Superoxide dismutase

RADIOBIOLOGICAL SIGNIFICANCE. OCCURRENCE IN HUMAN TISSUES, TUMOURS AND TUMOUR CELL-LINES.

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av

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ABSTRACT
Superoxide dismutases (SOD) are enzymes found in all aerotolerant organisms, catalyzing the dismutation of superoxide radicals, in the fastest enzyme-catalyzed reaction known:

\[ 2 \text{H}^+ + 2 \text{O}_2^- \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \]

Since superoxide radicals are formed in large amounts during aerobic irradiation, an experimental investigation on the possible significance of SOD in radiobiology and radiotherapy was of interest.

In the thesis the concept was studied in two different ways:

1. The activities of SOD (and for comparison also the activities of the hydrogen peroxide degrading enzymes catalase and glutathione peroxidase) were determined in human tissues, tumour and normal and neoplastic human cell-lines. The investigation gives comprehensive data on the enzyme activities in these systems and shows that CuZn SOD and glutathione peroxidase activities are evenly distributed, while Mn SOD and catalase activities vary considerably between different tissues, tumours and cells. However, no obvious relation was found between the enzyme activities and empirically assumed radiosensitivities.

II. A procedure was developed for inhibition of CuZn SOD in cells in vitro, by the chelating agent diethyldithiocarbamate, (DDTC). Temporary inhibition of CuZn SOD activity at the time of irradiation or in the first post-irradiation hours was shown to increase radiation damage to Chinese hamster cells, whilst no increase was seen for radiation-induced haemolysis of erythrocytes.

It is concluded that inhibition of CuZn SOD in actively dividing and metabolizing cells increases radiation damage probably through mechanisms related to potential lethal damage.

Key words: Superoxide dismutase, Radiation sensitivity, Diethyldithiocarbamate, Haemolysis, Catalase, Glutathione Peroxidase, Potential lethal damage.
SUPEROXIDE DISMUTASE

RADIOBIOLOGICAL SIGNIFICANCE. OCCURRENCE IN HUMAN TISSUES, TUMOURS AND TUMOUR CELL-LINES.

by
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This dissertation is based on the following papers referred to in the text by their Roman numerals:


1 GENERAL BACKGROUND

1.1. Introduction

Oxygen is absolutely essential for the life of higher organisms. Nevertheless life on earth began in the absence of oxygen, in a reducing atmosphere. The appearance of the first blue-green algae approximately 2 billion years ago and the subsequent oxygenation of the biosphere through photosynthesis, imposed a strong evolutionary pressure on many organisms that up to then had lived and evolved in the world. The presence of oxygen was dangerous for most compounds containing carbon, hydrogen and nitrogen, which in the presence of oxygen easily oxidize to the corresponding oxides, C to CO₂, N to NO₂ and H to H₂O. In parallel to developing mechanisms for the utilization of oxygen for energy production the organisms also had to develop defenses against the toxicity of oxygen.

However, oxygen is still toxic to most organisms at concentrations that are only slightly greater than those in normal air. Oxygen toxicity is seen in whole animals, plants and bacteria, and also in cell culture, homogenates and isolated organelles. Breathing pure oxygen rapidly damages lungs and causes retinal damage in newborn babies. Different aspects of oxygen toxicity has, therefore, attracted considerable interest since more than a century (19, 55, 81).

Oxygen toxicity is intimately related to the generation of free radicals, and the discovery by McCord and Fridovich (109) in 1969 of the enzyme superoxide dismutase (SOD), has opened new horizons for the research on oxygen toxicity. SOD is the first enzyme found which catalyzes a reaction between free radicals, the superoxide radicals, which are the first univalent reduction products of molecular oxygen.

Free radicals are of great scientific interest in a broad field of biology. In human biology and pathology they are associated with ageing (79), inflammatory processes (111), autoimmune diseases (49), ischemic diseases (132), and with neoplastic diseases (107, 108, 166, 167); with the effects of
radiation (31), phagocytic killing of microorganism (10), killing of tumour cells by NK cells (144) and with the action and toxicity of several drugs and chemicals (11,35,68,141,152,164).

Intermediates of oxygen reduction, therefore, are common in the cell and consequently knowledge of our defenses against these deleterious species is an important field in scientific research. The present study contribute to this field with special emphasis on the occurrence of protective enzymes in tissues and cells and the possible significance of SOD in radiobiology and radiotherapy.

1.2. Free radical chemistry

A free radical (R*) is defined as any atom or molecule with one unpaired electron occupying an outer orbital. A radical can be charged, e.g. H₃N⁺ (protonated amine radical), O₂⁻ (superoxide anion radical, superoxide radical, superoxide), or uncharged, e.g. NO (nitrous oxide). Free radicals are generally very reactive; some like the superoxide radical being considered less dangerous than others, like for example the hydroxyl radical (OH*). Free radical reactivity is accounted for by the strong tendency of the unpaired electron to interact with other electrons to form an electron pair. Radical reactions are usually divided into three types, initiation, propagation and termination reactions.

Initiation means the dissociation of a covalent bond,

\[ R:R \rightarrow R^* + R^* \]

and may be accomplished by thermal energy, electromagnetic energy (e.g. radiation), or by energy derived from oxidation-reduction coupling with a source of free radicals (e.g. H₂O₂).

a) thermal decomposition: \[ H₂O₂ \rightarrow 2 OH* \]

b) radiolysis of water: \[ H₂O \rightarrow OH^*, e^-, H^*, H₂O₂ \]

......
c) redoxcoupling with Fe: \[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH}^- + \text{OH}^+ \]

Reaction rates are modified by the products formed - (i.e. the effect of resonance stabilisation), heat of formation and presence of catalyzing compounds.

After formation of free radicals they may be propagated by several mechanisms, e.g. addition and transfer reactions:

d) atom transfer reaction: \[ \text{R}^* + \text{R}'\text{H} \rightarrow \text{RH} + \text{R}'^* \]

e) addition reaction: \[ \text{R}^* + -\text{C} = \text{C} \rightarrow \dot{\text{R}} - \text{C} - \text{C} - \]

Propagation finally ends with termination reactions, e.g. combination or disproportionation reactions:

f) combination reaction: \[ \text{R}^* + \text{R}^* \rightarrow \text{R-R} \]

g) disproportionation reaction: \[ \text{R}^* + \text{R}'\text{CH}_2 -\text{C} - \text{HR''} \rightarrow \text{RH} + \text{R}'\text{CH} = \text{CHR''} \]

1.3. **Intermediates in oxygen reduction**

1.3.1. **Molecular oxygen, O\(_2\)**

Molecular oxygen in its ground state is a biradical with two unpaired electrons with parallel spins in its outer orbital. This parallel electron spin arrangement prevents the direct addition of a pair of electrons with antiparallel spins, and an unfavourable so-called spin inversion is necessary for this process to occur. Spin inversion is a slow process and so there is seldom enough time for it to occur, during the life-time of the collisional complex. Therefore, if possible the one electron reduction of oxygen, involving no spin inversion, would dominate over the two electron reduction, (162).
1.3.2. Superoxide radical, $O_2^-$

The first step in the univalent reduction of oxygen results in formation of the superoxide radical. Superoxide is the conjugate base of a weak acid $HO_2^-$, with a $pK_a$ of about 4.8. Thus at the pH of the living cell about 7.4, the superoxide radical dominates over the perhydroxyl radical by almost a factor of $10^3$ (18).

Superoxide acts both as a reducing agent and as an oxidizing agent, i.e. donates or accepts electrons. In general the reactivity is low compared to other free radical species, and the damaging effects of superoxide therefore must be accounted for by the secondary production of other, more reactive species. The low reactivity on the other hand permits a long lifetime and a large diffusion capacity. The superoxide radicals can, therefore, be considered to play the role of a transmitter or generator of 'site-specific' hydroxyl radicals (24,50).

Superoxide radicals are generated in biological systems in many different ways. Several enzymes produce $O_2^-$ during oxidation-reduction reactions (e.g. xanthine oxidase, aldehyde dehydrogenase, dihydroorotic dehydrogenase and several flavin dehydrogenases (55). Drugs and chemical compounds produce superoxide radicals on their autoxidation or metabolism in the cell. Hemoglobin and myoglobin in their oxygenated form slowly liberate superoxide radicals when they are transformed to methemoglobin and metmyoglobin (55). In the cell superoxide generation has been demonstrated in fragments of organelles such as mitochondria (42), microsomes (8), chloroplasts (130) and also in nuclear preparations (14).
The concentration of superoxide radicals in the aerobic cell is maintained at about $10^{-11}$ M (168). Most of the radicals are formed in mitochondria, and the largest generation of radicals take place in the liver. On the whole, probably only a few percent of the total amount of oxygen consumed by the cell is converted to superoxide radicals (55). This is nevertheless a considerable quantity of radicals, due to the large amount of oxygen metabolized per unit time in the cell.

1.3.3. **Hydrogen peroxide, $H_2O_2$**

Hydrogen peroxide is generated when oxygen is reduced by two electrons (divalent reduction) or when superoxide is reduced by one electron (univalent reduction). Hydrogen peroxide in itself seems to be quite unreactive in the cell (501). This is due to two factors: i) most of the peroxide is generated in small special subcellular organelles called peroxisomes, where the hydrogen peroxide degrading enzyme catalase is also localized, ii) the low concentration in other parts of the cell ($10^{-8}$ M (130). High concentrations of hydrogen peroxide on the other hand is cytotoxic.

Hydrogen peroxide is produced by several enzymes: for example urate oxidase, L-amino acid oxidase and glycollate oxidase (in the peroxisomes) which transfer two electrons from their substrates onto each oxygen molecule. Most of the hydrogen peroxide in the cell is, however, produced by univalent reduction of superoxide, e.g. by the enzyme, xanthine oxidase.

