EFFECT OF BREATHING HYPOXIC GAS MIXTURES FOLLOWED BY IRRADIATION ON TUMOUR CELL SURVIVAL IN EXPERIMENTAL MOUSE TUMOURS

André Stephen Hendrikse

A THESIS

Submitted to The University of Cape Town for the Degree of

DOCTOR OF PHILOSOPHY

1989

To my father and to the memory of my mother

CONTENTS

ABSTRACT

It is generally accepted that most tumours contain radioresistant hypoxic cells, which limit the effectiveness of photon radiation. This dissertation outlines an attempt to increase the sensitivity of mouse tumours to X- and gamma radiation by reducing the fraction of hypoxic cells in tumours. It is proposed that this can be achieved by making the tumour-bearing animals breathe a hypoxic gas mixture for a period of time and then returning them to a normoxic or hyperbaric oxygen (HBO) environment just prior to and for the duration of delivery of radiation.

The effect of breathing 8% oxygen for 72 hours prior to radiation (single X-ray dose of 11 Gy) in air or in HBO on the regrowth delay of CaNT tumours and 3-methylcholanthrene-induced murine rhabdomyosarcomas was compared with radiation alone. No differences in regrowth delay were observed in the case of the CaNT tumour between the mice that received pretreatment and radiation and those that received radiation alone. In the rhabdomyosarcoma an increase in regrowth delay was observed in the mice that were exposed to the 8% oxygen environment for a 72 hour period prior to being irradiated. These findings are discussed with reference to the different hypoxic cell fractions which were determined for each tumour type (CaNT 54%; rhabdomyosarcoma 27%).

The response of the Fib/T tumour grown in WHT mice to ^{60}Co gamma rays (delivered in air or in HBO) where mice were exposed to different hypoxic pretreatments (8%, 10% or 15% oxygen) lasting either 48 hours or 72 hours was compared to that obtained where mice were pretreated with air, using an in vitro colony forming excision assay. The response of the

Fib/T tumour to radiation was improved by a 48 hour and 72 hour exposure of the WHT mice to 8%, 10% and 15% oxygen. However, the greatest sensitization was achieved where mice were kept in an 8% oxygen environment for 48 hours before radiation. These results are interpreted and discussed in relation to two adaptation mechanisms, viz. increased haemoglobin levels and increased 2,3-DPG concentrations, that were shown to operate where mice were exposed to a reduced oxygen environment. Furthermore, the importance of the "increased oxygen availability" model relative to the "reduced cord radius" model is assessed. Where mice, pretreated with air, were irradiated in HBO, a similar tumour response was observed compared to where mice were pretreated with 8% oxygen for 48 hours but irradiated in air. Where mice were exposed to two equal fractions of radiation, spaced by an interval of 24 hours, the greatest tumour response to radiation was observed where the mice were pretreated with 8% oxygen for 48 hours and then returned to this environment for the 24 hour interval between fractions. If both fractions of radiation were delivered in HBO, an increase in tumour radiation damage was produced compared to where radiations were delivered in air.

The response of the Fib/T tumour to single dose neutron radiation (delivered to air-breathing mice) was determined where mice were either pretreated for 48 hours with 8% oxygen or with air. Results indicated that a 48 hour 8% oxygen pretreatment was less efficacious in sensitizing the Fib/T tumour to neutron radiation than it was in sensitizing the Fib/T tumour to ^{60}Co gamma radiation.

The activity of the scavenger enzymes, catalase and glutathione peroxidase, and a related enzyme in the anti-oxidant system (glutathione reductase), as well as the content of glutathione were determined in the Fib/T tumour of mice before and after exposure to 8% oxygen. This hypoxic environment was found to produce no significant change in the

activity of either of the three enzymes or in glutathione levels.

Finally, the findings reported in this thesis are discussed in relation to possible adaptation in the clinical radiotherapy situation.

ACKNOWLEDGEMENTS

It is with sincere gratitude that I acknowledge my supervisor, Dr Gerry Blekkenhorst. Without his scientific knowledge and technical skills this project could not have been brought to fruition.

To Professor G.R.H. Sealy, I owe a great debt of thanks. His advice, criticisms and suggestions were invaluable - his patience inexhaustible.

I am deeply indebted to Professor I.D. Werner, Head of the Department of Radiotherapy at Groote Schuur Hospital, for placing the resources of his department at my disposal.

It is a pleasure to acknowledge the contributions from Dr R.P. Abratt, whose advice and constructive criticisms are deeply appreciated.

A number of my colleagues in the Radiobiology Laboratories played an active role in many facets of this project:

- Mrs P. Dowman for her assistance in tissue culture.
- Messrs C. Kent, A. Hunter, J. Madhoo and R. Duffett for helping me become computer literate.
- Messrs G. Nongalaza and S. Sokanyile for their care and treatment of the mice.

My thanks are also due to Dr R. Schall for his statistical interpretation of my fractionated irradiation data, to Mr J. Slabbert for his always-ready-to-help attitude in performing my alpha-beta analyses, and to Mr T. Elliott for his determination of the oxygen dissociation curves.

It is my pleasant duty to thank the physicists, both at Groote Schuur Hospital namely Dr E.R. Hering and at the

National Accelerator Centre namely Dr D. Jones and Mr M. Yudelev, who were involved in numerous beam calibrations.

To Mrs D. Godley, for her secretarial help and to Mrs S. Blyth, for typing parts of the manuscript, I acknowledge my indebtedness.

I particularly wish to express my special appreciation to Astrid for her tireless and meticulous care in proofreading, and for her friendship and support.

Finally, I would like to thank the National Cancer Association of South Africa for supporting this work financially.

ABBREVIATIONS USED IN THIS THESIS

ADP	-	Adenosine 5'-diphosphate
ATP	-	Adenosine 5'-triphosphate
1,3-DPG	-	1,3-Diphosphoglycerate
2,3-DPG	-	2,3-Diphosphoglycerate
DTNB	-	5'5'-dithiobis-(2-nitrobenzoic acid)
EDTA	-	Ethylenediaminetetra-acetic acid
FAD	-	Flavine adenine dinucleotide
GSSG	-	Glutathione, oxidized form
GSH	-	Glutathione, reduced form
NAD	-	Nicotinamide adenine dinucleotide
NADH	-	Nicotinamide adenine dinucleotide, reduced form
NADP	-	Nicotinamide adenine dinucleotide phosphate
NADPH	-	Nicotinamide adenine dinucleotide phosphate, reduced form
$K_3Fe(CN)_6$	-	Potassium ferricyanide

CHAPTER 1

INTRODUCTION

The "reproductive integrity", or the capacity of a cell for infinite proliferation in a suitable environment, is an important concept to the radiotherapist. It is the cardinal aim in radiotherapy to destroy the reproductive integrity of every cancer cell in a tumour in order to achieve local control. Hypoxic cells may survive a dose of radiation because of the protective effect of the lack of oxygen and may then regenerate the tumour in the better nutritional conditions that follow the death and absorption of their more radiosensitive aerobic neighbours. Thus, if radiation is delivered to a tumour in sufficient amounts to be lethal to all aerobic cells but in inadequate amounts to kill the resistant hypoxic cells, then following reoxygenation these hypoxic cells may be in a position to sustain life and thus initiate a recurrence. This provides a possible explanation why recurrence after tumour resolution and healing occurs. Of all the failures faced by the radiotherapist the most distressing is, probably, the local recurrence of persistent disease after an apparent but temporary period of healing.

The idea behind the investigations carried out in this thesis was first conceived in the early 1960's. Two investigators, Ellis[1] and Thomlinson[2], reasoned that if under normal conditions of respiration there was a layer(s) of hypoxic cells forming the interface between intact and necrotic tumour tissue, then by reducing the oxygen diffusion distance from a capillary these radiation-insensitive cells could be completely starved of oxygen and thus die. It was further

speculated that if the oxygen diffusion distance was then restored to its former value, adequate oxygenation of all living tumour cells would ensue resulting in a hypoxic cell-free tumour which should be very amenable to radiation therapy. Thomlinson tested this hypothesis by comparing the radiation response elicited in the tumours of rats that were pretreated with 8% oxygen for 24 hours with that of animals that were kept in an air-breathing environment prior to radiation. This experiment revealed that in the case of animals pretreated with 8% oxygen, a single dose of 20 Gy delivered in air had an equivalent effect to 29 Gy given as a single dose in air to animals pretreated with air.

In 1979, Siemann et al[3] demonstrated that the exposure of tumour-bearing mice to 12% oxygen for 15 hours prior to radiation in air, resulted in a reduction in tumour cell survival by a factor of 3-4 over that found for the tumours of air-breathing mice not given a low oxygen pretreatment. Furthermore, if tumours were irradiated while the animals breathed oxygen or carbogen after exposure to 12% oxygen, tumour cell survival was reduced to a value of about 10 times lower than was obtained in the tumours of air-breathing mice not given the low oxygen pretreatment.

In recent years, there has been an increasing awareness of how physiological parameters affecting oxygen delivery could influence the sensitivity of tumours to radiation (Oxygen and Radiation Responses - In Proc.8 Int. Cong. Rad. Research, pp 695-742). The work described in this thesis is, in fact, a variation on this theme. It involves combining physiological manipulations with radiotherapy - the rationale of the physiological manipulations is to significantly reduce the

proportion of hypoxic cells in the tumour before the commencement of radiotherapy.

Briefly, the specific aims of this project can be itemized as follows:

a) To investigate in a number of mouse tumour systems the sensitizing effect of a low oxygen pretreatment.

b) To determine the sensitizing efficiency of a range of low oxygen pretreatments, and to establish the duration of pretreatment that yields optimal tumour sensitization.

c) To investigate the response of tumours to two fractions of radiation with or without a low oxygen pretreatment. The efficacy of maintaining hypoxia between fractions, as well as varying the time interval between fractions, are also investigated.

d) To determine the effect of a low oxygen pretreatment where radiation (single dose and split-dose) is delivered in hyperbaric oxygen.

e) To compare the sensitivity of tumours to neutron radiation where the tumour-bearing animals are either given an air or a low oxygen pretreatment.

In summary, this thesis presents an experimental approach whereby hypoxia, which is seen to be a problem in radiotherapy, may be utilized to result in an enhanced tumour cell kill. The ultimate aim is to extend this approach to the radiotherapy clinic, if it is feasible, and to cure primary tumours with an associated decrease in the radiation dose and, thus, radiation morbidity.

CHAPTER 2

IONIZING RADIATION, AND THE BIOLOGY AND PHYSIOLOGY OF HYPOXIA

2.1 Oxygen and Radiosensitivity - Historical Aspects.

Is there evidence to support the contention that the sensitivity of biological systems toward ionizing radiation is higher in the presence of oxygen than in its absence? The dependence of radiosensitivity on the concentration of oxygen dissolved in tumour and normal tissue at the time of radiotherapy has been comprehensively investigated. Experimental data pertaining to this relationship have been reported in the literature from an early date. Holthusen in 1921[4] found that the radiation dose required to prevent Ascaris eggs from hatching was three times as great in an anaerobic environment as compared to air. Mottram[5,6] reported that reducing the blood supply to an irradiated area of skin decreased the amount of radiation damage produced there, although he was not aware that oxygen per se was involoved in the effect. Hypoxia has been shown to confer some degree of protection to animals receiving whole body radiation. A reduction in radiation injury has been demonstrated in cases where the circulation and breathing of rats have been impaired by strapping their chests.[7] Dowdy et al[8] investigated the effects of hypoxia on the $LD_{50/30}$ (i.e. the dose required to kill 50% of the animals exposed within a time period of 30 days) in rats after whole body X-radiation. The $LD_{50/30}$ for rats irradiated under hypoxic conditions increased approximately by a factor of 2 as compared to that obtained for the irradiated air-breathing control animals. The sensitizing

effect of oxygen on radiation has not been restricted to animal systems. Broad bean roots (Vicia faba) irradiated in water equilibrated with nitrogen have been shown by Read[9] to be markedly more radioresistant than normally aerated roots. Gray et al[10] in 1953 showed that cells, which were anoxic at the time of radiation, were generally much less damaged by a given dose of X-rays than those which were well oxygenated. These results indicated that the viability of Ehrlich mouse ascites tumour cells was depressed about 2.5 to 3 times as much by X-radiation in air or oxygen as it was in nitrogen. Deschner and Gray,[11] using visible chromosomal damage as a biological end-point, observed that the relative radiosensitivity of Ehrlich ascites tumour cells rose rapidly from a minimum value of 1 for anoxic cells to about 2.3 for cells in fluid equilibrated with oxygen at a pressure of 20 mm Hg. Hewitt and Wilson[12] in 1959 demonstrated that leukemia cells irradiated under anoxic conditions (in recently killed mice) were more radioresistant by a factor of 2.3 than cells irradiated in mice breathing air. A similar result was recorded a year later by Dewey[13] who irradiated human liver cells in medium equilibrated with either air or nitrogen. The relationship between radiosensitivity and oxygen tensions of varying intermediate values has been elucidated by a number of investigators. Radiation survival curves were established by Alper and Howard-Flanders[14] for E. Coli B. under nitrogen, oxygen and a range of nitrogen-oxygen mixtures. It was found that the radiosensitivity of the bacteria was increased by oxygen concentrations as low as 1 umol/l. This was very small compared with the concentration required to accomplish a similar change in other materials used in earlier radiobiological experiments.[9,10,15,] However, Gray in 1961,[16]

on analysing the data from a prior joint experiment with Deschner,[11] found the dependence of radiosensitivity on oxygen tension to be most pronounced at oxygen tensions below about 20 - 30 mm Hg. This resulted in a curve which relates the radiosensitivity of cells to the oxygen tension around them at the time of radiation (Fig.2.1). This curve showed that maximal radiosensitivity can be achieved at PO_2 values > 20 - 30 mm Hg.

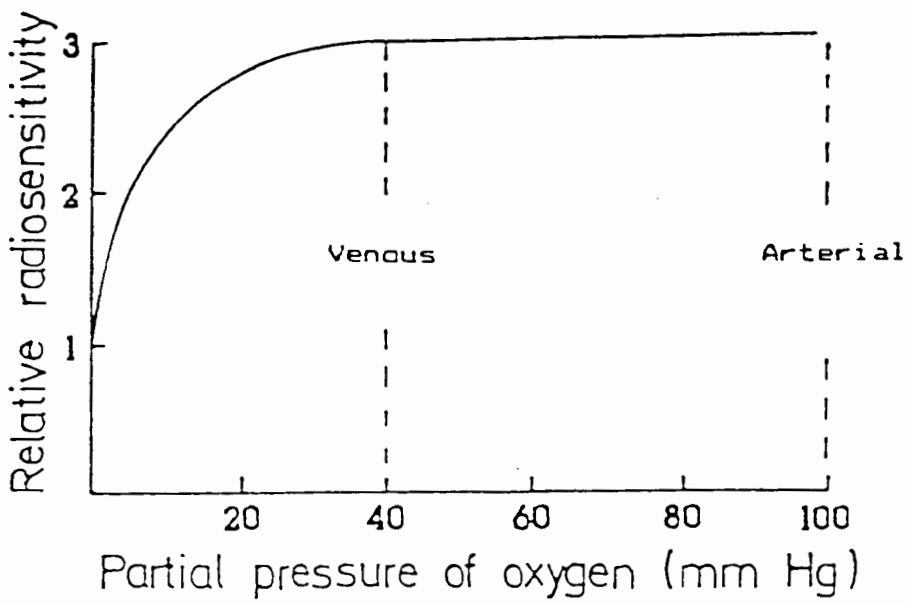


Fig.2.1 Idealized diagram, adapted from data of Gray,[16] to illustrate the influence of oxygen concentration on radiosensitivity.

It was also estimated that the concentration of dissolved oxygen at which radiosensitivity was exactly midway between the anoxic value and the maximum oxic value was 5 umol/l, which corresponds to a partial pressure of 2.8 mm Hg.

2.2 Radiolysis, Free Radicals and Oxygen.

The proposition that radiation damage is enhanced in the presence of oxygen stands firmly on its foundation in radiation

chemistry and radiation physics. Since water forms a major constituent of living cells, the radicals formed when water is exposed to ionizing radiation have been investigated using pulse radiolysis. The processes involved are discussed briefly below.[17] Exposure of water to ionizing radiation produces ionizations and excitations within 10^{-16} s

$$H_2O \text{ ------> } H_2O^+ + e^- + H_2O^*$$

where e^- represents an electron and H_2O^* an excited water molecule. Such excited molecules undergo homolytic fission in 10^{-14} - 10^{-13} s to give hydrogen atoms (which can equally well be called hydrogen radicals since they contain one unpaired electron) and hydroxyl radicals

$$H_2O \text{ ------> } H\cdot + OH\cdot$$

Within the same timescale H_2O^+ also reacts to give $OH\cdot$

$$H_2O^+ + H_2O \text{ ------> } H_3O^+ + OH\cdot$$

The electrons become surrounded by clusters of water molecules within 10^{-12} - 10^{-11} s. These hydrated electrons may be denoted as e^-_{aq}. Hence three different radicals are produced on pulsing an aqueous solution: $H\cdot$, $OH\cdot$, and e^-_{aq}.

In the presence of oxygen, the hydrated electrons formed by ionizing radiation can produce the superoxide radical (O_2^-)

$$e^-_{aq} + O_2 \text{ ------> } O_2^-$$

The superoxide radical, in turn, can interact with hydrogen peroxide to form more of the highly reactive hydroxyl radical[18] according to the overall reaction

$$H_2O_2 + O_2^- \text{ ------> } O_2 + OH^- + OH\cdot$$

Much of the initial damage done is due to the formation of the hydroxyl radical, $OH\cdot$, which can react with other cellular components to produce organic radicals. Often these organic radicals can be "repaired" by reaction with, for

example, glutathione. If R· is used to denote them, the "repair" can be represented by the equations

$$R\cdot + GSH \text{ ------> } RH + GS\cdot$$

$$2GS\cdot \text{ ------> } GSSG$$

However, the presence of oxygen may "fix" the damage by forming other radicals that cannot be repaired, e.g.

$$R\cdot + O_2 \text{ ------> } RO_2\cdot \text{ (peroxy radical)}$$

So, resulting from the direct interaction of oxygen with the target biomolecules in competition with GSH, any possible restitution or repair processes are prevented.

Some experiments,[19] do indeed support the view that the oxygen effect is due to radiochemical reactions. On the other hand, other investigators[20,21] have postulated that any difference in radiation-induced DNA strand breakage observed in oxygenated and hypoxic cells is caused by a novel rapid repair mechanism, involving DNA polymerase 1 and probably ligase, which preferentially rejoins hypoxic-type breaks.

2.3 Experimental Evidence for the Existence of Hypoxic Cells.

If it is accepted that hypoxia renders cells more resistant to ionizing radiation, then does it necessarily follow that it is a factor of importance as regards the radiosensitivity of tumours? In other words, is there data to affirm the presence of hypoxic cells in tumours? Evidence of the presence of tumour hypoxic cells was convincingly offered by Gray in an excellent article in 1961,[16] as well as by Churchill-Davidson in an address at the First Annual San Francisco Cancer Symposium.[22]

This evidence is based on:

1) Circulation physiology, and the histological structure of tumours.

Histological examination of tumour tissue reveals many vessels having walls characteristic of capillaries. The development of a blood supply to a tumour is controlled by the elaboration of a chemical mediator called the "tumour angiogenesis factor".

Tumours are able to grow bigger only by displacing the surrounding tissues, and this can be done only by generating within themselves sufficient pressure to overcome the resistance of these tissues. These pressures may cause a tendency to obstruction in veins and lymphatics, with a resulting increase in resistance to the flow of blood.[23] The nature of tumour growth may, therefore, lead to unfavourable conditions for oxygen delivery. Oxygen reaches the cells of tumours by way of the blood vessels of the stroma. Thomlinson and Gray[24] found that tumour cells of human lung carcinomas grew in solid masses or cords surrounded by, but not penetrated by, capillaries. Some of the more salient features of these tumour cords were that

a) all tumour cords more than 200 um in radius showed central necrosis.

b) no central necrosis was seen in any tumour cord less than 160 um in radius.

c) no apparently intact tumour cell was seen more than 180 um from the stroma.

This tumour cord structure was consistent with a theory of limited oxygen diffusion and it, therefore, followed that close to the area of central necrosis there were likely to be areas of living cells in various degrees of oxygen deficiency.

2) Direct and indirect measurements of oxygen tension.

Polarographic measurements of oxygen tension in tumours by Cater and Silver[25,26] provided evidence that there were cells within tumours that were protected from radiation injury by low oxygen tensions.

The presence of hypoxic cells was also predicted by Vaupel et al[27] on the basis of their cryophotometric studies. Cryophotometry has been shown to be a reliable method for quantifying the oxygenation status of solid tumours both in animals and in humans.

Radioactively-labelled hypoxic cell sensitizers, such as ^{14}C-misonidazole, have been used to demonstrate the presence of hypoxic cells in tumours.[28,29] ^{14}C-misonidazole is bound selectively to metabolizing hypoxic cells and the activity of this radionuclide can readily be detected by autoradiography.

3) Changes in the response of tumours irradiated under different oxygen tensions.

A number of investigators[30,31,32,33] have carried out experiments in which tumours, mostly in mice, have been irradiated under increased or reduced oxygen tensions. The fact that these tumours showed a radiobiologic response to increased or decreased oxygen tensions was indicative that they comprised cell populations existing at widely varying oxygen tensions and containing a significant proportion of radiobiological hypoxic cells. Powers and Tolmach[34] found that mouse lymphosarcoma cells irradiated in vivo yielded a survival curve that was biphasic in nature. They determined the ratio of the terminal exponential slope to the initial exponential slope to be 2.3:1, which was typical for the oxygen enhancement ratio observed in earlier studies by Gray et al.[10] It was concluded that the terminal slope was characteristic of

a subpopulation of cells in the tumour which, because of their lack of oxygen, showed an enhanced radioresistance. From these experiments the authors proposed that about 1% of the cells of solid, subcutaneous mouse lymphosarcomas, 1 - 2 cm in diameter, were hypoxic. However, if mouse lymphosarcoma cells were irradiated under oxic or anoxic conditions, the resultant survival curves had only one exponential slope.

4) Physiological and pathological factors[35].

Our vascular systems have evolved to maintain a tissue oxygen tension that fully saturates the mitochondrial cytochrome system. Studies on single mitochondria have shown that their critical PO_2 ranges from 1 to 3 mm Hg. It is, therefore, not hard to see why some cells, even in normal tissues, are radiobiologically hypoxic, that is, have PO_2 values < 20 - 30 mm Hg. The unfavourable conditions for oxygen delivery, which often exist in tumours, may be further exacerbated by cardiovascular and pulmonary disease.

From the preceding discourse it can be concluded that tumours contain some hypoxic and, therefore, radioresistant cells. It follows from this that it would be desirable to be able to overcome the relative radioresistance of hypoxic cells in tumours.

2.4 **Methods to Overcome the Radioresistance of the Hypoxic Cell.**[35,36,37]

2.4.1 Avoidance of the "oxygen effect".

a) Use of radiations with a high linear energy transfer, e.g. fast neutrons, heavy charged particles.[38]

b) Low dose-rate X- and gamma radiations: Experiments performed by Hall and Cavanagh[39] as well as by

Berry[40] suggest that low dose-rate radiations (< 0.45 Gy/hour) may well carry with them the inherent advantage of a lowered oxygen enhancement ratio.

c) Palcic et al[275] have demonstrated that the oxygen effect is reduced at lower radiation doses.

2.4.2 Decrease of oxygen tension in normal tissues.

The sensitivity of normal tissue to radiation is often the dose-limiting factor in clinical radiotherapy. The approach aimed at reducing the oxygen tension in normal tissue is based on the rationale that if normal tissue can be rendered hypoxic and thus more radioresistant, then a greatly increased radiation dose can be given to the tumour. Yarmonenko et al[41] were able to show that protection may be achieved when radiation of mice is performed in a 5-10% oxygen environment. Not only was a reduction in the damage to normal tissue noted but also the paradoxical effect on malignant tissue - i.e. an increased regression of allogeneic transplanted tumours (Sarcoma 37 and Ehrlich solid carcinoma) after radiation under conditions of "mild" total-body hypoxia.

2.4.3 Increase of oxygen tension in tumours.

a) Elevation of arterial partial pressure of oxygen (PaO_2).

This can be achieved by giving the subject pure oxygen to breathe at increased pressure, usually 3 atmospheres absolute pressure. An alternative method of raising the PaO_2 involves the intra-arterial infusion of buffered hydrogen peroxide solutions.[42] The introduction of hydrogen peroxide into blood results in rapid dissociation of the hydrogen peroxide into

oxygen and water by the action of erythrocyte and plasma catalase.

b) Inhibitors of oxidative metabolism.

A reduction in the rate of oxygen consumption by tumour cells would permit further diffusion of oxygen from the blood vessels. High glucose concentrations and specific metabolic inhibitors such as the nitrobenzene derivatives[43] have been shown to be effective in vitro but have not achieved consistent radiosensitization in vivo. Mild hypothermia, by lowering the tumour requirement for oxygen to a greater extent than the reduction in oxygen supply that is normally associated with hypothermia, may cause an overall rise in the oxygen tension of tumours.[44]

c) Alteration in the oxygen transport properties of the blood and vascular system.

An increase in the concentration of haemoglobin and a decrease in its affinity for oxygen are some of the known compensatory mechanisms that occur in response to a reduction in oxygen delivery. A reduction in oxygen delivery may be achieved by:

- inducing anaemia
- reducing the partial pressure of oxygen in the inspired gas. This may be accomplished either by a change in the percentage of oxygen breathed from the normal 21%, by a change in the pressure of the gas mixture from the normal 1 atmosphere or by a combination of the two.

Perfluorochemical emulsions (e.g. Fluosol-DA 20%) have been shown to increase the non-haemoglobin-bound oxygen carrying capacity of whole blood.[45] After intravenous administration of Fluosol-DA 20%, oxygen is carried in the blood bound to haemoglobin, dissolved in the plasma, and dissolved in the perfluorochemical micelles. The amount of

oxygen carried by the perfluorochemicals is directly related to the partial pressure of oxygen and the concentration of perfluorochemicals in the blood. A number of investigators have shown that Fluosol DA 20% can improve the radiosensitivity of several experimental animal tumours, provided the animals breathe 100% oxygen or carbogen during radiation.[46,47,48]

2.4.4 Decrease of oxygen tension in tumours.

The rationale behind this method is to obtain a reduction in the distance between capillaries and necrotic regions of tumour cords before conventional or hyperbaric oxygen radiotherapy. This may be achieved by

- bleeding the experimental animal or patient
- breathing of oxygen at subnormal tensions
- using drugs such as hydralazine which reduce tumour blood flow,[49] or agents which inhibit the dissociation of oxyhaemoglobin, e.g. BW12C.[50,51,52]

2.5 **Oxygen Transport.**

In this section, an examination is made of some of the physiological aspects of oxygen transport, tracing its passage from alveolar gas to the cells' mitochondria.

Diffusion causes oxygen molecules to move from alveolar gas into pulmonary capillary blood and also from peripheral capillary blood into contiguous cells. Diffusion may be defined as the passive movement of matter from a region of higher to one of lower concentration as a result of random molecular motion.[53] From the atmosphere to the alveoli, and again from the lung capillaries to the tissue capillaries, oxygen is transported largely by bulk flow.

2.5.1 Oxygen uptake along the pulmonary capillary.

The partial pressure of oxygen (PO_2) of mixed venous blood entering a pulmonary capillary is normally about 40 mm Hg. Across the blood-gas barrier, the alveolar PO_2 (PAO_2) is of the order of 100 mm Hg. Because of the prevailing differences in concentrations, oxygen floods down this large pressure gradient from the alveoli into the pulmonary capillary blood. As a result, the PO_2 in the blood rapidly rises. Under normal circumstances, the difference in PO_2 between alveolar gas and end-capillary blood is very small. Oxygen must pass several layers of heterogeneous material on its path from alveolar gas to the interior of the red blood cell. The length of this diffusion path varies from less than 0.5 um to more than 2 um.

2.5.2 Oxygen transport in the blood.

Oxygen is carried in the blood dissolved in plasma as well as bound to haemoglobin. The initial movement of oxygen across the blood-gas barrier takes place between alveolar gas and plasma.[54] As soon as oxygen molecules enter and begin to accumulate in plasma, a new concentration difference is established between oxygen in the plasma and that in the interior of the circulating red blood cell. This difference causes oxygen to move through the plasma and across the cell's membrane and within its interior to the sites of chemical reaction with haemoglobin.

2.5.2.1 Dissolved oxygen.

Dissolved oxygen, also termed oxygen in physical solution, comprises little more than 1% of the total blood oxygen content.[55] The amount dissolved is small but crucial

for the transfer of oxygen between air and haemoglobin and between haemoglobin and tissue. The amount of oxygen dissolved in blood is directly proportional to the PO_2 to which it is exposed (Henry's law of solubility of gases). For each mm Hg of PO_2, there is 0.003 ml oxygen/100 ml of blood. Thus, normal arterial blood that has a PO_2 of 100 mm Hg contains 0.3 ml oxygen/100 ml (sometimes written 0.3 vols%). In healthy man at rest the amount of oxygen extracted from the blood by the tissues is about 4 vols%. It is, therefore, apparent that the amount of oxygen carried in the blood in solution is inadequate to supply the oxygen requirements of the tissues in man.

2.5.2.2 Oxygen combined with haemoglobin.

The molecular weight of haemoglobin is 64,500. Haem is an iron-porphyrin compound and this is joined to the protein (globin) which consists of two pairs of polypeptide chains. The chains are of two types: the alpha-chains containing 141 amino acid residues and the beta-chains containing 146. Together, the peptide chains form a tetramer in which the four subunits are held together by bonds between positive and negative ions. Each polypeptide chain is folded to form a pocket on the surface of haemoglobin, into which the haem group fits, easily accessible to oxygen from outside. The ferrous atom is bound to a nearby site on the globin chain as well as to haem. Crystallographic techniques have shown that oxyhaemoglobin has a slightly different configuration to that of deoxyhaemoglobin in that in the former the pair of alpha chains and the pair of beta chains are slightly closer together.[56] Oxygen forms an easily reversible combination with haemoglobin to give oxyhaemoglobin. The combination of

an oxygen molecule with a haem group alters the position of the ferrous ion in the haem ring, changing in turn the position of certain amino acids and changing the affinity for oxygen of the haem group in the neighbouring subunit chain. Hence, in effect, the uptake of each oxygen molecule in turn enhances the uptake of more.[57]

The reversible chemical reaction between oxygen and haemoglobin is defined by the oxygen-haemoglobin dissociation curve which relates the % saturation of haemoglobin to the PO_2. The characteristic sigmoid shape of the oxygen-haemoglobin dissociation curve indicates that the affinity for oxygen progressively increases as successive molecules of oxygen combine with haemoglobin. The curved shape of the oxygen-haemoglobin dissociation curve is physiologically advantageous. The flat upper portion means that even if the PAO_2 falls somewhat, loading of oxygen will not be affected to any large degree. In addition, as the red cell takes up oxygen along the pulmonary capillary, a large partial pressure difference between alveolar gas and blood continues to exist even when much of the oxygen has been transferred. As a result the diffusion process is hastened. The middle steep portion enables the peripheral tissues to withdraw large amounts of oxygen for only a small drop in capillary PO_2.[58] This maintenance of blood PO_2 assists the diffusion of oxygen into the tissue cells.

Each gram of haemoglobin can react with and serve as the carrier for 1.34 ml of oxygen, and since normal blood has about 15 g of haemoglobin/100 ml, the oxygen capacity is about 20 ml oxygen/100 ml of blood. Thus, the oxygen capacity of blood is determined by the concentration of haemoglobin in the blood that is chemically able to combine with oxygen. Not every

oxygen molecule reacts with haemoglobin, and a small quantity of oxygen is present in physical solution in the bloodstream. The oxygen content of the blood is the actual amount of oxygen present in the blood, both chemically combined with haemoglobin and dissolved in plasma. The oxygen saturation of haemoglobin is given by

$$\frac{\text{oxygen combined with haemoglobin}}{\text{oxygen capacity}} \times 100$$

The oxygen saturation of human arterial blood with a PO_2 of 100 mm Hg is about 97.5% while that of mixed venous blood with a PO_2 of 40 mm Hg is about 75%. For example, in an anaemic patient with a haemoglobin concentration of +/- 7.5 g% with normal lungs, and arterial PO_2 of 100 mm Hg, the oxygen saturation will be 97.5%, but the oxygen combined with haemoglobin will be only 10.4 ml/100 ml of blood.

The position of the oxygen-haemoglobin dissociation curve is shifted by pH, PCO_2 and temperature. A fall in pH, rise in PCO_2 and rise in temparature all shift the curve to the right. Opposite changes shift it to the left (Fig.2.2). Although pH, PCO_2 and temperature affect the affinity of haemoglobin for oxygen, these variables are close to constant in healthy persons. Organic phosphates in red blood cells, in particular 2,3-DPG (see Section 2.7.4), profoundly affect the oxygen-haemoglobin dissociation curve. Addition of 2,3-DPG, which binds to the intact haemoglobin molecule, moves the curve to the right. Because the synthesis of 2,3-DPG increases during anaerobic conditions, red blood cells are equipped with their own inbuilt mechanism for responding to hypoxia in a manner that facilitates oxygen release to the tissues.

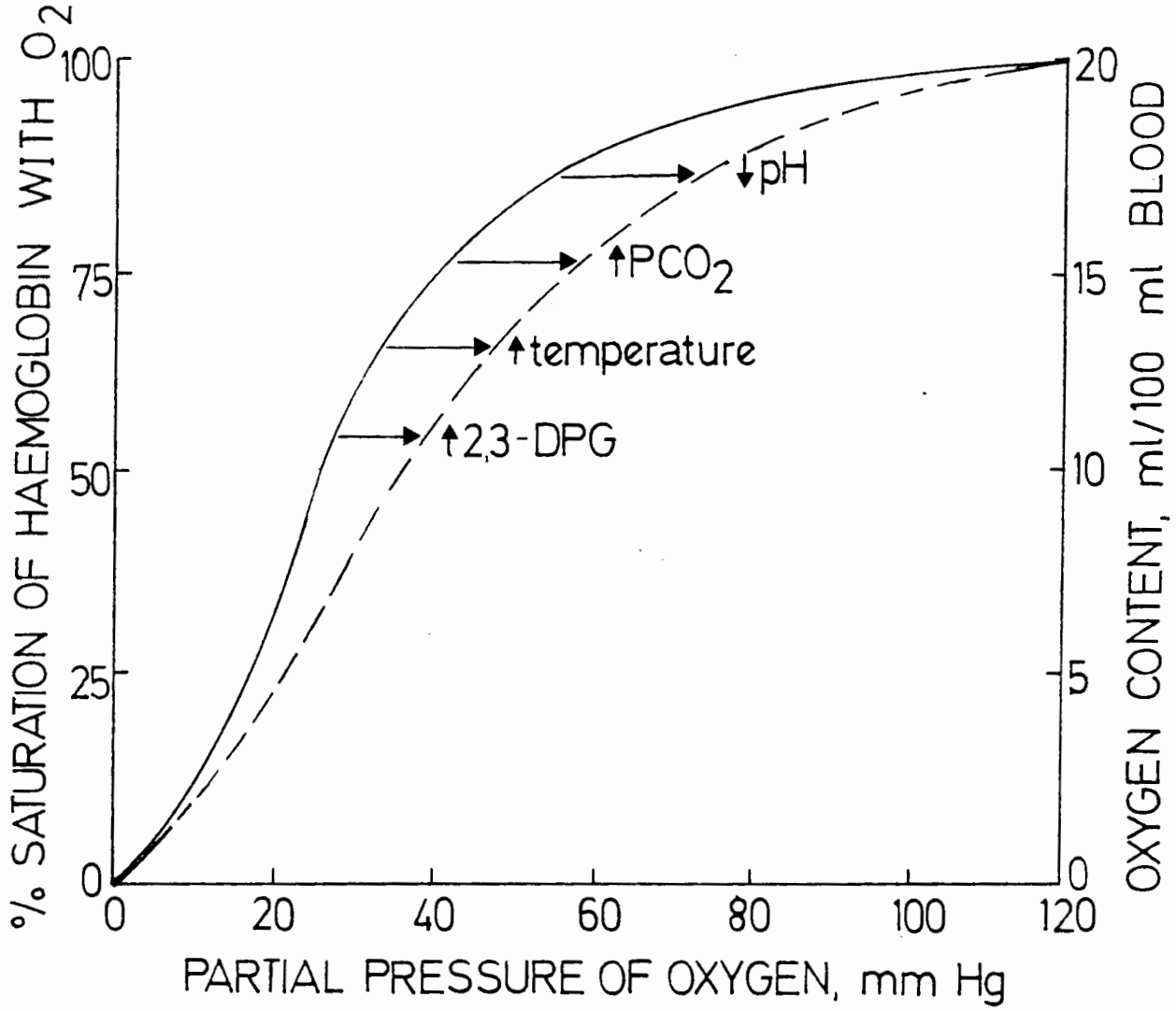


Fig. 2.2 The effect of pH, CO_2, temperature and 2,3-DPG on the dissociation of O_2 from haemoglobin.

