals.** 18-20 July 1990, Johannesburg, South Africa.

HALLIWELL B. and GUTTERIDGE J.M.C. (1985). Free radicals in Biology and Medicine, **Oxford University Press**, Oxford, U.K.

HANDLER P., BERNHEIM M.L.C. and KLEIN J.R. (1941). The oxidative demethylation of sarcosine to glycine. *J.Biol.Chem.* **138**, 211-218.

HARRIS J.W. and PHILLIPS T.L. (1971). Radiobiological and biochemical studies of thiophosphate radioprotective compounds related to cysteamine. *Radiat.Res.* **46**, 362-379.

HEIKKILA R.E. (1984) Inactivation of Superoxide dismutase by diethyldithiocarbamate. in *CRC Handbook of methods for oxygen radical research.* **CRC Press**, USA. 387-390.

HEIKKILA R.E., CABBAT F.S. and COHEN G. (1976). *In vivo* inhibition of superoxide dismutase in mouse by diethyldithiocarbamate. *J.Biol. Chem.* **251**, 2182-2185.

HEIKKILA R.E. and COHEN G. (1977) The inactivation of CuZn SOD by DDC. in *Superoxide and superoxide dismutases.* MICHELSON (ed). " **Academic Press**. New York, USA.

HEIMBERG M., FRIDOVICH I. and HANDLER P. (1953). The enzymatic oxidation of sulfite. *J.Biol.Chem.* **204**, 913-926.

HEWITT H.B. and BLAKE E.R. (1968). The growth of transplanted murine tumours in pre-irradiated sites. *Br.J.Cancer.* **12**, 808-824.

HILL R.P. (1980). An appraisal of in vivo assays of excised tumours. *Br.J.Cancer.* **41**, Suppl IV 230-239.

HOLMES R.S. and MASTERS C.J. (1970). Epigenetic interconversions of the multiple forms of mouse liver catalase. *FEBS.Lett.* **11**(1), 45-48.

HORECKER B.L. and KORNBERG A. (1948). The extinction co-efficients of the reduced band of pyridine nucleotides. *J.Biol.Chem.* **175** 385.

HUGHSON M., BALENTINE J.D. and DANIELL H.B. (1977). The ultrastructural pathology of hyperbaric oxygen exposure : Observations on the heart. *Lab.Invest.* **37**, 516-525.

ISSELS R.D., BIAGLOW J.E., EPSTEIN L. and GERWECK L.E. (1984). Enhancement of cysteamine cytotoxity by hyperthermia and its modification by catalase and superoxide dismutase in Chinese hamster ovary cells. *Cancer.Res.* **44**, 3911-3915.

JUNG H., BECK H.P., BRAMMER I. and ZYWIETZ F. (1981). Depopulation and repopulation of the R1H rhabdomyosarcoma of the rat after X-irradiation. *Eur.J.Cancer.* **17**, 375-386.

KAPLAN E.L. and MEIER P. (1958). Nonparametric estimation from incomplete observations. *J.Amer.Statist.Assoc.* **53**, 457-481.

KAPP D.S. and HAHN G.M. (1979). Thermosensitization by sulphydryl compounds of exponentially growing Chinese Hamster cells. *Cancer Res.* **39**, 4630-4635.

KENT C. and BLEKKENHORST G.H. (1988). In vivo radiosensitization by diethyldithiocarbamate. *Radiat.Res.* **116**, 539-546.

KIMURA Y., TADANO T., SAKAI K., MIYAMOTO T. and KISARA K. (1979). Changes of ejaculation due to suppression of noradrenaline biosynthesis by diethyldithiocarbamate. *Andrologia* **12**, 85-91.

KLUG D., RABANI J., and FRIDOVICH I. (1972). Direct demonstration of the catalytic action of superoxide dismutase through the use of pulse radiolysis. *J.Biol.Chem.* **247**, 4839-4842.

KOCH C.J. and HOWELL R.L. (1981). Combined radiation protective and radiation sensitizing agents. II. Radiosensitivity of hypoxic or aerobic Chinese hamster fibroblasts in the presence of cysteamine and misonidazole. Implications for the 'oxygen effect'. *Radiat.Res.* **87**, 265-283.