1.3.4. **Hydroxyl radical, $OH^*$**

The hydroxyl radical is generated by univalent reduction of hydrogen peroxide, or directly from water by ionizing radiation. This radical is very reactive and a very strong oxidizing agent (50). It removes electrons from other molecules and reacts with almost every other compound in a very short time. In view of its high reactivity ($k=10^{11}$ to $10^{12}$), the existence of free hydroxyl radicals in the cell has been questioned (24).
1.3.5. **Transition metal catalysis**

The presence of transition metals, e.g. iron, in the cell, is potentially dangerous as iron may catalyse several types of toxic redox reactions. Iron within the cell is not "free" but is chelated with ubiquitous cellular constituents as amino acids or specific proteins. Iron chelates are more soluble than free ferrous iron at or near neutral pH. Ferrous ions chelated with ADP, phosphate or other chelating substances undergo rapid autoxidation with generation of both hydrogen peroxide and hydroxyl radicals (36,75). Ferric iron complexes react rapidly with superoxide, which by donating its electron to the iron reduces it to the ferrous form and in the presence of hydrogen peroxide, hydroxyl radicals are formed. This reaction has been called the iron-catalyzed Haber-Weiss reaction and is of central importance in the discussion of the dangers of superoxide in biology (78).

\[
\begin{align*}
O_2^- + Fe^{3+} & \rightarrow O_2 + Fe^{2+} \\
Fe^{2+} + H_2O_2 & \rightarrow Fe^{3+} + OH^- + OH^*
\end{align*}
\]

**Figure 2.** The iron-catalyzed Haber-Weiss reaction.

1.4. **Enzymatic defense mechanisms**

The oxygen intermediates are too reactive to be tolerated by living organisms. Considering the common evolutionary pressure on a varied biota it is not surprising that multiple defense mechanisms evolved, (figure 3). However, in anaerobic organisms these mechanisms are often absent or may exist at an insignificant level (110).

**Figure 3.** Enzymatic defense mechanisms to bypass and prevent the accumulation of reactive oxygen intermediates.
1.4.1. Superoxide dismutase

Superoxide dismutase, was first isolated as a 35,000 molecular weight copper protein in erythrocytes and liver by Mann and Keilin in 1939 (101). In the mid-1960 the protein was isolated (without knowledge of its dismutating function) as an antiinflammatory protein by Huber et al. (87). An enzymic function of the enzyme was discovered in 1969 by I. Fridovich and J. McCord at Duke University (109). The enzyme catalyzes the disproportionation of superoxide radicals:

\[ 2H^+ + 2O_2^- \rightarrow O_2 + H_2O_2 \]

Thus the reaction occurs in a sequential pathway involving consecutive reactions in which two identical substrate molecules dismutate to higher and lower oxidation states. The reaction is the fastest enzyme reaction known with a reaction rate constant \( k \) for the CuZn form of the enzyme of:

\[ k = 2 \times 4 \times 10^9 \, M^{-1}s^{-1} \text{ at pH 7} \, (55) \]

The enzyme works efficiently even at the low intracellular enzyme concentrations.

Several types of the enzyme exist: in the eukaryotic cytosol there is a CuZn SOD with a molecular weight of 33,000, and in mitochondria a Mn SOD with a molecular weight of about 85,000. Recently a new extracellular CuZn containing enzyme was isolated from human lungs, the enzyme being distinctly different from the ordinary CuZn SOD with a molecular weight of about 135,000 (105). Prokaryotic organisms have both a Mn SOD and a Fe SOD. The CuZn SOD and Mn SOD are readily distinguished, even in crude extracts, since the former is inhibited by cyanide and is resistant to treatment by a mixture of chloroform and ethanol, whereas the latter is resistant to cyanide but is denatured by chloroform and ethanol. Hydrogen peroxide inactivates CuZn SOD in vitro at alkaline pH, through oxidative attack on a histidine residue in the active site of the enzyme (85).
Superoxide dismutases have been found in all aerotolerant organisms but not in obligate anaerobic organisms (110). The manganoenzyme is induced by oxygen in microorganisms and is also inducible in mammalian cells and tissues. The mitochondrial and bacterial Mn SOD have homologous amino acid sequences (153) in agreement with the hypothesis that mitochondria have evolved from an endocellular symbiosis between a prokaryote and a protoeukaryote.

It may be questioned as to why an enzyme is needed for the catalysis of a reaction that is so rapid, even in the absence of catalysis. As Fridovich (55) has discussed, however, the presence of $1 \times 10^{-5}$ M CuZn SOD in the cell increases the rate of the dismutation of $1 \times 10^{-10}$ M superoxide by a factor of $10^9$.

1.4.2. Catalase

Catalase (see review 32), is a hemoprotein (i.e. it contains iron), and its existence has been known since the beginning of the 19th century. It is present in virtually all mammalian cell types and catalyze the disproportionation of hydrogen peroxide to oxygen and water:

$$2H_2O_2 \rightarrow O_2 + 2H_2O$$

Catalase works equally well at high and low concentrations of oxygen but can not utilize other peroxides than hydrogen peroxides. The enzyme is localized in subcellular organelles such as the peroxisomes (microbodies) of the liver and the kidney and in other cells in much smaller aggregates such as the microperoxisomes and sometimes in the mitochondria.

1.4.3. Glutathione peroxidase

Glutathione peroxidase (see review 53), was discovered in 1957 by Mills (116), but the presence of selenium in the active site of the enzyme was not reported until 1972 (52). This enzyme catalyzes the reaction between several types of hydroperoxides (apart from hydrogen peroxide) and requires reduced glutathione (GSH) as a hydrogen donator. Also lipid
hydroperoxides may be inactivated by the enzyme, preventing thereby the propagation of the radical chain reactions during lipid peroxidation. During the reduction of the peroxide, GSH is oxidized to GSSG (oxidized glutathione). The reduction of GSSG back to GSH is accomplished by the enzyme glutathione reductase using NADPH as hydrogen donator. The NADPH is then regenerated by the hexose monophosphate pathway (HMP shunt).

\[
\text{GSHPX} \\
\begin{align*}
2 \text{GSH} + \text{ROOH} & \rightarrow \text{GSSG} + \text{ROH} + \text{H}_2\text{O} \\
\text{GSHRED} \\
\text{GSSG} + \text{NAD(P)H} + \text{H}^+ & \rightarrow 2 \text{GSH} + \text{NAD(P)}^+
\end{align*}
\]

Glutathione peroxidase works well at low concentrations of hydrogen peroxide but becomes saturated at higher concentrations. The subcellular distribution of glutathione peroxidase is complementary to that of catalase, with two thirds of the enzyme in the cytosol and one third in the mitochondria (32). The relative contribution of catalase and glutathione peroxidase to hydrogen peroxide degradation probably varies from cell type to cell type.

1.5. **Nonenzymatic defensive mechanisms**

Probably the most important hydrophilic defensive mechanism in the cell is reduced glutathione (GSH), which is a small tripeptide (γ-Glu-Cys-Glu). Glutathione (see review 91) has several functions in the cell. In connection with protection against free radical damage it is able to: i) scavenge hydroxyl radicals, ii) donate its hydrogen to radical-damaged target molecules, and iii) function as hydrogen donor for the action of glutathione peroxidase.

The normal concentration of GSH in the cell is quite high \(1-50 \times 10^{-4}\) M. As oxidized glutathione GSSG, is very toxic to the cell (through mixed disulfide formation) the cell tries to maintain the ratio GSH/GSSG at a constant level. Other thiol molecules in the cell as cysteine and protein
bound sulfhydryls also contribute, although to a minor extent, to the
defensive action of intracellular sulfhydryls.

Ascorbic acid reacts rapidly with both superoxide and hydroxyl radicals. It
has been found to be radioprotective in vitro (129) and in vivo (2), but like
oxidized GSH, ascorbic acid that has donated its hydrogen, may also be
deleterious since it combines with or damages cellular constituents,
especially if traces of metal ions are present. Therefore animal cells
possess a NADH semidehydro ascorbate reductase that presumably
functions in vivo to regenerate ascorbate from its oxidation products (61).

Compounds that form complexes with transition metal ions, as transferrin,
caeruloplasmin, ferritin and albumin are also of importance, as they may
prevent metal-catalyzed hydroxyl radical generation. Artificially added
simple aliphatic alcohols, thiourea and other thiols may also scavenge
hydroxyl radicals.

The hydrophobic regions of the cell are the lipid membranes with their
content of polyunsaturated fatty acids (PUFA). Since oxygen is 7-8 times
more soluble in non-polar media and metal complexes such as heme are
present in membranes, membranes are prone to undergo lipid peroxidation
(vide infra) with subsequent structural damage to the cell. The major
defensive mechanisms against this peroxidation are vitamin E (a-toco-
pherol) and vitamin A (a retinoid, retinol), and the enzyme glutathione-
peroxidase.

Vitamin E (see review 176), functions by interfering with the propagation
of radical chain reactions in the membranes, and perhaps also by quenching
singlet oxygen (an excited state of molecular oxygen). The vitamin E
radical formed is not very reactive.

Vitamin A (see review 38) and other retinoids also scavenge singlet oxygen-
dependent reactions in membranes. There is also a growing interest in the
utilization of these substances for the prevention of neoplastic
transformation of epithelial cells.
1.6. **Lipid peroxidation**

Lipid peroxidation (8,32,39,59,77,152), is a phenomenon of central importance for the understanding of the damaging effects of free radicals. Biological material contains a wide variety of polyunsaturated fatty acids (PUFA), in particular in membrane phospholipids. A characteristic property of such unsaturated fatty acids is that in the presence of free radical initiators and oxygen they undergo oxidative deterioration. Food products as milk, cheese, and meat fat all contain large amounts of PUFA. When they undergo lipid peroxidation this is called rancidity and is accompanied by a very unpleasant odour that makes food unpalatable.