2.5.3 Oxygen transport in the tissues.

Each 100 ml of arterial blood passes to the tissues carrying about 0.3 ml of oxygen in solution, and about 19 ml in combination with haemoglobin. The tension of oxygen in arterial blood is about 100 mm Hg; it must be remembered that tension is a property solely of the gas in solution. The oxygen tension in resting tissues is probably just a little lower than that found in venous blood, i.e. about 35 mm Hg. Owing to the great difference of oxygen pressure, oxygen molecules rapidly pass out of the plasma, through the capillary wall and the extracellular fluid, and through the cell membrane to the mitochondrial cristae. The oxygen tension in the blood falls to about 40 mm Hg. The oxyhaemoglobin is now exposed to a decreasing gradient of oxygen tensions as the blood progresses from the arterial to the venous end of the capillary. As a result, dissociation occurs and about 4.5 ml of oxygen from each 100 ml of blood is liberated from haemoglobin. This volume of gas cannot remain in solution in the plasma, which already holds as much oxygen as it can. Oxygen liberated from the red blood cell must, therefore, diffuse out into the tissue fluid. As oxygen diffuses away from the capillary, it is consumed by tissue and the tissue PO_2 falls. There are, therefore, oxygen partial pressure gradients, decreasing as one moves away from a capillary, in every tissue. For this reason, the search for an average oxygen tension in tissues is not meaningful. It is, however, important that oxygen tensions must exist in capillary blood to ensure adequate oxygen transfer to all cells. With isolated mitochondria, a minimum oxygen tension of 0.5 mm Hg is required. Because of the particular pressure gradients within a tissue, the capillary oxygen tension must be considerably

higher to maintain this minimal value. A capillary oxygen tension of about 20 mm Hg is probably close to the lower limit that will prevent significant tissue hypoxia under most conditions in most normal tissues.

Hypoxia is oxygen deficiency at the tissue level. It must be distinguished from anoxia, which refers to the absence of oxygen in tissues. Traditionally, hypoxia has been divided into 4 types:[59]

1) Hypoxic hypoxia, in which the PaO_2 is reduced.

2) Anaemic hypoxia, in which the PaO_2 is normal but the amount of haemoglobin available to carry oxygen is reduced.

3) Stagnant or ischaemic hypoxia, in which the blood flow to a tissue is so low that adequate oxygen is not delivered to it despite a normal PaO_2 and haemoglobin concentration.

4) Histotoxic hypoxia, in which the amount of oxygen delivered to a tissue is adequate but, because of the action of a toxic agent, the tissue cells cannot utilize the oxygen supplied to them.

Within the context of this dissertation, hypoxia will refer to those oxygen tensions at which a reduction in cellular sensitivity to radiation occurs.

2.6 Anaemia and Radiosensitivity.

Reducing the partial pressure of oxygen in the inspired air or lowering the haemoglobin level may have comparable physiological effects on tumours and as a consequence similarly affect tumour radiosensitivity. On the basis of the pre-radiation protocol invoked in this thesis (Chapter 1), it is of relevance to review both the clinical and animal studies pertaining to the effect of anaemia on radiosensitivity.

Anaemia is a common complication of human cancer and a wide variety of factors such as haemorrhage, nutritional deficiencies and chemotherapy-induced bone marrow depression are implicated in its pathogenesis.[60,61,62] Neither clinical nor animal experimental studies have yielded unequivocal evidence as to whether the average haemoglobin level during radiation therapy is a significant prognostic factor. Another pertinent question to be answered is whether reconstitution of the haemoglobin to "non-anaemic" levels by transfusions will improve the local relapse rate and consequent survival of chronically anaemic patients receiving radiation therapy.

Many authors have found that low haemoglobin levels, not corrected before radiation, were associated with reduced tumour radiocurability. Mottram and Eidinow,[63] in 1932, investigated the effect of anaemia on the reactions of the skin and of tumours to radiation. Their results indicated that both skin and tumours were rendered less sensitive to radiation by previous bleeding of the animal. Evans and Bergsjo[64] carried out a retrospective study of the radiation response of carcinoma of the cervix in patients treated in the Norwegian Radium Hospital during the years of the 2nd World War. The five-year survival rate, without apparent recurrence, was 29% in stage 2 patients whose haemoglobin levels were 70-78% of the accepted normal value. This compared to a survival rate of 50% for stage 2 patients with normal haemoglobin levels. The authors also suggested that pre-radiation transfusion may have improved the prognosis.

The relationship between anaemia and poor prognosis was further substantiated by Vigario et al.[65] A definite correlation was shown to exist between low haemoglobin levels and the five-year survival rate in patients receiving

radiotherapy for cancer of the cervix. The five-year cancer-free survival rate decreased from 75% in patients with haemoglobin levels above 13 g% to 55% in patients with haemoglobin levels of 10 g% or less. The poorer prognosis observed in anaemic patients was not significantly improved when the low haemoglobin levels were normalized by transfusions before radiotherapy. A decrease of approximately 20% in the five-year survival rate for carcinoma of the cervix patients with a mean haemoglobin level of less than 11 g%, compared to the rate for patients with a mean haemoglobin in the range 11 to 12 g%, was noted by Hierlihy et al.[66]

Schreiner et al[67] investigated the relationship between haemoglobin level and survival and/or recurrence rate in cancer of the uterine cervix patients treated with radiation. Treatment results were evaluated with respect to two haemoglobin levels, namely Hb > 12 g% and Hb < 12 g%, which were estimated as a mean of tests made on admission and during radiotherapy. This study indicated that anaemia altered unfavourably the effectiveness of radiotherapy with respect to both survival and recurrence rates.

Other tumours occurring in the anaemic patient have also been shown to be less amenable to treatment by radiation. The presence of anaemia (red blood cells less than $3.5 \times 10^6/mm^3$) was found to significantly reduce the 5-year survival rate in women receiving radiotherapy for inoperable endometrial cancer.[68]

Overgaard et al[69] investigated the influence of the haemoglobin concentration on the success of treating patients with carcinoma of the pharynx and larynx with radiation. None of the patients were anaemic, but even within the normal range the haemoglobin concentration was found to significantly affect

the success of the radiotherapy among patients with pharyngeal tumours. No correlation between pre-radiation haemoglobin levels and the probability of local tumour control and survival was detected in patients with glottic tumours.

In patients receiving radiation therapy for carcinoma of the bladder, it has been reported that haemoglobin levels of 12 g% or more were associated with a significant improvement in both complete local tumour regression at 6 months and durable local tumour control.[70] This, however, applied only to patients with T3 cancer or a cancer of grade 3 histology. According to these authors, T1 or grade 1 cancers with their good blood supply could be expected to be less affected by changes in the haemoglobin levels.

A possible explanation for such results is that anaemia leads to a reduction in the supply of oxygen to the tumour, and that this, in turn, leads to an increase in the fraction of hypoxic, and hence radioresistant cells. To test this hypothesis, Hill et al[71] determined an in vivo radiation survival curve for tumour cells when the host animal had a haemoglobin level of about 9.5 g%. This curve was then compared to that obtained for tumour cells in mice with normal haemoglobin levels. From these experiments it was calculated that a haemoglobin level in the host animal of 9.5 g% resulted in the animal having twice the number of hypoxic cells compared to that present when the host animal had a normal haemoglobin.

Hewitt and Blake[72] showed that a substantial proportion of the leukemia cells in the liver of CBA mice changed from an oxic to a hypoxic status within 48 hours of the institution of a phenylhydrazine-induced anaemia. This was also accompanied by the disappearance of large numbers of tumour cells. The proportion of hypoxic leukemia cells in anaemic mice was,

however, significantly reduced when the host animal was transfused with packed erythrocytes or allowed to breathe oxygen under high pressure, with high pressure oxygen showing a possible superiority over transfusion. Transfusion was also shown to have little effect on the proportion of hypoxic cells in a solid sarcoma grown in anaemic mice.

Hirst et al[73] have investigated the effect of acute and chronic anaemia on the radiosensitivity of three mouse tumours. They found that a short duration of anaemia resulted in a resistant tumour with each cell line, but the resistance was gradually lost as the anaemia was prolonged, although the animals were not transfused before radiation. In his excellent review, Hirst[74] suggests that adaptation mechanisms, which come into operation during chronic anaemia, should restore the proportion of hypoxic cells in a tumour to near pre-anaemic levels. Adaptation might involve a decreased requirement for oxygen resulting from a reduction in the radius of the tumour cord ("Reduced cord radius" model) or an increased unloading of oxygen from haemoglobin resulting from an increase in 2,3-DPG ("Increased oxygen availability" model) or both (see Fig.6.1). If adaptation entails a shrinkage of the tumour cord, then the expectation would be that the tumour could become even more easily controlled than the tumour occurring in non-anaemic patients.

In a clinical trial involving stage 2B and 3 carcinoma of the cervix patients, Bush et al[75] observed that patients whose haemoglobin levels were maintained above 12.5 g% showed a 10% higher survival than those with levels between 10 and 12 g%. These authors conclude that the haemoglobin level during radiotherapy has a direct relationship with the risk of local tumour relapse and dispute Hirst's contention[74] that the poorer

prognosis occurring in anaemic patients is purely because the anaemia is a concomitant symptom of a cancer with more aggressive biological properties. Bush and his colleagues[75] furthermore maintain that the decrease in the local tumour control rate observed in carcinoma of the cervix patients with low haemoglobin levels is due to chronic anaemia effecting an increased level of hypoxia in these tumours.[76] Rojas et al[77] have, however, obtained results which lend support to Hirst's theory. Chronic anaemia was induced in CaNT tumour-bearing CBA mice by bilateral radiation of their kidneys. It was found that the tumours irradiated in the anaemic, untransfused mice were more radiosensitive compared to that of normal non-anaemic animals. In addition to the two adaptation mechanisms stated before, a further one has been proposed in order to explain this result, that is, that oxygen delivery to poorly perfused regions is favoured by the fall in blood viscosity which accompanies chronic anaemia.

Transfusing the severely anaemic patient before radiotherapy has been shown in some instances to be of benefit - an improvement in the patient's general well being may result enabling them to better withstand the radiation treatment. However, a closer inspection of the "reduced cord radius" and "increased oxygen availability" models might suggest that transfusing the anaemic patient or allowing the patient to breathe 100% oxygen under pressure or a combination of the two could further reduce and even eliminate the hypoxic cell component in the tumour. What may be a factor of critical importance is the time interval between transfusion and radiation. Hirst[74] has proposed that tumours can adapt to an improvement in their oxygenation just as they do to the reduced oxygen supply of anaemia. Adaptation by cell proliferation

and/or a reduction in 2,3-DPG may negate any previous improvement in the tumour's radiosensitization. Rojas et al[77] report that the increase in the CaNT tumour's radiosensitivity produced by chronic anaemia was further enhanced by red blood cell replacement. Dische et al[78] have shown that anaemic patients receiving transfusions show far better local tumour control when irradiated in high pressure oxygen than in air. The explanation propounded by the authors is consistent with the "reduced cord radius" model. Chronic anaemia induces adaptation mechanisms which result in the hypoxic cell layer(s) being brought closer to the capillary. When the anaemia is corrected by transfusion, and if in addition radiation takes place in high pressure oxygen, effective oxygenation of the hypoxic cells may result. This finding was reproduced by Rojas' group using a murine tumour.[77] Mice with chronic anaemia corrected by transfusion prior to radiation in high pressure oxygen showed the most sensitive overall response.

2.7 **Physiological Responses to High Altitude.**

2.7.1 Preamble.

A high altitude environment imposes on the human body physical factors which are not operative at sea level. The most important of these is hypoxia.[79] The barometric pressure, which depends upon the molecular concentration of the air, decreases with distance above the earth's surface in an approximately exponential manner. This in turn means that the PO_2 in the ambient air at high altitude is reduced. At sea level the barometric pressure is 760 mm Hg and hence the PO_2 in the air is 20.93% of that value, namely 159 mm Hg. When air is breathed into the bronchial tree, it becomes saturated with

water vapour which exerts a pressure of 47 mm Hg so that the PO_2 in the inspired air in contrast to ambient air is 20.93% of (760-47) mm Hg, i.e. 149 mm Hg. At an altitude of 5,500 m, the barometric pressure is one-half the normal 760 mm Hg so the PO_2 of moist inspired air is (380-47) x 0.2093 = 69 mm Hg.

At sea level, the PO_2 at the venous end of the capillary is of the order of 40 mm Hg. Studies on single mitochondria have shown that their critical PO_2 ranges from 1 to 3 mm Hg.[80] A drop of 10 to 15 mm Hg in the PO_2 at the venous end of the capillary, which occurs at high altitude, would seriously endanger the survival of cells at the periphery of a cylinder of tissue being provided with oxygen. In spite of the hypoxia associated with high altitude, some 15 million people live at elevations of 3,000 m or more. A remarkable degree of acclimatization (this refers to those changes which occur in a lowlander after residence for a prolonged period at high altitude) and accommodation (this refers to those physiological changes which occur in the recently arrived lowlander who is acutely exposed to hypoxia) occurs when man ascends to these altitudes. By these processes, oxygen reaches the mitochondria at an adequate partial pressure. Here the oxygen is reduced to water by hydrogen ions and electrons accepted from the respiratory chain. Phosphate is utilized to combine with ADP to give rise to the energy-rich ATP. Thus, the biochemical significance of the delivery of oxygen at adequate partial pressure by the process of acclimatization is to allow the formation of energy-rich chemical bonds which are available for the vital functions of the cell.[81] Some of these compensatory mechanisms, which occur during acute exposure to high altitude, might pertain to the situation where

experimental animals are allowed to breathe gas mixtures containing low PO_2's.

2.7.2 Hyperventilation.

Hyperventilation is an important ventilatory response to hypoxia in so far that it maintains an adequate oxygen tension in the alveolar spaces in spite of the low PO_2 in the ambient air.[82,83] The partial pressure of oxygen in the alveolar spaces, PAO_2, is lower than atmospheric PO_2. As stated in section 2.7.1, the PO_2 of moist inspired air is 149 mm Hg in contrast to that of ambient air which is 159 mm Hg. Once in the alveolar spaces, oxygen diffuses through the alveolar walls to the pulmonary capillaries while carbon dioxide diffuses out from them into the alveoli. Mixture with venous blood then follows resulting in the PAO_2 at sea level approximating to 100 mm Hg and, hence, already a third of the oxygen gradient transporting the gas to the mitochondria has been lost. At each breath only some 15% of the alveolar air is replaced by fresh ambient air. However, with hyperventilation, the steepness of the gradient in PO_2 from ambient to alveolar air falls. So hyperventilation replaces more of the alveolar air by freshly inspired air thus elevating PAO_2 by as much as 50%.[81] Pulmonary hyperventilation in the newcomer to high altitude occurs within a few hours of arrival if the arterial partial pressure of oxygen, PaO_2, is reduced below about 55 mm Hg.[84] The initial hyperventilation on first exposure to high altitude is due to stimulation of the carotid bodies by hypoxaemia.[85]

2.7.3 Polycythaemia.

The quantity of oxygen in the blood depends not only on the ability of the lungs to oxygenate it but also on the concentration of haemoglobin in the blood.

At sea level haemoglobin leaving the lungs is 97% saturated but at the venous end of capillaries this saturation has fallen to 70%. The oxygen content of 100 ml of blood leaving the lungs is 19.4 ml but that of blood leaving the capillaries of the tissues is 14.4 ml. Thus each 100 ml of blood delivers 5 ml of oxygen to the tissues. Under conditions of high altitude where the supply of oxygen is restricted by the diminished barometric pressure of the ambient air, the haemoglobin concentration assumes considerable importance.

The effect of altitude is to raise the level of functional haemoglobin and the number of circulating erythrocytes. This rise constitutes the classical phenomenon called polycythaemia, the magnitude of which depends on the degree of hypoxaemia.[86] The processes leading to altitude polycythaemia have been extensively investigated. One of the most comprehensive experimental studies carried out on animals was that of Lord and Murphy.[87] They analysed the kinetics of erythropoietic production in mice exposed to a simulated altitude of 6,680 m for periods ranging from 1 to 15 days. A striking finding was the sharp increase in the haematocrit which occurred within the first hours of exposure. At an altitude of 2,000 m the haemoglobin level was approximately 1g/dl higher than at sea level; at 3,000 m it was about 2g/dl higher. In permanent residents of Morococha (4,540 m) the PaO_2 is only 45 mm Hg and the corresponding arterial oxygen saturation only 81%. Ordinarily this would considerably decrease the arterial oxygen content, but because of the polycythaemia, the haemoglobin level is increased from 15.6 to 20.1 g/dl,[88] giving an arterial oxygen content of 22.4 ml/100 ml of blood which is above the normal sea level value.

However, the increase in haematocrit will cause a increase in blood viscosity and a fall in tissue perfusion, thus vitiating its beneficial effects.

High altitude polycythaemia is the result of increased erythropoietin activity which occurs within two hours of exposure to hypoxia.[89] The increased erythropoietic activity induced by increases of erythropoietin in the circulating plasma has been demonstrated in the study of bone marrow biopsies and by changes in plasma-iron turnover.[90,91,92] The elevation in the number of circulating red blood cells and haemoglobin which occurs at high altitude is due to an absolute polycythaemia associated with a greater red cell volume and a normal or slightly reduced plasma volume.[93,94]

2.7.4 Modifications in Oxygen Release to the Tissues.

As 98.5% of the oxygen is carried in the erythrocyte and only 1.5% in the plasma, the increased concentration of haemoglobin in the blood at high altitude means that it transports an increased amount of oxygen to the tissues. However, its availability there depends on the ease with which it will be liberated from haemoglobin. Such affinity can be expressed as the familiar oxygen-haemoglobin dissociation curve. The position of the curve is given by the PO_2 associated with 50% oxygen saturation of blood (i.e. when half the total haem groups are combined with oxygen) at $37^{o}C$ and pH 7.4. This particular partial pressure is known as the P_{50}. The role of the position of the curve in unloading oxygen is to maintain a relatively high PO_2 when oxygen has been removed from haemoglobin during passage through a capillary. The amount of oxygen per minute that will diffuse from the blood to the tissue cells varies directly with the difference in PO_2

between these two regions. A shift of the oxygen-haemoglobin dissociation curve to the right yields a higher PO_2 for every value of saturation and is, therefore, favourable in maintaining an adequate level of oxygen diffusion in the tissues.[95] It has been shown that the oxygen-haemoglobin dissociation curve can be shifted to the right by an increase in the erythrocyte 2,3-DPG.[96,97] 2,3-DPG seems to affect the affinity of haemoglobin for oxygen both by direct action (binding) and by lowering the intracellular pH (Bohr effect). It is now clear that the concentration of 2,3-DPG in the red blood cells is a major determinant of the affinity of the blood for oxygen, a fact that was recognized at about the same time by Chanutin and Curnish[98] and Benesch and Benesch.[99] An understanding of the factors influencing erythrocyte 2,3-DPG is important. 2,3-DPG must be regarded as an intermediate of the glycolytic pathway of the erythrocyte. It is formed in the Rapoport-Luebering cycle[100] (Fig.2.3) which by-passes the phosphoglycerate kinase step. Phosphoglycerate kinase catalyzes the transfer of the high-energy phosphate from 1,3-DPG to ADP, forming ATP and releasing 3-phosphoglycerate. 2,3-DPG results from the activity of the enzyme diphosphoglycerate mutase, which catalyzes a transfer of the phosphate from the first to the second carbon of 1,3-DPG. The size of the 2,3-DPG pool in the erythrocytes is determined by a dynamic equilibrium between its synthesis and its degradation. The rate of synthesis depends primarily on the concentration of 1,3-DPG, which in turn is governed by several glycolytic enzymes - phosphofructokinase, glyceraldehyde phosphate dehydrogenase, phosphoglycerate kinase and pyruvate kinase. The rate of synthesis, however, depends not only on the concentration of 1,3-DPG but also on the concentration of 2,3-

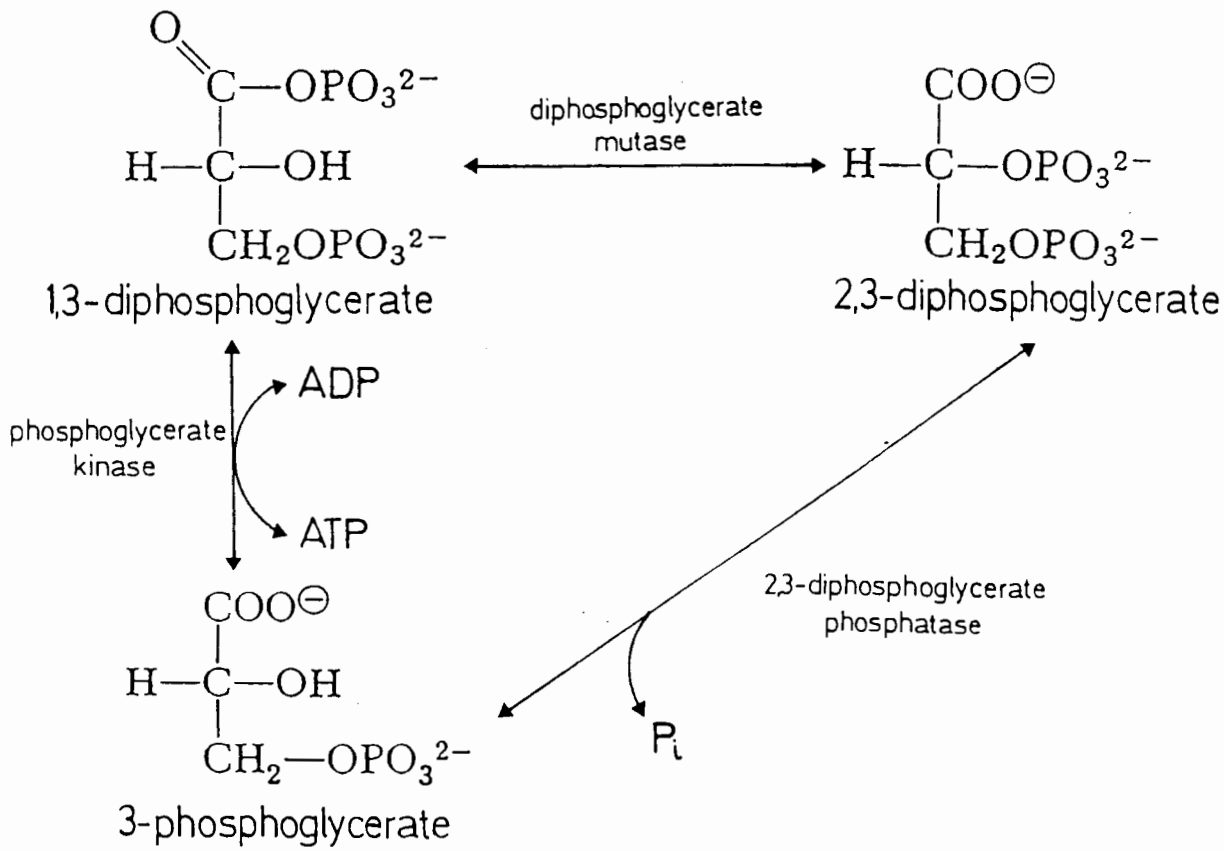


Fig. 2.3 The formation of 2,3-DPG in the Rapoport Luebering Cycle.

DPG itself; diphosphoglycerate mutase is strongly inhibited by 2,3-DPG[101] and at physiological levels of 2,3-DPG, inhibition is about 99%. The rate of 2,3-DPG degradation is independent of its concentration because of the low Michaelis constant, K_M, of 2,3-DPG phosphatase, which is 1 uM. This enzyme has a pH optimum at 6.5 so any decrease in pH from 7.2 will accelerate 2,3-DPG degradation. An increased pH and increased inorganic phosphate concentration also stimulate glycolysis and hence increase the concentration of 2,3-DPG.[102]

Each erythrocyte contains about 15 umol of 2,3-DPG per gram of haemoglobin. 2,3-DPG affects the affinity of haemoglobin for oxygen by entering the core of the haemoglobin molecule, when it is in the deoxy form, between the B chains and binding itself to each.[103] Thus binding of oxygen and 2,3-DPG is mutually exclusive. The stabilization of the deoxy form favours oxygen release thus making more oxygen available to the tissues.

2,3-DPG levels have been shown to increase in people living at high altitude. This is thought to be due to an increase in the plasma pH which in turn is secondary to haemoglobin desaturation and respiratory alkalosis. According to Lenfant et al[104] 2,3-DPG levels altered quickly (during the first 36 hours of exposure to altitude hypoxia) and the change was of significant proportions (an increase from 27 to 38%). The values of the P_{50} have been shown by Mulhausen et al[105] to increase from 26.7 mm Hg to 30.2 mm Hg within 24 hours at an altitude of 3,500 m. The authors also noted that the shift of the oxygen-haemoglobin dissociation curve to the right was already significant even after 6 hours exposure to 3,500 m.

2.7.5 Cardiac Output.

A number of studies have shown that cardiac output increases on acute exposure to high altitude. Cardiac output increased on the first day at 3,800 m to a maximum, and decreased the following days to values slightly lower than at sea level according to Klausen.[106] Vogel and Harris[107] also observed an increase in cardiac output with altitude. In their investigations, the cardiac output response appeared to follow an exponential response with altitude. Cardiac output rose from 71 at 610 m to 84 and 123 ml/min per kg at 3,355 m and 4,575 m respectively. Their results indicated that a peak cardiac output was reached within a matter of hours of exposure to high altitude. A sharp increase in cardiac output upon ascent to 4,509 m and a return within 48 hours to pre-exposure values has been noted by Lenfant et al.[108]

During the initial phase of exposure to high altitude the predominant factor which works in the direction of increasing cardiac output is tachycardia. It has been suggested that the tachycardia is in response to the increased activity of the sympathoadrenal system which is reflected by a rise in the levels of both plasma and urinary catecholamines.[109]

However, conflicting results to that reported above have emanated from the studies of Hoon et al.[110] They reported an immediate and sharp fall in cardiac output at high altitude with a return to normal on return to sea level. Although the effect of high altitude on cardiac output has produced disparate results, a general finding has been that changes in cardiac output, which may occur at high altitude, do not appear to persist on return to sea level.

2.8 Cancer and Altitude.

There has long been a tradition in Spanish South American medicine that a number of diseases, including cancer, are rare or nonexistent in natives of high-altitude. In fact, the people in the state of Hunza (above 4,575 m) are reputed never to develop cancer.[111] Loewy and Wittkower[112] have observed that the carcinoma mortality rate was decidedly lower in La Paz, Bolivia (3,510 m above sea level) than in areas nearer sea level. However, a number of workers, notably Hever Kruger and Arias-Stella[113] as well as Mori-Chavez and Hellriegel[114] have shown the existence of malignant tumours in natives and long-term residents of altitudes of 3,510 m and higher. A closer analysis of the former authors' reports suggests, however, that cancer may be less frequent at high altitude than might be expected. The existence of an inverse relationship between altitude and cancer mortality has since been reaffirmed by several investigators. Eckhoff et al[115] used published demographic data and leukemia mortality data to correlate the leukemia mortality rate with altitude. The findings of this study indicated that above 610 m the leukemia mortality rate decreased significantly with increasing altitude. A similar altitude effect on mortality from cancers other than leukemia was examined by Mason and Miller,[116] who attributed finding reduced lethality at high altitude to "rurality" rather than to altitude. The relationship of altitude to cancer has been investigated by Amsel et al.[117] They used techniques that minimized the possible confounding effects of industrialization, urbanization and ethnicity to compare site-specific cancer mortality rates for high and low altitude populations. For most malignancies a decrease in mortality in high altitude counties (in the USA) was observed. The largest

differences between the low and high altitude groups were found for melanoma and for cancers of the upper aerodigestive tract. These findings were quite consistent for both males and females, across virtually all levels of industrialization, urbanization and ethnicity.

The picture is complicated by mouse studies, where there is evidence of both increased and decreased spontaneous and radiation-induced tumour development at high altitude. Mori-Chavez[118] has analysed the spontaneous development of pulmonary tumours in the highly susceptible strain A mice at sea level and at high altitude (4,545 m above sea level). The incidence of spontaneous pulmonary tumours was 61.4% at high altitude compared to 53% at sea level - this difference was not statistically significant. However, the average diameter was greater in the high altitude group which also contained a higher incidence of large tumours of 4 mm and above. Subsequent studies were undertaken by Mori-Chavez et al[119] using RF/Un mice in which tumours could be induced with 1.5 Gy whole-body X-radiation. An enhancement in the induction of granulocytic leukemia was observed when mice were housed at high altitude (4,540 m) for 3 months after radiation as compared to mice kept at low altitude (150 m). On the other hand when mice, following radiation, were kept at high altitude into old age, a decrease in the incidence of lymphomas, ovarian tumours, pituitary and adrenocortical adenomas, sarcomas and endometrial carcinomas was noted. Experiments carried out on C58 mice by Mori-Chavez[120] indicated a lower incidence of spontaneous leukemia at high altitude than at sea level. These investigations also showed that leukemia developed at an earlier age at sea level than at altitude. Furthermore, the anatomical lesions observed at high altitude were not so

extensive or prominent as those observed at sea level. Blatteis[121] recorded no difference in the growth of hepatomas in rats kept at a 4,500 m simulated altitude and those animals kept at sea level.

A number of theories have been advanced in an attempt to explain why the incidence of cancer could be lower at high altitude than at sea level. Blatteis et al[122] have suggested that increases in the activities of tumour lysosomal enzymes at altitude may be implicated in the reported growth retardation. Their hypothesis is that tumour growth might be proceeding at a normal rate at high altitude, but concomitant autolytic destruction resulting from increased lysosomal hydrolase activities might modulate the tumour's development. To test this hypothesis, Buffalo strain rats were inoculated with Morris hepatomas, after which they were either exposed for eight weeks to a 4,500 m simulated altitude in a hypobaric chamber or kept at sea level. At the conclusion of the eighth week of exposure to their respective environments, the tumours' dimensions were determined. The tumours were then excised and assayed for hydrolase activities. Although the altitude hepatomas were smaller than the sea level tumours, the hydrolase activities were unchanged at 4,500 m as compared to sea level. These results would seem to suggest that, to the contrary, lysosomally-mediated, progressive autolytic destruction of the growing tumours was probably not a factor in causing their smaller mass at altitude.

It has been documented that changes in the pH of the external medium have a marked effect on growth and cellular division in tissue culture.[123] Sensitivity to changes in pH is such that a 0.2 unit shift in pH is usually sufficient to reduce the growth rate to half the maximum value. Burton[124]

has proposed that interference with tumour growth at high altitude may be pH related. During acclimatization to high altitude, hyperventilation occurs which results in excessive loss of CO_2 and a rise of blood pH. Although the blood pH may return to normal in about a week, the alkali reserve of the body will remain markedly abnormal. This will result in a disturbance of acid-base relations within the cell.

Direct hypoxia may be a factor in affecting tumour growth at high altitude. This statement is based on the premise that the rate of tumour cell proliferation depends on the oxygen tension within the tumour. The oxygen tension within the tumour depends on, amongst other things, the oxygen tension in the blood. In spite of a compensatory mechanism such as hyperventilation, which operates at high altitude, the mean capillary oxygen partial pressure in the animal at high altitude remains well below the normoxic value.[125] It is also to be expected that the oxygen tension within the tumour would be lower at high altitude than at sea level [81] (notwithstanding short term adaptation mechanisms such as an increase in erythrocyte 2,3-DPG). It, therefore, follows that a slower rate of tumour cell proliferation is to be expected at high altitude. Tannock and Steel[126] have shown that mouse mammary tumours grew more slowly in animals kept in 10% oxygen than in animals kept in air. To assess the rate of tumour cell proliferation, the mean thymidine labeling indices for the two groups of tumours were estimated. The labeling index was significantly reduced when the host animal breathed 10% oxygen implying a lower rate of cell proliferation in these tumours.

CHAPTER 3

MATERIALS AND METHODS

The subject matter of this chapter has been grouped into 4 sections. The first section describes the experimental animals and tumours used, the radiation sources and techniques, and the tissue culture techniques. In the second section, the protocols used to investigate the response of experimental tumours to different treatment regimens are detailed. The third section details the different methods used to determine the hypoxic fractions of tumours. Section four examines the biochemical assays carried out on tumour tissue, blood and on cultured tumour cells.

3.1 Experimental Material, Radiation and Tissue Culture Techniques.

3.1.1 Experimental Animals.

Male BALB/c, CBA and WHT mice, aged from 6 to 8 weeks at the beginning of all experiments, were used throughout this study. Experimental animals were bred in the Animal Unit of the University of Cape Town's Medical School for the early experiments, then for the remainder in the specific pathogen free facility of the Radiobiology Laboratory. The experimental animals were kept in the animal laboratory of the Radiobiology Department. The mice were allowed food and water ad libitum. The temperature in the animal laboratory was maintained at 22 +/- 1^{o}C.

3.1.2 Experimental Mice and Tumours.

3.1.2.1 CBA Mice and the CaNT Tumour.

The CaNT tumour was originally obtained from Professor R. Berry of the Middlesex Hospital Medical School, University of London, UK. The tumour was maintained by serial passage by the inoculation of 0.1 ml of a tumour cell suspension (prepared as detailed in section 3.1.2.5) in Mc Coy's 5A medium and containing approximately 2×10^6 cells subcutaneously in the sternal area of the CBA mice.