KUMAR K.S., SANCHO A.M. and WEISS J.F. (1986). A novel interaction of diethyldithiocarbamate with the glutathione/glutathione peroxidase system. *Int.J.Radiat.Oncol.Biol.Phys.* **12**, 1463-1467.

LAVOISIER A.L. (1782-83). Sur les altérations qui arrivent à l'air dans plusieurs circonstances où se trouvent les hommes réunis en société. Mémoirs de Médecine. *Hist.Soc.Med.* **5**, 569-582.

LAWRENCE R.A. and BURK R.F. (1976). Glutathione peroxidase activity in selenium-deficient rat liver. *Biochem.Biophys.Res.Com.* **71**(4), 952-958.

LEPCOCK J.R., ARNOLD L.D., TORRIE B.H., ANDREWS B. and KRUUV J. (1985). Structural analyses of various Cu^{2+}, and Zn^{2+} - superoxide dismutases by differential scanning calorimetry and Raman spectroscopy. *Arch.Biochem.Biophys.* **241**, 243-251.

LIN P.S., KWOCK L. and BUTTERFIELD C.E. (1979a). Diethyldithiocarbamate enhancement of radiation and hyperthermic effects on Chinese hamster cells in vitro. *Radiat.Res.* **77**, 501-511.

LIN P.S., KWOCK L., LUI P. and HEFTER K. (1979b). Enhancement of oxygen toxicity by diethyldithiocarbamate on Chinese hamster and mouse cells. *Int.J.Radiat.Biol.Oncol.Phys.* **5**, 1699-1703.

LIN P.S., QUOMO S., HO K.C. and BAUR K. (1985). The diethyldithiocarbamate concentration effects and interactions with other cytotoxic agents on Chinese Hamster Cells (V79). *Radiat.Res.* **102**, 271-282.

LIN W.S., ARMSTRONG D.A. and LAL M. (1978). Effects of superoxide dismutase, dithiothreitol, and formate ion on the inactivation of papain by hydroxyl and superoxide. *Int.J.Radiat.Biol.* **33**, 231-243.

LOVEN D.P., DOWNES D.L. and McLENDON D.L. (1988). Heat shock increases superoxide production by thermosensitive, but not thermotolerant CHO cells. (Abstract) 36th Conference of the American Radiation Research Society and the 8th Annual Meeting of the North American Hyperthermia Group. April 16-21 1988 p47.

LOWN J.W. and WEIR G. (1978). Studies related to antitumour antibiotics. Part XIV. Reactions of mitomycin C with DNA. *Can.J.Biochem.* **56**, 296-304.

LOWRY O.H., ROSEBROUGH N.J., FARR A.L. and RANDALL R.A. (1951). Protein measurement with folin phenol reagent. *J.Biol.Chem.* **193**, 265-275.

LUNEC J. (1981). A cautionary note on the use of thiol compounds to protect normal tissues in radiotherapy. *Br.J.Radiol.* **54**, 428-429.

MACQUER (1777) Dictionaire de Chymie, contenant la théorie et la practique de cette science, son application à la médicine, et aux arts dépendants de la chymie. (Paris 2nd ed. P.Fr Didot jeune, Libraire de la Faculté de Médecine.) Quoted in Partington J.R. (1962) A History of Chemistry v.3. **St. Martins Press**, New York, USA.

MALINOWSKI D.P. and FRIDOVICH I. (1979). Subunit association and side chain reactivities of bovine erythrocyte superoxide dismutase in denaturing solvents. *Biochemistry* **18**, 5055-5060.

MANERS A.W., WALKER E.M., BAKER M. and PAPPAS A. (1985). Diethyldithiocarbamate as a radiation modifier. *Ann.Clin.Lab.Sci.* **15**, 343-344.

MANN T. and KEILEN D. (1938). *Proc.Roy.Soc.(Lond).* **B126**, 303-315.