A simplified scheme for the reactions involved in lipid peroxidation is shown in the figure in which R* is the radical initiator. In the first reaction, R*abstracts hydrogen from the unsaturated fatty acid (I) to yield the free radical (II). This is followed by the uptake of oxygen by (II) to produce the fatty acid peroxy radical (III). This radical (III) reacts with another molecule of (I) in a propagation step, to yield a further molecule of (II) and the stable end product the unsaturated hydroperoxide (IV).

\[
\begin{align*}
(-\text{CH}=\text{CH}-\text{CH}_{2}-) + \text{R}^* & \quad \rightarrow \quad (-\text{CH}=\text{CH}-\text{CH}) + \text{RH} \\
(\text{I}) & \quad (\text{II}) \\
(-\text{CH}=\text{CH}-\text{CH}) + \text{O}_2 & \quad \rightarrow \quad (-\text{CH}=\text{CH}-\text{CH}) \\
(\text{II}) & \quad (\text{III}) \quad \text{O-O}^* \\
(-\text{CH}=\text{CH}-\text{CH}) + (-\text{CH}=\text{CH}-\text{CH}_{2}-) & \quad \text{O-O}^* \quad (-\text{CH}=\text{CH}-\text{CH}_{2}-) \\
(\text{III}) & \quad (\text{I}) \\
(-\text{CH}=\text{CH}-\text{CH}) + (-\text{CH}=\text{CH}-\text{CH}) & \quad \text{O-OH} \quad (-\text{CH}=\text{CH}-\text{CH}) \\
(\text{IV}) & \quad (\text{II})
\end{align*}
\]

**Figure 4.** A simplified scheme for the chain reaction involved in the formation of unsaturated lipid hydroperoxide (IV).
Lipid peroxidation may be initiated by several types of free radicals among which are the oxygen free radicals. Lipid peroxidation is associated with a loss of unsaturated bonds, conjugation of double bonds (diene conjugation) and finally fragmentation with production of ethane, pentane and malonaldehyde (152, 161). The biochemical consequences of lipid peroxidation include perturbation of membrane architecture (8,152) with alteration of membrane function and permeability and enzyme inhibition. Examples of physiological consequences are hemolysis of erythrocytes, swelling of mitochondria, accumulation of lipofuscin in lysosomes, and generation of lipid peroxides in microsomes. It has been repeatedly shown that lipid peroxidation is much more likely to occur in membranes of animals who have been kept on diets deficient in vitamin E or selenium, and lipid peroxidation is also increased in animals fed a diet rich in polyunsaturated lipids (152,177). In vitro lipid peroxidation is greatly stimulated by ferrous iron and reducing substances (8,39), and some chelating agents that bind iron may paradoxically increase lipid peroxidation (77).

1.7. Radiation and oxygen free radicals

In aqueous dilute systems such as the cell, most of the energy of radiation is deposited in water molecules, which lyse to yield water radicals. The primary radicals formed are the hydroxyl radical (OH\(^\cdot\)), the electron (e\(^{-}\)), the hydrogen atom (H\(^\cdot\)), and some others in minor amounts (see table 1).

<table>
<thead>
<tr>
<th>(G_{e_{aq}}^+G_{H^+})</th>
<th>(G_{e_{aq}})</th>
<th>(G_{H^*})</th>
<th>(G_{OH^*})</th>
<th>(G_{H_2})</th>
<th>(G_{H_2O_2})</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.13-3.31</td>
<td>2.65-2.83</td>
<td>0.48-0.64</td>
<td>2.59-2.75</td>
<td>0.45</td>
<td>0.67-0.72</td>
</tr>
</tbody>
</table>

When oxygen is present (as in oxygenated water) the hydrogen atom and the electron reacts rapidly with the oxygen molecules, Eq. 1-3.
The reaction between H and O\(_2\), Eq. 1, generates perhydroxyl radicals (HO\(_2\)), but at physiologic pH this species rapidly loses the proton, and is transformed to the conjugate base O\(_2^-\), Eq. 2. Thus at pH 7.4 the superoxide radical dominates over the perhydroxyl radical by a factor of almost 10\(^3\).

The hydrated electron directly forms superoxide with molecular oxygen, Eq. 3.

Since the 1950s it has been known that oxygen enhances the biological effects of ionizing radiation by a factor of 2.5-3. Despite considerable research efforts the precise mechanism is still unknown. The most accepted theory, however, is the oxygen-fixation hypothesis (3), which states that molecular oxygen by binding to the target (T) molecule transformed by radiation (T'), fixes the target molecule damage:
T + aerobic irradiation $\rightarrow$ T' + O$_2$ $\rightarrow$ TOO (schematically)

Under hypoxic conditions the transformed target molecule can be reconstituted by donation of a hydrogen atom from reduced glutathione.

T + hypoxic irradiation $\rightarrow$ T' + GSH $\rightarrow$ TH + GSSG (schematically)

Recently this theory received a strong support by the demonstration of a reduced ability of genetically glutathione deficient fibroblasts to withstand anoxic irradiation, and consequently to have a reduced OER (114).

1.8. **Diethyldithiocarbamate (DDTC)**

Diethyldithiocarbamic acid sodium salt: White crystals, m.p. 90-2°C; soluble in water, ethanol, methanol, and acetone, insoluble in benzene and ether. Relatively stable at room temperature in neutral or alkaline solution, but unstable in acid solution. Reacts slowly with atmospheric oxygen to form insoluble oxidation products. Chelates most divalent and trivalent cations (21). For a review on older literature on DDTC see reference 163.

Analysis of DDTC in biological material has been performed using different analytical methods but today high performance liquid chromatography (HPLC) is probably the most sensitive and accurate method (89,121).
1.8.1. Inhibition of CuZn superoxide dismutase by DDTC

In 1976 Heikkila et al. (83) demonstrated that DDTC could inhibit CuZn SOD both in vitro and in vivo, and later Heikkila et al. (84) also described other compounds that were inhibitory. The DDTC inhibition was demonstrated in vivo in the mouse by administration of 1.5g/kg body weight of DDTC intraperitoneally. By this procedure liver enzyme activity and blood enzyme activity were at the lowest level (20% of initial activity) one hour after the DDTC administration and the enzyme activity then slowly returned. By 24 hours the enzyme activity in the liver was depressed to about 60% of initial value. In vitro the enzyme inhibition was shown to be essentially complete by incubation for 60 minutes with 2 mM DDTC (200 ng CuZn SOD/ml) and it could not be reversed by dialysis of the inhibited enzyme. However, enzyme activity was restored by dialysing in buffer containing 0.5 mM CuSO₄. Diethyldithiocarbamate formed a yellow complex with SOD, similar in appearance to the complex formed between DDTC and cupric ion complex. In contrast to inorganic Cu-DDTC, the complex between the Cu of CuZn SOD and DDTC was not extractable with organic solvents, which lead to the suggestion that DDTC was bound to the active site of the enzyme. Misra (118) suggested the formation of a ternary complex between enzyme-Cu and DDTC. Cocco et al. (34), in a recent study repeated the experiments of Misra and reached a divergent conclusion, demonstrating that DDTC removed Cu from the active site of the enzyme, in a slow process, leaving the apoenzyme. The failure to remove the comlex by dialysis, gel filtration or solvent extraction was thought to depend on interaction of Cu-DDTC with the protein part of the enzyme, but not involving the native copper coordination ligands of the active site. Lengfelder (93) demonstrated that the DDTC-Cu complex has no superoxide dismutase activity.

1.8.2. Inhibition of enzymes other than CuZn SOD

Purified beef heart cytochrome oxidase was inhibited by 25% with 1 mM DDTC (72). A 50% inhibition of isolated rat liver and beef heart cytochrome oxidase activity by 5-10mM DDTC has also been reported (60).
Metabolic activation of DDTC gives carbon disulphide, which can be oxidized by cytochrome P-450 to carbonylsulphide. This compound then releases atomic sulphur which can bind covalently to microsomal proteins in particular the cytochrome P-450 and destroy them. Therefore DDTC in vivo may inhibit cytochrome P-450. It has also been demonstrated that DDTC in vitro may inhibit benzo[a/- pyrene monoxygenase (BP monoxygenase), and in vivo a reduction of both liver and intestinal BP monoxygenase has been shown (67).

When DDTC is administered in vivo into rats the microsomal hydroxylation system is inhibited whilst the the glucuronic acid pathway enzymes are stimulated (90,106).

It has been found that DDTC in vitro and in vivo inhibits dopamine-b-hydroxylase and tyrosine hydroxylase (160).

Diethyldithiocarbamate can be enzymatically oxidized to its disulphide form and subsequently inhibit many other enzymes, preferentially SH-group containing enzymes through formation of mixed disulphides, e.g. glucose hexokinase (156), and xanthine oxidase (56).

Inhibition of CuZn SOD leading to increased concentration of superoxide radicals has also been proposed to cause inhibition of glutathione peroxidase in vivo and in vitro (64).

1.8.3. Effects of DDTC on subcellular organelles

Ultrastructural changes have been found in the mouse hepatocyte after DDTC feeding (1% in the diet) (7). Changes included reduction of liver weight and pale appearance of the livers. In the cells there was formation of megamitochondria evenly distributed in the hepatocyte, dilatation of the endoplasmic reticulum and disappearance of fat and glycogen droplets. The mitochondria were too large to be accounted simply by a swelling of each mitochondrion. In the mitochondria the matrix and cristae were normal as far as their quantity and appearance were concerned. These authors found
no other changes in cells apart from in the hepatocytes. In a study on malignant 3T3 cells similar changes in ultrastructure were noted, however, after incubation for 2 hours with 0.1mM DDTC (96).

In another study DDTC (1.2g/kg) caused a marked reduction in rat cardiac mitochondrial SOD, considerably decreased the oxygen consumption and respiratory control and induced a slower oxidative phosphorylation. Diethyldithiocarbamate also caused increased malondialdehyde content in mitochondria, indicating an increased lipid peroxidation of mitochondrial membrane (73).

Gallagher and Reeve (60) studying mitochondrial phospholipid synthesis noted that DDTC 5-10mM (as did copper deficiency) caused a reduction in the binding of ADP to the inner mitochondrial membrane. This was proposed to impair the ATP production. The impaired ATP formation then would cause a decrease in the translocation of ATP to the outer mitochondrial membrane, and a depression of ATP-dependent phospholipid synthesis.

It was demonstrated that DDTC induces synthesis of four proteins in normal chick embryo and human foreskin cells, and in the presence of copper DDTC was found to bind to HeLa cell DNA and Rous sarcoma virus 70S genome RNA (93).