3.1.2.2 BALB/c Mice and the 3-Methylcholanthrene (3-MC)-Induced Transplantable Rhabdomyosarcoma.

A transplantable rhabdomyosarcoma was induced in the BALB/c mice by injection of 0.1 ml of a 1 mg/ml solution of 3-MC in arachis oil into their right flanks. The tumours took approximately 3 to 4 months to develop after which they were maintained by serial passage in the sternal area, as described for the CaNT tumour.

3.1.2.3 WHT Mice and the Fib/T Tumour.

The Fib/T tumour was originally obtained from Dr N. Mc Nally of Mount Vernon Hospital, Northwood, UK. The tumour was maintained by serial passage by the inoculation of a tumour cell suspension into the right gastrocnemius muscle. It was intended to maintain the Fib/T tumour subcutaneously in the sternal region, but the tumour showed poor and eratic growth at that site.

3.1.2.4 Measurement of Volume of Experimental Mouse Tumours.

Mouse tumours were assumed to be spherical and their volumes were calculated after measurement in three perpendicular directions, a,b,c, with Vernier calipers according to the formula:

$$\text{Volume} = 4/3\pi(a+b+c)^3/6$$

3.1.2.5 Tumour Propagation Procedure in Experimental Animals.

Tumour-bearing mice were killed by prolonged exposure to ether, following which their tumours were surgically excised. The tumours were then placed into approximately 5 ml of McCoy's 5A medium containing $NaHCO_3$ to adjust the pH to between 7.2 and 7.4. The tumours were then mechanically minced to a brei using a sterile scalpel and forceps.

After completion of the mincing procedure the preparation was allowed to settle for about one minute. The supernatant containing tumour cells was then aspirated into a sterile syringe. Subsequently, mice were inoculated with 0.1 ml of the supernatant using a 0.45 x 13 mm needle. All three tumour types took approximately 10 days to appear.

3.1.3 Radiation Techniques.

3.1.3.1 Restraining Devices for Mice.

Mice bearing tumours in the sternal region were restrained without anaesthesia in the prone position using an acrylic plastic jig as shown in Fig.3.1. Rubber bands were used to gently tie the front and back legs to the jig which ensured immobilization during the radiation period.

WHT mice with Fib/T tumours were immobilized without anaesthesia in the prone position on an acrylic plastic

restraining jig containing an aperture through which the tumour-bearing leg was made to pass (Fig.3.2). The leg bearing the tumour was taped down with masking tape.

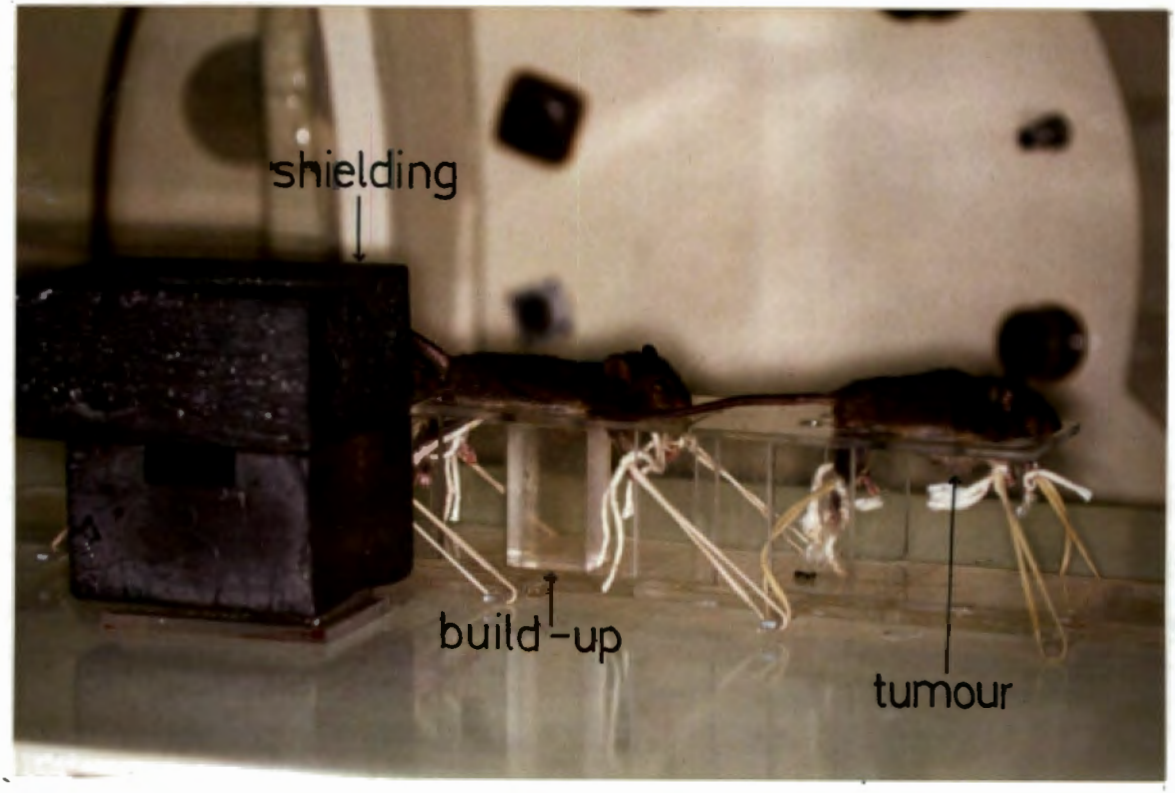


Fig.3.1 Irradiation of mice with 8-MV X-rays.

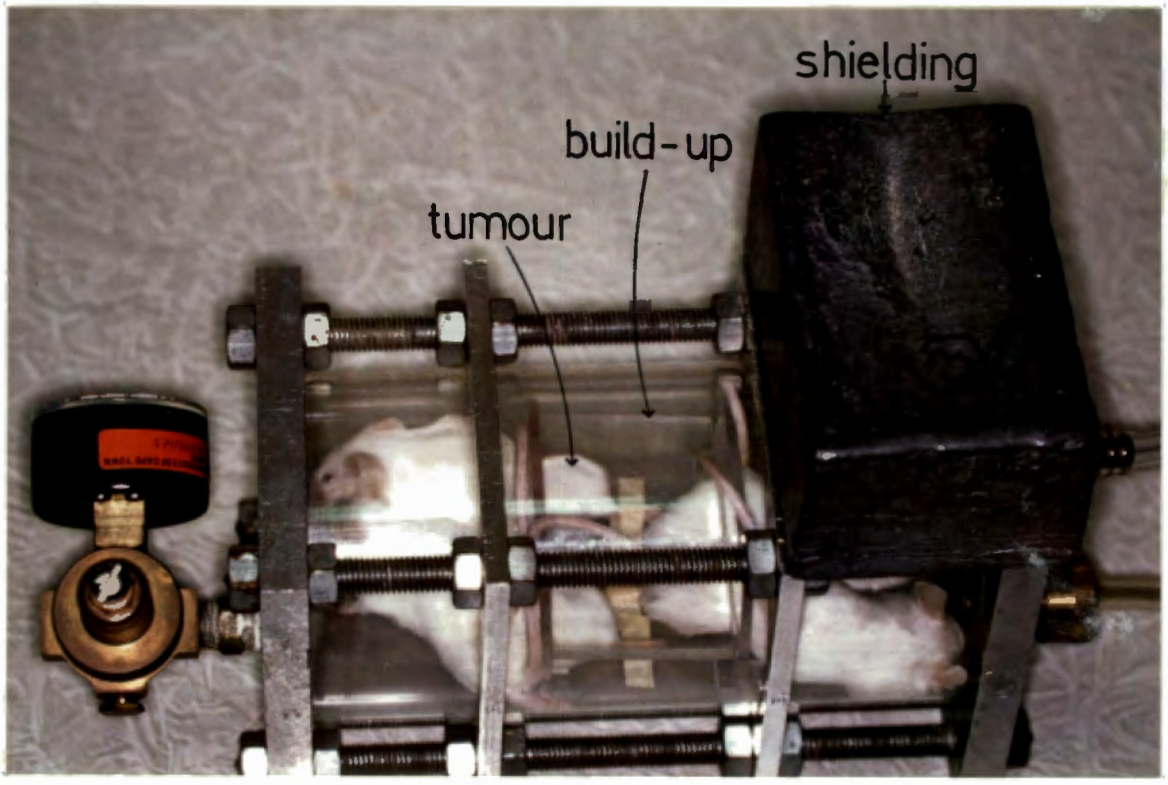


Fig.3.2 Irradiation of mice in "small animal" hyperbaric chamber.

3.1.3.2 100 kVp X-Ray Unit.

All experiments carried out to determine the hypoxic fraction of mouse tumours involved radiation with a Philips RT100 X-ray machine, 100 kVp, 3.0 mm HVL, operating at 8 mA. At the time of performing hypoxic fraction determinations, the 100 kVp X-ray unit was the only source that was conveniently available to the Radiobiology Department for experimental purposes. Tumours in the sternal region were irradiated at a focus to surface distance (FSD) of 12.5 cm whereas a FSD of 10 cm was used for the thigh Fib/T tumours (due to the design of the jigs). A circular field of 2.5 cm diameter was used for all tumour radiations.

The 100 kVp X-ray unit was calibrated with a flat (small volume 0.3 cm^3) ionization chamber connected to a Baldwin-Farmer sub-standard dosimeter and placed at the end of a circular applicator, diameter 2.5 cm, FSD 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 FSD using a circular applicator, diameter 5 cm. The output was measured weekly at 30 cm FSD with the circular 5 cm diameter applicator, and changes in output were corrected for in the calculation used to determine the dose to be given.

Tumours on the sternum were irradiated horizontally in such a way that half the dose was given from one side and half the dose from the other. This aided dose homogeneity. Thigh tumours were irradiated from above with the applicator vertically aligned and in contact with the tumour. The variation in X-ray dose across the tumour was calculated to be not more than 1.0%.[127] All parts of the mouse apart from the tumour were shielded with 3 mm thick lead.

3.1.3.3 ^{60}Co Gamma Ray Unit.

WHT mice with the Fib/T tumour in the gastrocnemius muscle were irradiated with a Eldorado 6 cobalt-60 unit, which was made available to the Radiobiology Department for the exclusive purpose of radiobiological experimentation. This machine has been modified with a cast-lead collimator which results in a fixed 40 x 40 cm field at a SSD of 80 cm. Only the inner 20 x 20 cm area of this field was used for tumour radiations. The dose at the edge of the 20 x 20 cm field was within 5% of the maximum dose. A 0.5 cm thick sheet of acrylic plastic was placed above and just in contact with the tumours to provide build-up. Lead blocks, 7.5 cm thick, shielded all but the tumours from radiation. In order to calibrate the Eldorado 6 for irradiating mouse tumours both in air and in a hyperbaric oxygen chamber, a flat ionization chamber connected to a substandard Baldwin-Farmer dosimeter was placed in the identical geometrical position as for the tumours. Wax blocks were used as mouse phantoms. Outputs of 0.5868 and 0.5598 Gy/min were measured on 18 November 1986 at distances corresponding to those of the mouse tumours positioned in the hyperbaric chamber and in air respectively.

3.1.3.4 8-MV X-Ray Unit.

The CaNT tumour and the 3-MC-induced rhabdomyosarcoma grown on the sternum of CBA and BALB/c mice respectively were irradiated horizontally with 8-MV X-rays from a Phillips SL 75-20 linear accelerator. Lead blocks, 7.5 cm thick, were placed so that all parts of the mice were shielded except for the tumours. Blocks of acrylic plastic, 2 cm thick, were positioned in front of the tumours as build-up (Fig.3.1).

(These experiments were carried out before the Radiobiology Department acquired its own cobalt source. The use of the Vickers Medical, single patient, hyperbaric oxygen chamber necessitated that all radiations involved in the growth delay studies were delivered using a linear accelerator).

Calibration of the beam was performed with a Baldwin-Farmer ionization chamber in the same geometrical arrangement as for the mouse tumours. This applied to mice irradiated in air and mice irradiated in the Vickers Medical, single patient, hyperbaric oxygen chamber.

3.1.3.5 .Neutron Therapy Unit.

WHT mice with the Fib/T tumour in the gastrocnemius muscle were irradiated using the neutron therapy isocentric unit of the National Accelerator Centre situated at Faure near Cape Town.

Neutrons are produced by the reaction of 66 MeV protons on a 19.6 mm beryllium target in which the proton beam dissipates 40 MeV, the remaining energy being dissipated in the graphite backing of the target and the cooling water. This reaction is thus designated p(66)/Be(40). The quality of the neutron beam is modified by the use of iron flattening filters and a 2.5 cm thick polyethylene hardening filter.

Tumours were irradiated at a surface to axis distance of 150 cm. The beam was used in the vertical mode. A 29 x 29 cm field was used but all tumours were always positioned within the inner 20 x 20 cm area of this field, thus ensuring uniformity of dose. Although the entire mouse was in the field without shielding, mice were sacrificed immediately after radiation. A 20 x 20 x 2 cm closed perspex box (perspex thickness = 3 mm) containing de-ionized water was used as

build-up. The build-up was placed above and just in contact with the tumours.

Calibration of the beam for mouse tumour radiation was performed using a tissue equivalent (A150 plastic) calibration phantom into which ionization chambers could be fitted at the identical geometrical positions as for tumour radiation. For the radiation conditions used, the gamma dose was 4% of the total dose.

3.1.4 Reduced Oxygen Environment.

Unanaesthetized mice with food and water ad libitum were kept in a closed plastic container (5/container) through which a mixture of oxygen and nitrogen was passed at a flow rate of 2 l/min. The carbon dioxide concentration of the effluent gas was not measurable. The oxygen concentration was measured in the container using a Critikon Oxychek oxygen monitor. The purities of the oxygen and nitrogen were 99.6% and 99.995% respectively.

3.1.5 Compression of Experimental Animals in the Hyperbaric Chambers.

To investigate the response of the CaNT tumour and the 3-MC-induced rhabdomyosarcoma to 8-MV X-rays under conditions of high pressure oxygen, the mice were compressed in a single patient hyperbaric oxygen chamber (Vickers Medical).

Fib/T tumour-bearing mice that were to be irradiated with ^{60}Co gamma rays were compressed in a "small animal" hyperbaric chamber manufactured in conjunction with the Department of Medical Physics of the University of Cape Town (Fig.3.2). This chamber was large enough to accommodate 4 mice.

The hyperbaric chamber into which the mice were introduced was initially flushed with pure oxygen in order to eliminate all but 2-3% of nitrogen. Pressurization then proceeded at such a rate that 3 atmospheres absolute (3 ATA) was reached in 10 minutes. This pressure was maintained for 15 minutes before radiation was commenced. Decompression was effected without complications in 2 minutes.[128]

3.1.6 Tissue Culture Techniques.

3.1.6.1 Cell Line and Cell Culture Conditions.

The B16 mouse melanoma cell line is an established line and can be cultured in vitro using the method outlined by Puck and Marcus.[129] The cells were maintained routinely in 250 ml tissue culture flasks in McCoy's 5A medium (Gibco) supplemented with 10% foetal calf serum (Gibco), 10 U/ml penicillin, 10 ug/ml streptomycin and 10 ug/ml neomycin. The tissue culture flasks were incubated in a humidified atmosphere containing 95% air/5% carbon dioxide at 37^{o}C.

3.1.6.2 Sub-Culture of B16 Cells.

3.1.6.2.1 Reagents.

a) EDTA in phosphate buffered saline was made up to 10 times the required concentration as follows:

NaCl, 82 g; Na_2HPO_4, 12.78 g; $NaH_2PO_4.2H_2O$, 2 g; and EDTA disodium salt, 2 g dissolved in distilled water and made up to 1 litre. The pH was adjusted to 7.2 - 7.4 with concentrated NaOH, and the solution was filter sterilized through a sterile 0.22 um Millipore filter. Stock EDTA was frozen in 10 ml aliquots and diluted 10 fold with sterile water before use.

b) Trypsin in phosphate buffered saline was made up as a 10 times concentrated stock solution. The trypsin solution was prepared thus:

NaCl, 82 g; Na_2HPO_4, 12.78 g; NaH_2PO_4, 2 g; EDTA disodium salt, 1 g; and trypsin, 5 g dissolved in distilled water and made up to 1 litre. The pH was adjusted to 7.4 with concentrated NaOH and then filter sterilized through a sterile

0.22 um Millipore filter. Stock solutions were stored frozen, then were thawed and diluted 10 fold before use.

3.1.6.2.2 Procedure.

Confluent B16 melanoma stock cultures were detached from their flasks using the following procedure. Medium was poured off, and the cells rinsed twice with 5 ml 0.02% EDTA solution. Cell cultures were then trypsinized by the addition of 5 ml 0.05% trypsin solution, which was left in contact with the cells for 5 minutes at 37^{o}C in a 95% air/5% carbon dioxide incubator. The cells became detached from the bottom of the culture flasks by this procedure. The contents of the flasks were transferred to 10 ml plastic centrifuge tubes and 5 ml of McCoy's 5A medium containing 10% foetal calf serum and antibiotics was added to each tube. The tubes were then centrifuged for 5 minutes at 200 x g in a Runne bench-top centrifuge. The supernatants were discarded and the cell pellets resuspended in 8 ml McCoy's 5A medium. The cell concentrations were obtained by counting a sample of the cell suspension in a haemocytometer. After appropriate dilutions were made, 2 ml of McCoy's 5A medium containing approximately 1x10^{4} cells was pipetted into 15 mm diameter glass petri dishes. The petri dishes were kept in a 95% air/5% carbon dioxide incubator at 37^{o}C until the cells grew to confluence.

3.2 Treatment Protocols.

3.2.1 Tumour Regrowth Delay Assay.

In the BALB/c and CBA mice bearing the 3-MC-induced rhabdomyosarcoma and the CaNT tumour, respectively, the tumour response was assessed by a regrowth delay assay.[130,131] The

tumour-bearing mice from both strains were divided into 6 groups consisting of from 6 to 10 mice per group and treated in the following manner:

Group 1) Mice were placed in an 8% oxygen environment for 72 hours. Within 1 hour of removal from the 8% oxygen environment, the mice were restrained in a jig (as detailed in section 3.1.3.1) and irradiated in air.

Group 2) Mice were kept for 72 hours in a container through which 21% oxygen (air) was passed at a flow rate of 2 l/min. Mice were then removed and irradiated in air.

Group 3) These mice were kept in an 8% oxygen environment for 72 hours. Within 1 hour of removal from the 8% oxygen environment, the mice were pressurized to 3 ATA with 100% oxygen in a hyperbaric oxygen chamber and then irradiated.

Group 4) Mice were kept in a 21% oxygen environment for 72 hours, and were then irradiated after being pressurized to 3 ATA with 100% oxygen.

Group 5) Mice were kept in 8% oxygen for 72 hours, but received no radiation. These mice served as controls for groups 1 and 3.

Group 6) Mice were kept in a 21% oxygen environment for 72 hours, but were not irradiated thus serving as controls for groups 2 and 4.

A control group, which consisted of mice pressurized to 3 ATA only, was not included in these investigations. Therefore, where mice were irradiated in hyperbaric oxygen, any direct effect of the 3 ATA exposure on tumour growth delay was incorporated in the radiosensitivity effect induced by the hyperbaric oxygen environment.

Mice in groups 1-4 were all irradiated with a single dose of 11 Gy, 8-MV X-rays (as described in section 3.1.3.4).

After treatment, mice were kept under ambient conditions and tumour volume measured three times per week. The time taken for the tumours to reach four times their initial volume could be determined on regrowth curves. Initial tumour volume was defined as that volume measured immediately following removal of the mouse from its 72 hour period in a given oxygen environment. Initial tumour volumes were all in the order of 0.2 +/- 0.03 cm^3. The regrowth delay due to radiation was determined as the mean time required for the animals within an radiation group to develop tumours four times their initial size minus the mean time taken for the appropriate unirradiated control group animals to do likewise.

Significance of differences was assessed using the Student's t-test.

3.2.2 Treatment Protocols for the Fib/T Tumour.

The response of the Fib/T tumour, grown in the right gastrocnemius muscle of WHT mice, to different treatment protocols was assessed using an in vivo-in vitro excision assay.

The experiments performed can conveniently be divided into 5 groups.

3.2.2.1 Single Dose Gamma Radiation in Air of Experimental Mouse Tumours.

Groups of mice, consisting of from 5 to 7 per radiation dose point, were treated in the following manner:

Group 1) Mice kept in 8% oxygen for 24 hours.

Group 2) Mice kept in 8% oxygen for 48 hours.

Group 3) Mice kept in 8% oxygen for 72 hours.

Group 4) Mice kept in 10% oxygen for 48 hours.

Group 5) Mice kept in 10% oxygen for 72 hours.

Group 6) Mice kept in 15% oxygen for 48 hours.

Group 7) Mice kept in 15% oxygen for 72 hours.

Group 8) Mice kept in 21% oxygen (air) for 24 hours.

Group 8) Mice kept in 21% oxygen for 48 hours.

Group 9) Mice kept in 21% oxygen for 72 hours.

Group 10) Mice kept in 8% oxygen for 48 hours followed by a 24 hour exposure to 21% oxygen.

Within 1 hour, following removal of the mice from their respective oxygen environment, the tumours were irradiated while the mice breathed air. In the case of mice in group 10, tumours were irradiated following removal of the mice from the 21% oxygen environment. Doses ranging from 5 to 25 Gy (at 5 Gy intervals) were given. Immediately after radiation, the mice were sacrificed and their tumours removed. Control groups of mice (i.e. mice that received the same pretreatment as the irradiated groups but were not subsequently irradiated) were sacrificed within 1.5 to 2 hours following removal from their oxygen environment, and their tumours excised. The tumours of the control groups served for the determination of plating efficiencies.

3.2.2.2 Single Dose Neutron Radiation in Air of Experimental Mouse Tumours.

Two groups of mice, consisting of 5 per radiation dose point, were treated in the following manner:

Group 1) Mice kept in 8% oxygen for 48 hours.

Group 2) Mice kept in 21% oxygen for 48 hours.

Within 1 hour, following removal of the mice from their respective oxygen environment, the tumours were irradiated while the mice breathed air. Doses ranging from 2 to 10 Gy

(at 2 Gy intervals) were given. Immediately after radiation, the mice were sacrificed and their tumours removed. Unirradiated mice, which received the appropriate pretreatment, served as controls for plating efficiency determinations.

3.2.2.3 Single Dose Gamma Radiation in Hyperbaric Oxygen of Experimental Mouse Tumours.

Two groups of mice, consisting of from 5 to 7 per radiation dose point, were treated in the following manner:

Group 1) Mice kept in 8% oxygen for 48 hours.

Group 2) Mice kept in 21% oxygen for 48 hours.

Within 1 hour of removal from their particular pretreatment environment, the mice were pressurized to 3 ATA with 100% oxygen in a hyperbaric oxygen chamber and irradiated. Mice received doses ranging from 5 to 25 Gy to their tumours. Immediately after decompression, the mice were sacrificed and the tumours removed. Unirradiated mice, which received the appropriate pretreatment, served as controls.

3.2.2.4 Fractionated Gamma Radiation delivered while the Tumour-bearing Mice breathed Air.

Two equal fractions of radiation, separated by either 4 or 24 hours, were delivered in air to the mouse tumours to give total doses of 8, 16 and 24 Gy.

3.2.2.4.1 Split-dose radiation of experimental mouse tumours allowing a 4 hour interval between the two exposures.

The mice were divided into 3 groups consisting of 5 mice per radiation dose point and treated in the following manner:

Group 1) These mice were kept in 8% oxygen for 48 hours. Within 1 hour, following removal of the mice from the 8% oxygen environment, the first fraction of radiation was delivered to the tumours while the mice breathed air. The mice were then kept in air and the second fraction of radiation was delivered 4 hours later. The mice were then sacrificed and their tumours removed.

Group 2) Mice received the same pretreatment as in group 1 except that they were replaced in 8% oxygen for the 4 hour period between fractions.

Group 3) Mice were pretreated with 21% oxygen for 48 hours, and kept in this environment for the 4 hour period between radiation fractions.

3.2.2.4.2 Split-dose radiation of experimental mouse tumours allowing a 24 hour interval between the two exposures.

The mice were divided into 4 groups consisting of 5 mice per radiation dose point and treated in the following manner:

Group 1) Mice were kept in 8% oxygen for 48 hours. Subsequently, two equal doses of radiation separated by 24 hours were delivered in air to the tumours. The mice were kept in air during the 24 hour radiation-free interval. Immediately following the second fraction of radiation, the mice were sacrificed and their tumours removed.

Group 2) These mice received the same overall treatment as in group 1 with one exception, viz. the mice were pretreated with 21% rather than 8% oxygen.

Group 3) Mice were kept in 21% oxygen for 48 hours. After the first fraction of radiation was delivered in air, the mice were placed in 8% oxygen for 24 hours. Then following

removal of the mice from the 8% oxygen environment, the second radiation fraction was given in air. Mice were then sacrificed and their tumours removed.

Group 4) The mice were kept in 8% oxygen for 48 hours prior to their receiving the first fraction of radiation. Thereafter, treatment was as in group 3.

For all four groups, mice were included that were not irradiated but otherwise received the same treatments as their irradiated counterparts. These mice acted as controls.

3.2.2.5 Fractionated Gamma Radiation delivered while the Tumour-bearing Mice breathed 100% Oxygen under Pressure.

Two equal fractions of radiation, separated by 24 hours, were delivered under conditions of high pressure oxygen to tumour-bearing mice to give total doses of 8, 16 and 24 Gy.

The mice were divided into 4 groups consisting of 5 mice per radiation dose point and each group treated as groups 1 to 4 in section 3.2.2.4.2 above, except that all radiations were in hyperbaric oxygen.

3.2.3 Excision Assay Procedure.

The cell dispersion technique used is based on that employed by several investigators.[132,133,134] The mice were sacrificed by prolonged exposure to ether and the tumours surgically excised. The tumours were then placed into approximately 5 ml of Alpha modified Eagle's medium (Gibco) containing $NaHCO_3$ to adjust the pH to between 7.2 and 7.4. In addition the medium contained 100 U/ml penicillin, 100ug/ml streptomycin and 100 ug/ml neomycin. The tumours were then transferred to Alpha modified Eagle's medium containing 10 U/ml

penicillin, 10 ug/ml streptomycin and 10 ug/ml neomycin. In this medium the tumours were minced using a sterile scalpel and forceps. Trypsin and collagenase were added resulting in a final concentration of 0.25% (w/v) and 0.06% (w/v) respectively. This preparation was incubated for 30 minutes at 37^{o}C in a shaking water bath, after which it was filtered through 1 cm of loosely packed, sterile, surgical gauze. After centrifugation for 5 minutes at 200 x g in a Runne bench-top centrifuge, the supernatant was decanted and the pellet resuspended in 8 ml of Alpha modified Eagle's medium containing 10% foetal calf serum (FCS), 10 U/ml penicillin, 10 ug/ml streptomycin and 10 ug/ml neomycin. This process was repeated twice. Trypan Blue, 30 ul, was mixed with 30 ul of the Fib/T suspension, and an aliquot was then counted with a haemocytometer, with trypan blue exclusion used as the indicator of cell integrity. The above procedure resulted in a single-cell suspension which was greater than 95% dye-excluding. The cell yield was, in general, greater than 5 x 10^{7} cells/g tumour. Tumours were always assayed individually. After appropriate dilutions were made, known numbers of cells were plated in the same medium as used above into 25 cm^{2} tissue culture flasks which were then incubated for 7 days at 37^{o}C in a 95% air/5% carbon dioxide incubator. The colonies were washed with phosphate buffered saline, stained with Gentian Violet and counted with the aid of a microscope.

The resultant number of colonies, each consisting of more than 50 cells, represented the number of surviving cells in the plated suspension.[135] The fraction of cells that retain the capacity for unlimited proliferation after exposure to doses of radiation could then be determined from the formula:

Surviving Fraction = colonies counted/cells seeded x (plating efficiency/100)

From this data dose survival curves were constructed showing the relationship between radiation dose and tumour cell survival for the different treatment regimes.

3.2.4 Dose Response Data Analysis.

For single-dose studies, survival data were fitted by non-linear least squares regression to the linear quadratic equation:[136]

$$\text{Survival fraction} = \exp(-\alpha D - \beta D^2)$$

The linear quadratic equation gave a reasonable fit to the single radiation dose data - that being the reason for using this kind of analysis. The dose modifying factor (DMF) could be calculated at specified biological end-points (i.e. survival levels) from the ratio of the doses following pretreatment with air to the doses following an hypoxic pretreatment. In these investigations, for each pair of curves, the DMF was determined at 18 survival levels (from 0.9 to 0.01) and the mean DMF then calculated. The method used to determine the DMF did not enable an error on the value to be calculated.

For split-dose studies, when the natural logarithm (ln) of survival fraction was plotted against radiation dose, the data appeared to be approximated well by a straight line. In other words, the ln of survival was a linear function of radiation dose. The lines were, therefore, fitted by the method of least squares. For each pair of curves, the DMF was determined at 16 survival levels (from -1.4 to -4.4, where survival fraction was plotted as ln) and the mean DMF then calculated. DMF's were also calculated using slope-ratio and parallel line assays, as discussed in Chapter 5. All the DMF's quoted in the results of Chapter 4 were derived using the parallel line assay.

3.3 Hypoxic Fraction Measurements of Experimental Mouse Tumours.

The hypoxic fractions of the three tumour types used in these studies were determined by indirect techniques that compare the response of the tumours to large single doses of radiation given under normal aerated and artificially-induced hypoxic conditions.[137,138] The clamped tumour growth delay method was used to determine the hypoxic fraction of all three tumour types. In the case of the Fib/T tumour, the hypoxic fraction measured with the above method was compared to that obtained using the paired survival curve method.

3.3.1 Clamped Tumour Growth Delay Method.

In the clamped tumour growth delay assay, the hypoxic fraction was determined by comparing the post-radiation growth of normally aerated and artificially hypoxic tumours and analysing the time required for the irradiated tumours to reach a specified size minus the time for the untreated tumours to reach the same size - the "growth delay".

Three groups of six tumour-bearing mice were taken from each of the CBA, BALB/c and WHT strains. One group was untreated, whilst the second and third groups each received 12 Gy X-rays (100 kVp) to the tumours. (12 Gy was chosen in that this dose was shown to produce no tumour cures, yet appreciable tumour growth delay). In the third group of mice, tumour hypoxia was induced by clamping the blood supply to the tumours 5 minutes before radiation. At the end of the radiation period, the clamp was removed. The procedure adopted to clamp the tumours was to retract the tumour away from the body of the mouse and to tie a thin string firmly around the skin between the host and tumour.

For all groups of mice, tumour volumes were determined three times per week. From this data the tumour volume doubling time and the time taken for the tumours to reach four times their initial size of 0.2 +/- 0.03 cm^3 were ascertained. The hypoxic fractions could then be calculated from the observed growth delays (see Appendix A.1).

3.3.2 Paired Survival Curve Method.

The paired survival curve assay was used to determine the hypoxic fraction of the Fib/T tumour grown in the gastrocnemius muscle of WHT mice. As the site of a tumour's implantation may influence its hypoxic fraction, the Fib/T tumour was also grown subcutaneously in the sternum of a second group of WHT mice. The hypoxic fraction of the tumour at this site was then measured using the above technique and compared to that obtained when the tumour was grown intra-muscularly.

In the paired survival curve assay, survival curves were determined for cells in normally aerated and artificially-induced hypoxic Fib/T tumours. The hypoxic fraction could then be calculated by a comparison of the curves (see Appendix A.2).

Mice bearing tumours of 0.2 +/- 0.03 cm^3 in size were used in this series of investigations. For hypoxic radiations, the animals were gassed with nitrogen for 5 minutes prior to being irradiated. Both the normally aerated, unanaesthetized and the nitrogen asphyxiated animals were given doses ranging from 6 to 30 Gy (100 kVp X-rays) locally to their tumours. The tumours were then surgically excised and the same assay procedure followed as described previously (see excision assay procedure, section 3.2.3).

3.4 Biochemical Assays.

Experimental designs will explicitly indicate the protocol according to which the experimental animals were treated, as well as the procurement and preparation of the tissues investigated. The treatment of B16 melanoma cells grown in culture and the preparation of these cells for ATP determination are also described. Following this section, the experimental techniques involved in each assay will be detailed with appropriate discussion.

3.4.1 Experimental Designs.

3.4.1.1 Treatment of Experimental Animals.

All biochemical studies were carried out using WHT mice bearing the Fib/T tumour, 0.2 +/- 0.03 cm^3 in size, in the right gastrocnemius muscle.

The mice used for tumour biochemical assays were divided into 4 groups, consisting of from 6 to 10 mice per group, and treated in the following manner:

Group 1) Mice kept in 8% oxygen for 24 hours.

Group 2) Mice kept in 8% oxygen for 48 hours.

Group 3) Mice kept in 8% oxygen for 72 hours.

Mice from Group 4) were kept in air.

Tumours were excised within 1 hour following completion of the treatment procedure.

The mice used for haemoglobin and 2,3-DPG studies were divided into 14 groups, consisting of from 6 to 10 mice per group, and treated in the following manner:

Group 1) Mice kept in 8% oxygen for 24 hours.

Group 2) Mice kept in 8% oxygen for 48 hours.

Group 3) Mice kept in 8% oxygen for 72 hours.

Group 4) Mice kept in 10% oxygen for 24 hours.

Group 5) Mice kept in 10% oxygen for 48 hours.

Group 6) Mice kept in 10% oxygen for 72 hours.

Group 7) Mice kept in 15% oxygen for 24 hours.

Group 8) Mice kept in 15% oxygen for 48 hours.

Group 9) Mice kept in 15% oxygen for 72 hours.

Group 10) Mice kept in 8% oxygen for 48 hours, then in air for 24.5 hours.

Group 11) Mice kept in 8% oxygen for 48 hours, then in air for 0.5 hour followed by a further 24 hours in 8% oxygen.

Group 12) Mice kept in 8% oxygen for 48 hours, then in air for 4.5 hours.

Group 13) Mice kept in 8% oxygen for 48 hours, then in air for 0.5 hour followed by 4 hours in 8% oxygen again.

Mice from Group 14) were kept in air.

Blood samples were taken within 0.5 hour following completion of the treatment procedure.

The mice used for recording blood oxygen-haemoglobin dissociation curves were divided into 4 groups and treated in the following manner:

Group 1) Mice kept in 8% oxygen for 48 hours.

Group 2) Mice kept in 8% oxygen for 48 hours, then in air for 24.5 hours.

Group 3) Mice kept in 8% oxygen for 48 hours, then in air for 0.5 hour followed by a further 24 hours in 8% oxygen.

Mice from Group 4) were kept in air.

Blood samples were taken within 0.25 hour following completion of the treatment procedure.

3.4.1.1.1 Procurement of Tumour Tissue.

Tumours were surgically excised after the mice had been sacrificed in ether. The skin and surrounding tissue were removed, as was any overtly necrotic tissue. The tumours were halved and each half treated in a different way as described in sections 3.4.1.3.1 and 3.4.1.3.2.

3.4.1.1.2 Procurement of Blood from Mice.

Blood samples from WHT mice with Fib/T tumours in the right gastrocnemius muscle were used for haemoglobin, 2,3-DPG and oxygen-haemoglobin dissociation curve determinations. The mice were anaesthetized by the intra-peritoneal injection of 0.02 ml Pentobarbitone Sodium (60 mg/ml). The peritoneum was opened, the inferior vena-carva exposed and 0.2 +/- 0.03 ml of blood drawn through a 0.45 x 16 mm needle into a heparinized 1 ml syringe.