MANTEL N. (1966). Evaluation of survival data and two new rank order statistics arising from its consideration. *Cancer.Chemother.Rep.* **50**, 163-170.

MANTEL N. and HAENSZEL W. (1959). Statistical aspects of the analysis of data from retrospective studies of disease. *J.Natl.Cancer.Inst.* **22**, 719-48.

MARKLUND S. and WESTMAN G. (1980). Biological and Clinical aspects of superoxide and superoxide dismutase. W.H. Bannister & J.V. Bannister (eds) **Elsevier** Holland. p318.

MARKOSSIAN K.A., POGHOSSIAN A.A., PAITIAN N.A. and NALBANDYAN R.M. (1978). Superoxide dismutase activity of cytochrome oxidase. *Biochem.Biophys.Res.Comm.* **81**, 1336-1343.

MARKOWITZ H., CARTWRIGHT G.E. and WINTROBE M.M. (1959). Studies on copper metabolism XXVII. The isolation and properties of an erythrocyte cuproprotein (erythrocuprein). *J.Biol.Chem.* **239**, 40-45.

McCORD J.M., BEAUCHAMP C.O., GOSEIN S., MISRA H.P. and FRIDOVICH I. (1973). Proceedings of the second International Symposium on oxidases and related redox systems. T.O. King, H.S. Mason, and M.Morrison (Eds), **University Park Press**. Baltimore, USA. 51-76.

McCORD J.M., CRAPO J.D. and FRIDOVICH I. (1977). 'Superoxide dismutase assays : A review of methodology.' in *Superoxide and superoxide dismutases.* A.M. Michelson (ed) **Academic Press** New York, USA.

McCORD J.M. and FRIDOVICH I. (1968). The reduction of cytochrome c by milk xanthine oxidase. *J.Biol.Chem.* **243**, 5753-5760.

McCORD J.M. and FRIDOVICH I. (1969a). Superoxide dismutase. An enzyme function for erythrocuprein (hemocuprein). *J.Biol.Chem.* **244**, 6049-6055

McCORD J.M. and FRIDOVICH I. (1969b). The utility of superoxide dismutase in studying free radical reactions I. Radicals generated by the interactions of sulfite, dimethyl sulfoxide and oxygen. *J.Biol.Chem.* **244**, 6056-6063.

McCORD J.M. and FRIDOVICH I. (1977). Superoxide dismutases : A history in *Superoxide and superoxide dismutases.* A.M. Michelson. (ed) **Academic Press.**

McCORD J.M., KEELE B.B.Jr. and FRIDOVICH I. (1971). An enzyme based theory of obligate anaerobiosis - The physiological function of superoxide dismutase. *Proc.Nat.Acad.Sci.USA.* **68**, 1024-1027.

McGILVERY R.W. and GOLDSTEIN G.W. (1983). Biochemistry, A functional approach. 3rd edition, **W.B. Saunders Co.** Philadelphia, USA.

MICHAEL B.D., ADAMS G.E., HEWITT H.B., JONES W.B.G. and WATTS M.E. (1973). Post effect of oxygen in irradiated bacteria - a submillisecond fast mixing study. *Radiat.Res.* **54**, 239-251.

MIJNHEER B.J., WOOTON P., WILLIAMS J.R., ENMAA J. and PARNELL C.J. (1987). Uniformity in dose protocols for therapeutic applications of fast neutron beams. *Med.Phys.* **14**, 1020-1026.

MILAS L., ITO H., HUNTER N., JONES S. and PETERS L.J. (1986). Retardation of tumour growth caused by radiation induced injury of the tumour bed stroma : Dependency on tumour type. *Cancer.Res* **46**, 723-727.

MILLER S.L. (1953). A production of amino acids under possible primitive earth conditions. *Science* **117**, 528-529.

MILLS G.C. (1957). Haemoglobin catabolism : 1. Glutathione peroxidase, an erythrocyte enzyme which protects haemoglobin from oxidative breakdown. *J.Biol.Chem.* **229**, 189-197.

MISRA H.P. and FRIDOVICH, I. (1972a). The role of superoxide anion in the autoxidation of epinephrine and a simple assay for superoxide dismutase. *J.Biol.Chem* **247**, 3170-3175.