1.8.4. Other biological effects

There are multiple studies of different biological effects of DDTC, some of these being initiated by the fact that DDTC is the reduced monomer of Antabuse (Disulfiram). Mutagenic effects were studied in Ames test, and although ZnDDTC and CdDDTC showed direct mutagenic effects, pure DDTC was not tested, and disulfiram was not mutagenic (82). In several studies DDTC (and disulfiram) has been shown to have anticarcinogenic effects when administered before carcinogens as benzo/a/pyrene (67), and dimethylhydrazine (167). Diethyldithiocarbamate has also been used to prevent the hepatotoxic action of carbon tetrachloride (100,164). In rats
DDTC prevented the nephrotoxic action of the cancer chemotherapeutic agent, cis-platinum (22) without abrogating its antineoplastic effect (23). In rats and humans it reduces the toxicity of cadmium (57), nickel carbonyl (158) and mercury vapor (157). Humans exposed to nickel carbonyl vapours were cured without side effects with DDTC 1 g twice daily for 10 days (158), while a nontreated individual died of nickel poisoning. On the other hand DDTC has also been shown to have toxic effects on cells in culture (140) and on spermatocytes (174) at low concentrations. It has a peculiar biphasic toxic effect on cells (41, 138). Various immunological effects of DDTC have been reported as the compound is related in structure to the thiol moiety of the immunoactive drug Levamisole (120). Diethyldithiocarbamate is also used in neuropsychological and neuropharmacological research (63) as a noradrenaline depleting agent (due to the inhibitory action of DDTC on the enzyme dopamine-b-hydroxylase).

Diethyldithiocarbamate decreased (40,54) short term (48h) survival of rats in normobaric hyperoxic (95-97%) atmosphere, and of mice in ozone (64), which was related to the inhibitory effect of DDTC on hyperoxia-induced increase in lung activities of SOD and glutathione peroxidase. However, DDTC was shown to increase long term (96h) survival of adult rats in 95-97% oxygen and potentiated the inducing effects of oxygen on the activities of glutathione peroxidase, glutathione reductase and glucose-6-phosphate dehydrogenase without any effect on the oxygen-induced increase of SOD (40).

Diethyldithiocarbamate is a radioprotector both in vitro and in vivo (4,12,17,170,171,). In these studies the compound was added just prior to the irradiation. The protective effect was as good as that of cysteine or cysteamine (4). After the discovery of DDTC as an inhibitor of CuZn SOD, a radiosensitizing effect of DDTC (95,96,143,155) was demonstrated in vitro, when the compound was added 30 to 90 minutes before irradiation. Diethyldithiocarbamate has also been found to protect against the lethal effect of nitrogen mustard (29) in vivo, but in vitro it was shown to increase the toxic effect of Bleomycin (97) and hyperthermia (95).
2 PRESENT INVESTIGATION

2.1 Aims

The interest in oxygen toxicity increased dramatically after the discovery of superoxide dismutase (SOD), as an obligatory defense against oxygen derived radicals. Oxygen also enhances the damaging effects of ionizing radiation and is therefore of great general interest in basic radiobiological science and particularly in radiotherapy. In view of this, an experimental investigation on the possible significance of SOD and superoxide radicals in radiobiology and radiotherapy was of interest. In the present thesis, different experimental approaches were taken in investigating this concept.

A. To determine whether inhibition of intracellular CuZn SOD would increase radiation damage because of decreased protection against radiation-generated superoxide radicals. For this purpose cell culture using Chinese hamster V 79 fibroblasts (I,V) which contain CuZn SOD and Mn SOD, and human erythrocytes (IV) which contain only CuZn SOD were used.

B. To determine whether an increase in extracellular activity of CuZn SOD would decrease radiation damage because of increased protection against radiation-generated superoxide radicals. For this purpose, bovine CuZn SOD was added prior to irradiation (IV).

C. To assess the activities of CuZn SOD and Mn SOD in human tissues, tumours, and cell lines with the aim of detecting an obvious relationship between the enzyme activities and assumed (albeit not tested) radio-sensitivity.
2.2 Materials and methods

A short description and some additional data on each method used in the publications (I-V) are presented in this section, but more detailed descriptions are found in the relevant publications or in the original publications on the methods given in the reference list.

2.2.1 Superoxide dismutase

Superoxide dismutase (SOD) activity (both CuZn SOD and Mn SOD) in homogenates (I-IV), was studied by a direct method (102), following the disappearance of superoxide radicals in a spectrophotometer. The radicals were produced from small pieces of solid potassium superoxide at pH 9.5, (the superoxide radical is more stable at alkaline pH); and the enhancement of the disproportionation reaction brought about by the addition of homogenates containing SOD was determined. The activity of Mn SOD was determined after inhibition of CuZn SOD by 1 mM KCN, and CuZn SOD as total SOD minus Mn SOD activity:

\[
2H^+ + 2O_2^- \xrightarrow{\text{SOD}} O_2 + H_2O_2
\]

This assay is direct and more sensitive than other assays, but is 10 times more sensitive for CuZn SOD than for Mn SOD. For this reason the Mn SOD activities in the thesis have been multiplied by a factor of 10.

Superoxide dismutase was also analysed by agarose gel electrophoresis in several of the tissues and the mesothelioma cell lines (III), and this semiquantitative method agreed in every case with the enzymic activities.

The accuracy of the SOD assay was determined from a control sample consisting of the same human haemolysate (stored at -80°C), over a period of almost a year. The standard deviation of the mean was about 5% both on a daily (n=54) and on a day-to-day (n=39) basis. This variation is valid for CuZn SOD and Mn SOD.
2.2.2 Glutathione peroxidase

Glutathione peroxidase (GSHPX) in homogenates (III, IV) was determined according to Günzler et al. (74), with some modifications, by following the consumption of NADPH at 340 nm in a spectrophotometer:

\[
\text{GSHPX} \quad \text{ROOH} + 2\text{GSH} \longrightarrow \text{ROH} + \text{GSSG} + \text{H}_2\text{O}
\]

GSSG is reduced back to GSH by the enzyme glutathione reductase (GSR):

\[
\text{GSR} \quad \text{GSSG} + \text{NADPH} + \text{H}^+ \longrightarrow 2\text{GSH} + \text{NADP}^+
\]

The variation of this method was about 7% (SD) on a daily basis (n=10). In parallel determinations the variation according to Günzler (74) was about 3%.

2.2.3 Catalase

Catalase (CAT) was determined by two different methods. In (I) and (III) the catalase activity was measured by following the catalase-catalyzed initial liberation of oxygen from hydrogen peroxide, using an oxygen electrode, essentially as described by Del Rio (37). This assay is 50 times more sensitive than ordinary spectrophotometric or titration methods. The variation of the method was around 10% on a daily basis (n=10).

\[
\text{CAT} \quad \text{H}_2\text{O}_2 + \text{H}_2\text{O}_2 \longrightarrow \text{O}_2 + 2\text{H}_2\text{O}
\]

Catalase activity in the erythrocytes (IV) was determined by following the decrease of absorption of hydrogen peroxide at 240 nm in a cuvette containing 10 mM hydrogen peroxide at pH 7.4. The accuracy of this assay is sufficient for the determination of catalase in erythrocytes since the enzyme is abundant in these cells.
2.2.4 Protein

Protein in the homogenates (I-IV) was determined using the blue colour compound Coomassie Brilliant Blue G-250 (27). This method is more sensitive, technically less complicated and more specific than the conventional Lowry method, and was shown to give comparable results in control experiments.

2.2.5 Cell culture

A substrain of the Chinese hamster cell line (V79-379A) was cultured in an ordinary CO\(_2\) cell culture incubator at 37°C, pH about 7.4 and at a relative humidity of more than 90%. The cells were grown in Eagle's minimal essential medium containing Earle's salts supplemented with 15% fetal calf serum and antibiotics in plastic (I) or glass (V) Petri dishes. For radiation experiments, exponentially growing cells were trypsinized, monodispersed, counted and seeded into dishes of 6 cm diameter. Treatment with DDTC was never started earlier than about 3 hours after explantation, and irradiation was given about 5 hours after explantation. Control dishes were treated in the same way. Control experiments with feeder cells demonstrated that the plating efficiency was not influenced by the number of cells initially explanted. Surviving clones were fixed, stained and counted after 7-8 days. Colonies had to contain at least 50 cells to be considered as representative of a surviving cell.

2.2.6 Cellular irradiation techniques

In experiments on clonogenic survival after irradiation, the number of cells explanted were adjusted according to each radiation dose in such a way that 50-100 cells would be expected to survive. In no case were more than 15,000 cells allocated to a 6 cm dish. Before the irradiation the cells in (I) were gassed with CO\(_2\) at 37°C, in tight plastic boxes, and then transferred to the radiation department for irradiation. The temperature during the irradiation was around 30°C. In the so-called postirradiation experiments (V), the cells were irradiated at room temperature without CO\(_2\) gassing. In experiments determining clonogenic survival under various conditions of oxygenation (V), the cells adhering to Petri dishes in only 1 ml of medium
were irradiated at room temperature; the small quantity of medium facilitating gas exchange. The dishes with their covers removed were placed in an air-tight plastic box and exposed before and during irradiation to a gust of either argon, containing less than 2 ppm oxygen impurity (total exposure time 15 min.), air (5 min.) or pure oxygen (5 min.). Carbon dioxide was added to the gas flow to maintain pH at a normal level.

2.2.7 Erythrocytes

Erythrocytes (IV) were collected from myself, washed three times with Eagle's MEM and diluted to a haematocrite value of 1/20. This suspension was then treated with DDTC as described below. In irradiation experiments aliquots of 0.4 ml were added into wells (diameter 2cm) in a multiwell dish, irradiated and the cells incubated overnight in a refrigerator before the determination of radiation-induced haemolysis.

2.2.8 Radiation-induced haemolysis

Erythrocyte haemolysis (IV) was determined by the median glycerol haemolysis procedure of Gottfried and Robertson (66). Irradiated erythrocyte suspension (20-50 ul) was added to 3 ml 0.2M glycerol in 50 mM NaCl buffered with 3 mM potassium phosphate, pH 7.4. The haemolysis was monitored as the fall in turbidity at 625. The median glycerol haemolysis time (GLT 50) was taken as the time required for the optical density to fall to half the initial value.

The mean GLT 50-value of control erythrocytes when assayed immediately after sham-irradiation was 53.5 ± 1.5s (SD, n=6) on a day to day basis. The GLT 50-value of control cells after sham-treatment and 20 hour incubation in the refrigerator varied more, 44.3 ± 3.7s (SD, n=19) between the experiments. The mean difference between double determinations of GLT 50 varying from 16.8 to 47.0 s within the radiation experiments, was around 2 per cent.
2.2.9 Radiation dose-effect curves

The survival curves from the experiments on the V79 cells (I, V) were plotted for each experiment. In these curves the logarithm of the surviving fraction is represented on the y-axis and radiation dose is represented on the x-axis. The survival curve may also be represented by an equation, and many different equations exist, based on different theories and yielding different parameters for the equations. The most commonly utilized equation, based on the so-called single-hit multitarget theory, is used in the present investigation:

\[ S = 1 - (1 - e^{-D/Do})^n \]

In this equation S is the surviving fraction, Do is the radiation dose that causes a decrease of the surviving fraction by a factor 1/e (about 37%) along the final straight portion of the curve. D is the actual dose administered and n the extrapolation number, is defined as the intercept of the extrapolated straight portion of the curve on the y-axis. The dose-modifying factor (DMF) or dose-modifying effect, is often defined as the ratio between the slope constants or Do-values of the straight parts of two survival curves in paired experiments. When oxygen is used as a modifying factor the DMF of oxygen is called the oxygen enhancement ratio, OER.