3.4.1.2 Treatment of B16 Melanoma Cells for ATP Determination.

B16 melanoma cells were grown to confluence in 15 mm diameter glass petri dishes as described in section 3.1.6.2.2.

3.4.1.2.1 Induction of Hypoxia in B16 Melanoma Cells.

Medium was removed from the petri dishes using a sterile disposable Pasteur pipette and replaced with 0.8 ml McCoy's 5A medium, pH 6.25. The small amount of medium was used to facilitate deoxygenation. The pH was achieved by adding appropriate amounts of $NaHCO_3$ to bicarbonate-free medium.

Each petri dish was sealed in an aluminium chamber which was connected via a copper manifold to a cylinder of 95% nitrogen/5% carbon dioxide. This gas mixture was certified to

contain less than 10 ppm oxygen. Hypoxia was effected by initially allowing the 95% nitrogen/5% carbon dioxide gas mixture to perfuse all the chambers for 10 minutes. The inlet to a chamber was then closed, and gas within that chamber extracted by a vacuum pump connected to the chamber's outlet. The duration of the extraction process was approximately 1 minute. Perfusion of the chamber with 95% nitrogen/5% carbon dioxide was then restored whilst the extraction process was repeated in the next chamber. A total of 8 exchanges, lasting about 1 hour, was completed for each chamber. Thereafter, all the chambers were continuously flushed with 95% nitrogen/5% carbon dioxide for a further 15 hours. This entire procedure was carried out at room temperature.

3.4.1.2.2 Reoxygenation of Hypoxic Cells.

At the end of the 16 hour period of hypoxia, the chambers were opened and the petri dishes removed. Reoxygenation was achieved by pipetting off the medium, replacing it with 2 ml of McCoy's 5A medium, pH 7.2 - 7.4 and returning the petri dishes to the 95% air/5% carbon dioxide incubator.

3.4.1.2.3 The treatment protocols which preceded ATP determinations were as follows:

Protocol 1) Cells were exposed to hypoxia and low pH for 16 hours. Cells were then reoxygenated and ATP measured at 0.5, 1, 3 and 5 hours after initiation of reoxygenation.

Protocol 2) Cells were exposed to hypoxia and low pH for 16 hours. Cells were then incubated for 0.5 hour at pH 7.2 - 7.4 in 95% air/5% carbon dioxide. Following this, hypoxia was reinduced at pH 6.25 through a series of eight 95% nitrogen/5% carbon dioxide gas exchanges. ATP was then measured at 1 hour

(i.e. immediately following completion of the eight exchanges) and 4.5 hours (cells were flushed with 95% nitrogen/5% carbon dioxide for 3.5 hours following completion of the eight exchanges) after initiation of the second hypoxic episode.

Protocol 3) ATP was determined in cells that were maintained at pH 7.2 - 7.4 in a 95% air/5% carbon dioxide incubator.

3.4.1.3.1 Tumour Tissue Preparation for Catalase, Glutathione Reductase and Glutathione Peroxidase assays.

Tumours were homogenized in 2 ml ice cold homogenizing medium, which was 10 mM in respect of KH_2PO_4 and 30 mM in respect of KCL, pH 8. The homogenization was carried out using a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 17210 x g at 4^oC for 20 minutes in a Sorvall RC-5B centrifuge. The supernatant was decanted and stored under ice for not longer than 5 hours before commencement of any of the assays.

3.4.1.3.2 Tumour Tissue Preparation for Glutathione Determination.

Tumour tissue was weighed out and homogenized 1:5 w/v with ice cold homogenizing medium, which was 10 mM in respect of KH_2PO_4 and 30 mM in respect of KCL, pH8. The homogenization was carried out using a Potter-Elvehjem homogenizer. A volume of 5% trichloroacetic acid equal to that of the homogenizing medium was added, and following further homogenization the preparation was centrifuged at 17210 x g for 20 minutes at 4^oC in a Sorvall RC-5B centrifuge. The supernatant was decanted and its volume measured. Ten ml of the supernatant was diluted to 1000 ml with buffer which was

0.1 M in NaH_2PO_4 and 0.005 M in EDTA, pH 7.5. The dilutant was stored under ice for not longer than 4 hours before commencement of the assay.

3.4.1.3.3 Preparation of B16 Melanoma Cells for ATP Determination.

B16 melanoma cells grown in 15 mm diameter petri dishes were trypsinized as detailed in section 3.1.6.2.2 and resuspended in 2 ml serum-free McCoy's 5A medium. Because of the smaller volume of the petri dishes, the amounts of EDTA and trypsin were reduced accordingly. After the cells were counted in a haemocytometer, the volume containing 1 x 10^5 cells was placed in a plastic tube. One ml $HClO_4$ (6% w/v) was added, then the sample was freeze-thawed three times by immersion of the closed tube into liquid nitrogen followed by warming at 37°C in a temperature- controlled water bath. After the last thawing, the preparation was centrifuged at 17210 x g for 20 minutes at 4°C in a Sorvall RC-5B centrifuge. The supernatant was removed, made up to 20 ml with distilled water and the pH adjusted to 7.5 with 5 M K_2CO_3. This was followed by recentrifugation at 17210 x g for 20 minutes at 4°C. The supernatant was removed, its volume measured and used for the determination of cellular ATP levels using the highly sensitive luciferin-luciferase system (see section 3.4.2.9).

3.4.2 Experimental Techniques.

Methods used for the assay of the following will be discussed:

Tumour Assays:

1) Catalase activity

2) Glutathione Reductase activity

3) Glutathione Peroxidase activity

4) Protein determination

5) Glutathione levels

Blood Assays:

1) Haemoglobin concentrations

2) 2,3-DPG levels

3) Oxygen-Haemoglobin Dissociation Curves

B16 Melanoma Cell Assay:

1) ATP levels

Each of the experimental techniques used will be presented in the following format:

a) Principles of the particular assay will be discussed.

b) A detailed account of materials and methods will be given.

c) The assay procedure will be succinctly described.

d) Comments on any particular assay will be made when necessary.

All light absorbance readings were made using a Pye Unicam 5P8-400 UV/V15 double beam spectrophotometer. ATP was determined using a Beckman Liquid Scintillation Counter, Model LS 1801, in the single photon mode.

The methods for calculation of results from the experimental data are given in Appendix B.

3.4.2.1. Catalase.

a) Principles of Assay

Most aerobic cells contain catalase activity. In animals catalase is present in all major body organs, being especially concentrated in the liver and erythrocytes.

Catalase was first isolated and obtained in crystalline form from ox liver.[139] Most purified catalases have been shown to consist of four protein subunits, each of which contains a haem (Fe(3)-protoporphyin) group bound to its active site.

For a number of years it has been known that catalase has a double function. It can catalyse

1) the decomposition of H_2O_2 to give H_2O and O_2 (catalase activity; see equation 1).

2) the oxidation of various substrates, for example methanol, ethanol, phenols with the consumption of 1 mole of peroxide (peroxidase activity; see equation 2).

$2H_2O_2$ ----------->$2H_2O + O_2$ (eq.1)

$ROOH + AH_2$---------->$H_2O + ROH + A$ (eq.2)

Catalase activity can be measured by following either the decomposition of H_2O_2 or the liberation of O_2. The method of choice for biological material is the UV spectrophotometric method.[140] In the ultraviolet range H_2O_2 shows a continual increase in absorption with decreasing wavelength. The decomposition of H_2O_2 can be followed directly by the decrease in absorbance at 240 nm. The change in absorbance per unit time is a measure of the catalase activity.

b) Materials and Methods

The sample of the Fib/T tumour was freshly prepared as detailed in section 3.4.1.3.1 and kept under ice ready to utilize for the enzymatic assay. The different reagents required for the assay had the following concentrations:

1) Tris (hydroxymethyl) methylamine, 100 mM, buffered to pH 7.4 with concentrated HCl.
(Tris (hydroxymethyl) methylamine, 1.21 g dissolved in distilled water and made up to 100 ml).

2) H_2O_2, 100 mM.

(30% H_2O_2, 1.07 ml diluted with distilled water to 100 ml).

c) Assay Procedure

The total volume of 3 ml was contained in 4 ml quartz glass cuvettes. For the measurement of enzyme activity the reference cuvette contained: Tris (hydroxmethyl) methylamine, 150 umol; H_2O_2, 30 umol; distilled water, 1.2 ml.

The sample cuvette contained: Tris (hydroxmethyl) methylamine, 150 umol; H_2O_2, 30 umol; distilled water, 1.1 ml; tumour sample, 0.1 ml. The decrease in absorbance was monitored at 25^{o}C for 3 minutes.

d) Comments

To avoid inactivation of the enzyme during the assay or the formation of bubbles in the cuvette due to the liberation of oxygen, it was necessary to use a relatively low H_2O_2 concentration (10 mM). The H_2O_2 concentration was critical as there is a direct proportionality between the substrate concentration and its rate of decomposition.

3.4.2.2 Glutathione Reductase.

a) Principles of Assay

Glutathione reductase contains two protein subunits, each with the flavin FAD at its active site. The enzyme catalyses the reaction between GSSG and NADPH according to the reaction:

GSSG + NADPH + H^+ ---------> 2 GSH + $NADP^+$

The NADPH required is mainly provided in animal tissues by the oxidative pentose phosphate pathway. The first enzyme in this pathway is glucose-6-phosphate dehydrogenase, which catalyses the reaction

glucose-6-phosphate + $NADP^+$ ---------> 6-phosphogluconate + NADPH + H^+

This is followed by 6-phosphogluconate dehydrogenase which catalyses

6-phosphogluconate + $NADP^+$ ---------> CO_2 + NADPH + H^+ + ribulose-5-phosphate

As glutathione reductase operates and reduces the $NADPH/NADP^+$ ratio, the flux through the pentose phosphate pathway increases in order to replace depleted NADPH.

In the presence of GSSG the oxidation of NADPH by glutathione reductase can be followed by monitoring the decrease in absorbance at 340 nm.

b) Materials and Methods

The sample of the Fib/T tumour was freshly prepared as detailed in section 3.4.1.3.1 and kept under ice ready to utilize for the enzymatic assay. The method used for assay of glutathione reductase was based on that of Worthington et al.[141] The different reagents required for the assay had the following concentrations:

1) Potassium phosphate buffer, 46 mM (pH 7.0).
(KH_2PO_4, 2.79 g and K_2HPO_4, 4.61 g dissolved in distilled water and made up to 1000 ml).

2) KCl, 2 M.
(KCl, 1.49 g dissolved in distilled water and made up to 10 ml).

3) EDTA, 30 mM.
(EDTA, 112 mg dissolved in distilled water and made up to 10 ml).

4) GSSG, 30 mM.
(GSSG, 184 mg dissolved in distilled water and made up to 10 ml).

5) NADPH, 3 mM.
(NADPH, 25 mg dissolved in distilled water and made up to 10 ml).

c) Assay Procedure

The total volume of 3 ml was contained in 4 ml quartz glass cuvettes. For the measurement of enzyme activity the reference cuvette contained: potassium phosphate buffer, 110.4 umol; KCl, 0.6 mmol; EDTA, 3 umol; GSSG, 3 umol; NADPH, 0.3 umol.

The sample cuvette contained: potassium phosphate buffer, 109.48 umol; KCl, 0.6 mmol; EDTA, 3 umol; GSSG, 3 umol; NADPH, 0.3 umol; tumour sample, 0.02 ml. The decrease in absorbance was monitored at 25^{o}C for 3 minutes.

3.4.2.3 Glutathione Peroxidase.

a) Principles of Assay

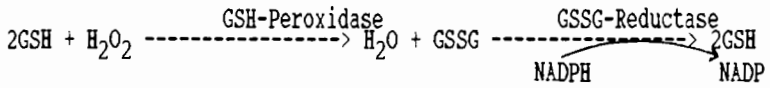
Glutathione peroxidase was first discovered in erythrocyte lysates as a consequence of its ability to protect haemoglobin from oxidative breakdown.[142] It is made up of four protein sub-units, each of which contains one atom of the element selenium at its active site.[143] The enzyme's preferred substrate is the low-molecular-weight thiol compound glutathione. Glutathione peroxidase catalyses the oxidation of GSH to GSSG at the expense of hydrogen peroxide:

H_2O_2 + 2GSH ---------> GSSG + $2H_2O$

Although the enzyme is specific for GSH as a hydrogen donor, it will accept other peroxides in addition to hydrogen peroxide, including cumene hydroperoxide, several steroid hydroperoxides and thymine hydroperoxide.[144]

Enzyme activity was measured by a modification[145] of the coupled assay procedure of Paglia and Valentine.[146] In this assay the progressive loss of GSH is not measured as a means to assess glutathione peroxidase activity. Rather, GSH is maintained at a constant concentration by the addition of

exogenous glutathione reductase and NADPH, which results in the conversion of any GSSG produced to the reduced form:



The rate of GSSG formation is then measured by following the decrease in absorbance of the reaction mixture at 340 nm as NADPH is converted to NADP.

b) Materials and Methods

The sample of the Fib/T tumour was freshly prepared as detailed in 3.4.1.3.1 and kept under ice, ready to utilize for the enzymatic assay. The different reagents required for the assay were as follows:

1) Potassium phosphate buffer, 0.2 M (pH 7.0).
(KH_2PO_4, 12.13 g and K_2HPO_4, 20.04 g dissolved in distilled water and made up to 1000 ml).

2) EDTA, 10 mM.
(EDTA, 37.2 mg dissolved in distilled water and made up to 10 ml).

3) NaN_3, 10 mM.
(NaN_3, 6.5 mg dissolved in distilled water and made up to 10 ml).

4) NADPH, 2 mM.
(NADPH, 16.7 mg dissolved in distilled water and made up to 10 ml).

5) GSH, 10 mM.
(GSH, 30.7 mg dissolved in distilled water and made up to 10 ml).

6) Glutathione reductase, 150 U/mg

7) H_2O_2, 2.5 mM.
(30% H_2O_2, 0.267 ml diluted with distilled water to 1000 ml).

c) Assay Procedure

The total volume of 2 ml was contained in 4 ml quartz glass cuvettes. For the measurement of enzyme activity the reference cuvette contained: potassium phosphate buffer, 100 umol; EDTA, 2 umol; NaN_3, 2 umol; NADPH, 0.4 umol; GSH, 2 umol; glutathione reductase, 2.27 units; distilled water, 0.499 ml.

The sample cuvette contained: potassium phosphate buffer, 100 umol; EDTA, 2 umol; NaN_3, 2 umol; NADPH, 0.4 umol; GSH, 2 umol; glutathione reductase, 2.27 units; distilled water, 0.479 ml; tumour sample, 0.02 ml.

The reaction mixture of both cuvettes was allowed to incubate for 2 minutes at 37°C before initiation of the reaction by the addition of 0.5 umol H_2O_2 solution to both cuvettes. The decrease in optical density at 340 nm was monitored for 3 minutes.

3.4.2.4 Protein Determination.

These were made by the method of Lowry et al[147] using bovine serum albumin, fraction V as a standard. All protein determinations were made so that the amounts measured ranged from 0.05 mg to 0.25 mg, thus falling within the linear range of concentration as measured spectrophotometrically at 750 nm.

3.4.2.5 Glutathione.

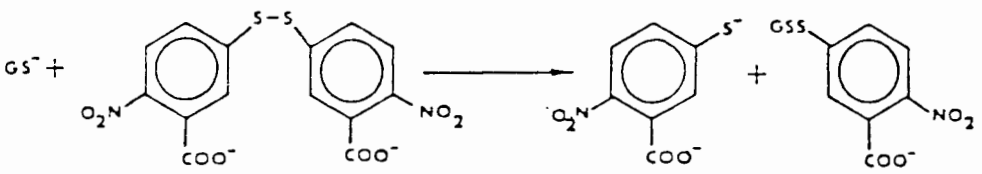
a) Principles of Assay

The widespread distribution of glutathione and its apparent involvement in a multitude of biological functions have generated a continual interest in methods of analysis of this cellular component ever since its discovery and isolation 60 years ago. The tripeptide (glutamylcysteinylglycine) can

exist in both a reduced (sulphydryl) and an oxidized (disulfide) form, but the ratios of GSH/GSSG in normal cells are kept high. This is because of the equally ubiquitous enzyme glutathione reductase which reduces GSSG back to GSH.

A method for measuring total glutathione (GSH + GSSG), which is not subject to appreciable interference by the presence of other thiol compounds, has been described by Tietze.[148] It is based on the catalytic action of GSH or GSSG in the reduction of Ellman reagent (5,5'-dithiobis-(2-nitrobenzoic acid) by a mixture of NADPH (TPNH) and glutathione reductase (see equations 1 and 2).

$$GSSG + TPNH + H^{+} \xrightarrow{GR} 2\,GSH + TPN^{+} \qquad (1)$$



(2)

The chromophoric product resulting from reaction of Ellman reagent with GSH, viz. 2-nitro-5-thiobenzoic acid, can be determined spectrophotometrically by measurement of absorbance at 412 nm.

b) Materials and Methods

The sample of the Fib/T tumour was freshly prepared as detailed in section 3.4.1.3.2 and kept under ice ready to utilize for the assay. The reagents used in the assay were:

1) Sodium phosphate-EDTA buffer which was 0.1 M in NaH_2PO_4 and 0.0005 M in EDTA, pH 7.5.
(12 g NaH_2PO_4 and 1.86 g EDTA dissolved in distilled water and made up to 1000 ml).

2) Glutathione reductase, 150 U/mg.

3) DTNB, 0.01 M.

(39.6 mg DTNB dissolved in phosphate -EDTA buffer and made up to 10 ml).

4) GSH, 0.033 mM.

(1 mg GSH dissolved in 0.01 N HCl and made up to 100 ml).

5) NADPH, 11 mM.

(9.2 mg NADPH dissolved in phosphate-EDTA buffer and made up to 1 ml).

c) Assay Procedure

The total volume of 1 ml was contained in 1.5 ml quartz glass cuvettes. For the measurement of glutathione the reference cuvette contained: DTNB, 0.6 umol; glutathione reductase, 1.5 units: NADPH, 0.2 umol; 0.921 ml phosphate-EDTA buffer.

The sample cuvette contained: DTNB, 0.6 umol; 0.1 ml deproteinized tumour sample; glutathione reductase, 1.5 units; NADPH, 0.2 umol; 0.821 ml phosphate-EDTA buffer.

Glutathione levels in the tumour samples were determined by comparison against a standard curve prepared when the tumour samples in the sample cuvette were replaced by 1 - 100 ng GSH. The rate of reaction at 25^{o}C was expressed as the change in absorbancy per minute at 412 nm.

d) Comments

Initiation of the reaction by the addition of NADPH resulted in a rate of colour development at 412 nm that was linear for well beyond 6 minutes.

3.4.2.6 Haemoglobin.

a) Principles of Assay

Haemoglobins were determined by the spectrophotometric determination of cyanmethaemoglobin after conversion.[149] In an alkaline condition, ferricyanide converts haemoglobin iron from the ferrous to the ferric state to form methaemoglobin. Methaemoglobin then combines with KCN to produce stable cyanmethaemoglobin. The absorbance of a known Acuglobin Haemoglobin Standard containing a known concentration of cyanmethaemoglobin was determined at 540 nm. The exact equivalent of haemoglobin in mg per 100 ml was obtainable from each ampul of Standard. Calculations of haemoglobin concentrations were made by comparing the absorbance of the unknown with the absorbance of the known Acuglobin Haemoglobin Standard.

b) Materials and Methods

Drabkin's solution was prepared approximately one hour before the commencement of haemoglobin determinations. It contained the following components:

1) $K_3Fe(CN)_6$,121.5 umol
2) KCN, 153.6 umol
3) KH_2PO_4, 205.7 umol
4) Triton X, 0.2 ml

These components were dissolved in distilled water and made up to 200 ml.

c) Assay Procedure

Whole blood, 0.02 ml, taken from the WHT mice as detailed in section 3.4.1.1.2, was added to 5 ml of Drabkin's solution. The mixture was allowed to stand for 10 minutes to permit cyanmethaemoglobin to form. The absorbance was then determined at 540 nm against Drabkin's solution as a blank.

Readings were compared to that obtained for Acuglobin Haemoglobin Standard against Drabkin's solution, for calculation of the haemoglobin concentrations.

3.4.2.7 2,3-Diphosphoglycerate (2,3-DPG).

a) Principles of Assay

In 1964, Lowry et al[150] developed a method for the determination of 2,3-DPG levels in brain extracts. In this method, 2,3-DPG was measured by the disappearance of NADH upon enzymatic conversion to glyceraldehyde-3-phosphate. This procedure was later adapted by Keitt[151] for measuring this compound in red cells.

The method used in this thesis is based on that of Lowry et al,[150] but the reaction is monitored spectrophotometrically instead of fluorometrically. In this procedure, the test is performed on a protein-free supernatant. The three enzymatic reactions involved in the described procedure are as follows:

1) 2,3-DPG is hydrolysed to 3-phosphoglycerate (3-PGA) and inorganic phosphorus. The enzyme which catalyses this reaction is present in purified preparations of phosphoglycerate mutase (PGM) and is termed 2,3-DPG phosphatase. 2-Phosphoglycolic acid is needed as a stimulator for this reaction:

$$\text{2,3-DPG} \xrightarrow[\text{2-Phosphoglycolic acid}]{\text{2,3-DPG phosphatase}} \text{3-PGA} + \text{Pi}$$

2) 3-PGA reacts with ATP in the presence of 3-phosphoglycerate phosphokinase (PGK) to form 1,3-DPG and ADP

$$\text{3-PGA} + \text{ATP} \xrightarrow{\text{PGK}} \text{1,3-DPG} + \text{ADP}$$

3) 1,3-DPG oxidizes NADH to NAD in the presence of glyceraldehyde-3-phosphate dehydrogenase (GAPD) and is reduced to glyceraldehyde-3-phosphate (G-3-P)

$$1,3\text{-DPG} + \text{NADH} \xrightarrow{\text{GAPD}} \text{G-3-P} + \text{NAD}$$

Measurement of the decrease in absorbance at 340 nm caused by the oxidation of NADH to NAD then reflects the amount of 2,3-DPG originally present.

b) Materials and Methods

Red cell 2,3-DPG in whole blood was measured using the 2,3-DPG Reagent Kit (Sigma Diagnostics). The individual reagents had the following concentrations:

1) Triethanolamine buffer solution, 0.2 M.

2) NADH, 0.176 mM.

(NADH, 1 mg, was dissolved in 8 ml triethanolamine buffer).

3) ATP, 0.181 M.

(ATP, 500 mg, was dissolved in 5 ml triethanolamine buffer).

4) GAPD/PGK enzyme mixture containing 800 U/ml GAPD and 450 U/ml PGK.

5) Phosphoglycerate mutase, 2400 U/ml.

6) Phosphoglycolic acid, trimonocyclohexylamine salt, 20 mM.

7) Trichloroacetic acid solution, 8% (w/v).

c) Assay Procedure

A known volume (+/- 0.2 ml) of whole blood taken from the WHT mice as detailed in 3.4.1.1.2 was added to three times that volume (+/- 0.6 ml) of cold trichloroacetic acid. The mixture was vigorously shaken for several seconds and kept cold for an additional 5 minutes to assure complete precipitation. After centrifugation for 10 minutes at 600 x g in a Sorvall RC-5B

refrigerated centrifuge at 4°C, the supernatant was decanted and kept under ice (2,3-DPG in trichloroacetic acid supernatants have been shown to be stable at least 2 weeks when kept at 2°C).

For the measurement of 2,3-DPG the sample cuvette contained: NADH, 176 nmol; ATP, 7.24 umol; GAPD, 6.4 units/PGK, 3.6 units; phosphoglycerate mutase, 19.2 units; protein-free supernatant, 0.02 ml; distilled water, 0.08 ml. An initial absorbance was determined at 340 nm against distilled water as reference. Phosphoglycolic acid solution, 800 nmol, was added to the sample cuvette which was then kept at 37°C for 15 minutes to allow the reaction to go to completion. The absorbance was again determined at 340 nm against distilled water as blank. Concentration was determined from the absorbance change.

d) Comments

Venous blood collected in heparin was recommended for use in this procedure. 2,3-DPG has been shown to be stable in heparinized blood for at least 2 hours when stored in an ice bath. A negligible decrease in 2,3-DPG levels has been demonstrated in heparinized blood samples after 6 hours at 4°C.[152]

3.4.2.8 Oxygen-Haemoglobin Dissociation Curves.

a) Principles of Assay

The determination of oxygen-haemoglobin dissociation curves depends upon two measurements: the PO_2 with which the blood is equilibrated, and the proportion of haemoglobin that is saturated with oxygen. Methods for determining the dissociation curve fall into three main groups:[153]

1) The PO_2 is set by the experimental conditions and the percentage saturation of haemoglobin is measured.

2) The percentage saturation is predetermined by mixing known proportions of oxygenated and deoxygenated blood and the PO_2 is measured.

3) The percentage saturation of haemoglobin is plotted continuously against PO_2 during deoxygenation. This was the method utilized in these investigations, making use of a Hemox-Analyzer.

The Hemox-Analyzer is an automatic system for the recording of blood oxygen dissociation curves. The operating principle of the Hemox-Analyzer is based on dual-wavelength spectrophotometry for the measurement of the optical properties of haemoglobin, and a Clark electrode for measuring the PO_2 in mm Hg. The signals from both measuring systems are fed to a X-Y recorder, which plots the resulting function.

The absorption spectrum of haemoglobin provides the basis for the observation of oxygen saturation of haemoglobin in whole blood. The absorption spectra of oxyhaemoglobin and deoxyhaemoglobin are different. In the Hemox-Analyzer, a beam of polychromatic light is passed through a plastic cuvette containing the sample, after which the beam is made monochromatic prior to reaching the photomultipliers. In the case of haemoglobin, the wavelength of maximum absorbance is the measuring wavelength (560 nm), whilst a wavelength of 570 nm (i.e. the wavelength corresponding to the crossover point of the absorption spectra of oxy - and deoxyhaemoglobin) is the reference wavelength. During deoxygenation of haemoglobin, the absorbance at 570 nm remains practically unchanged but the absorbance at 560 nm undergoes a change. Since the differential extinction coefficient of oxygenated and

deoxygenated blood is known, the percentage oxygenated haemoglobin in the sample can be determined.

The Hemox-Analyzer uses a Clark electrode for determining the PO_2 in the cuvette where the absorbance is also being monitored. For full scale calibration of the recorder prior to starting the plotting of the curve, air is passed through the sample in the cuvette thus generating a PO_2 of 158 mm Hg. When oxygen in the sample is replaced by an inert gas, such as nitrogen, in a continuous process, haemoglobin becomes deoxygenated.

b) Materials and Methods

1) Stock phosphate buffer, 0.15 M (pH 7.4).

(NaH_2PO_4, 23.4 g dissolved in distilled water and made up to 1000 ml - Solution A.

Na_2HPO_4, 21.3 g dissolved in distilled water and made up to 1000 ml - Solution B.

Solution A was added slowly to solution B until the pH reached 7.4).

2) Working phosphate buffer

(One volume of stock phosphate buffer was added to 9 volumes of physiological saline just prior to the start of the assay)

3) Additive-A

4) Anti-foaming agent

c) Assay Procedure

Whole blood, 0.05 ml, taken from the WHT mice as detailed in 3.4.1.1.2 was added to a vial which contained: working phosphate buffer, 4 ml; additive-A, 0.02 ml; anti-foaming agent, 0.01 ml. The vial was gently inverted to ensure mixing of its contents, which were then drawn into the cuvette via a intake tube. The "air" position of the gas selector switch was chosen initiating the flow of air through the cuvette and

thus oxygenating the sample. The recording was then started. The gas selector switch was then changed to the "N_2" position, which caused the flow of nitrogen through the sample and started the deoxygenation process. The completion of the recording was achieved when the pen reached zero on the X-axis, i.e. the PO_2 was zero and haemoglobin was completely deoxygenated. Deoxygenation being complete, the sample was removed through the bottom outlet port to drain.

A line could be drawn through the 50% point on the Y-axis parallel to the X-axis until it intercepted the dissociation curve. From that point, a line was drawn parallel to the Y-axis until it intercepted the X-axis. The point where the line intercepted the X-axis was the P_{50} value in mm Hg. Similarly, the P_{10} values could also be determined.

d) Comments

After the sample solution was drawn into the cuvette, the temperature of the solution was equilibrated until it reached $37^{o}C$.

3.4.2.9 Adenosine-5'-Triphosphate.

a) Principles of Assay

Of all the naturally occurring phosphates, ATP is the most widely distributed. This energy-rich nucleotide serves as an "energy pool" to meet the energy requirements of cellular metabolism.

A highly sensitive method for the determination of ATP has been developed using luciferase from the American firefly, Photinus pyralis. This enzyme catalyses a chain of reactions, where finally luciferin is activated by adenylation followed by oxidation with atmospheric oxygen. On decarboxylation, oxyluciferin is formed in the excited singlet state, which

returns to the ground state by emission of a photon. This reaction has a very high quantal yield, of the order of 90%, so that the system is eminently suitable for making highly sensitive measurements of ATP concentrations.

b) Materials and Methods

Reagents required for the assay were:

1) Luciferin-luciferase dissolved in distilled water to result in a concentration of 8 mg/ml. This solution was wrapped in aluminium foil and stored in the dark at 4°C for between 5 and 24 hours before use.

2) Tris-acetate buffer was prepared by adjustment of the pH of tris-(hydroxymethyl) aminomethane solution to 7.75 with glacial acetic acid. The final concentration of the buffer was 0.25 M.

3) A stock solution of ATP (0.5 mM) was prepared by dissolving 27.56 mg ATP (from equine muscle) in 100 ml distilled water. The stock solution was diluted 1:1000 just prior to the start of the assay.

Bioluminescence was measured using a Beckman Liquid Scintillation Counter, Model LS 1801, which was programmed to make use of the single photon monitor. Samples were counted for 0.1 minutes. Individual samples were counted immediately after preparation, and it was found that good reproducibility was achieved if sample preparation time and exposure to light were limited to the shortest possible periods.

c) Assay Procedure

Luciferin-luciferase solution, 0.01 ml, was added to tris-acetate buffer and from 0.01 to 0.02 ml ATP preparation pipetted into a plastic scintillation counting vial. The final assay volume was 0,5 ml. The sample was gently shaken to ensure good mixing of the reagents, then taken immediately

to the scintillation counter and the counts recorded. With each set of ATP determinations, a calibration curve was prepared. It was found convenient to add 0.003, 0.005, 0.0075, 0.01 and 0.02 ml of the 1:1000 dilution of stock ATP solution (corresponding to 1.5 x10-13, 2.5 x 10-13, 3.75 x 10-13, 5 x 10-13 and 1 x 10-12 moles ATP) to tris-acetate buffer in a counting vial to result in a volume of 0.49 ml. Luciferin-luciferase solution (0.01 ml) was then added before the samples were counted. The counts obtained were plotted against ATP content on a log-log scale, and the line of best fit was obtained by regression analysis. The concentration of ATP in the "unknown" samples could then be ascertained from the calibration plot. The ATP content was expressed as moles ATP per cell.

It should be noted that ATP in each sample was assayed in duplicate at two different concentrations, namely using 0.01 ml and 0.02 ml of sample in the manner described above. The mean ATP content of each sample was calculated from the mean of the four separate determinations.

CHAPTER 4

RESULTS

In the first part of this chapter, tumour regrowth studies will be reported, followed by results obtained from the excision assays. Tumour hypoxic fraction measurements then follow, with results pertaining to the various biochemical assays concluding the chapter.

4.1 Tumour Regrowth Studies.

4.1.1 Tumour Regrowth after 72 Hours Pretreatment with 8% or 21% Oxygen.

Mice were pretreated with

a) 8% oxygen for 72 hours or

b) 21% oxygen for 72 hours.

They were then maintained in air. The number of days taken for tumours to grow to four times their initial volume was recorded, where the initial volume of 0.2 +/- 0.03 cm^3 was the size of the tumours at the time of removal of the mice from the 72 hour pretreatment environment.

The mean times for the CaNT tumours, after the 8% and the 21% oxygen pretreatment, to reach four times their initial size were 6.2 +/- 0.1 days and 5.9 +/- 0.3 days respectively. There was no significant difference between the two values.

In the case of the 3-MC-induced rhabdomyosarcoma, the times after the 8% and the 21% oxygen pretreatments were respectively 6.7 +/-0.1 days and 6.5 +/- 0.3 days. Again there was no significant difference between these two values. The data is shown in Fig.4.1. The trend was for the 72 hour

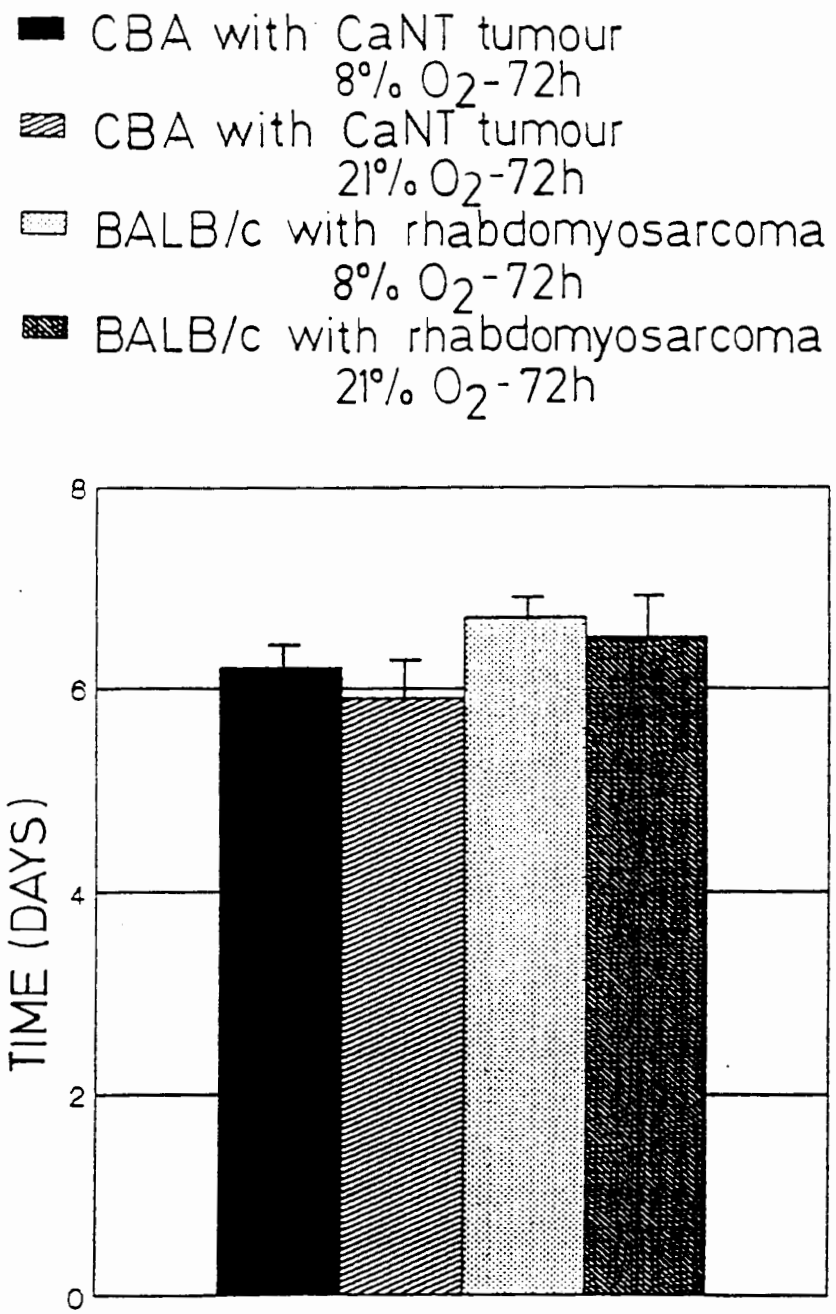


Fig.4.1 Time for tumours to reach four times initial volume following a 72h pretreatment with 8% O_2 or 21% O_2. The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars represent standard deviations.

pretreatment in 8% oxygen to slightly delay growth in both tumour types. It was also noteworthy that both the CaNT tumour and the 3-MC-induced rhabdomyosarcoma showed similar growth rates (Fig.4.1).