MISRA H.P. and FRIDOVICH, I. (1972b). The purification and properties of superoxide dismutase from *neurospora crassa*. *J.Biol.Chem* **247**, 3410-3414.

MISRA H.P. and KEELE B.B.Jr. (1975). The purification and properties of superoxide dismutase from a blue-green alga. *Biochim.Biophys.Acta.* **379**, 418-425.

MITCHELL J.B. and RUSSO A. (1983). Thiols, thiol depletion and thermosensitivity. *Radiat.Res.* **95**, 471-485.

NOHL H. and HENGER D. (1978). Do mitochondria produce oxygen radicals in vivo ? *Eur.J.Biochem.* **82**, 562-567.

NOWAK K., PECKHAM M.J. and STEEL G.G. (1978). Variation in response of xenografts of colorectal carcinoma to chemotherapy. *Br.J.Cancer.* **37**, 576-584.

OPARIN A.I. (1936). *The origin of life.* Translated by S. Margolis. **Dover**, New York, USA.

PACKER J.E. and WINCHESTER R.V. (1970). ^{60}Co gamma radiolysis of oxygenated aqueous solutions of cysteine at pH 7. *Canad.J.Chem.* **48**, 417.

PAGLIA D.E. and VALENTINE W.N. (1967). Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. *J.Lab.Clin.Med.* **70**, 158-169.

PAREDES J., HONG W.K., FELDER T.B., DIMERY I.W., CHOKSI A.J., NEWMAN R.A., CASTELLANOS A.M., ROBBINS K.T., McCARTHY., ATKINSON N., KRAMER A.M., HERSH E.M. and GOEPFERT H. (1988). Prospective randomised trial of high dose cisplatin fluorouracil infusion with and without sodium diethyldithiocarbamate in recurrent and/or metastatic squamous cell carcinoma of the head and neck. *J.Clin.Oncol.* **6**, 955-962.

PARKINS C., FOWLER J.F. and DENEKAMP J. (1983). Low radioprotection by thiol in lung : The role of local tissue oxygenation. *Eur.J.Cancer.Clin.Oncol.* **19**, 1169-1172.

PATT H.M. (1953). Protective mechanisms in ionizing radiation injury. *Physiol.Rev.* **33**, 35-76.

PATT H.M., TYREE E.B., STRAUBE R.L. and SMITH D.E. (1949). Cysteine protection against X-irradiation. *Science* **110**, 213.

PETKAU A. and CHELACK W.S. (1974). Radioprotection of model phospholipid membranes by superoxide dismutase. *Fed.Proc.* **33**, 1505.

PETKAU A. and CHELACK W.S. (1976). Radioprotective effect of superoxide dismutase on model phospholipid membranes. *Biochim.Biophys.Acta.* **380** 445-456.

PETO J. (1984). The calculation and interpretation of survival curves in *Cancer Clinical Trials : Methods and Practice.* **Oxford University Press**, Oxford U.K. 350-380.

PRIESTLEY J. (1772). Observations on different kinds of air. *Phil.Trans.* **65**, 384-394.

PUGET K. and MICHELSON A.M. (1974). Isolation of a new copper containing superoxide dismutase bacteriocuprein. *Biochem.Biophys.Res.Comm.* **58**, 830-838.

QUEENSBERRY P., MORLEY A., HAWARD D. and STOHLMAN F. (1973). The effects of endotoxin on murine stem cells. *J.Cell.Physiol.* **82**, 239-244.

RIGAS D.A., EGNITIS-RIGAS C., BIGLEY R.H., STANKOVA L. and HEAD C. (1980). Biphasic radiosensitization of human lymphocytes by diethyldithiocarbamate : possible involvement of superoxide dismutase. *Int.J.Radiat.Biol.* **38**, 257-266.

RIGAS D.A., EGNITIS-RIGAS C. and HEAD C. (1979). Biphasic toxicity of diethyldithiocarbamate, a metal chelator to T-lymphocytes and polymorphonuclear granulocytes : Reversal by zinc and copper. *Biochem.Biophys.Res.Comm.* **88**, 373-379.