In erythrocyte experiments the logarithm of the GLT 50 (as percent of initial GLT 50) was plotted against radiation dose and the slope and intercept values were calculated by linear regression analysis.

2.2.10 Radiation split-dose survival

Split-dose experiments were used to study the capacity of the irradiated cells to recover from so-called sublethal radiation damage (48). In such experiments the surviving fraction after a single dose treatment is compared to the surviving fraction after administering the same dose, divided into two equal fractions (i.e. half the single dose administered twice). As expected, the survival after split-dose treatment is higher than after single dose treatment. The enhancement of survival is usually
presented as the split-dose ratio, which is defined as the surviving fraction after split-dose treatment divided by the surviving fraction after single-dose treatment, for example:

\[
\text{Split dose ratio} = \frac{\text{Survival after } 2 \times 4.0 \text{ Gy}}{\text{Survival after } 8.0 \text{ Gy}}
\]

By varying the time-interval between the split-dose fractions the kinetics of recovery can also be studied. In the split-dose experiments in (V) the time-interval between the split-doses was 18 hours. This interval has previously been found to be shorter than the time necessary to complete a cell division under the prevailing conditions (98).

2.2.11 Statistics

Statistical testing of the differences between the slopes and intercepts were performed by (one-way) analysis of variance in (I), by multiple regression analysis in (IV) and by Student's t-test in (V). The results of (I) has also been evaluated later by Student's t-test and multiple regression analysis with no change of the conclusions made in (I).

2.2.12 Diethyldithiocarbamate (DDTC)

The DDTC solution was freshly prepared every day. Since the pH of the solution was about 7.8, no relevant decomposition of DDTC was possible for several hours (21).

2.2.13 Diethyldithiocarbamate-inhibition of superoxide dismutase

Essentially the same procedure was used when treating V 79 cells and erythrocytes with DDTC. The ordinary medium in which cells were suspended was exchanged with medium containing 3mM DDTC, followed by incubation for 90 min in an incubator. In the erythrocyte experiments DDTC was added to the erythrocytes to give a final concentration of 3mM. DDTC was then removed by 4 successive medium changes (in erythrocyte experiments centrifugations were used), whereafter the cells were irradiated. In the experiments on postirradiation treatment of V 79 cells the irradiation was given at various intervals before the DDTC-treatment (V).
For the determination of SOD activity in DDTC treated V 79 cells, several cell culture dishes were treated with DDTC and at appropriate time intervals the cells were washed twice with buffered saline containing a diethylenetriamine pentaacetic acid (DTPA), a copper-chelating substance (to prevent enzyme reactivation), scraped off and stored at -80°C until enzyme assay. After 90 min DDTC was removed as described above. Control plates were treated identically. In the case of erythrocytes it was sufficient to use only one dish and take aliquotes (0.4ml) at predetermined intervals during and after the DDTC treatment.

2.2.14 Removal of DDTC
Removal of DDTC appeared essential in the radiation experiments as free DDTC in vivo and in vitro is an efficient radioprotector, and as removal would minimize effects on other enzymes in the cells. The free DDTC concentration after the washings therefore was determined in separate experiments (I), where about 15x10^6 cells were exposed to 15 mM DDTC for 90 minutes. The remaining DDTC after successive medium changes was estimated by spectrophotometry from homogenates, after extraction with isoamylalcohol. After four washings no remaining free DDTC (the lower detection level of the assay was 50 uM) could be found in the cell homogenates. In the radiation experiments, as a few thousand cells were exposed to only 3 mM DDTC, the free DDTC concentration in these cells would have been even lower.

2.2.15 Cell cycle analysis of DDTC-treated cells
In order to assess other effects of DDTC on the cells of possible importance for radiation response, separate experiments were performed aiming to detect a cell-cycle effect of DDTC on the V 79 cells. Such an effect of DDTC could change the radiosensitivity (76) of the cells.

In these experiments about 1 million cells were seeded into the Petri dishes, incubated for adherence for 3-4 hours, and then incubated with 3 mM DDTC for a further 90 minute period. After removal of DDTC by four successive medium changes, the cells were washed twice with phosphate
buffered saline and 1-2 ml of a propidium iodide solution containing RNAses and a detergent (173) was added. DNA analysis was performed by flow cytometry as described by Roos et al. (145). The fraction of cells in the different phases (G0-G1, G2-M and S) of the cell-cycle and the fraction of diploid cells were determined from the DNA histograms of DDTC-treated and untreated cells. The results from 4 separate experiments showed no significant difference between DDTC-treated and untreated cells. See table 2.

Table 2. DDTC effects on the distribution of V 79 cells in the various phases of the cell cycle and the fraction of diploid cells. Figures are mean and SEM of four paired experiments.

<table>
<thead>
<tr>
<th>DDTC O mM</th>
<th>DDTC 3 mM</th>
<th>DDTC 0 mM</th>
<th>DDTC 3 mM</th>
</tr>
</thead>
<tbody>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>G0-G1</td>
<td>S</td>
<td>G2-M</td>
<td>G0-G1</td>
</tr>
<tr>
<td>64.0</td>
<td>24.0</td>
<td>11.9</td>
<td>62.0</td>
</tr>
<tr>
<td>1.5</td>
<td>2.1</td>
<td>2.6</td>
<td>0.9</td>
</tr>
</tbody>
</table>

2.2.16 Ultrastructural effects of DDTC

The effect of DDTC on ultrastructure of V 79 cells was investigated in a separate experiment. In this experiment about 5 million cells were used and treated with DDTC for 90 minutes as described earlier. After removing the DDTC and washing the cells twice with isotonic NaCl the cells were fixed in 2.5% glutar aldehyde in 0.1 M sodium-cacodylate + 0.1 M sucrose, after-fixated in 1% osmium tetroxide in 0.15 M sodium-cacodylate, embedded in Epon, sectioned and finally contrasted with uranyl acetate and lead citrate. A JEOL 100 CX electron microscope was utilized for morphological assessment.

No ultra-structural aberration could be detected in the cells treated with 3 mM DDTC compared to the DDTC-untreated cells, see figure 5.
Figure 5a. Electron micrograph of a DDTC-untreated Chinese hamster V 79 cell. M = mitochondrion, N = nucleus.
Figure 5b. Electron micrograph of a Chinese hamster V 79 cell treated with 3 mM DDTC for 90 min.
2.2.17 Cell lines

Cell lines used in (III) consisted of 31 human cell lines of different origins. Most lines are well known and are characterized in a number of earlier investigations. The mesothelioma cell-lines were established from pleural effusions and have neoplastic properties, as judged by chromosome analysis and cell growth characteristics (154). The JC-1 line, derived from a giant cell tumour, had reached passage 21 and could not yet be considered as a permanent cell line. The cells were grown in Eagle's minimal essential medium (anchorage dependent lines) or Ham's F-10 medium (suspension growing lines) supplemented with 10% fetal calf serum, and with antibiotics. The cells were harvested 3-7 days after explantation, washed twice with 0.15 M NaCl containing 3 mM DTPA, centrifuged and stored at -80°C until assay of enzymes.

2.2.18 Tissue specimens

Tissue specimens were collected from accident victims without known physical disease within 24 hours after death. Homogeneous tumour pieces were cut out of surgical specimens within 1 hour after their removal from the patients. The tissues were then stored at -80°C until homogenized.

2.2.19 Correction for tissue erythrocyte contamination

Erythrocytes contain large amounts of three of the enzymes assayed in (III). The content of glutathione peroxidase and especially catalase is larger than in several of the tissues. It was, therefore, necessary to compensate for the enzyme activities in the homogenates contributed by the contaminating erythrocytes. The hemoglobin was specifically determined (103) in terms of the differential protective activity of serum (haptoglobin) and egg white on the peroxidatic activity of the hemoprotein. Correction of the erythrocyte contamination of tissue homogenates was thus made according to the enzymic activities of the erythrocytes from the corresponding individuals.
2.2.20 Post-mortem stability of tissue enzymes

The possibility of a decrease of enzyme activities in tissues before freezing was investigated in experiments on swine liver. Small pieces of the liver were removed from an anesthetized animal, and kept in test tubes (in the dark) at room temperature (+22°C) and in a refrigerator (+4°C) for various periods of time, after which the tubes were transferred to -80°C. Enzyme activities were determined later on the thawed samples after homogenization and sonication according to the procedures utilized in (II) and (III). No enzyme inactivation was seen for at least 30 hours at either temperature, see table 3. Similar findings have been published in a study on the post-mortem stability of rat brain catalase and glutathioneperoxidase (28).

Table 3. Post-mortem stability of swine liver CuZn superoxide dismutase, Mn superoxide dismutase, catalase and glutathione peroxidase at two different temperatures. Each figure represents the mean of two samples from the same liver.

<table>
<thead>
<tr>
<th>Time after death (h)</th>
<th>CuZn SOD +4°C</th>
<th>CuZn SOD +22</th>
<th>Mn SOD +4</th>
<th>Mn SOD +22</th>
<th>Catalase +4</th>
<th>Catalase +22</th>
<th>GSH-peroxidase +4</th>
<th>GSH-peroxidase +22</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>87.90</td>
<td>87.90</td>
<td>1.35</td>
<td>1.34</td>
<td>155.40</td>
<td>155.40</td>
<td>13.20</td>
<td>13.20</td>
</tr>
<tr>
<td>6</td>
<td>93.40</td>
<td>97.70</td>
<td>1.40</td>
<td>1.53</td>
<td>158.10</td>
<td>167.60</td>
<td>15.90</td>
<td>14.10</td>
</tr>
<tr>
<td>18</td>
<td>97.00</td>
<td>90.60</td>
<td>1.44</td>
<td>1.46</td>
<td>167.40</td>
<td>155.90</td>
<td>15.60</td>
<td>11.70</td>
</tr>
<tr>
<td>30</td>
<td>84.30</td>
<td>104.10</td>
<td>1.46</td>
<td>1.42</td>
<td>163.10</td>
<td>153.80</td>
<td>14.50</td>
<td>12.80</td>
</tr>
</tbody>
</table>

2.2.21 Strength of extraction medium and enzyme activities

The variation of enzyme activities due to different strengths of the extraction media was studied on mouse kidney homogenates. The strength of the extraction medium was increased by addition of extra KCl or by addition of a detergent (Triton X-100). The results are presented in table 4. Only in the case of glutathione peroxidase an increase of the strength of the extraction medium enhanced the yield of the enzyme.
Table 4. Influence of extraction medium strength on the yield of enzyme activity from mouse kidney homogenates. Each figure represents the mean of two samples.