4.1.2 The Relative Effects of a 72 Hour 8% Oxygen and a 72 hour 21% Oxygen Exposure on Tumour Growth.

Mice with tumours were divided into 2 groups, viz. group a) where mice were kept in 8% oxygen for 72 hours and group b) where mice were kept in 21% oxygen for 72 hours.

Tumour volumes were determined immediately before the mice were exposed to either 8% or 21% oxygen and then again 72 hours later, after they were removed from these environments.

In the case of the CaNT tumour, where the tumour-bearing mice were exposed to 8% oxygen, tumour volume increased from 0.2 +/- 0.02 cm^3 to 0.28 +/- 0.03 cm3, whereas where mice were exposed to 21% oxygen, tumour volume increased from 0.21 +/- 0.03 cm3 to 0.42 +/- 0.03 cm3 (Fig.4.2). The difference in growth over the 72 hour period as a result of the different environments was significant ($p < 0.05$).

For the mice bearing the 3-MC-induced rhabdomyosarcoma and exposed to 8% oxygen, tumour volume increased from 0.19 +/- 0.02 cm^3 to 0.26 +/- 0.04 cm^3, whereas where mice were exposed to 21% oxygen, tumour volume increased from 0.2 +/- 0.01 cm^3 to 0.38 +/- 0.03 cm^3 (Fig.4.2). The difference in growth over the 72 hour period as a result of the different environments was significant ($p < 0.05$).

4.1.3 Tumour Regrowth Delay.

The regrowth delay of the CaNT tumour and 3-MC-induced rhabdomyosarcoma due to 11 Gy X-radiation (8-MV) was determined

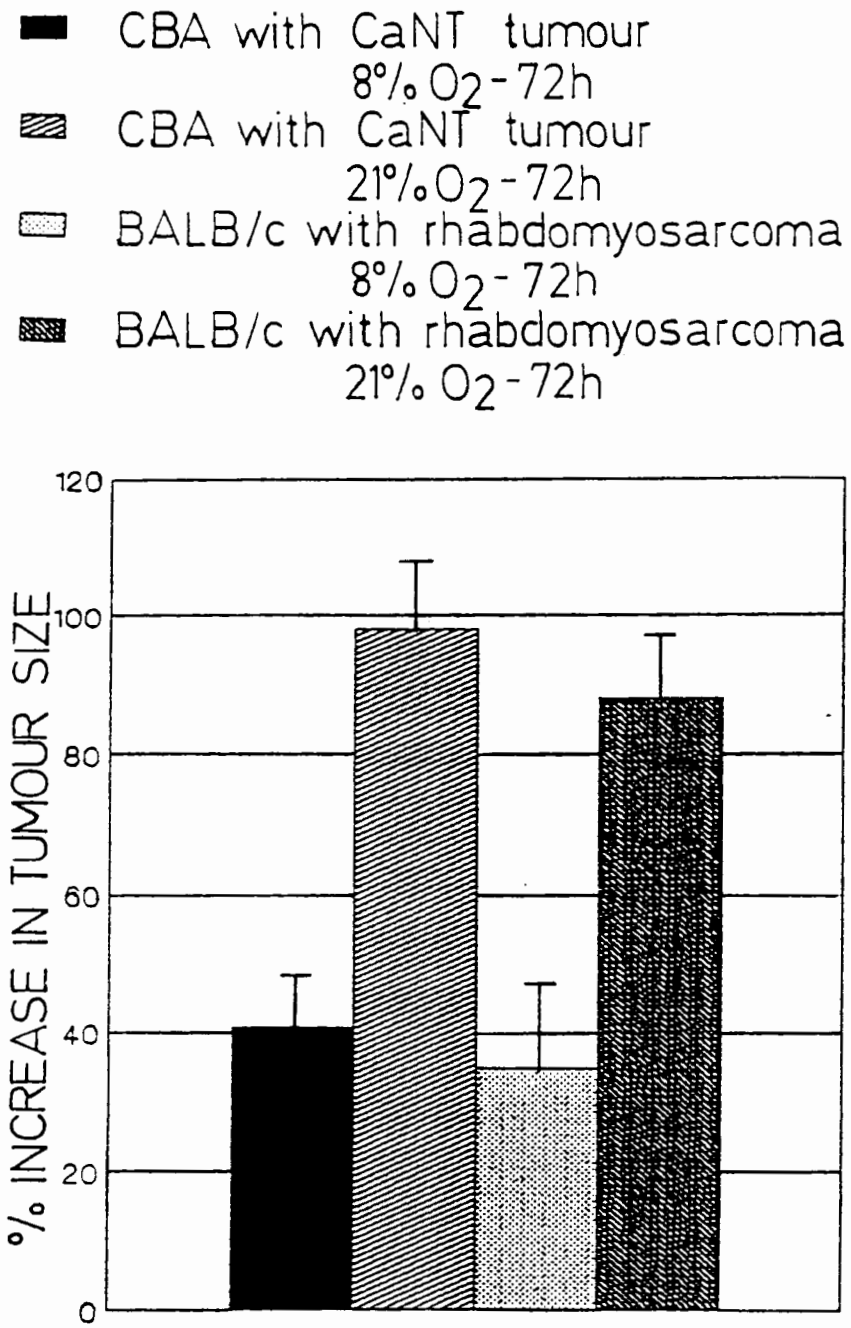


Fig.4.2 Percentage increase in tumour size for mice kept for 72h in 8% O_2 or 21% O_2.
The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars represent standard deviations

as detailed in 3.2.1.

4.1.3.1 CaNT Tumour Regrowth Delay.

Results of tumour regrowth delay after various pretreatments and radiation are shown in Fig.4.3. In group 1, which was exposed to 8% oxygen for 72 hours and then irradiated (11 Gy) in air, the regrowth delay was 8.6 +/- 1.6 days; in group 2, exposed to 21% oxygen for 72 hours, then irradiated (11 Gy) in air, the regrowth delay was 6.1 +/- 1 days. Group 3, which was exposed to 8% oxygen for 72 hours and then irradiated (11Gy) under 100% oxygen at 3 ATA, showed a regrowth delay of 7.5 +/- 0.9 days, whilst Group 4, exposed to 21% oxygen for 72 hours, then irradiated (11 Gy) under 100% oxygen at 3 ATA, manifested a regrowth delay of 7.5 +/- 1.7 days. In all cases, the regrowth delay refers to the time taken for tumours to grow to four times their initial size (section 3.2.1), and there was no significant difference in regrowth delay between any of the four groups.

4.1.3.2 3-MC-Induced Rhabdomyosarcoma Regrowth Delay.

Fig.4.4 shows the delay in growth of the 3-MC-induced rhabdomyosarcoma in the various groups treated as detailed in section 3.2.1. An increased regrowth delay was observed in those two groups that were exposed to 8% oxygen for 72 hours before receiving radiation either in air (Group 1-regrowth delay 10.7 +/- 1.8 days) or in 100% oxygen at 3 ATA (Group 3-regrowth delay 8.7 +/- 1.8 days). This was significantly different ($p < 0.05$) when compared to either of those groups that received no pretreatment with 8% oxygen and were irradiated in air (Group 2-regrowth delay 4.2 +/- 1 days) or in hyperbaric oxygen (Group 4-regrowth delay 4.3 +/- 1.6 days).

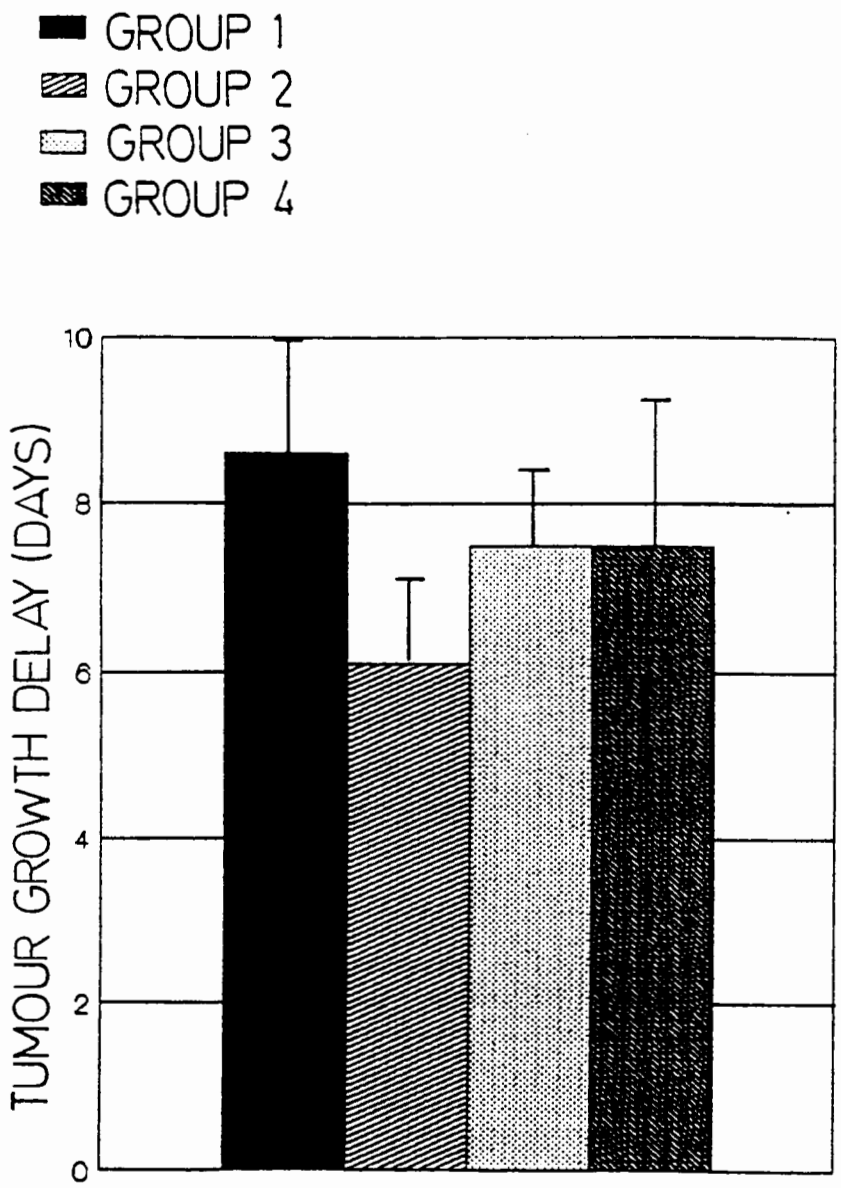


Fig.4.3 CaNT tumour growth delay following treatments as detailed in the text in sections 3.2.1 and 4.1.3.1.
The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars represent standard deviations

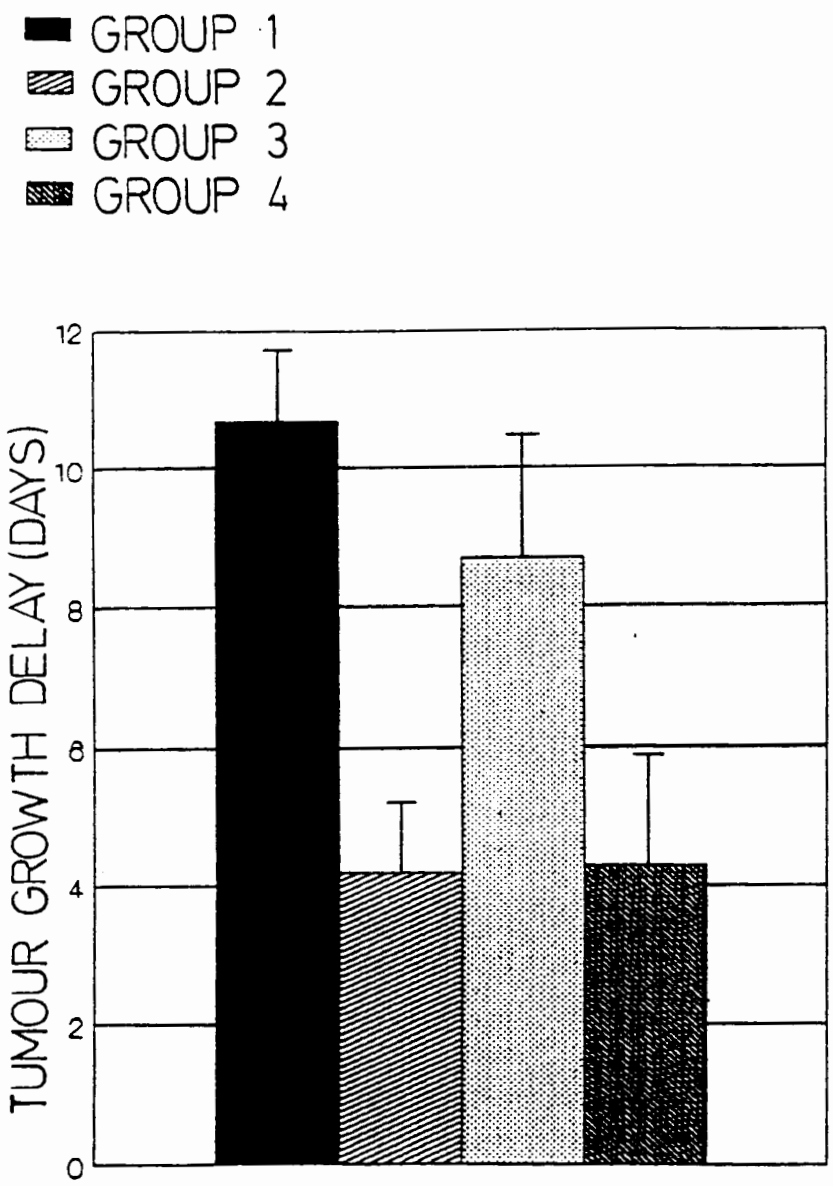


Fig.4.4 Three-MC-induced rhabdomyosarcoma growth delay following treatments as detailed in sections 3.2.1 and 4.1.3.2. The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars indicate standard deviations

There was no significant difference in tumour regrowth delay between those groups irradiated in air and those irradiated under conditions of hyperbaric oxygen, when the same pretreatment was given. It was, however, observed that the longest delay in tumour regrowth occurred in the group of mice that was exposed to an 8% oxygen environment for 72 hours prior to radiation in air, but this was not significantly different from the group that received the same pre-radiation treatment but was then irradiated in hyperbaric oxygen.

In all subsequent figures where data points are indicated as the mean of a number of experiments/values, each experiment/value equals one tumour.

4.2 **Excision Assay.**

The following section relates to results from the excision assays performed on WHT mice bearing the Fib/T tumour and subjected to various treatment schedules as detailed in section 3.2.2.

4.2.1 Single Dose Radiations.

4.2.1.1 Single Dose Gamma Radiation in Air of Experimental Mouse Tumours.

Fig.4.5 shows the effect of radiation on the clonogenic survival of Fib/T cells where the host animal was pretreated with either 8% oxygen or with air for 24, 48 or 72 hours. The 24, 48 and 72 hour hypoxic exposures caused a left shift of the dose survival curve to lower doses such that the DMF's were 1.36, 1.72 and 1.44 respectively.

Fig.4.6 shows the effect of radiation on the clonogenic survival of Fib/T cells where the host animal was either

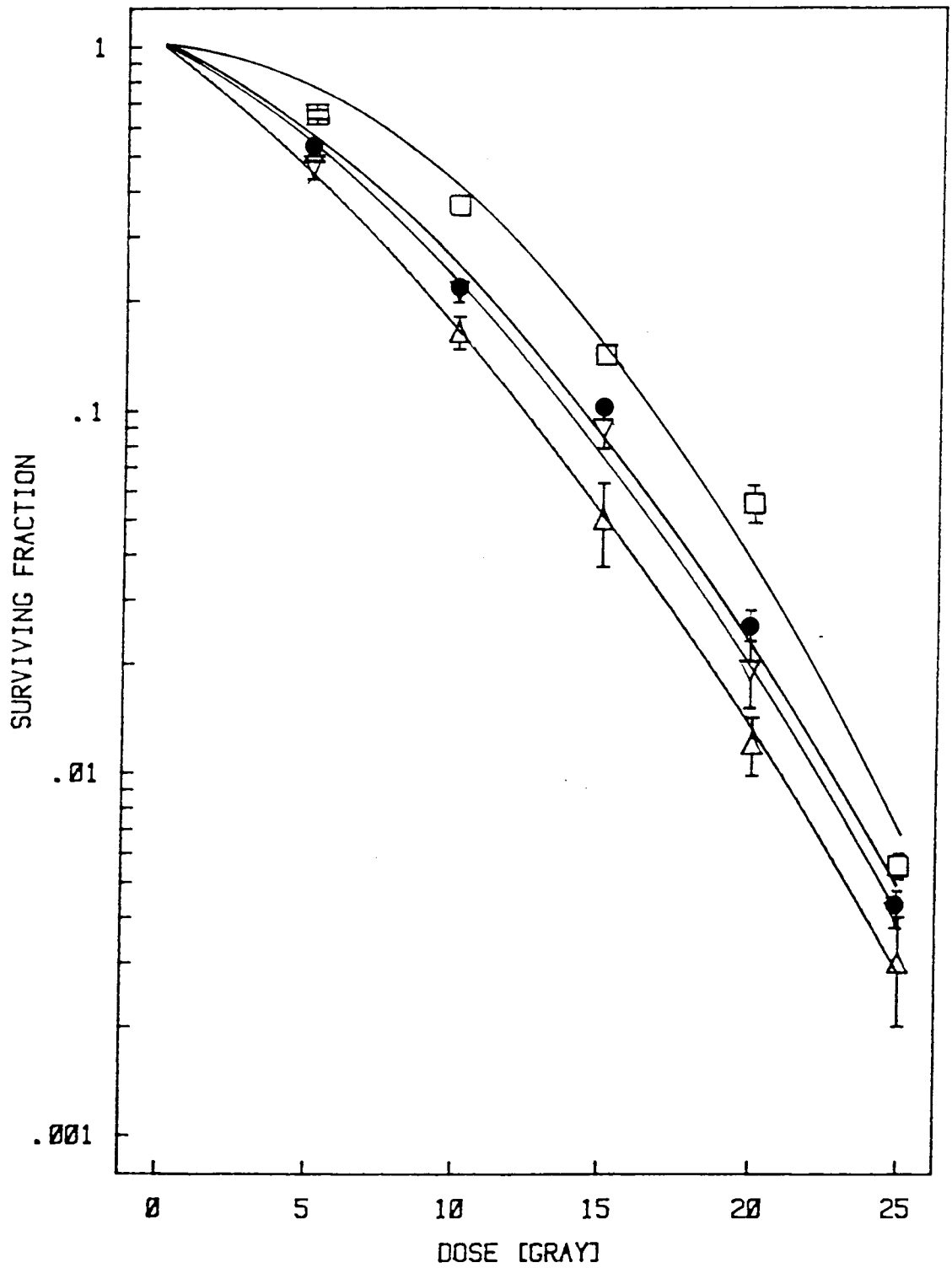


Fig.4.5 Radiation dose response curves for Fib/T tumours where WHT mice were pretreated with air (—□—), or with 8% O_2 for 24h (—●—), 48h (—△—) and 72h (—▽—). Gamma irradiation was in air.
Data points are the mean of not less than 5 experiments and error bars represent standard deviations.

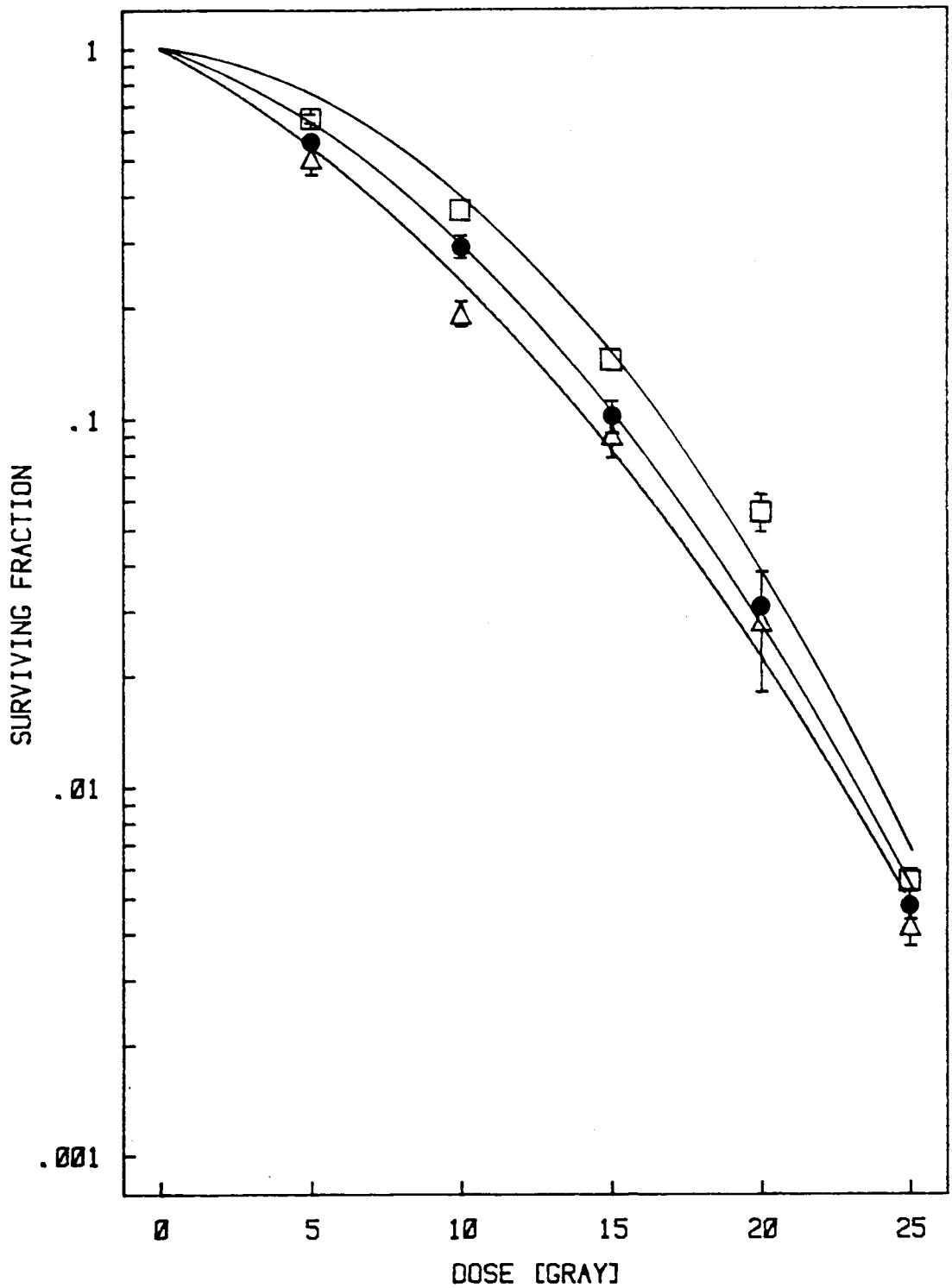


Fig.4.6 Radiation dose response curves for Fib/T tumours where WHT mice were pretreated with air (—□—), or with 10% O_2 for 48h (—△—) and 72h (—●—). Gamma irradiation was in air. Data points are the mean of not less than 5 experiments and error bars represent standard deviations.

pretreated by exposure to an atmosphere containing 10% oxygen for 48 hours or 72 hours, or to air. Pretreatment with 10% oxygen for 48 hours gave a DMF of 1.4 whilst a 72 hour pretreatment with 10% oxygen produced a DMF of 1.22.

The relative effects of radiation on the clonogenicity of the Fib/T tumour, where mice were either pretreated with 15% oxygen for 48 hours or 72 hours, or with air, are shown in Fig.4.7. The pretreatment of mice with 15% oxygen for 48 and 72 hours produced DMF's of 1.24 and 1.18 respectively.

The relative effects of radiation on the clonogenicity of the Fib/T tumour, where mice were either pretreated with 8% oxygen for 48 hours and then with 21% oxygen for 24 hours, or with air, are shown in Fig.4.8. The former pretreatment produced a DMF of 1.20.

It must be noted that for each experiment involving the exposure of mice to low oxygen, a control experiment (i.e. mice pretreated with air) was run concurrently. These control lines were found to be highly reproducible over a period of 1.5 years. The control curve indicated in Figs.4.5-4.10 is, therefore, the mean of a number (i.e. 11) of control series of experiments.

4.2.1.2 Single Dose Neutron Radiation in Air of Experimental Mouse Tumours.

Fig.4.9 shows the effect of radiation on the clonogenic survival of Fib/T cells where the host animal was either pretreated with 8% oxygen for 48 hours, or with air. The 48 hour hypoxic exposure caused a left shift of the dose survival curve to lower doses such that the DMF was 1.17.

The biological effectiveness of neutrons relative to ^{60}Co gamma rays for the tumour response of mice kept and irradiated

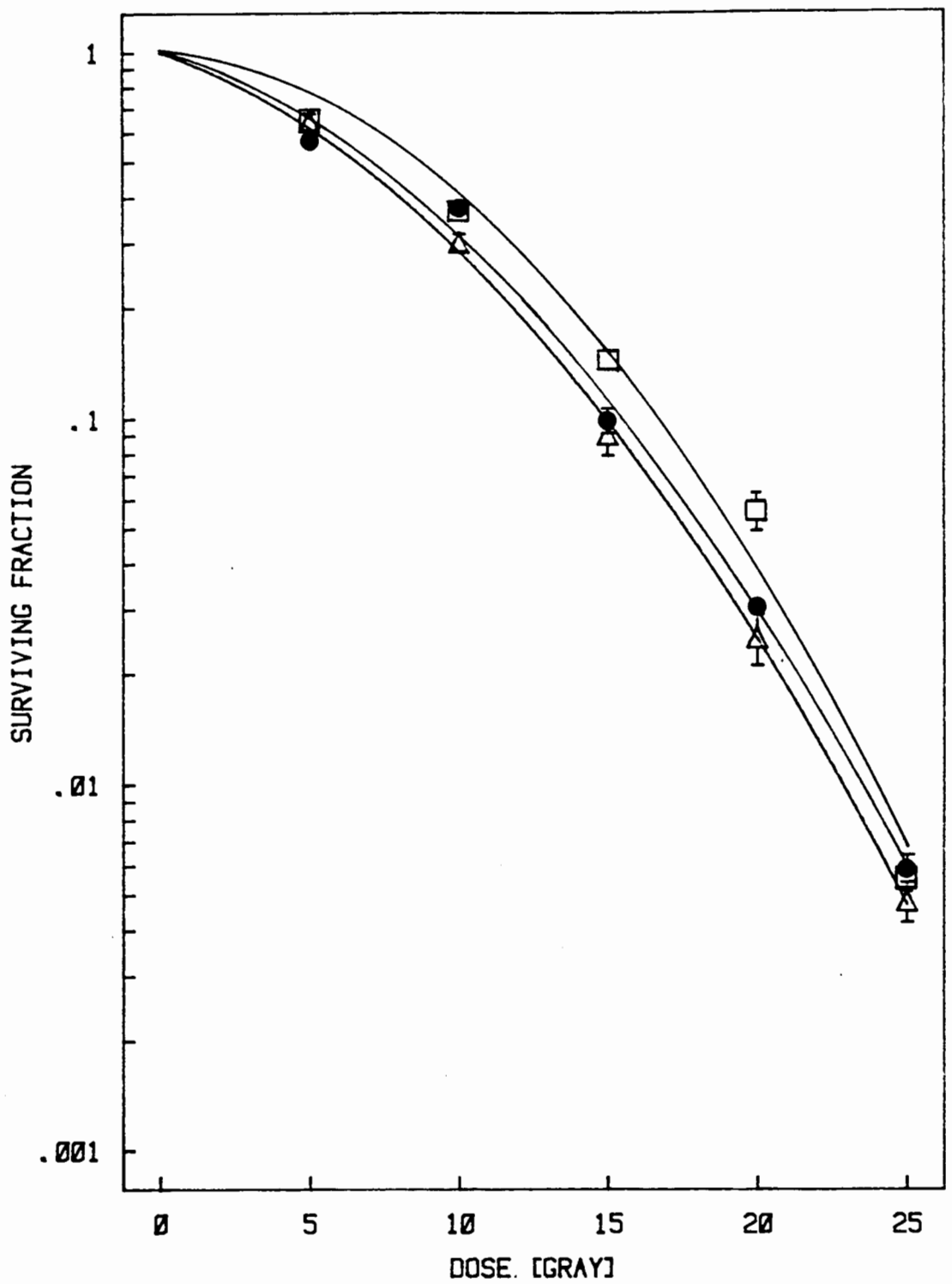


Fig.4.7 Radiation dose response curves for Fib/T tumours where WHT mice were pretreated with air (—□—), or with 15% O_2 for 48h (—△—) and 72h (—●—). Gamma irradiation was in air. Data points are the mean of not less than 5 experiments and error bars represent standard deviations

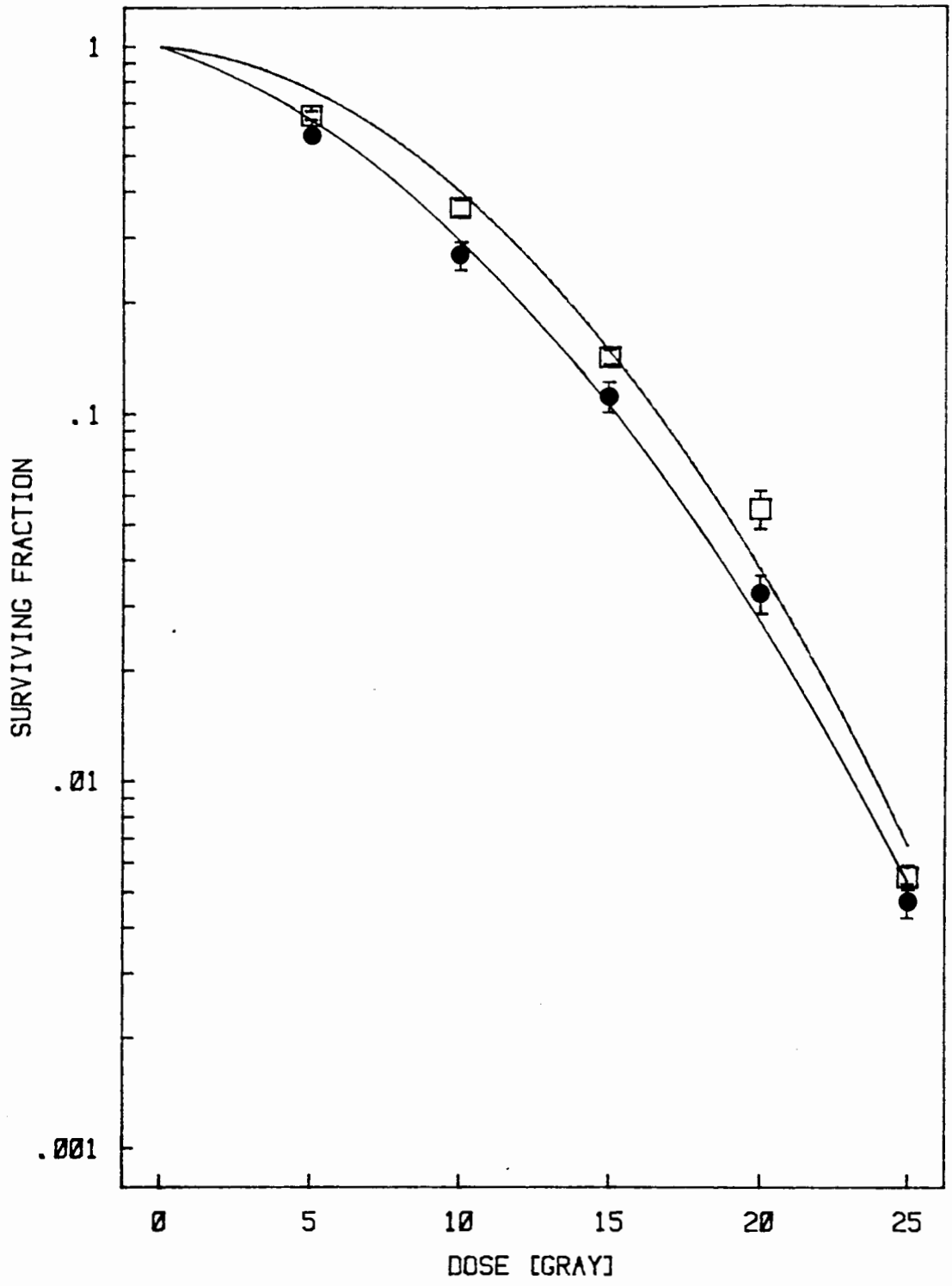


Fig.4.8 Radiation dose response curves for Fib/T tumours where WHT mice were either pretreated with air (—□—), or with 8% O_2 for 48h followed by a 24h exposure to air (—●—). Data points are the mean of not less than 5 experiments and error bars represent standard deviations.