ROBINSON J.E., WIZENBURG M.J. and McCREADY W.A. (1974). Radiation and hyperthermal response of normal tissue in situ. *Radiology* **113**, 195.

ROE J.A., BUTLER A., SCHOLLER D.M., VALENTINE J.S., MARKY L. and BRESLAUER K.J. (1988). Differential scanning calorimetry of Cu,Zn superoxide dismutase, the opoprotein, and its zinc substituted derivatives. *Biochemistry* **27**, 950-958.

SAMUNI A., CHEVION M., HALPERN Y.S., ILAN Y.A. and CZAPSKI G. (1978). Radiation induced damage in bacteriophage T4. Effect of superoxide radicals and molecular oxygen. *Radiat.Res.* **75**, 489-496.

SAMUNI A. and CZAPSKI G. (1978). Radiation induced damage in *Escherichia coli* B - Effect of superoxide radicals and molecular oxygen. *Radiat.Res.* **76**, 624-632.

SAWADA S. and OKADA S. (1970). Cysteamine, cystamine and single strand breaks of DNA in cultured mammalian cells. *Radiat.Res.* **44**, 116-132.

SCHEELE C.W. (1777) Chemishe abhandlung von der luft und dem feuer. Uppsala, Leipzig. Sec 2. Chemical Treatise on air and fire. in Scheele C.W. *The collected papers of Carl Wilhelm Scheele.* **G.Bell & Sons**, 1931 New York, USA. 85-178.

SHELDON P.W. and SMITH A.M. (1975). Modest radiosensitization of solid tumours in C3H mice by the hypoxic cell sensitizer NDPP. *Br.J.Cancer.* **31**, 81-88.

SIGDESTAD C.P., CONNOR A.M. and SCOTT R.M. (1975). The effect of S-2-(3-aminopropylamino) ethylphosphorothioate (WR-2721) on intestinal crypt survival. I. 4 MeV X-rays. *Radiat.Res.* **62**, 267-275.

SIGDESTAD C.P., CONNOR A.M. and SCOTT R.M. (1976). The effect of S-2-(3-aminopropylamino) ethylphosphorothioate (WR-2721) on intestinal crypt survival. II. Fission neutrons. *Radiat.Res.* **62**, 430-439.

SIGDESTAD C.P., GRDINA D.J., CONNOR A.M. and HANSON W.R. (1986). A comparison of radioprotection from three neutron sources and ^{60}Co by WR-2721 and WR-151327. *Radiat.Res.* **106**, 224-233.

SMITH W.W., BRESCHER G., FRED S. and BUDD R.A. (1966). Effect of endotoxin on the kinetics of hemopoietic colony forming cells in irradiated mice. *Radiat.Res.* **27**, 710-717.

STANLEY J.A., SHIPLEY W.U. and STEEL G.G. (1979). The influence of tumour size on hypoxic fraction and therapeutic sensitivity of Lewis lung tumour. *Br.J.Cancer.* **36**, 107-108.

STEEL G.G. and ADAMS K. (1975). Stem-cell survival and tumor control in the Lewis lung carcinoma. *Cancer Res.* **35**, 1530-1535.

STEFFENS G.J., BANNISTER J.V., BANNISTER W.H., FLOHE L., GUNZLER W.A., KIM S-M. A. and OTTING F. (1983). The primary structure of Cu-Zn superoxide dismutase from *Photobacterium leiognathi* : Evidence for a separate evolution of Cu-Zn superoxide dismutase in bacteria. *Hoppe-Seyler's Z.Physiol.Chem.* **364**, 675-690.

STEINMAN H. (1978). Amino acid sequence of mangano-superoxide dismutase from *Escherichia coli* B. *J.Biol.Chem.* **253**, 8708-8720.

STONE D., LIN P.S. and KWOCK L. (1978). Radiosensitization of human erythrocytes by diethyldithiocarbamate. *Int.J.Radiat.Biol.* **33**, 393-396.