<table>
<thead>
<tr>
<th>Extraction medium</th>
<th>Centrifugation</th>
<th>Enzyme activities (10^3 units/g wet weight)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CuZn SOD Mn SOD Catalase GSH-peroxidase</td>
</tr>
<tr>
<td>I 10 mM K-phosphate pH 8.0 + 30 mM KCl</td>
<td></td>
<td>40.7 2.27 33.5 90.5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>37.2 2.56 36.6 89.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>36.7 2.52 35.4 71.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>42.0 2.58 36.7 74.3</td>
</tr>
<tr>
<td>II Same as I, plus extra 250 mM KCl</td>
<td></td>
<td>38.6 2.06 37.3 90.5</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>37.2 2.41 39.1 90.5</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>38.8 2.60 37.1 87.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>39.3 2.60 39.9 86.4</td>
</tr>
<tr>
<td>III Same as I, plus 0.3% Triton X-100</td>
<td></td>
<td>43.0 2.60 39.4 87.8</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>42.5 2.54 37.8 -</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>39.3 2.33 35.4 82.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>40.4 2.81 35.1 82.4</td>
</tr>
</tbody>
</table>

2.2.22 Strength of sonication and enzyme activities

To test the influence of variations in the homogenization and sonication procedures on enzyme activities, two separate experiments were done, using the suspension growing cell-line Namalwa (1-7x10^7 cells), that had been rinsed four times with 0.15 M NaCl containing 3 mM DTPA, collected and stored at -80°C. For sonication, the cells were then thawed and mixed with 1.5-4 ml, 10 mM K-phosphate buffer pH 7.4 + 30 mM KCl in test tubes and sonicated on ice for one minute. A Branson Model B 30 cell disruptor, operated with the micro tip at 10% 'duty cycle', with 'output control' varied from 1.4-4, and at 'continuous' mode was used for sonication. After sonication the cell suspension was left in air at +8°C for 30 minutes for extraction of enzymes, centrifuged, and enzymes in the supernatant assayed, see table 5.
Table 5. Influence of differences in sonication conditions on the activities of superoxide dismutases, catalase and glutathione peroxidase in homogenates of Namalwa cells. Figures are mean and SEM of 13 runs (experiment No. I) and 5 runs (No. II).

<table>
<thead>
<tr>
<th></th>
<th>CuZn SOD (U/mg protein)</th>
<th>Mn SOD (U/mg protein)</th>
<th>Catalase (U/mg protein)</th>
<th>GSH-peroxidase (U/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Experiment No.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>I</strong></td>
<td>Mean 83.6</td>
<td>1.87</td>
<td>45.7</td>
<td>50.4</td>
</tr>
<tr>
<td></td>
<td>SEM 5.8</td>
<td>0.23</td>
<td>8.53</td>
<td>9.50</td>
</tr>
<tr>
<td><strong>II</strong></td>
<td>Mean 95.7</td>
<td>1.96</td>
<td>33.9</td>
<td>57.9</td>
</tr>
<tr>
<td></td>
<td>SEM 13.6</td>
<td>0.10</td>
<td>4.40</td>
<td>5.60</td>
</tr>
</tbody>
</table>

As can be seen in the table the difference between the means of the first and second runs of the experiment were for CuZn SOD +14%, for Mn SOD +5%, for catalase -26% and for glutathione peroxidase +15%.

In conclusion the inherent variation in the determinations of the enzymes seem to be about 5-8%. The variations when the total procedure is included is much larger and for catalase and glutathione peroxidase may amount to 25%. However even if this variation seems large, it cannot explain the large variations in enzymic activities in the tissues, tumours and cells.

2.2.23 Irradiation

In (I) and (V) irradiation was produced from a 4 MV linear accelerator (Varian Clinac 4) with a dose rate of 3 Gy/min. In (IV) irradiation was produced from a 50 kV X-ray machine with a filtration of 1.0 mm Al, HVL 0.9 mm Al and a dose rate of 12 Gy/min at the surface of the solution. In (V) irradiation was produced from a 190 kV X-ray machine with 1.5 mm Al inherent filter, 1.0 mm Al additional filter, HVL 0.95 mm Cu, a dose rate of 235 R/min and a focus to target distance of 43 cm.
2.3 Results and discussion

2.3.1 Radiation experiments

Superoxide radicals are generated in cells through autooxidative and metabolic mechanisms. Radiolysis of water also yields a large amount of superoxide radicals. Superoxide radicals as well as radiation have been shown to damage DNA, virus, bacteria and higher cells.

Superoxide dismutase is the principal defense in the cell against the superoxide radical and it was therefore of interest to investigate the possible significance of an inhibition of CuZn SOD for the damaging effects of radiation.

Inhibition of CuZn SOD and specificity of the inhibition

It was demonstrated (I,IV) that in both fibroblasts and erythrocytes CuZn SOD was inhibited by more than 95% by treatment with 3mM DDTC for 90 minutes. Furthermore enzyme inhibition in both cell types was not reversed after removal of excess DDTC. At the start of irradiation, about 30-45 minutes after the end of the DDTC exposure, CuZn SOD activity in the cells was reduced by more than 90%; and in the following two hours the enzyme was still more than 80% inhibited.

Since DDTC has a wide range of biological effects (see section 1.8.), other factors of importance for the radiosensitivity may have been influenced. On the other hand, if DDTC was found not to influence such factors the relevance of the CuZn SOD inhibition for the radiation effects would increase.

Thus Mn SOD (I), catalase (IV,104), glutathione peroxidase (V) and cytochrome oxidase (104) activities were not changed by the present DDTC procedure and neither was the content of non-protein sulphydryl (104). There was no change in cell morphology as seen by electron microscopy (see section 2.2.16.), and no influence on distribution of cells in different phases of the cell-cycle as determined by flow cyto-fluorometry (see section 2.2.15.).
Radiation clonogenic survival after CuZn SOD inhibition

Pretreatment with DDTC decreased the clonogenic survival of V79 fibroblasts (I) as seen by a reduction of the Do value from 2.29 Gy in control cells to 1.87 Gy in DDTC pre-treated cells giving a DMF of 1.23. There was no influence on the extrapolation number. The results, therefore, were compatible with the proposition that superoxide radicals contributed to radiation damage.

Workers using Chinese hamster (DON) cells and mouse 3T3 cells (95,96), and using lymphocytes (143) have reported a radiosensitizing effect of DDTC. However, in contrast to the present investigation, they did not remove DDTC before irradiation and neither was the actual magnitude of SOD inhibition determined.

Significance of Mn SOD?

The Mn SOD activity in the V79 fibroblasts contributed to only 20% of the total SOD activity before DDTC treatment. In contrast, after DDTC treatment which had no effect on the activity of Mn SOD, this enzyme accounted for the largest part of functional SOD. Most of the Mn SOD is localized in the inner membrane and matrix space in the mitochondria, but in primates Mn SOD is also found in the cytoplasm. In rat liver cells about 20% has been found in the nuclear fraction (168). The distribution of Mn SOD in the V79 cells is not known, and it cannot be excluded that Mn SOD is also present in the cytoplasm and the nucleus of these cells. The superoxide anion is comparatively stable and as it permeates cell membranes it may be scavenged by Mn SOD not only in the mitochondria but also in other cell compartments. It is, therefore possible that though most of the CuZn SOD in the cells was inhibited by DDTC, a more complete SOD inhibition (including also inhibition of Mn SOD), could have brought about an even larger enhancement of radiation damage.

Trypsinization has been found to slowly reduce Mn SOD activity in normal WI-38 cells (human fibroblasts), but not in the SV-40 transformed WI-38 cells (177). In V79 cells, which are transformed cells, trypsinization would
probably exert the same effects as in transformed WI-38 cells. On the basis of this, this method of reduction of Mn SOD could not be utilized. The investigation on cell lines (III), showed that Mn SOD was not reduced in any of the cell lines either. As erythrocytes are known to lack Mn SOD, experiments were carried out to determine whether DDTC treatment would induce an even larger enhancement of radiation damage in these cells.

**Radiation response after CuZn SOD inhibition of Mn SOD deficient cells**

Since clonogenic survival could not be used as an endpoint in erythrocytes, haemolysis was chosen as the indicator of radiation damage (see IV). Pretreatment with DDTC of erythrocytes caused a substantial reduction in their CuZn SOD activity, now representative of the reduction of total SOD activity. Despite this, only a small increase in radiation damage was noted. It appeared as an increase of the slope of the dose response curve of the DDTC pre-treated cells giving a DMF of 1.04. (not significant), and a reduction of the intercept from 98.6% (of initial GLT 50) for control cells to 92% for DDTC pre-treated cells (p=0.005). These results were in contrast to the DDTC-enhancement of radiation haemolysis observed by Stone et al. (155).