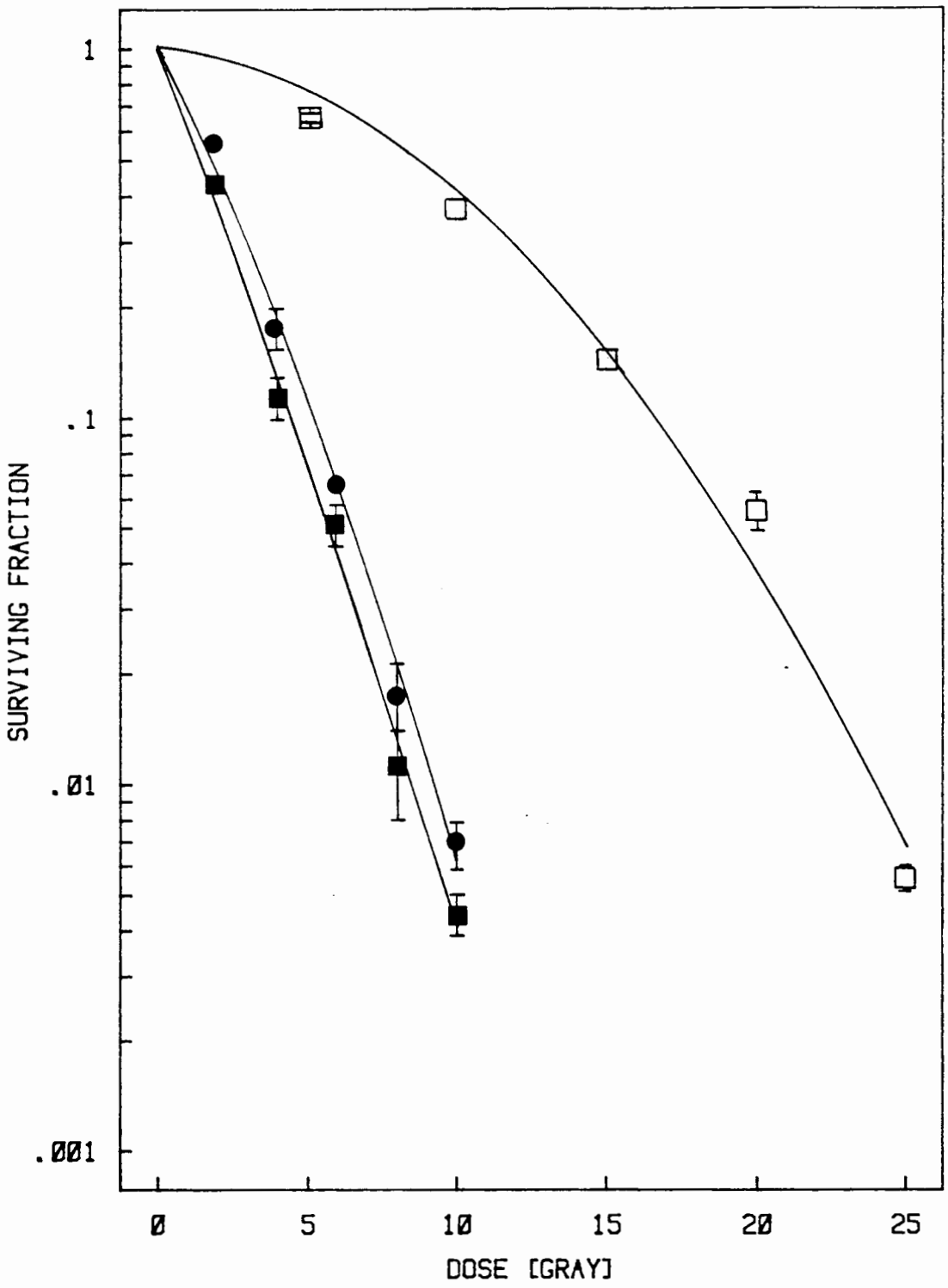


Fig.4.9 Radiation dose response curves for Fib/T tumours where WHT mice were pretreated with air and irradiated in air with ^{60}Co-gamma rays (—□—); pretreated with air and irradiated in air with neutrons (—●—); pretreated with 8% O_2 for 48h and irradiated in air with neutrons (—■—).
Data points are the mean of not less than 5 experiments and error bars represent standard deviations.

in air was 4.30 and the relevant dose survival curves are shown in Fig.4.9. (As was the case for DMF's, the relative biological effectiveness (RBE) was determined at a number of survival levels and the mean RBE then calculated. The statistical method yielded extremely high RBE's at the higher survival levels, e.g. at a survival level of 0.9, a RBE of 10.3 was determined).

4.2.1.3 Single Dose Gamma Radiation in Hyperbaric Oxygen of Experimental Mouse Tumours.

Where mice, pretreated with air, were irradiated in hyperbaric oxygen, a left shift of the dose survival curve to lower doses was observed when compared to the survival curve where tumours were irradiated in air, such that the DMF was 1.74 (Fig.4.10). The exposure of mice to 8% oxygen for 48 hours before radiation in hyperbaric oxygen had little effect on the improvement of the tumours' radiation response when compared to that obtained for mice irradiated in hyperbaric oxygen but pretreated with air (DMF of 1.90 vs. 1.74) (Fig.4.10).

4.2.1.4 Plating Efficiencies (P.E.'s).

The percentage of Fib/T tumour cells seeded in culture which grew into colonies was determined where tumour-bearing WHT mice were not irradiated but were treated in the following manner:

1) Mice kept in air (this served as the "control" P.E.).
2) Mice kept in 8% oxygen for 24 hours.
3) Mice kept in 8% oxygen for 48 hours.
4) Mice kept in 10% oxygen for 48 hours.
5) Mice kept in 15% oxygen for 48 hours.

The mean tumour cell P.E. for mice exposed only to an air-breathing environment was 0.58 +/- 0.04. Table 4.1 indicates the P.E.'s of tumour cells from mice that received different hypoxic treatments. These P.E.'s are expressed as a percentage (+/- standard deviation) of the "control" P.E. The tumour cell P.E.'s of mice exposed to 8% oxygen for periods of 24 hours and 48 hours were significantly different to those of mice exposed only to air ($p < 0.05$). There was no significant

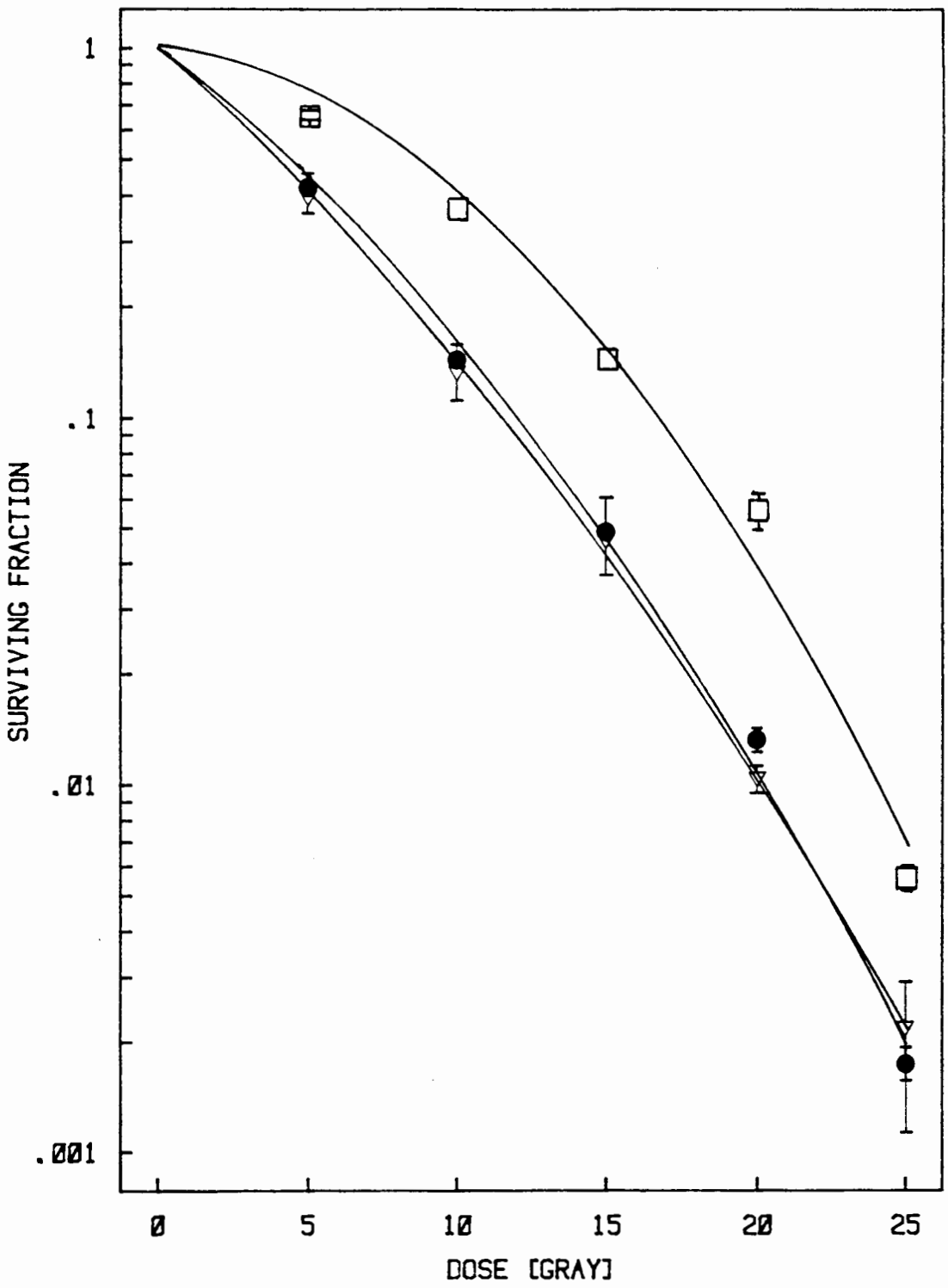


Fig.4.10 Gamma ray dose response of Fib/T tumours where WHT mice were pretreated with air and irradiated in air (—□—); pretreated with air but irradiated in hyperbaric oxygen (—●—); pretreated with 8% O_2 for 48h and irradiated in hyperbaric oxygen (—▽—).
Data points are the mean of not less than 5 experiments and error bars represent standard deviations.

difference in the tumour cell P.E. between mice kept in 10% oxygen for 48 hours and mice kept in air ($p > 0.1$). The tumour cell P.E. of mice exposed to 15% oxygen for 48 hours did not differ significantly from that of mice exposed only to air ($p > 0.5$).

Table 4.1 The effect of hypoxic treatments on plating efficiency.

Hypoxic Treatment	P.E.(% of "control" P.E.)
8% O_2-24h	0.82+/-0.03
8% O_2-48h	0.76+/-0.04
10% O_2-48h	0.82+/-0.09
15% O_2-48h	0.92+/-0.11

4.2.2 Fractionated Radiations.

4.2.2.1 Fractionated Gamma Radiation delivered while the Tumour-bearing Mice breathed Air.

4.2.2.1.1 Split-dose radiation allowing a 4 hour interval between the two exposures.

When the tumour radiation response of mice pretreated with 8% oxygen for 48 hours but kept in air for the period between the radiation doses was compared to that obtained where mice were pretreated with air and maintained in air between radiation doses, a DMF of 1.19 (1.11-1.28) was determined (Fig.4.11). If mice were pretreated with 8% oxygen for 48 hours and then returned to this environment for the radiation-free interval, a slightly greater DMF of 1.23 (1.14-1.33) was obtained (Fig.4.11).

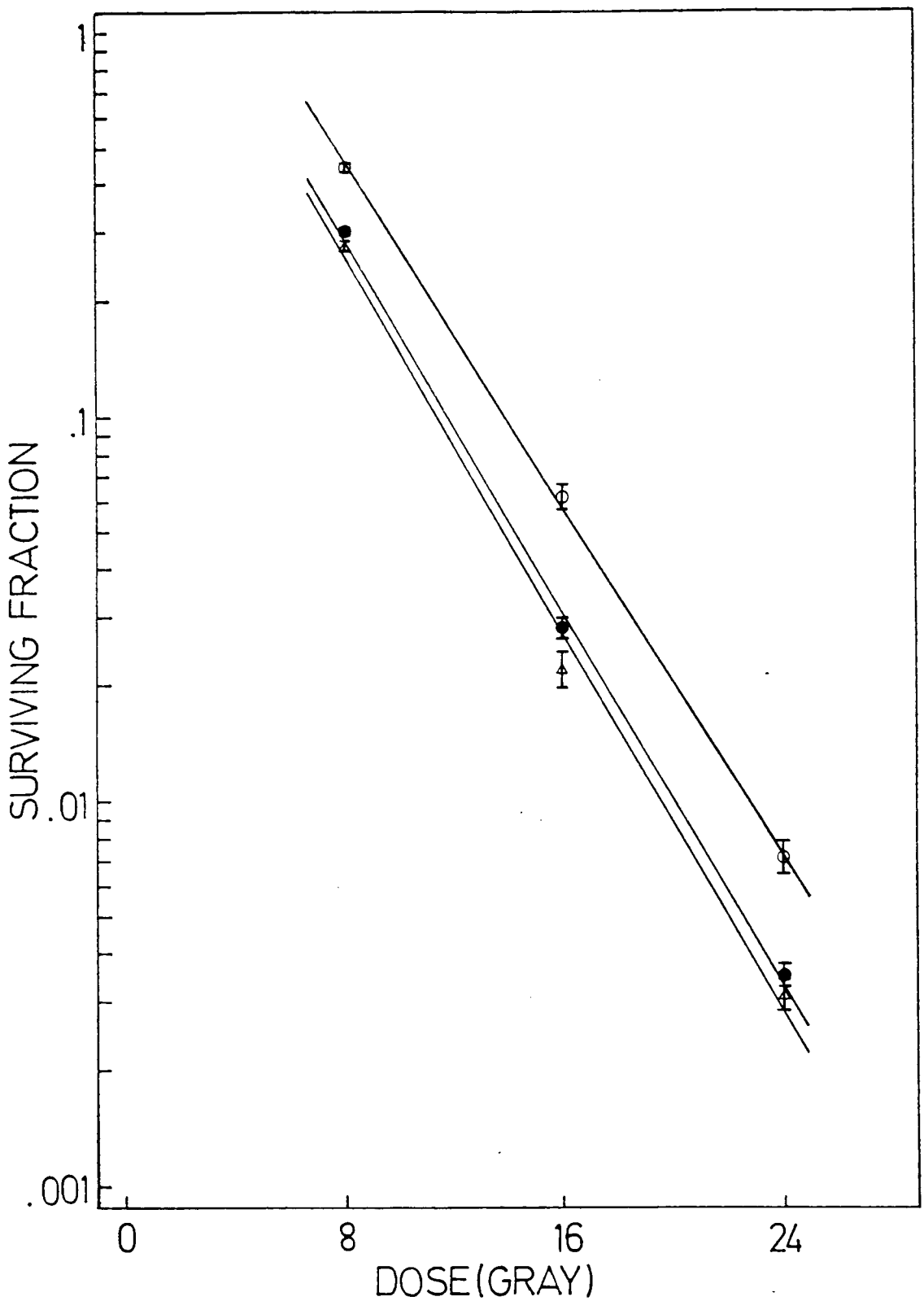


Fig.4.11 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation, separated by 4h. Mice that were pretreated with air were kept in air between fractions (—○—). Mice that were pretreated with 8% O_2 for 48h were either kept in air between fractions (—●—), or kept in 8% O_2 between fractions (—△—). All irradiations were in air.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

4.2.2.1.2 Split-dose radiation allowing a 24 hour interval between the two exposures.

Fig.4.12 shows the response of the Fib/T tumour to two equal fractions of radiation, separated by 24 hours, where the host animal was either pretreated with 8% oxygen or with air. Where the tumour radiation response of mice receiving a 48 hour hypoxic pretreatment but placed in air between radiation fractions was compared to that where mice were pretreated with air and kept in air between fractions, a DMF of 1.11 (1.05-1.17) was obtained. However, following a 48 hour hypoxic pretreatment, the replacement of mice into 8% oxygen between radiation fractions increased the DMF to 1.34 (1.26-1.42) (Fig.4.12). Mice pretreated with air but kept in 8% oxygen between radiation fractions did not show an improved tumour response as compared to mice similarly pretreated and maintained in air between fractions (Fig.4.13).

It is of interest to compare some of the responses described in this section and the one immediately preceeding, i.e. when the interfraction interval was increased from 4 to 24 hours. Figure 4.14 shows data from Figs.4.11 and 4.12. It was calculated that the tumour response of mice pretreated with air and kept in air for the 24 hour interval between radiation fractions was improved by a factor of 1.1 (1.02-1.18) by reducing the interval between fractions to 4 hours (Fig.4.14).

In a similar way, Fig.4.15 shows data from Figs.4.11 and 4.12. Here it was determined that mice pretreated with 8% oxygen but kept during the 4 hour radiation-free interval in air showed a similar tumour response to that of mice pretreated with 8% oxygen and kept in this environment for a 24 hour period between radiation fractions. When the responses were compared to the tumour response elicited in mice pretreated

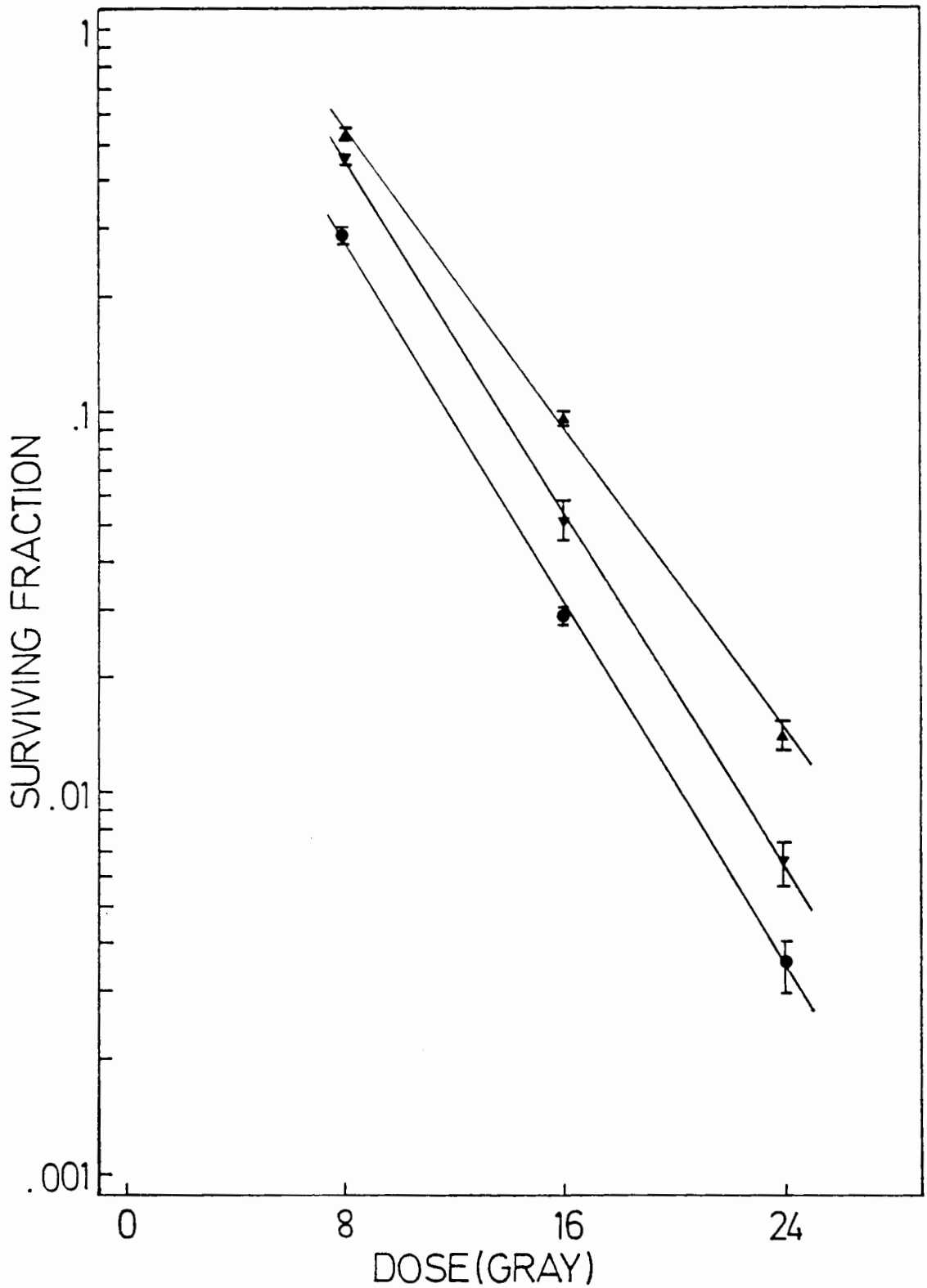


Fig.4.12 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation, separated by 24h. Mice that were pretreated with air were kept in air between fractions (—▲—). Mice that were pretreated with 8% O_2 for 48h were either kept in air between fractions (—▼—), or in 8% O_2 between fractions (—●—). All irradiations were in air.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

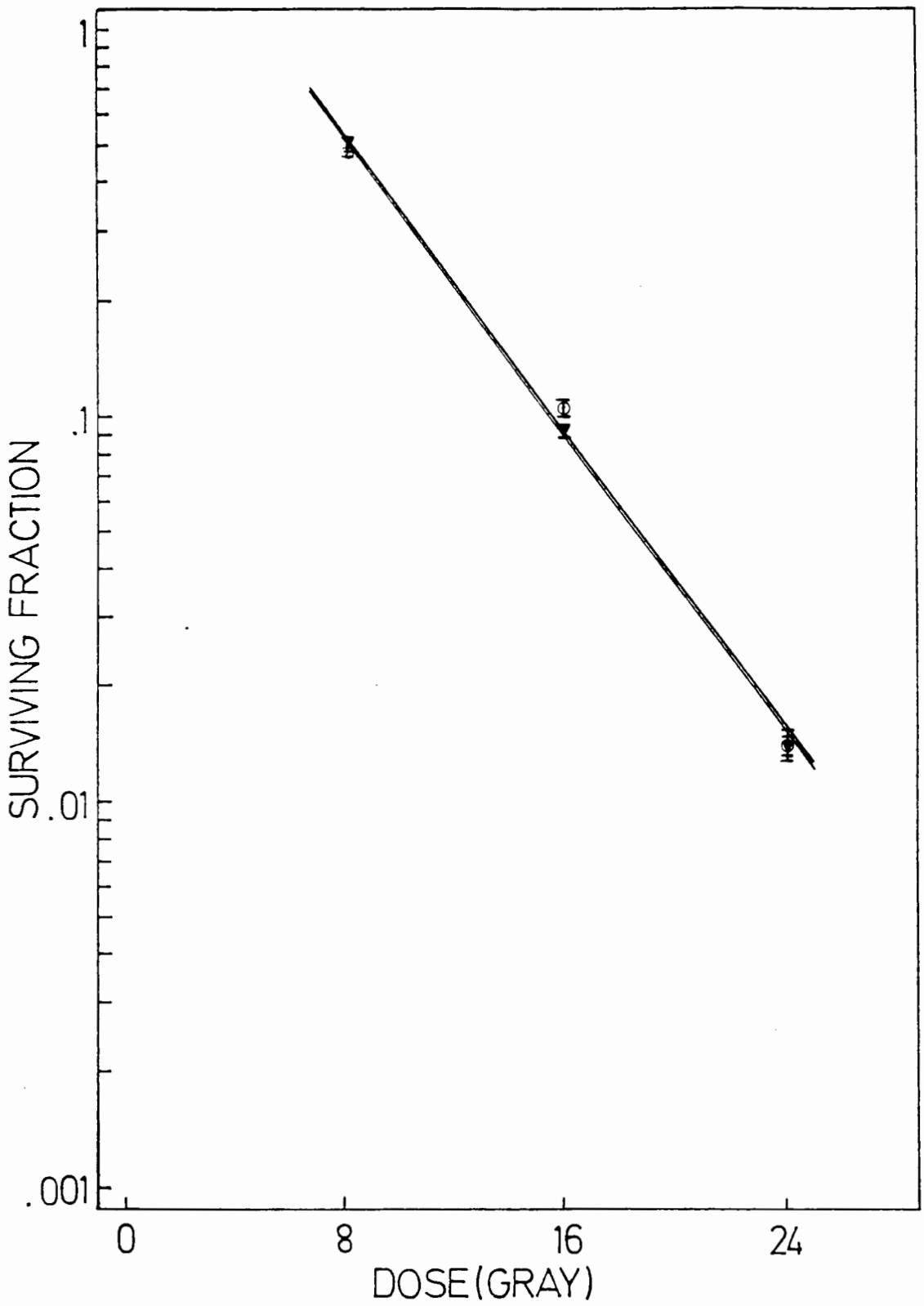


Fig.4.13 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation, separated by 24h. Mice pretreated with air were either kept in air between fractions (—▼—), or in 8% O_2 between fractions (—⊖—). All irradiations were in air.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

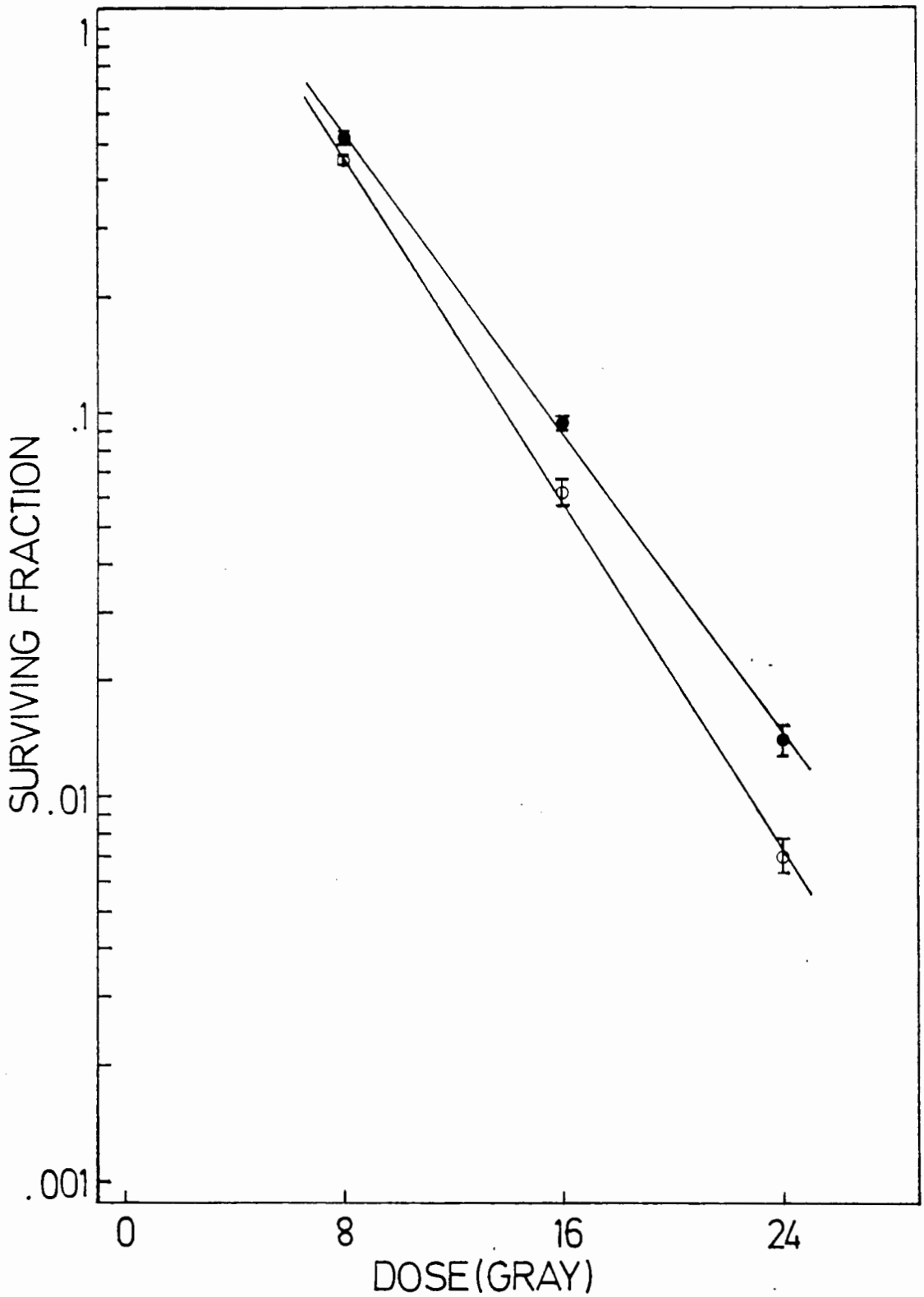


Fig.4.14 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation, separated by either 4h (—○—) or 24h (—●—). Pretreatments were with air and mice were kept in air between fractions. All irradiations were in air.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

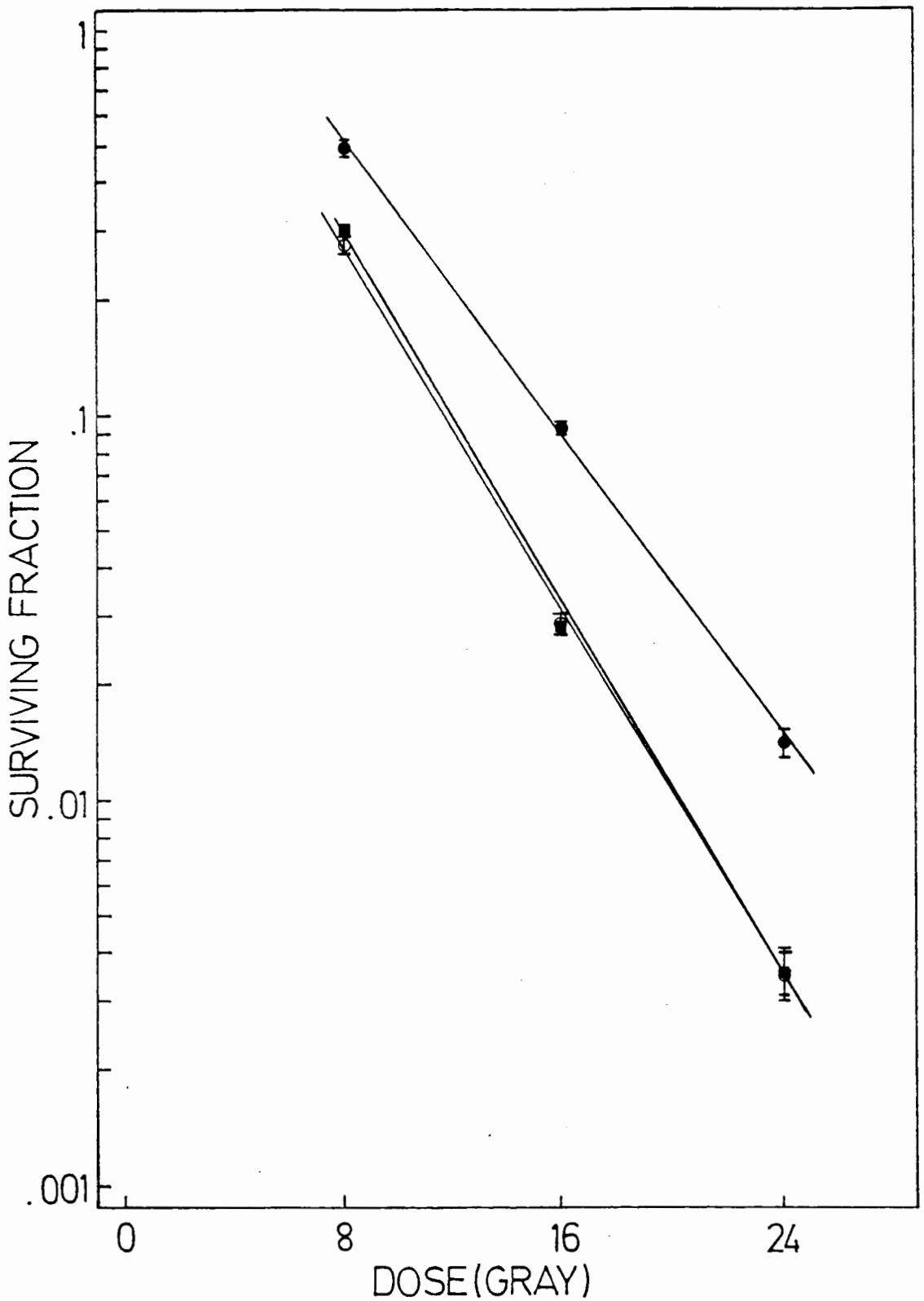


Fig.4.15 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation, separated by 4h or 24h. Mice pretreated with air were kept in air during the 24h interval between fractions (—●—). Mice pretreated with 8% O_2 for 48h were either kept in 8% O_2 for 24h between fractions (—○—), or in air for 4h between fractions (—■—). All irradiations were in air.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

with air and kept in air for 24 hours between fractions, DMF's of 1.33 (1.26-1.4) and 1.34 (1.26-1.42) respectively were calculated (Fig.4.15).

4.2.2.2 Split-Dose Radiation, separated by 24 hours, delivered while the Tumour-bearing Mice breathed 100% Oxygen under Pressure (3 ATA).

Fig.4.16 shows that the tumour response of mice pretreated with 8% oxygen but kept in air between the two radiation exposures was not significantly different to that of mice pretreated with air and kept in air between fractions The DMF was calculated to be 1.05 (0.96-1.15). However, if mice pretreated with 8% oxygen were kept in this hypoxic environment during the 24 hour interval between the two fractions, the DMF increased to 1.14 (1.07-1.21).

Fig.4.17 shows the effect of a split-dose radiation separated by 24 hours on the response of the Fib/T tumour where the host animal was either irradiated in hyperbaric oxygen or in air. Where mice were pretreated with air and kept in air between radiation fractions, hyperbaric oxygen caused a left shift of the dose survival curve to lower doses such that the DMF was 1.23 (1.16-1.31). The tumour response of mice pretreated with 8% oxygen but kept in air between radiation exposures was increased by a factor of 1.18 (1.09-1.28) when the radiations were given under conditions of hyperbaric oxygen rather than in air (Fig.4.18). A DMF of 1.08 (1.02-1.14) was obtained when the tumour response of mice pretreated with 8% oxygen and kept in 8% oxygen between radiation fractions was compared under hyperbaric oxygen and air conditions (Fig.4.19).