STROMME J.H. (1965a). Interactions of disulfiram and diethyldithiocarbamate with serum proteins studied by means of a gel filtration technique. *Biochem.Pharmacol.* **14**, 379-381.

STROMME J.H. (1965b). Metabolism of disulfiram and diethyldithiocarbamate in rats with demonstration of an *in vivo* ethanol induced inhibition of glucuronic acid conjugation of thiol. *Biochem.Pharmacol.* **14**, 393-408.

STROMME J.H. and ELDJARN L. (1966). Distribution and chemical forms of diethyldithiocarbamate and tetraethylthiuram disulphide (disulfiram) in mice in relation to radioprotection. *Biochem.Pharmacol.* **15**, 287-297.

STUSCHKE M., BUDACH V., BAMBERG M. and BUDACH W. (1990). Methods of analysis of censored tumour growth delay data. *Radiat.Res.* **122**, 172-180.

SUIT H.D., SHALEK R., FAGUNDES L., GOITEN M, and ROTHMAN K. (1978). Time distribution of recurrences of an immunogenic and non-immunogenic tumour following local irradiation. *Radiat.Res.* **73**, 251-266.

SUMMERS W.C., CLIFTON K.H. and VERMUND H. (1964). X-Irradiation of the tumour bed. I. A study of the indirect actions of radiation on transplantable tumors. *Radiology* **82**, 691-703.

SUMNER J.B. (1941). The chemical nature of catalase. *Adv.Enzymology* **1**, 163-176.

SUNDERMAN F.W. Jr, WHITE J.C., SUNDERMAN F.W. and LOCZYN G.W. (1963). Metabolic balance studies in hepatolenticular degeneration treated with diethyldithiocarbamate. *Am.J.Med.* **34**, 875-888.

SUNDERMAN F.W. (1979). Efficacy of diethyldithiocarbamate (dithiocarb) in acute nickel carbonyl poisoning. *Ann.Clin.Lab.Sci.* **9**, 1-10.

SUTHERLAND R.M. and PHIL A. (1968). Repair of radiation damage to erythrocyte membranes : The reduction of radiation induced disulphide groups. *Radiat.Res.* **34**, 300.

SYKES, J.A., McCORMACK F.X. and O'BRIEN, T.J. (1978). A preliminary study of the superoxide dismutase content of some human tumours. *Cancer Res.* **38**, 2759-2762.

SYLVESTER R.J., MACHIN D. and STAQUET M.J. (1978). A comparison of the alternative methods of calculating survival curves arising from clinical trials. *Biomedicine* **28**, 49-53.

TAGAKI Y., SHIKITA M., TERASIMA T. and AKABOSHI S. (1974). Specificity of radioprotective and cytotoxic effects of cysteamine in HeLa S_3 cells : Generation of peroxide as the mechanism of paradoxical toxicity. *Radiat.Res.* **60**, 292-301.

TAINER J.A., GETZOFF E.D., BEEM K.M., RICHARDSON J.S. and RICHARDSON D.C. (1982). Determination and analysis of the 2Å structure of bovine copper, zinc superoxide dismutase. *J.Mol.Biol.* **160**, 181-217.

TEGELSTROM H. (1975). Interspecific hybridisation *in vitro* of superoxide dismutase from various species. *Hereditas* **81**, 185-198.

THOMLINSON R.H. (1980). An experimental method for comparing treatments of intact malignant tumours in animals and its application to the use of oxygen in radiotherapy. *Br.J.Cancer.* **21**, 555-576.

THOMLINSON R.H. and CRADOCK E.A. (1967). The gross response of an experimental tumour to single doses of X-Rays. *Br.J.Cancer.* **21**, 108-123.

THOMSON J.F. (1963). Possible role of catalase in radiation effects on mammals. *Radiat.Res.* **Suppl 3**, 93-109.

TILL J.E. and McCULLOCH E.A. (1961). A direct measurement of the radiosensitivity of normal mouse bone marrow cells. *Radiat.Res.* **14**, 213-222.