**Increasing the extra-cellular CuZn SOD activity before irradiation**

Experiments aimed at determining whether addition of exogenous enzymes SOD and catalase would increase the radiation resistance of erythrocytes were carried out. It was also hoped that the addition of these enzymes would counteract a possible radiosensitizing effect of DDTC pre-treatment. This was, however, not found as only a small and insignificant reduction of haemolysis of both control, and DDTC pre-treated cells was observed. The results were thus very similar to those from the DDTC pre-treatment experiments and indicated that superoxide radicals are of only marginal importance for radiation haemolysis. Other studies (16) are in agreement with this, emphasizing instead the significance of the hydroxyl radical. Furthermore, the assumption that erythrocyte deficiency of Mn SOD should further increase the enhancement of radiation damage after DDTC pre-treatment was found to be wrong.
Importance of CuZn SOD for the radiation response of different cell types

The erythrocyte is a highly specialized nonproliferating, anucleate cell. In view of this, the effects of DDTC on cell compartments and cell functions of nucleated cells, such as the V79 cells are not representative for specialized cells like erythrocytes. Moreover, radiation haemolysis is determined within 24 hours in a metabolically inactive cell, whilst clonogenic survival is determined after 7 days in proliferating, metabolically active cells. A decreased defence against superoxide radicals was, therefore, still a possible explanation for the enhanced aerobic radiation damage in DDTC pre-treated V79 cells. However, participation of superoxide radicals in the immediate radical-dependent, physico-chemical radiation damage has been questioned by fast-kinetic studies (112), and by studies on T4 bacteriophage (149) and E. coli B (150). Attempts to reduce radiation damage in vivo and in vitro in mice or mouse cells respectively (131), by increasing the amount of extracellular SOD before irradiation have also failed. Even so, DDTC pre-irradiation treatment may be radiosensitizing, since the intracellular CuZn SOD activity, and thereby the defense against superoxide radicals was also markedly reduced during the first hours of the post-irradiation period.

Protection by SOD against immediate or delayed radiation damage?

Since the presence of oxygen during water radiolysis results in the formation of superoxide radicals from the hydrated electrons and the hydrogen atoms (see section 1.7), radiolysis in the absence of oxygen cannot yield superoxide radicals. To study the importance of superoxide radicals in the immediate effect of radiation, an attempt was made to prevent DDTC-enhancement, by anoxic irradiation of DDTC pre-treated cells. Irradiation in oxygen was also tried in order to further enhance the effect of DDTC.

In these experiments (V), however, DDTC-enhancement in argon, air or oxygen did not differ significantly, with a DMF in argon of 1.31, in air of 1.26 and in oxygen of 1.51. Accordingly, DDTC-enhancement could not be dependent on the generation of superoxide radicals during the irradiation
period suggesting that superoxide radicals do not seem to be associated with immediate radiation damage. The effect in argon was still compatible however, with increased damage from superoxide radicals, as in these experiments the cells were returned to oxygenated atmospheric conditions within 30 seconds after the end of the irradiation while CuZn SOD continued to be markedly inhibited for several hours after the irradiation.

Protection by SOD against delayed radiation damage

The results with the cells irradiated in anoxic conditions suggested that DDTC-enhancement was a post-irradiation enhancement. This in principle could be caused either by DDTC-interference with sublethal type of radiation damage (48), or by interference with so-called potential lethal damage (PLD), (138).

The results from experiments showing survival curves for DDTC pre-treated and untreated cells in air (I,V), argon (V) and oxygen (V), which had shown no effect of DDTC pre-treatment on the extrapolation numbers, however, indicated that DDTC did not interfere with the expression/repair of sublethal radiation damage. This was in accordance with the results of the split-dose experiments in (V), since the increase of the split-dose ratio of cells treated with DDTC only before the first fraction was incompatible with a DDTC-effect on sublethal radiation damage (see discussion in V).

Post-irradiation treatment of the cells with DDTC was therefore tried at various intervals after irradiation, and a post-irradiation enhancement was detected. The enhancement of radiation damage was of the same magnitude as could be seen from survival curves after DDTC pre-treatment, with a reduction of survival by a factor of about 2 at the dose level of 7.0 Gy. The effect was seen to diminish with time and had completely disappeared within 14 hours, probably even sooner.

The enhancement induced by DDTC is, therefore, a post-irradiation phenomenon, related to potentially lethal radiation damage, which can be accomplished either by treatment of the cells with DDTC immediately
before the exposure to radiation, or by DDTC-treatment in the post-irradiation period. The effect is seen as an increase of the slope of the survival curve by a DMF of about 1.3 whilst there is no change in the extrapolation number.

**Further indications of a post-irradiation importance of SOD**

Addition of SOD protects isolated bacteriophage DNA from inactivation by radiation (172), and increases viability and preserves morphology of irradiated foetal calf myoblasts (113). However, in these studies formate was added before irradiation to convert all radicals to superoxide radicals.

It has been reported (134) that addition of SOD to the medium reduced radiation damage to mycoplasma, and the maximal protective effect was seen when the enzyme was added 10 min after irradiation. Phospholipid membranes produced from soybean lipid fraction are also found to be protected by SOD, principally when this is added before irradiation, but some protective effect is also seen when SOD is added 5 minutes after irradiation (136).

Several studies, not always differentiating between immediate and delayed effects, also indicate the existence of toxic superoxide-dependent processes after irradiation. A reduction in the oxygen enhancement ratio (OER), at least when a dilute suspension of stationary-phase E. coli B is irradiated in the presence of SOD has been reported (117). The OER with SOD decreased from 2.3 to 1.5, but boiled, inactivated enzyme had no effect. Catalase, mannitol and histidine, so-called hydroxyl radical scavengers, were as effective as SOD. Other workers found an increase in OER in E.coli B cells containing lowered activities (about 60% of normal) of periplasmic Fe SOD of this bacteria, and this increase in OER could be prevented by addition of exogenous SOD to the medium before the irradiation (124).

There are also reports of SOD, catalase, or both, diminishing the number of radiation-induced chromosome aberrations in human lymphocytes; not
depending on lymphocyte cell-cycle phase and being even more pronounced when the enzymes were added after the irradiation than before the irradiation (122,123).

Neoplastic transformation of mouse embryo fibroblasts after irradiation or treatment with Misonidazole, have been found to be reduced if SOD is present during the post-irradiation/post-treatment period, but not if SOD is present only for a short period before or during irradiation/treatment with Misonidazole (115).

The results of Petkau et al. (135,137), show that SOD can raise the LD 50/30 of six-weeks old female Swiss mice by a factor of 1.35, and the most effective treatment is to inject the enzyme both before and after the irradiation. In agreement with this Abe et al., using a different mouse strain, found an increase of LD 50/30 but only with a DMF of 1.05. (1). Recently, Gray and Stull (69) described a protection by polyethylene glycol-attached CuZn SOD (PEG SOD) and PEG-catalase on mouse LD 50/30, but the effect was dependent on the mouse strain employed.

A transferable chromosome-damaging effect of plasma obtained from accidentally or therapeutically irradiated humans has been detected when this plasma is added to normal lymphocytes (65,86,151). This has been shown by Emerit (49) and in several studies she has shown that blood taken from patients suffering from auto-immune diseases, inflammatory bowel diseases, and congenital chromosome breakage syndromes, e.g. Bloom's syndrome and ataxia telangiectasia, also have a transferable clastogenic (chromosome breaking) effect. The effect seems to depend on the presence of a low molecular weight substance generated in the blood when it is exposed to superoxide radicals. The clastogenic effect on normal cells is, therefore, reduced in the presence of SOD (49).

Accordingly, these studies using addition of exogenous SOD to reduce radiation damage in the post-irradiation period in different systems, are in agreement with the results of the present investigation where radiation
damage is enhanced by reduction of intracellular CuZn SOD activity during the post-irradiation period.

Conclusions

A procedure for inhibition of intra-cellular CuZn SOD by DDTC was developed. An increased radiation damage of metabolically active cells was seen after such a treatment. The increased damage is compatible with the concept of an increased superoxide-radical mediated damage in the first post-irradiation hours. The post-irradiation enhancement is also in agreement with investigations suggesting that superoxide radicals do not participate in the immediate damaging effects of radiation (112,149,150).

As to the mechanism of the post-irradiation enhancement one can only speculate. It could involve increased metabolic production of superoxide radicals and derived species in irradiated cells compared to unirradiated cells, membrane lipid peroxidation, and extra-nuclear and nuclear events.

2.3.2 Superoxide dismutase in tissues, tumours and cell-lines

Many studies have been published on the activities of SOD and the other protective enzymes in tumours and tumour cells because of the oxygen radical scavenging abilities of these enzymes and the possibility of a relationship between SOD and radiosensitivity. The present study examined the activities of CuZn SOD and Mn SOD in human tumours and tumour cells, and for comparison in normal human tissues and cells. Catalase and glutathione peroxidase, which also are of conceivable interest in connection with free radicals were determined in the tissues and the cell-lines, but not in the tumours. The present study is much more extensive than most other studies published which have been performed only on single tumour specimens, or on experimental murine tumours.

Activities of CuZn superoxide dismutase

Tumours in general (II), had CuZn SOD activities around 10.000 U/g wet weight, which was low compared to metabolically more active normal tissues as the liver (100.000 U/g) and kidney (30 - 40.000 U/g), but similar
to most other organs and higher than adipose tissue (1.000 U/g). The high enzyme activity of the liver should indicate a very high radioresistance, but this is controversial, some studies have reported the liver to be only moderately radioresistant (88,90). On the other hand, skeletal muscle with a medium CuZn SOD activity of 11.000 also has been considered "radioresistant".

Most of the tumours had enzyme activities around 10.000 and there were several tumours, e.g. the lymphomas, and the Ewing sarcoma, tumours which usually are rather radiosensitive, that had quite similar enzyme levels to tumours which are generally more radioresistant like the malignant melanoma.

This suggests that there is no direct correlation between tumour or tissue enzyme activities and radiosensitivity. However the mechanisms that lead to destruction of tissues and tumours after irradiation are complex depending also for example, on the proliferative state of the cells and the vascularisation. Tissues are also a mixture of different cell types, with different enzyme activities and possibly different radiosensitivities. It was therefore of considerable interest to study the enzyme activities in more pure systems such as cell cultures.

This investigation approach (III) demonstrated that CuZn SOD activities did not on the whole vary much between the different cell lines, which was in accordance with the suggested obligatory requirement for the enzyme in aerobic cells. The total mean for the enzyme activity was 178 U/mg protein with a SEM of 11 U (6%). The highest enzyme activities were found in the Jurkat, and the RPMI 8226 lines with CuZn SOD activities of 300 U, and the lowest activities were found in the embryonal lung fibroblast with 83 U, and the renal carcinoma cell line HCV 29 with 72 U/mg protein.

Thus the findings of reduced activities of CuZn SOD (cytosolic SOD) as reported in studies on transplantable murine tumours (15,20,25,126,148), and in a study on a human tumour (58), was not substantiated here. Recently increased activities of CuZn SOD in tumours and tumour cells
have also been reported (178, 99). Furthermore, no relationship was found between the enzyme activities and assumed radiosensitivity in the present studies.