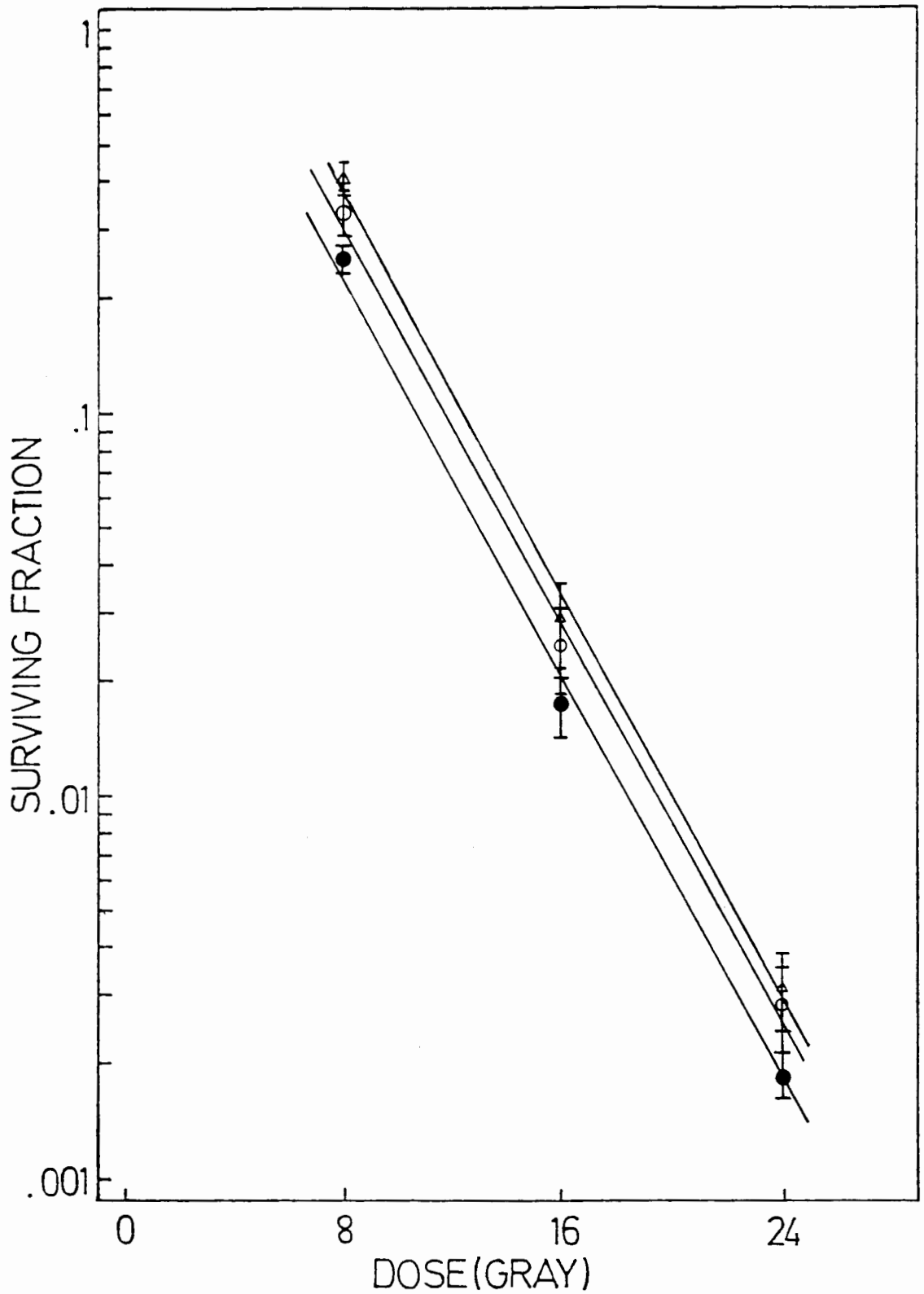


Fig.4.16 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation, separated by 24h. Mice that were pretreated with air were kept in air between fractions (—△—). Mice that were pretreated with 8% O_2 for 48h were either kept in air between fractions (—○—), or in 8% O_2 between fractions (—●—). All irradiations were in hyperbaric oxygen.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

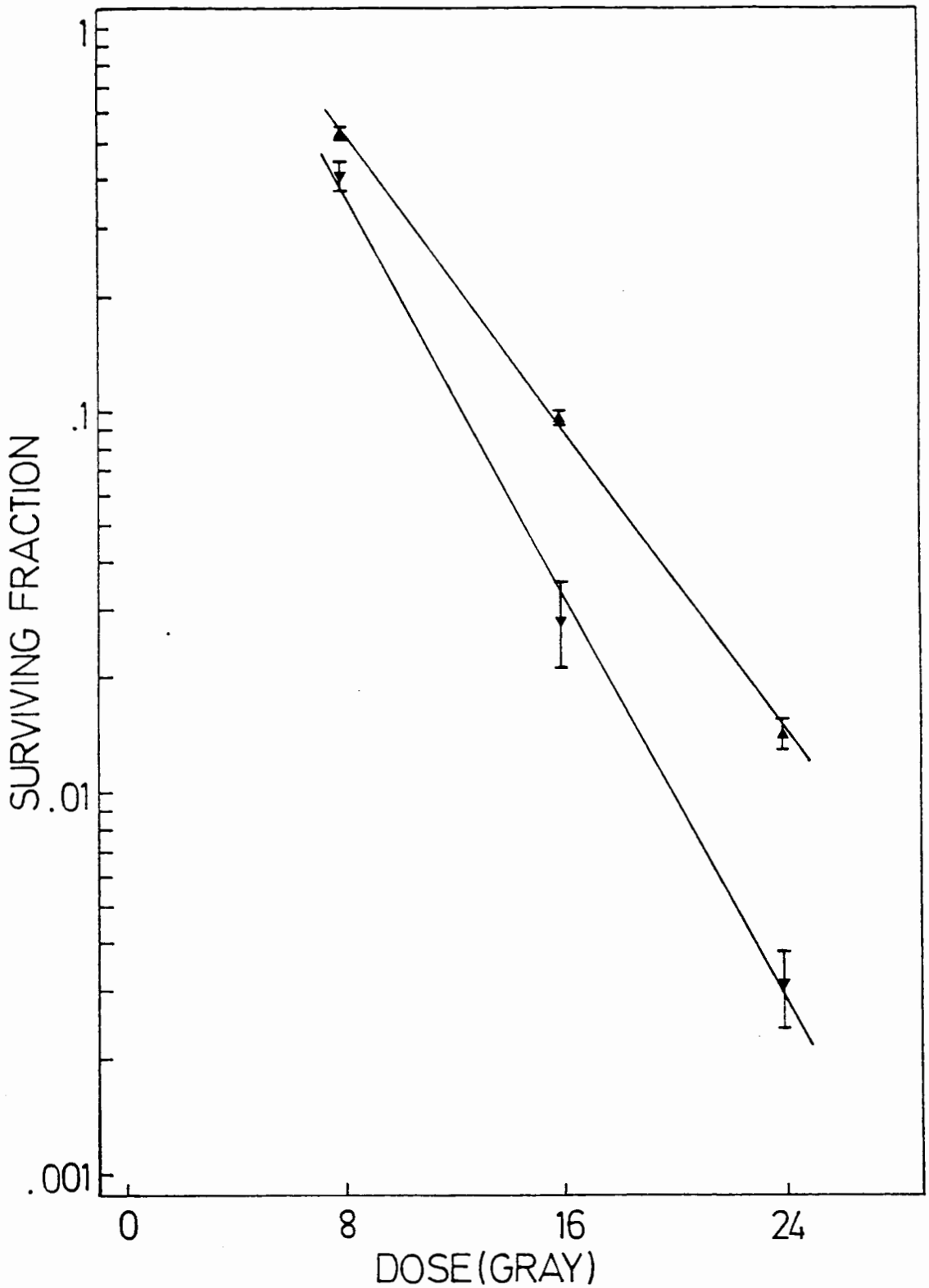


Fig.4.17 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation delivered either in air (—▲—) or in hyperbaric oxygen (—▼—). Mice were pretreated with air and kept in air during the 24h interval between fractions.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

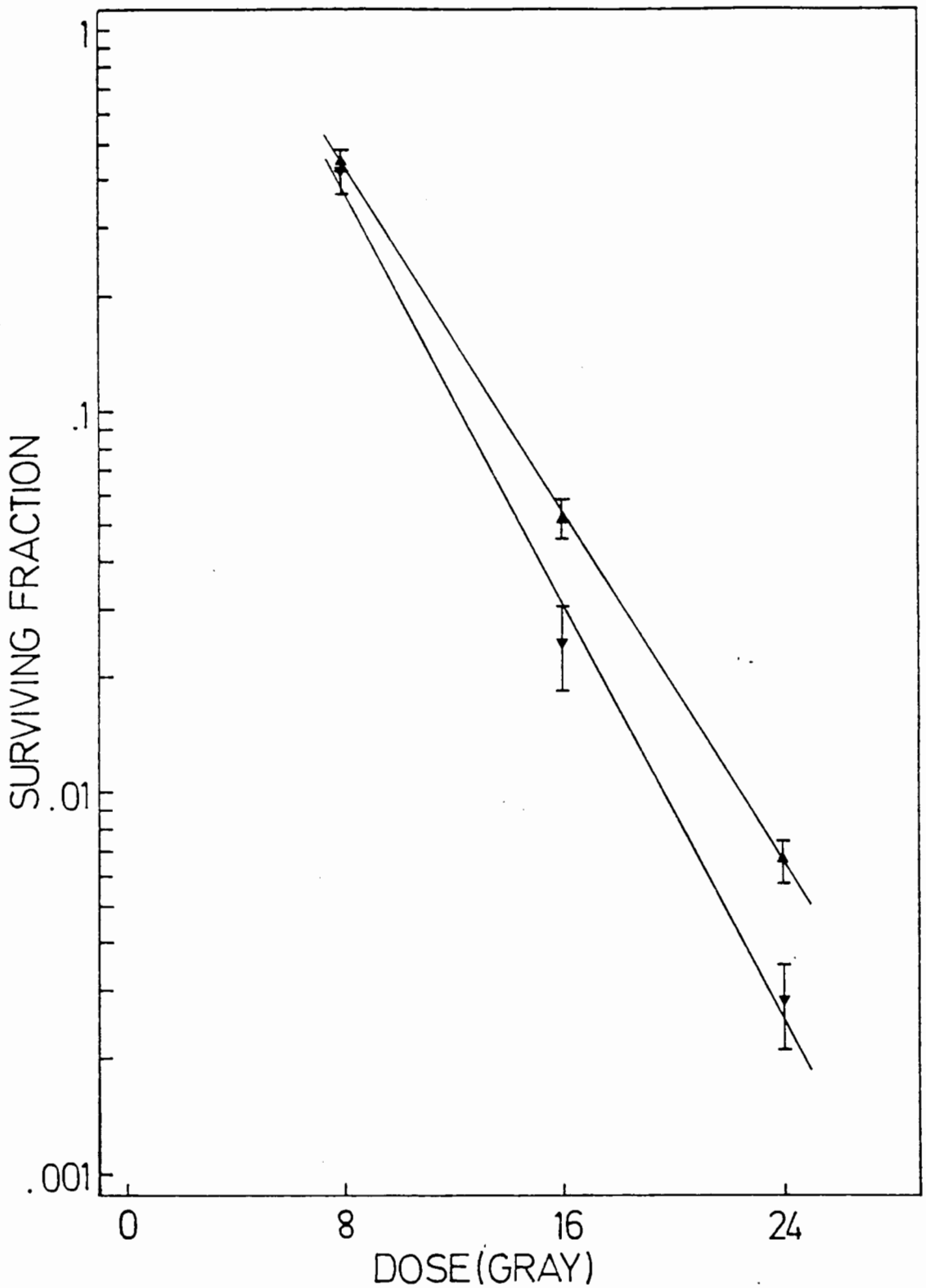


Fig.4.18 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation delivered either in air (—▲—) or in hyperbaric oxygen (—▼—). Mice were pretreated with 8% O_2 for 48h but kept in air during the 24h interval between fractions.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

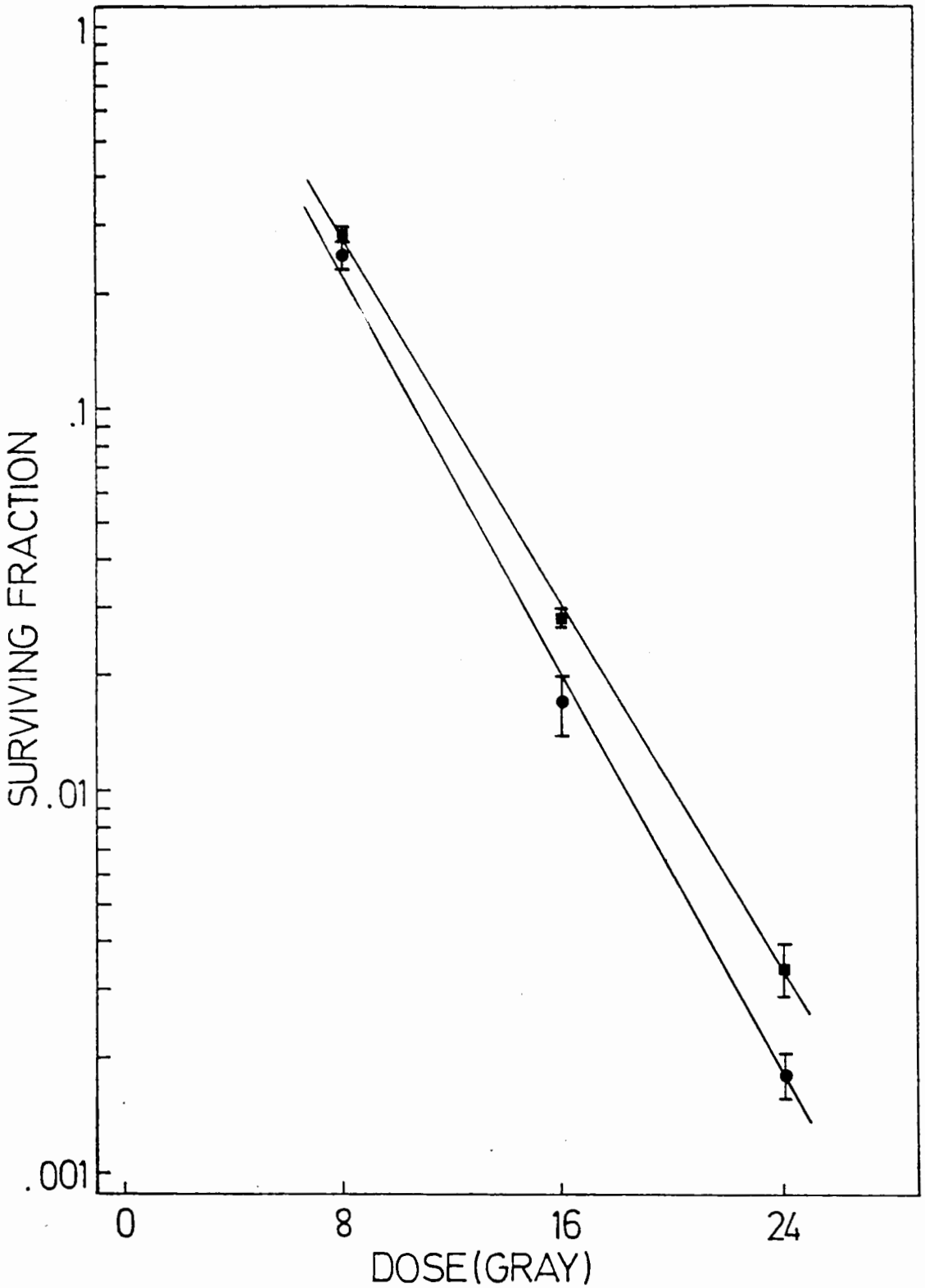


Fig.4.19 Dose response of Fib/T tumours where WHT mice received two equal fractions of gamma irradiation delivered either in air (—■—) or in hyperbaric oxygen (—●—). Mice were pretreated with 8% O_2 for 48h and kept in 8% O_2 during the 24h interval between fractions.
Data points are the mean of 5 experiments +/- standard deviation. Lines were fitted by the method of least squares.

4.3 Hypoxic Fraction Determinations.

4.3.1 Clamped Tumour Growth Delay Method.

The clamped tumour growth delay method as described in section 3.3.1 was used to determine the hypoxic fractions of the CaNT tumour, the 3-MC-induced rhabdomyosarcoma and the Fib/T tumour. As shown in Table 4.2, the hypoxic fractions of the CaNT and Fib/T tumours were similar, but the hypoxic fraction for the 3-MC-induced rhabdomyosarcoma was significantly lower than in both the aforementioned tumours ($p < 0.01$ and $p < 0.05$ respectively).

Table 4.2 Clamped tumour growth delay assays (for calculations, see Appendix A).

Tumour System	Ta+/-S (days)	Tc+/-S (days)	Td+/-S (days)	Hypoxic Fraction (95% C.I.)
CaNT	12.9+/-0.73	8.5+/-0.89	5+/-0.35	54(43-65)%
3-MC induced rhabdomyosarcoma	13.5+/-0.73	5.8+/-0.95	4.1+/-0.46	27(20-34)%
Fib/T	9.02+/-0.89	4.12+/-0.86	4.22+/-0.29	45(34-56)%

4.3.2 Paired Survival Curve Method.

The Fib/T tumour can be assayed for viability by an in vitro colony formation assay. It was, therefore, possible to determine the hypoxic fraction of this tumour using the paired survival curve method, as described in section 3.3.2.

Fig.4.20 illustrates the analysis of the hypoxic fraction of Fib/T tumours grown in the gastrocnemius muscle of WHT mice.

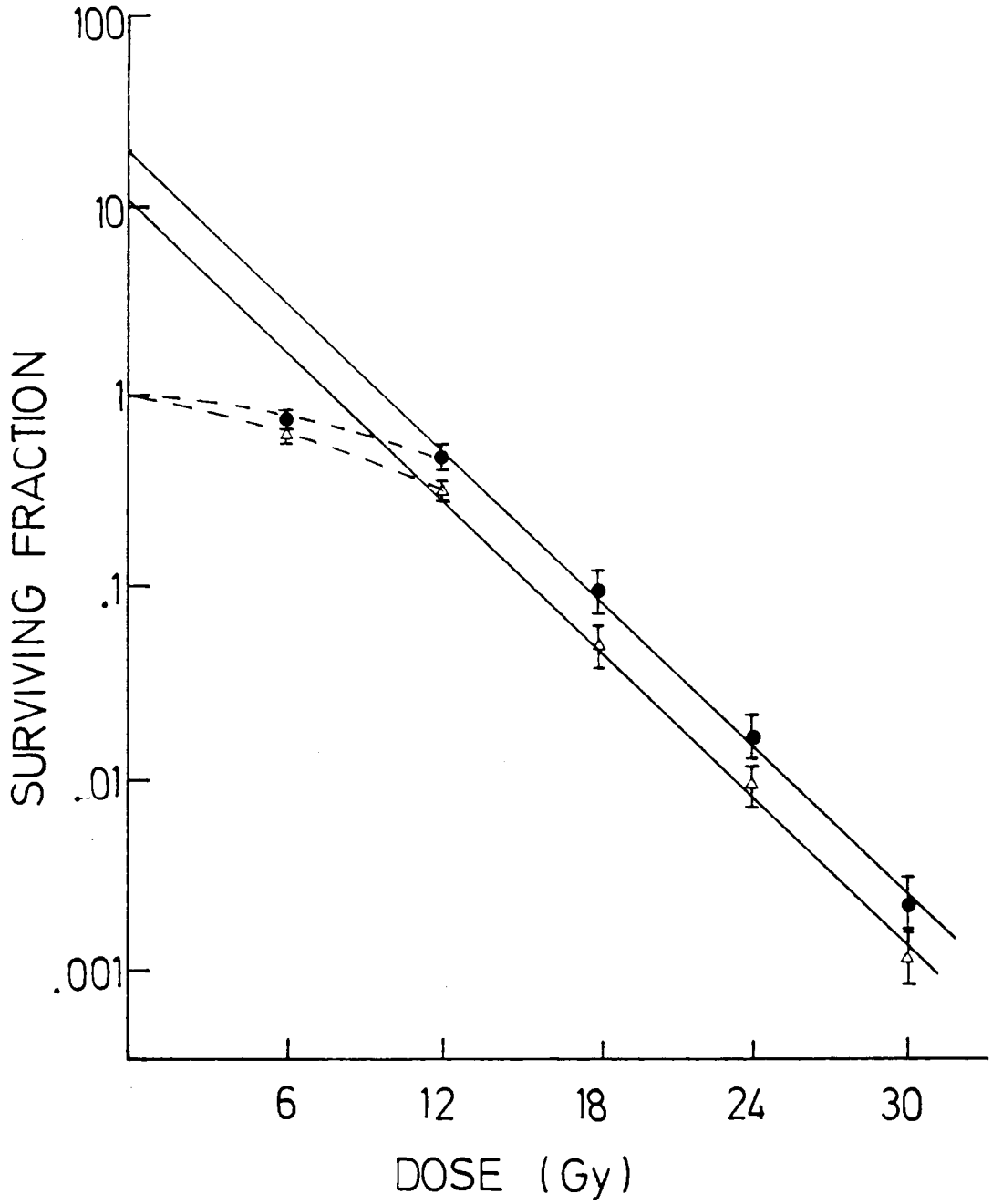


Fig.4.20 Survival curves for i.m. Fib/T tumours.
(—●—) tumours irradiated under hypoxia (clamped)
(—△—) tumours irradiated in air-breathing mice
Solid lines are the best parallel lines that could be fitted to the data for doses of 12 Gy and above. Data points are the mean of 6 experiments +/- standard deviation.

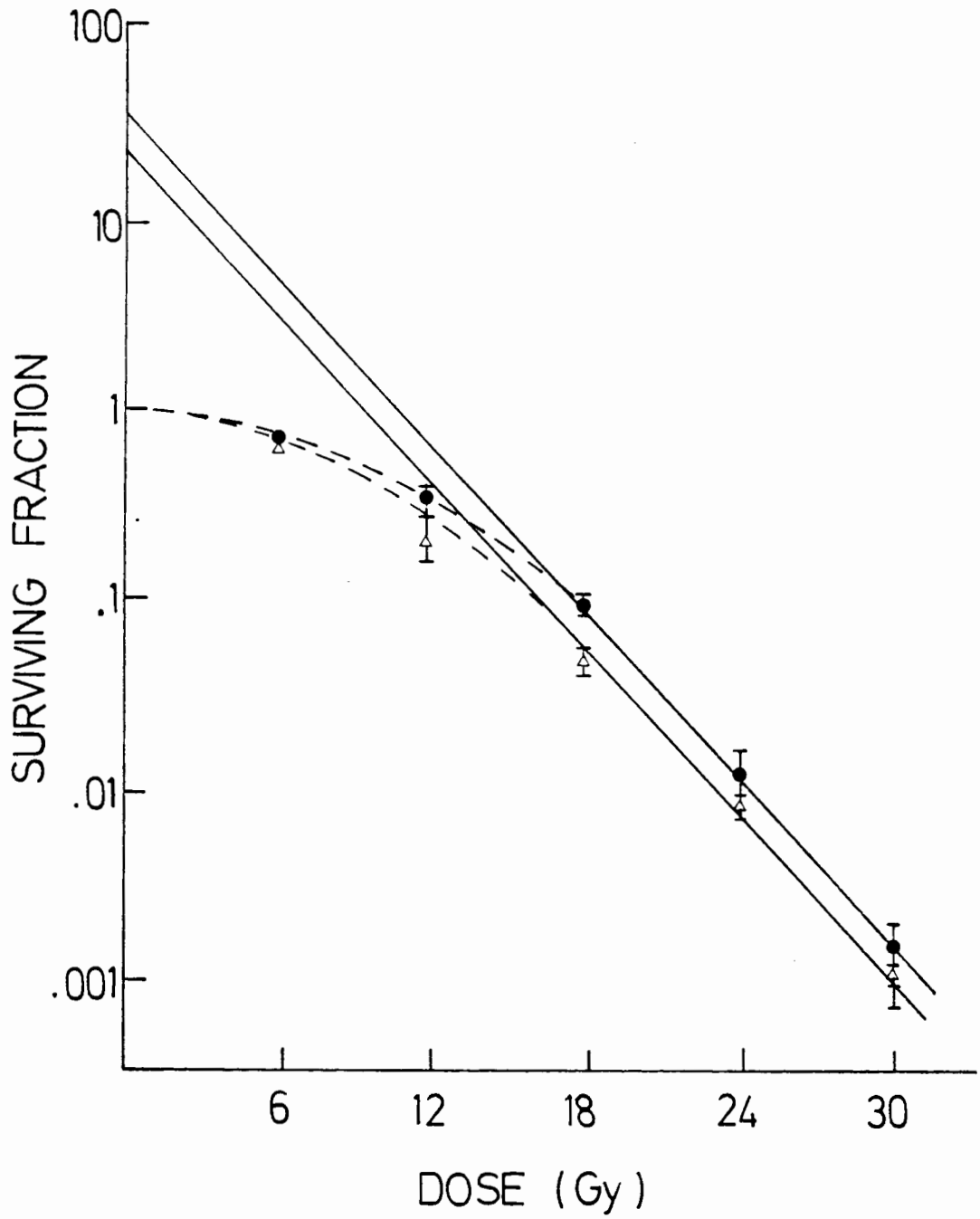


Fig.4.21 Survival curves for s.c. Fib/T tumours.
(—●—) tumours irradiated under hypoxia (clamped)
(—△—) tumours irradiated in air-breathing mice
Solid lines are the best parallel lines that could be fitted to the data for doses of 18 Gy and above. Data points are the mean of 6 experiments +/- standard deviation.

The best linear parallel fits to the air and hypoxic curves were found by linear regression after deleting low-dose points in the shoulder regions (< 12 Gy). From the vertical distance between the intercepts, a hypoxic fraction of 58% was determined (95% confidence interval: 52%-64%). The paired survival curve assay yielded a hypoxic fraction of 61% (95% confidence interval: 58%-64)%) for the Fib/T tumour grown subcutaneously in the sternum of the WHT mice (Fig.4.21). The best linear parallel fits to the air and hypoxic curves necessitated the deletion of dose points below 18 Gy.

4.4 **Biochemical Determinations.**

4.4.1 Tumour Enzyme Activity.

The activity of

a) Catalase

b) Glutathione reductase and

c) Glutathione peroxidase

was measured in the Fib/T tumour in

1) air-breathing WHT mice

2) WHT mice kept in 8% oxygen for 24 hours

3) WHT mice kept in 8% oxygen for 48 hours

4) WHT mice kept in 8% oxygen for 72 hours.

4.4.1.1 Catalase Activity in the Fib/T Tumour.

The variation of catalase activity in the Fib/T tumour in WHT mice exposed to 8% oxygen for 24, 48 and 72 hours is shown in Fig.4.22. Catalase activity is expressed as a percentage of that value determined in the tumours of air-breathing mice. The mean tumour catalase activity in air-breathing mice from 16 determinations was 0.471 +/- 0.031 umol H_2O_2 degraded/min/mg

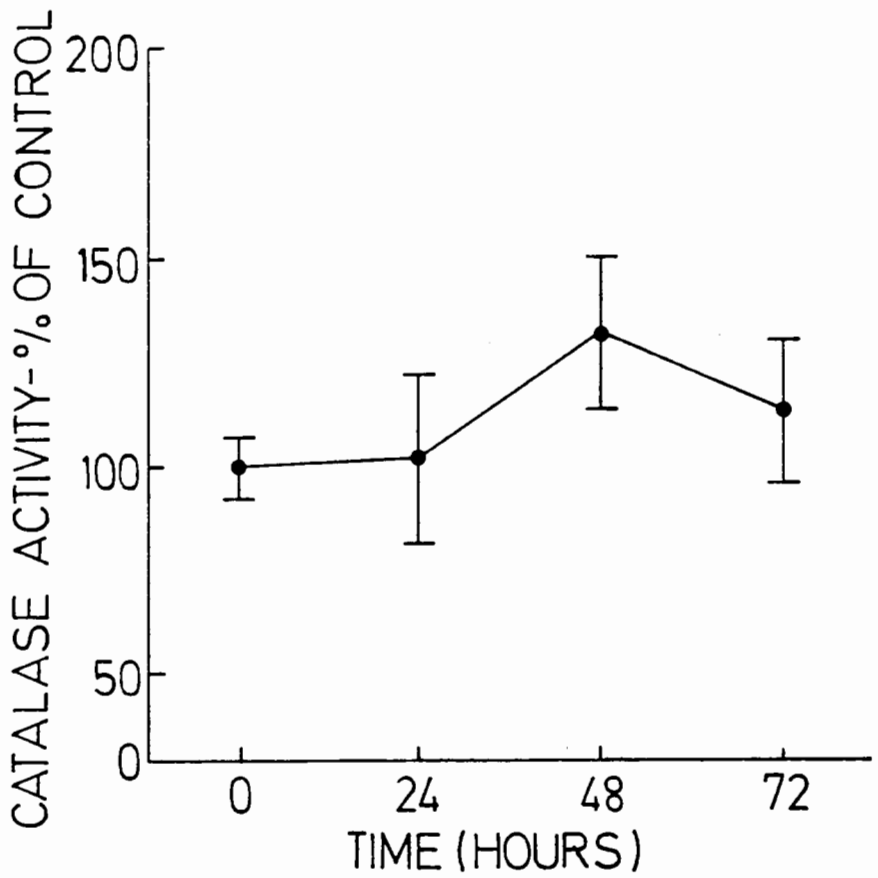


Fig.4.22 Fib/T tumour catalase activity before and during exposure of WHT mice to 8% O_2.
Each point represents the mean of not less than 7 values, and error bars represent standard deviations.

protein. The largest increase in tumour catalase activity was observed in those mice kept in 8% oxygen for 48 hours, viz. 1.32 times that of the air-breathing control group. However, this difference was not statistically significant ($p > 0.2$).

4.4.1.2 Glutathione Reductase Activity in the Fib/T Tumour.

The variation of glutathione reductase activity in the Fib/T tumour in WHT mice exposed to 8% oxygen for 24, 48 and 72 hours is shown in Fig.4.23. Glutathione reductase activity is expressed as a percentage of the activity measured in the tumours of air-breathing mice. The mean tumour glutathione reductase activity in air-breathing mice from 14 determinations was 0.041 +/- 0.004 umol GSSG degraded/min/mg protein. There was no significant difference in tumour glutathione reductase activity between mice exposed to 8% oxygen and mice kept in air, irrespective of the duration of exposure to 8% oxygen ($p > 0.45$) (Fig.4.23).

4.4.1.3 Glutathione Peroxidase Activity in the Fib/T Tumour.

The variation of glutathione peroxidase activity in the Fib/T tumour in WHT mice exposed to 8% oxygen for 24, 48 and 72 hours is shown in Fig.4.24. Glutathione peroxidase activity is expressed as a percentage of that measured in the tumours of air-breathing mice. The mean tumour glutathione peroxidase activity in air-breathing mice from 8 determinations was 0.057 +/- 0.002 umol GSSG degraded/min/mg protein. Tumour glutathione peroxidase activity of mice exposed to an 8% oxygen environment for varying times did not differ significantly when compared to that of air-breathing mice (Fig.4.24).

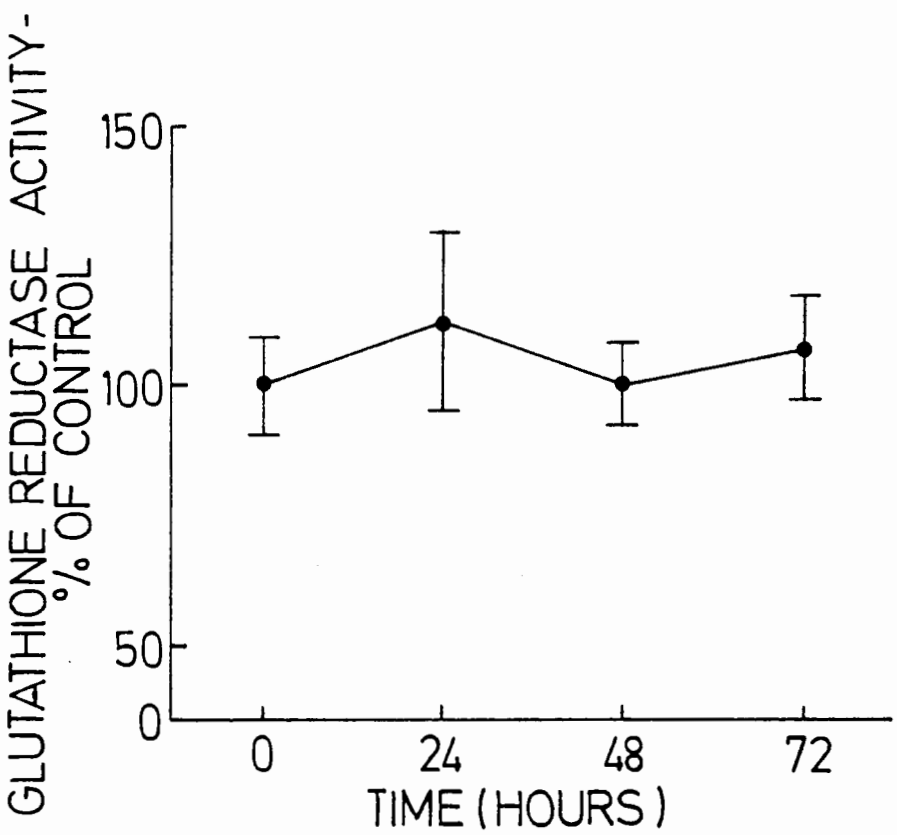


Fig.4.23 Fib/T tumour glutathione reductase activity before and during exposure of WHT mice to 8% O_2.
Each point represents the mean of not less than 6 observations, and error bars represent standard deviations.

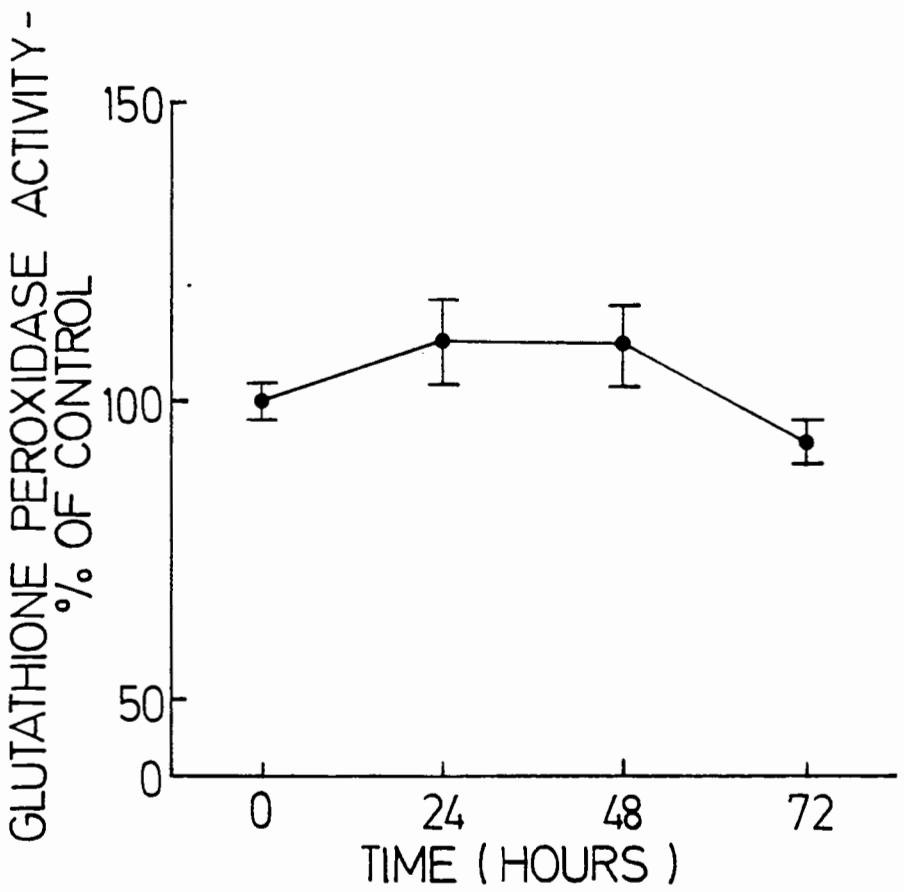


Fig.4.24 Fib/T tumour glutathione peroxidase activity before and during exposure of WHT mice to 8% O_2.
Each point represents the mean of not less than 6 values, and error bars indicate standard deviations.

4.4.2 Glutathione Levels in Deproteinized Samples of the Fib/T Tumour.

By using the enzymatic method of Tietze,[148] levels of glutathione (GSH and GSSG) were measured in the Fib/T tumours of

1) air-breathing WHT mice
2) WHT mice exposed to 8% oxygen for 24 hours
3) WHT mice exposed to 8% oxygen for 48 hours
4) WHT mice exposed to 8% oxygen for 72 hours.

Fig.4.25 shows the variation of glutathione levels in the Fib/T tumours before and after exposure of the mice to 8% oxygen. The glutathione levels are expressed as a percentage of that determined in the tumours of air-breathing mice. The mean tumour glutathione level in 8 air-breathing mice was 559 +/- 7 ng/mg. When compared to the control, no significant change in tumour glutathione levels was observed when mice were exposed to 8% oxygen for 24, 48 and 72 hours ($p > 0.45$).