URANO M., GERWECK L.E., EPSTEIN R., CUNNINGHAM M. and SUIT H.D. (1980). Response of a spontaneous murine tumor to hyperthermia: Factors which modify the thermal response in vivo. *Radiat.Res.* **83**, 312-322.

UREY H.C. (1952). On the early chemical history of the earth and the origins of life. *Proc.Nat.Acad.Sci.USA.* **38**, 351-363.

VAN BEKKUM D.W. (1956). The protective action of dithiocarbamates against the lethal effects of X-irradiation in mice. *Acta.Physiol.Pharmacol.Nederl.* **4**, 508-523.

VERGROESSEN A.J., BUTKE L. and VOS O. (1967). Protection against X-irradiation by sulphydryl compounds 2. Studies on the relation between chemical structure and protective activity for tissue culture cells. *Int.J.Radiat.Biol.* **13**, 77.

VERMUND H., STENSTROM K.W., MOSSER D.G. and JOHNSON E.A. (1956). Effects of Roentgen irradiation on the tumour bed II. The inhibiting action of different dose levels of local pretransplantation Roentgen irradiation on the growth of mouse mammary carcinoma. *Radiat.Res.* 5, 357-364.

WEISIGER R.A. and FRIDOVICH I. (1973). Superoxide dismutase - Organelle specificity. *J.Biol.Chem.* **248**, 3582-3592.

WEISS J.F., JACOBS A.J. and KUMAR K.S. (1985). Some immunological and biochemical effects of radioprotectant sulphydryl compounds. *Int.J.Immunopharmacol.* **7**, 338.

WESTMAN G. and MARKLUND S.L. (1980). Diethyldithiocarbamate, a superoxide dismutase inhibitor, decreases the radioresistance of Chinese hamster cells. *Radiat.Res.* **83**, 303-311.

WESTMAN N.G. and MARKLUND S.L. (1983). Diethyldithiocarbamate inhibition of CuZn-superoxide dismutase in human erythrocytes : No increase in radiation haemolysis. *Int.J.Radiat.Biol.* **43**, 103-109.

WESTMAN G. and MIDANDER J. (1984). Post irradiation diethyldithiocarbamate inhibition of CuZn-superoxide dismutase reduces clonogenic survival of Chinese hamster V79 cells. *Int.J.Radiat.Biol.* **45**, 11-20.

WHELDON T.E. and BRUNTON G.F. (1982). Relation of growth delay to cure for experimental tumour systems conforming to Poisson curve statistics. *Br.J.Cancer.* **45**, 1-9.

WILLIAMS M.V., ROJAS A. and DENEKAMP J. (1984). Tumour sensitization and protection : Influence of stromal injury on estimates of dose modification. *Int.J.Radiat.Oncol.Biol.Phys.* **10**, 1545-1549.

YOST F.J. and FRIDOVICH I. (1973). An iron containing superoxide dismutase from *Escherichia coli*. *J.Biol.Chem.* **248**, 4905-4908.

YUDELEV M. and JONES D.T.L. (1988). Evaluation of the gamma dose in a p(66) MeV/Be neutron therapy beam. *NAC Report* **88-15**. National Accelerator Centre, Faure, South Africa.

YUHAS J.M. (1973). Radiotherapy of experimental lung tumours in the presence and absence of a radioprotective drug, S-2-(3-aminopropylamino) ethylphosphorothioic acid (WR-2721). *J.Nat.Cancer.Inst.* **48**, 69-78.

YUHAS J.M. (1980). Active versus passive absorbtion kinetics as the basis for selective protection of normal tissues by WR-2721. *Cancer Res.* **40**, 1519-1524.

YUHAS J.M., SPELLMAN J.M. and CULO F. (1980). The role of WR2721 in radiotherapy and/or chemotherapy. in *Radiation sensitizers* L. BRADY (ed) 303-308. **Masson** New York, USA.

ZEMAITIS M.A, and GREENE F. (1979). In vivo and in vitro effects of thiuram disulphides and diethyldithiocarbamates on hepatic microsomal drug metabolism in the rat. *Toxicol.Appl.Pharmacol.* **48**, 343-350.