**Activities of Mn superoxide dismutase**

The Mn SOD activities in tissues and tumours were much more variable than the activities of CuZn SOD. Mn SOD was present in all of the tumours investigated, most of the tumours containing from 1.000 to 10.000 U/g wet weight (determined Mn SOD activities multiplied by 10, see section 2.2.1.), but some of the sarcomas contained less than 1.000 units. These enzyme activities were within the range of several normal tissues, where again the liver contained the highest enzyme activities (around 25.000). Erythrocytes were shown to lack Mn SOD, and adipose tissue contained rather little enzyme.

The investigation on cell lines yielded a similar picture with a large Mn SOD variation and with exceptionally high activities in two of the mesotheliomas, P27 with 2300 units/mg protein and P7 with 1260 U. The median enzyme activity was that of the glioma cell line 251 MG, with an enzyme activity of 57 U and the lowest activity was that of the T-lymphoblastic cell line Molt 4 with 13 U. The mean value of all of the 31 cell lines was 258 U with a SEM of 84 U (30%). The variability of the Mn SOD activities in cells and tissues may be related to the known inducibility of this enzyme (47,70,110).

There was no correlation between Mn SOD activities and CuZn SOD activities and again no direct relationship was found between the Mn SOD activities and the assumed radiosensitivities of the tissues, tumours or cell lines. A more reliable way of investigating the proposition would, however, be to determine the radiation sensitivity of the cell lines in parallel to enzyme determinations. Such studies are at present in progress.

Reduced or a total lack of Mn SOD (mitochondrial SOD) activity has been reported repeatedly, mostly in transplantable tumours (20, 42, 99, 126,
127, 148, 169) but also in human cells in culture (177) and in human leukemia cells in vivo (178). In two recent publications (15,99), a direct correlation between the growth rate of experimental rodent tumours and the decrease of Mn SOD activity was found. Even a proposition (125,128), that all neoplastic cells lack Mn SOD, and that this lack of Mn SOD is a prerequisite for malignant transformation, has been put forward. The present investigations, which were performed with human material do not support this proposition. Other studies have also found Mn SOD activities in tumours and tumour cells (99,127,178).

**Total superoxide dismutase activities**

Several studies have only measured total SOD activity. Reduced total activity of SOD has been reported in several transplantable mouse, rat and hamster tumours (13,20,30,127,169), in transformed cells in culture (51) and in a preliminary study on human tumours (159). Many of these results may be accounted for by the dependence of the total SOD activity on the activity of Mn SOD, and hence a reduction of Mn SOD results in a reduction of total SOD. In the light of the present studies, however, a reduction of total SOD is not a characteristic of human tumours.

**Catalase activities**

The catalase activities varied a lot in tissues, the highest activity being in the liver and in the erythrocyte which had almost 1.500 U/mg protein. Low activities were found in several other organs, for example the heart with 54 U/mg, and the spleen had a similar level. Even lower activities were found in skeletal muscle, brain grey and white matter and thyroid gland. Intermediate activities were found in kidney, adrenal, lung, pancreas and lymphatic node. Interestingly adipose tissue contained also intermediate catalase activities. Catalase levels were not determined in the tumours.

Catalase activities in the cell lines also varied considerably and were largest in the HL60 promyleocytic leukemia cell line with an enzyme activity of 920 U/mg protein, and were also high in the histiocytic lymphoma U937, the KG1 myeloblastic cell line and in HeLa. The lowest
catalase activities were found in the endothelial cell, EC, which is not neoplastic, with an enzyme activity of 5.0 U, and in the mesothelioma cell line P31 with 5.5 U. The mean catalase activity of the 27 cell lines which were analyzed for this enzyme was 125 U/mg protein. Due to the skewed distribution of the activities, most of the activities centered around 50 U/mg protein; the median value for the catalase activity in the myeloma cell line RPMI 8226 however being 67 U. Catalase is also known to be an inducible enzyme in several systems (80,119) which may explain the variation in the enzyme activities.

In the light of the present results, the tissues with the highest metabolism again seem to have the highest catalase activities. Among the cell lines most neoplastic cells appeared to have little catalase. Reduced catalase activities have also been reported in other studies on tumours (13,43,119). In conclusion the present study, however, failed to show a general correlation between catalase activities in tissues or tumour cells and assumed radiosensitivity. Furthermore no relationship between catalase activities and the neoplastic state 'per se' was found.

**Glutathione peroxidase activities**

In tissues, glutathione peroxidase activities (GSHPX) were comparatively more evenly distributed than the other enzymes with the highest activity in the liver (about 10,000 U/g wet weight or 150-200 U/mg protein). The difference between the liver and the other tissues was not as great as it was in the case of catalase and SOD; indeed several tissues as spleen, renal cortex and erythrocytes contained almost the same activity. Several other tissues e.g. adrenal gland, renal medulla, pancreas, lymphatic node, lung, heart, brain and adipose tissue had values about half or more of the liver enzyme activity. The glutathione peroxidase activities in the tumours were not determined.

The liver also contains a large amount of glutathione-S-transferase enzyme which possesses glutathione peroxidase activity (139), and this enzyme activity was not assessed by the present assay. The occurrence of this
transferase in the liver, thereby in reality raises the glutathione peroxidase activity of the liver above that of other organs.

Glutathione peroxidase activities were also more evenly distributed in the cell lines. The highest GSHPX activities were found in the U937 histiocytic lymphoma cell line and in the KG1 myeloblastic cell line which had enzyme activity of 250 U. The lowest activity was found in the Burkitt lymphoma cell line Namalwa with 1.5 U/mg protein. The mean value of 26 cell lines was 54 U, with an SEM of 11 U (20%). Most values centered around an enzyme activity of 50 U/mg protein.

Glutathione peroxidase removes not only hydrogen peroxide but also other hydroperoxides, and thus has a broader protective ability than catalase. The enzyme was discovered rather recently and investigations on the enzyme activities in human neoplastic cells have not yet been presented. Reduced activities of glutathione peroxidase have been reported in mouse and rat transplantable tumours (25,133).

Glutathione peroxidase activities in neoplastic cells were thus within the range of normal cells and tissues, and no relationship was detected between glutathione peroxidase activity and assumed radiosensitivity of tissues and cells.

**Hydrogen peroxide accumulation**

Rotilio et al. (25,26,147), have proposed that increased ratios of SOD to catalase or preferably SOD to glutathione peroxidase might be characteristic of some tumour cells. The significance of this would be that the decreased ability of the neoplastic cells to take care of hydrogen peroxide, coupled to an increased relative amount of SOD which generates hydrogen peroxide, could increase their sensitivity to hydrogen peroxide. In particular the sensitivity of such cells to increased levels of hydrogen peroxide, produced by irradiation or treatment with certain antitumour drugs, would be increased, and possibly influence their sensitivity towards these agents. In the present study a high ratio of SOD to GSHPX, or SOD to catalase was
present in some cells but it was not a general feature. The mesothelioma P31 ratios were both rather high, and in other cell lines there was a simultaneous occurrence of a low ratio of SOD to catalase and a high ratio of SOD to GSHPX, e.g. CCRF-CEM with a SOD/catalase ratio of 1.1 and a SOD/GSHPX ratio of about 40, and Namalwa with the ratios 0.6 and 141 respectively. On basis of this, the proposition of Rotilio is not contradicted by the present study, but it seems that it does not apply to all tumour cells.

Decreased ratios of hydrogen peroxide generating-enzymes to hydrogen peroxide-degrading enzymes was also looked for. However decreased SOD/catalase or SOD/GSHPX ratios was not a general characteristic of the tumour cell lines compared to the few normal cell lines (adult skin, embryonal lung, and endothelial cell line EC). Some tumour cell lines contained rather low ratios, e.g. U937 with a SOD/catalase ratio of 0.5 and a SOD/GSHPX ratio of 1.2, and HL60 with the corresponding ratios of 0.25 and 3.5 respectively.

In no case was there a low ratio of SOD/catalase in combination with a low SOD/GSHPX ratio. This indicates that in the addition to SOD the cell must contain at least one of the catalase degrading enzymes to be able to survive.

**Free radical producing cytotoxic drugs**

Although the principal aim of the Thesis was to answer the question of the importance of SOD for radiosensitivity, it is also evident that no simple, obvious relationship was observed for the enzyme activities in tumours or tumour cells and their assumed sensitivity to radical-producing antitumour agents.

The findings in heart tissue of low activities of catalase and glutathione peroxidase however, are in accordance with previous reports (45), and in fact the present measurements are more relevant as the enzymes were determined not only in the heart and the liver but in most tissues. The low activities of these enzymes may, therefore, contribute to the suscepti-
bility of the heart to be damaged by Adriamycin or other free radical producing anti-tumour drugs.

**Conclusions**

The activities of superoxide dismutase, catalase and glutathione peroxidase were determined in human tissues, human tumours (only superoxide dismutase) and human cell-lines. The metabolically most active tissues possessed the highest enzyme activities. In all of the investigated systems the variation of enzyme activities was large for Mn superoxide dismutase and catalase, and less for CuZn superoxide dismutase and glutathione peroxidase. No obvious correlation was found between superoxide dismutase activity and assumed radiation sensitivity.
3 CONCLUSIONS

1. Treatment of Chinese hamster V 79 cells or erythrocytes with diethyl-dithiocarbamate reduced substantially their CuZn SOD activity.

2. The DDTC-induced reduction of intracellular CuZn SOD increased the radiation damage (clonogenic survival) of proliferating, metabolically active, V 79 cells through a post-irradiation mechanism related to potentially lethal damage. However, DDTC-induced reduction of intracellular CuZn SOD did not modify the radiation damage (haemolysis) of non-proliferating, metabolically arrested erythrocytes, and no reduction in radiation haemolysis was seen when the extracellular activity of CuZn SOD was increased.

3. The activity levels of CuZn SOD and Mn SOD were determined in normal human tissues, human neoplastic tumours and normal and neoplastic human cell-lines. There was no obvious correlation between the enzyme activities and the assumed radio-sensitivities of the tissues, tumours and cell-lines.

4. The increased radiation sensitivity seen in DDTC-treated cells was too small to be detected in studies like (II) and (III).
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