4.4.3 Haemoglobin Determinations in WHT Mice.

4.4.3.1 Haemoglobin Determinations in Mice Exposed to a Single Oxygen Concentration.

Groups of WHT mice bearing the Fib/T tumour were each exposed to a different oxygen concentration for varying times as detailed in section 3.4.1.1.

The mean haemoglobin concentration in 10 air-breathing mice was 13.95 +/- 0.32 g/100 ml blood.

The haemoglobin values of mice exposed to 8% oxygen for 24, 48 and 72 hours are shown in Fig.4.26. The haemoglobin values increased with time and significant augmentations ($p < 0.05$) of 8% and 11.1% that of the control were observed at 48

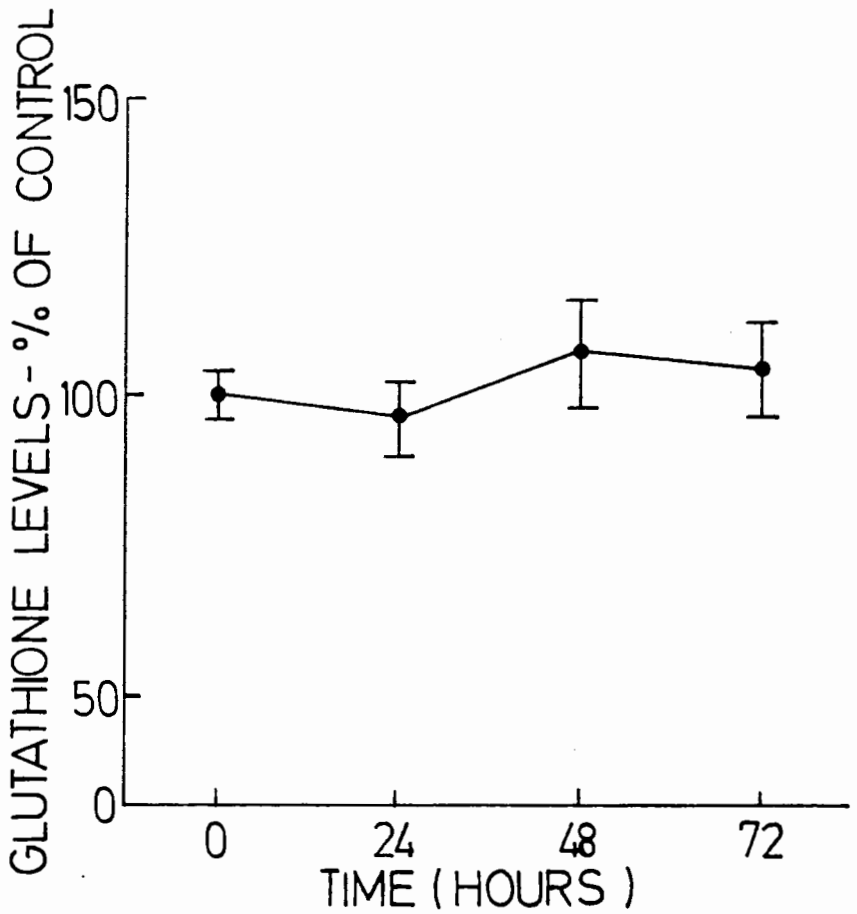


Fig.4.25 Fib/T tumour glutathione levels before and during exposure of WHT mice to 8% O_2.
Each point represents the mean of not less than 6 values, and error bars represent standard deviations.

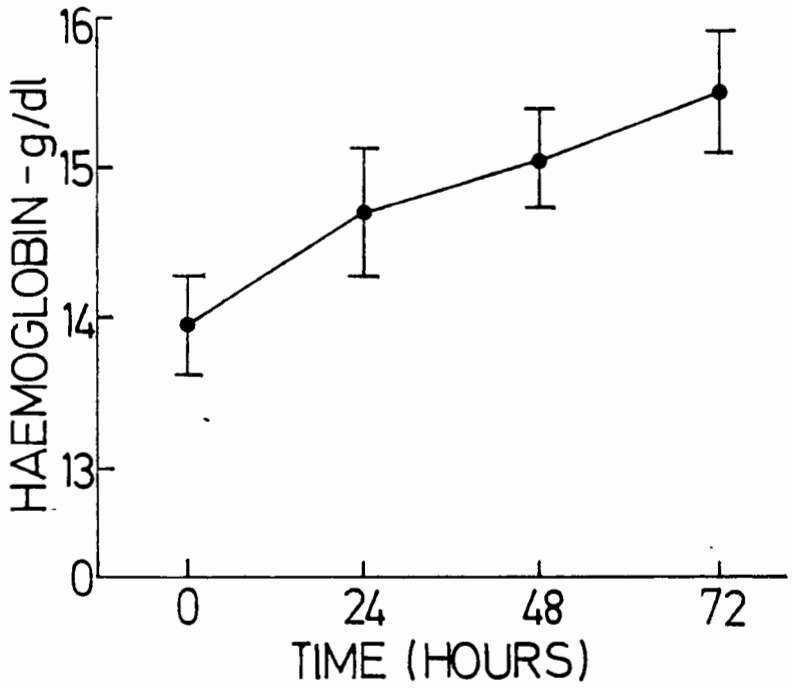


Fig.4.26 Haemoglobin concentration as a function of time of exposure of WHT mice to 8% O_2.
Each point is the mean of not less than 6 values, and error bars represent standard deviations.

and 72 hours respectively. Although the haemoglobin concentration of mice kept in 8% oxygen for 24 hours increased by 5.3% over that of the control, this increase was not significant ($p > 0.18$).

The haemoglobin values of mice exposed to 10% oxygen for 24, 48 and 72 hours are shown in Fig.4.27. The haemoglobin values increased with time but the haemoglobin concentration at 24 hours was not significantly different from that of the control ($p > 0.3$). However, at 48 and 72 hours, respective augmentations of 9% and 10.7% that of the control were observed, which were significant ($p < 0.05$).

The haemoglobin values of mice exposed to 15% oxygen for 24, 48 and 72 hours are shown in Fig.4.28. Although the haemoglobin values of mice exposed to 15% oxygen appeared to increase with time, they were not significantly different from that of the control at any of the times investigated ($p > 0.14$).

4.4.3.2 Haemoglobin Levels of Mice Exposed Sequentially to 8% Oxygen and Air.

4.4.3.2.1 Haemoglobin Determinations 24.5 Hours after Completion of a 48 Hour Exposure to 8% Oxygen.

For these investigations, mice were divided into 4 groups:

Group 1 mice were air breathing.

Group 2 mice were exposed to 8% oxygen for 48 hours.

Group 3 mice were pretreated with 8% oxygen for 48 hours and then kept in an air-breathing environment for 24.5 hours.

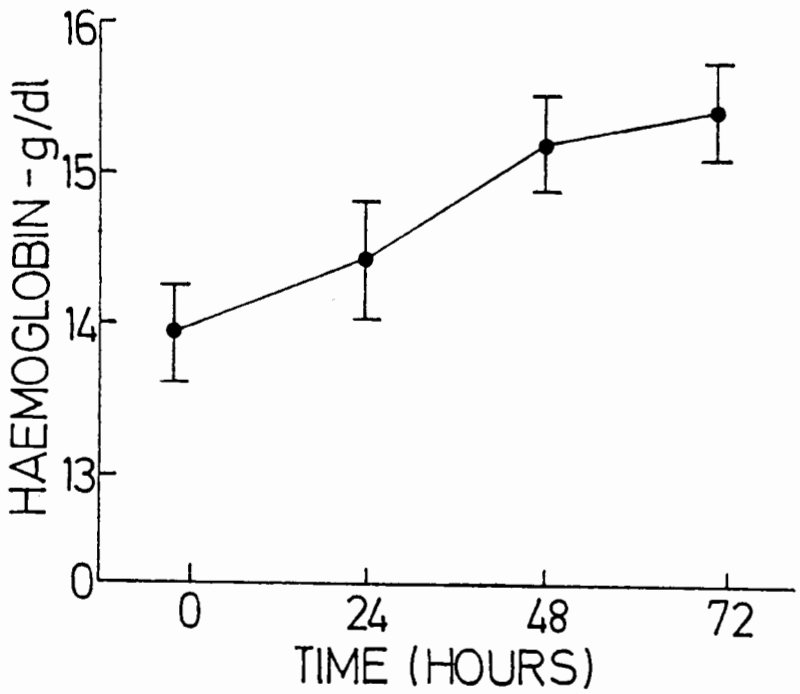


Fig.4.27 Haemoglobin concentration as a function of time of exposure of WHT mice to 10% O_2.
Each point is the mean of not less than 6 values, and error bars indicate standard deviations.

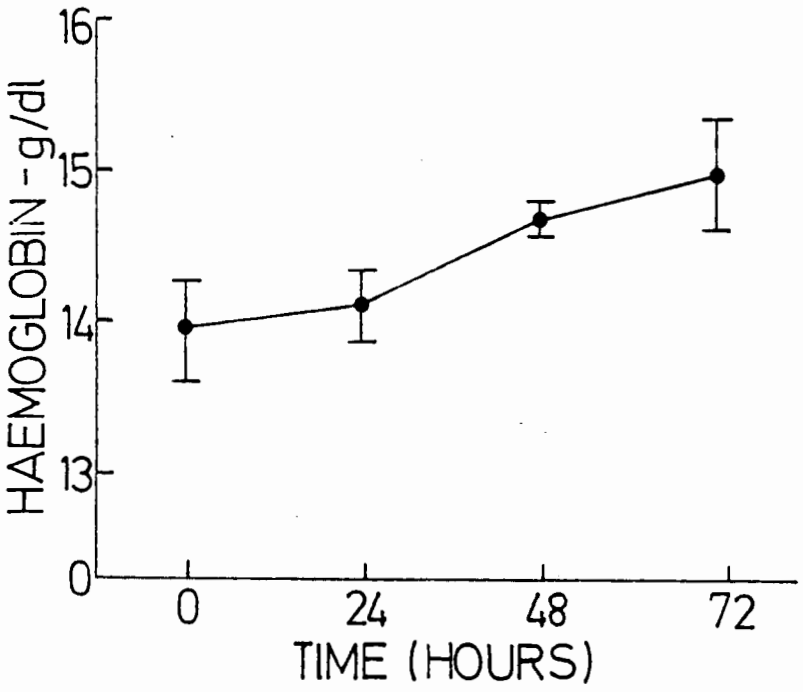


Fig.4.28 Haemoglobin concentration as a function of time of exposure of WHT mice to 15% O_2.
Each point is the mean of not less than 6 values, and error bars represent standard deviations.

Group 4 mice were exposed to 8% oxygen for 48 hours. Following this, the mice were kept in air for 0.5 hours and then replaced in 8% oxygen for a further 24 hours.

Fig.4.29 shows that the haemoglobin concentration of group 3 was not significantly different from that of the control group of air-breathing mice ($p > 0.5$). However, there was a significant difference in the haemoglobin concentrations between group 4 mice and air-breathing mice as well as between groups 3 and 4 mice ($p < 0.05$). Haemoglobin levels in groups 2 and 4 were not different ($p > 0.4$), but there was a significant difference between groups 2 and 3 ($p < 0.05$).

4.4.3.2.2 Haemoglobin Determinations 4.5 Hours after Completion of a 48 Hour Exposure to 8% Oxygen.

For these investigations, mice were divided into 4 groups:

Group 1 mice were air breathing.

Group 2 mice were exposed to 8% oxygen for 48 hours.

Group 3 mice were pretreated with 8% oxygen for 48 hours and then kept in air for 4.5 hours.

Group 4 mice were exposed to 8% oxygen for 48 hours, then kept in air for 0.5 hours and then returned to the 8% oxygen environment for 4 hours.

The haemoglobin concentration of group 3 was not significantly different from that of group 4 mice ($p > 0.7$). The haemoglobin concentrations of both groups 3 and 4 mice did, however, differ significantly from that of air-breathing mice ($p < 0.05$), but did not differ significantly from that of group 2 ($p > 0.2$) (Fig.4.30).

4.4.4 2,3-DPG Determinations in WHT Mice.

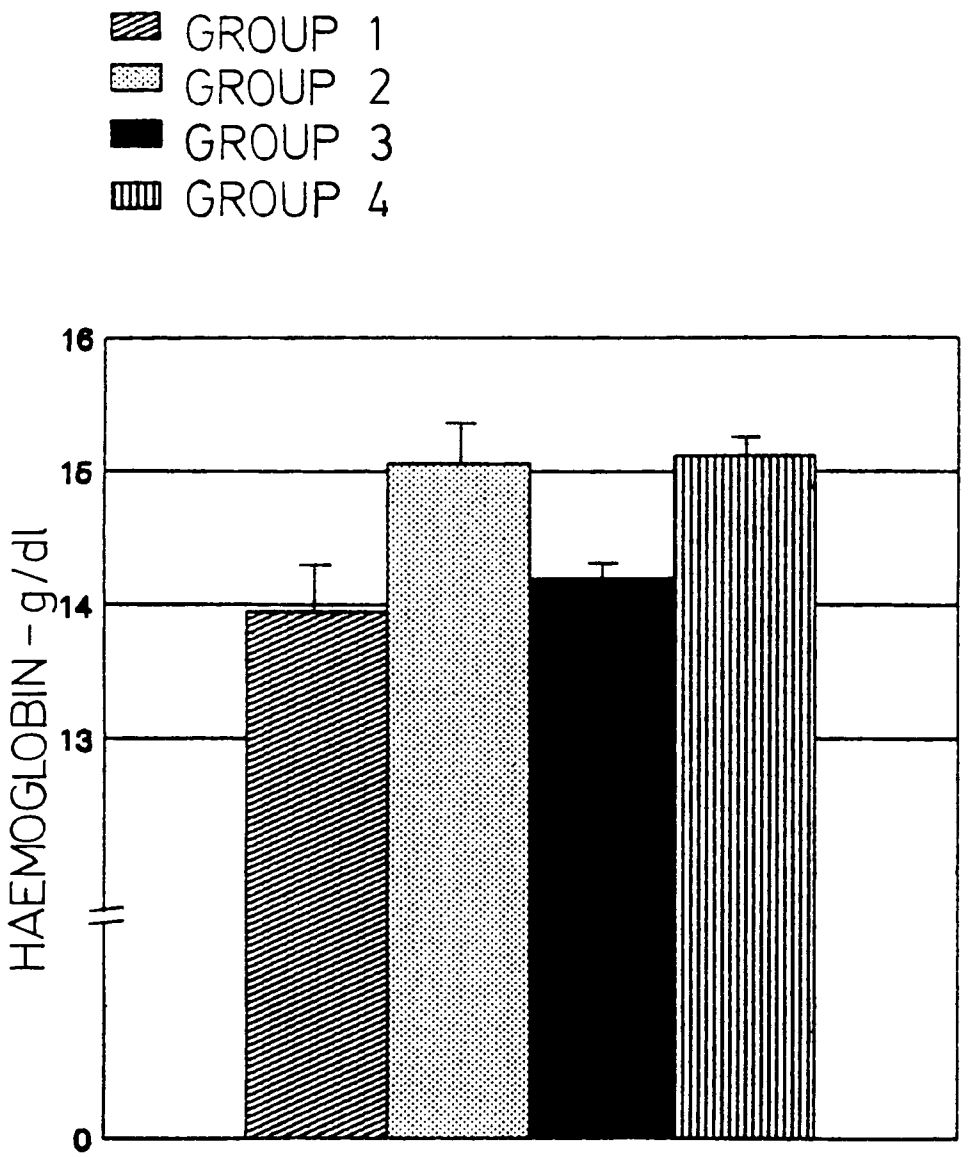


Fig.4.29 Haemoglobin concentrations in WHT mice treated as detailed in section 4.4.3.2.1.
The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars represent standard deviations.

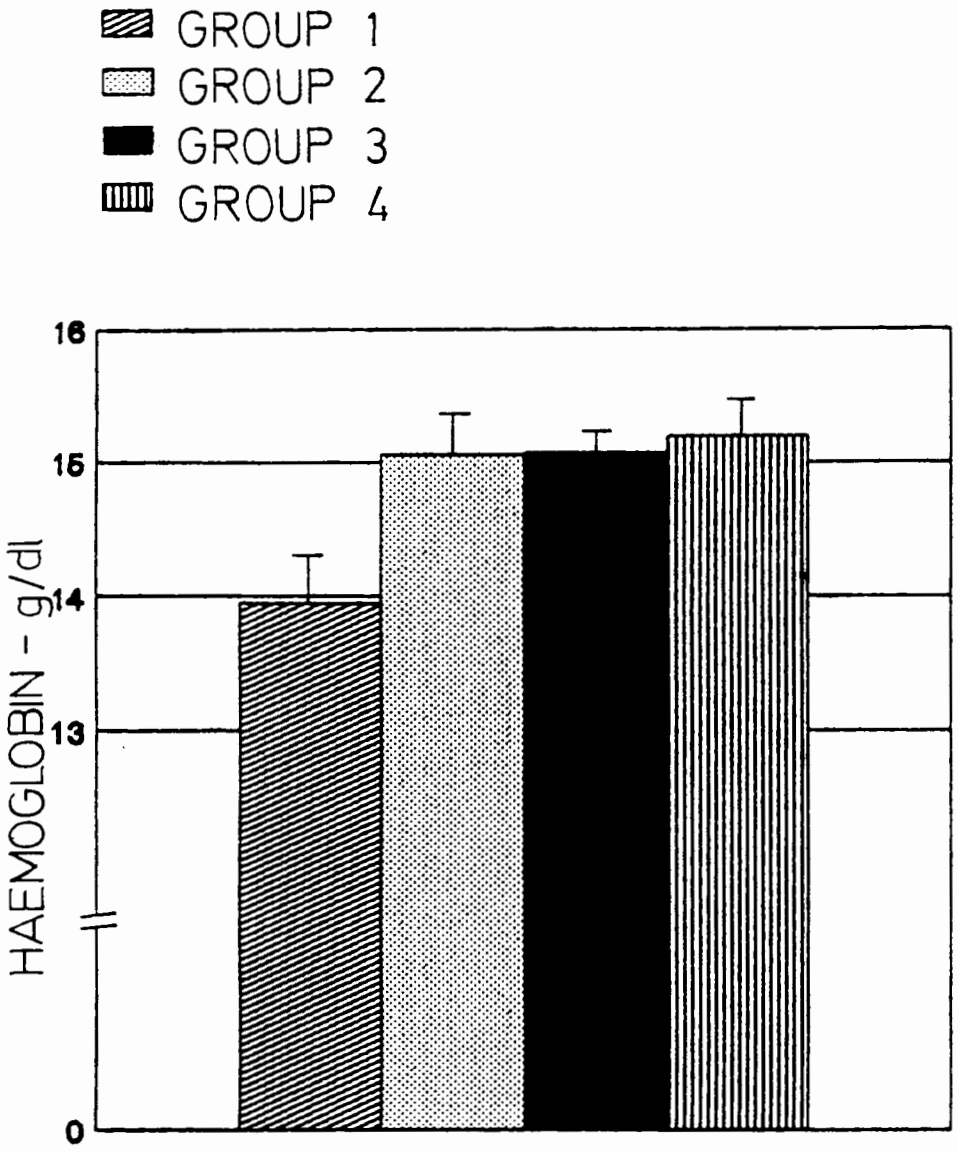


Fig.4.30 Haemoglobin concentrations in WHT mice treated as detailed in section 4.4.3.2.2.
The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars represent standard deviations.

4.4.4.1 2,3-DPG Concentrations of Mice Exposed to a Single Oxygen Concentration.

Red cell 2,3-DPG concentrations were determined in tumour-bearing WHT mice that had been exposed to different oxygen tensions for varying times as detailed in section 3.4.1.1. The mean 2,3-DPG concentration in 10 air-breathing mice was 4.18 +/- 0.14 umol/ml.

The 2,3-DPG concentrations of mice kept in 8% oxygen for 24, 48 and 72 hours are shown in Fig.4.31. The 2,3-DPG concentration for mice exposed to 8% oxygen for 24 hours increased significantly by 28.5% over that of the control value ($p < 0.001$). It continued to increase up to a maximum value of 37.8% that of the control at 48 hours ($p < 0.0001$). It then slightly decreased, for mice kept in 8% oxygen for 72 hours, to 0.97 times that of the 48 hour value. However, the level was significantly greater (33.7%) than that of the control group ($p < 0.001$).

The 2,3-DPG concentrations of mice kept in 10% oxygen for 24, 48 and 72 hours are shown in Fig.4.31. A significant increase of 26.6% over that of the control was noted for mice kept in 10% oxygen for 24 hours ($p < 0.001$). An increase to 29.2% at 48 hours ($p <0.001$) was observed, but 2,3-DPG concentrations fell to 14.1% in the case of mice kept for 72 hours in 10% oxygen, which was still significantly different from the concentrations in the control group ($p < 0.05$).

The 2,3-DPG concentrations of mice kept in 15% oxygen for 24, 48 and 72 hours are shown in Fig.4.31. The 2,3-DPG concentrations did not vary significantly compared with the control, at any of the times investigated ($p > 0.15$).

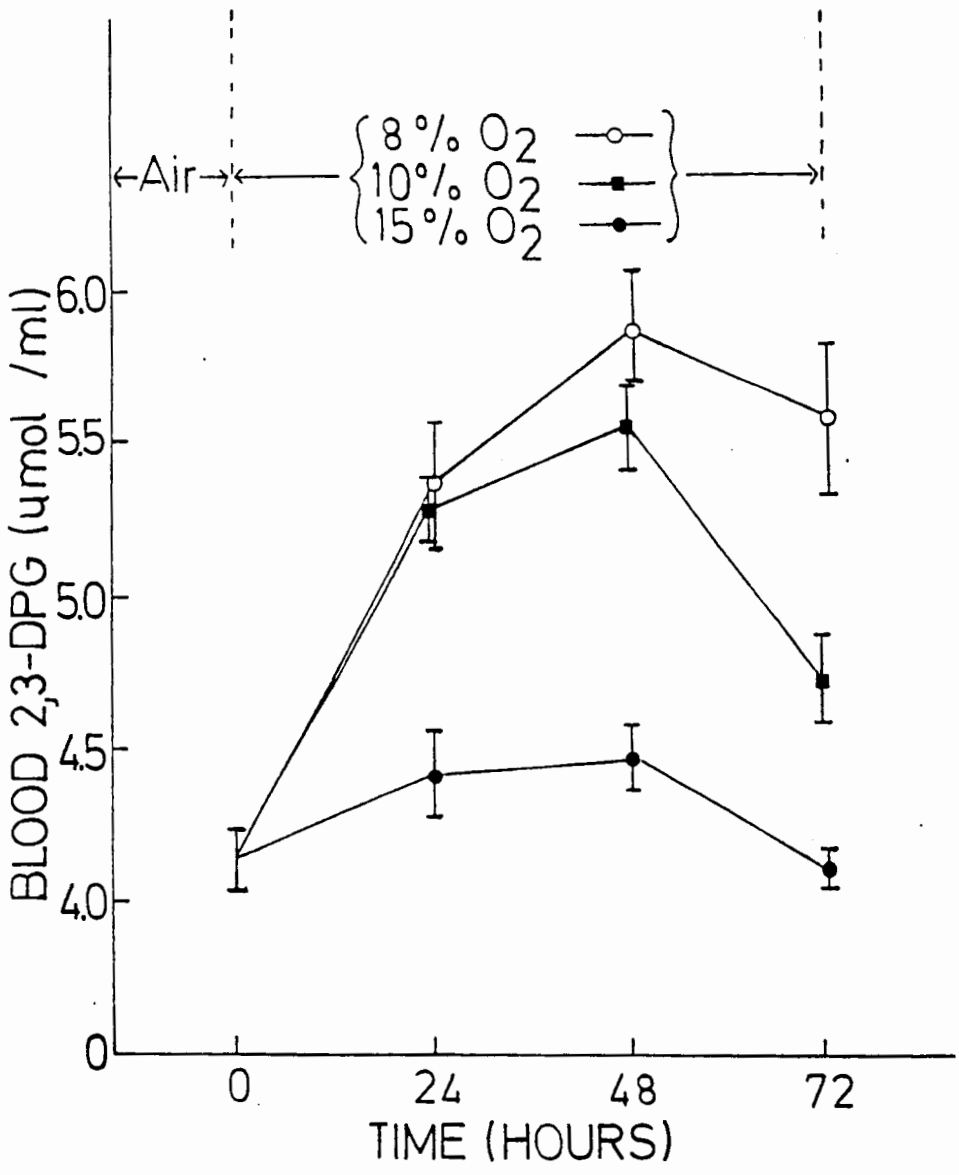


Fig.4.31 Blood 2,3-DPG values before and during exposure of WHT mice to low oxygen concentrations.
Each point is the mean of not less than 6 values. Standard deviations are represented by error bars.

4.4.4.2 2,3-DPG Concentrations of Mice Exposed Sequentially to 8% Oxygen and Air.

4.4.4.2.1 2,3-DPG Determinations 24.5 Hours after Completion of a 48 Hour Exposure to 8% Oxygen.

For these investigations, mice were divided into 4 groups:

Group 1 mice were air breathing.

Group 2 mice were exposed to 8% oxygen for 48 hours.

Group 3 mice were pretreated with 8% oxygen for 48 hours and then kept in air for 24.5 hours.

Group 4 mice were pretreated with 8% oxygen for 48 hours, then kept in air for 0.5 hours and then replaced in 8% oxygen for a further 24 hours.

The 2,3-DPG concentration of group 3 mice was not significantly different from that of the control group of air-breathing mice ($p > 0.5$). There was a significant difference in the 2,3-DPG concentrations between group 4 and air-breathing control mice as well as between groups 3 and 4 mice ($p < 0.05$). The 2,3-DPG concentrations in groups 2 and 4 were not statistically different ($p > 0.05$), but there was a significant difference between groups 2 and 3 ($p < 0.01$) (Fig.4.32).

4.4.4.2.2 2,3-DPG Determinations 4.5 Hours after Completion of a 48 Hour Exposure to 8% Oxygen.

For these investigations, mice were divided into 4 groups:

Group 1 mice were air breathing.

Group 2 mice were exposed to 8% oxygen for 48 hours.

Group 3 mice were pretreated with 8% oxygen for 48 hours and then were kept in air for 4.5 hours.

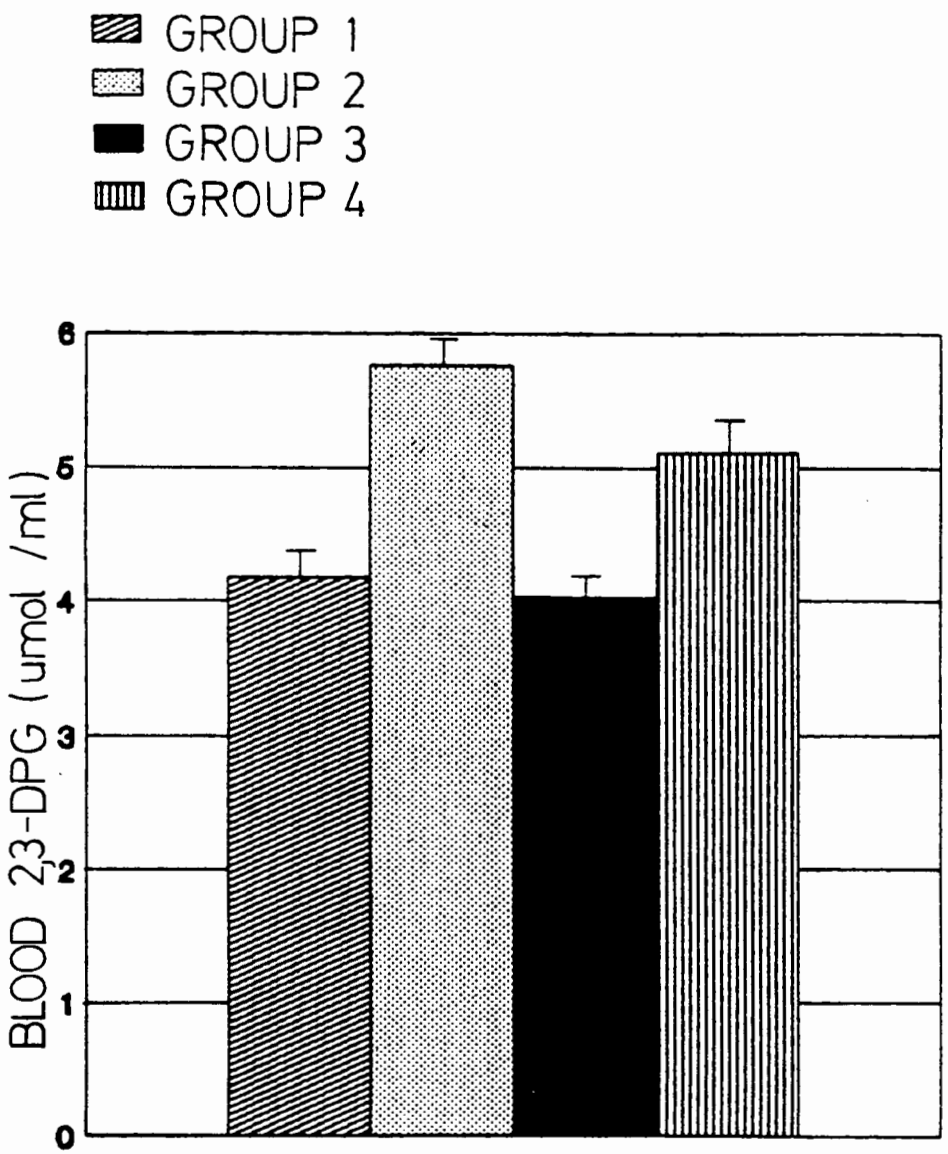


Fig.4.32 Blood 2,3-DPG values in WHT mice treated as detailed in section 4.4.4.2.1.
The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars represent standard deviations.

Group 4 mice were pretreated with 8% oxygen for 48 hours, then were kept in air for 0.5 hours and then returned to the 8% oxygen environment for 4 hours.

There was no significant difference in 2,3-DPG concentrations between groups 2, 3 and 4 mice ($p > 0.35$). However, groups 2, 3 and 4 mice all showed significantly increased concentrations of 2,3-DPG when compared to that measured in the air-breathing control group of mice ($p < 0.05$) (Fig.4.33).

4.4.5 Blood Oxygen-Haemoglobin Dissociation Curves.

The mice used for recording blood oxygen-haemoglobin dissociation curves were divided into 4 groups and treated in the following manner:

Group 1) Mice kept in 8% oxygen for 48 hours.

Group 2) Mice kept in 8% oxygen for 48 hours, then in air for 24.5 hours.

Group 3) Mice kept in 8% oxygen for 48 hours, then in air for 0.5 hours followed by a further 24 hours in 8% oxygen.

Group 4) Mice exposed only to an air-breathing environment.

Table 4.3 P_{10} and P_{50} values (+/- standard deviation) for WHT mice treated as detailed in section 4.4.5.

	P_{10}	P_{50}
Group 1	9.0+/-1.0	40+/-0.80
Group 2	5.5+/-0.05	34+/-0.18
Group 3	8.25+/-0.06	37.2+/-0.8
Group 4	5.3+/-0.47	33.5+/-0.29

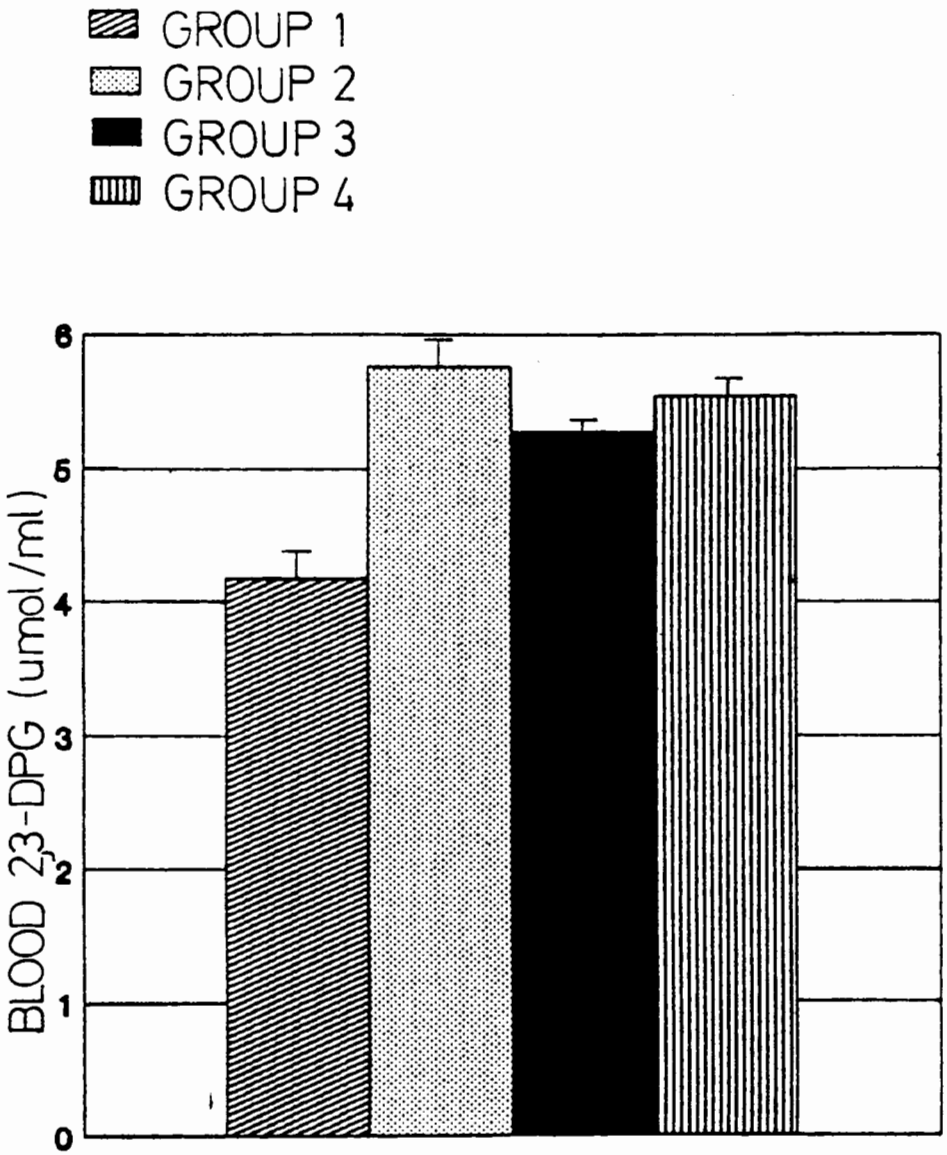


Fig.4.33 Blood 2,3-DPG values in WHT mice treated as detailed in section 4.4.4.2.2.
The heights of the histogram compartments represent the mean of not less than 6 determinations. Vertical bars represent standard deviations.

In the group of mice kept in 8% oxygen for 48 hours, P_{10} and P_{50} values were significantly increased over that obtained in the group of mice kept in air ($p < 0.05$ and $p < 0.001$ respectively). There was a significant difference in both the P_{10} and P_{50} values between the group of mice kept in 8% oxygen for 48 hours, then in air for 0.5 hours followed by a further 24 hours in 8% oxygen and the group kept in 8% oxygen for 48 hours, then in air for 24.5 hours ($p < 0.001$ and $p < 0.05$ respectively). The P_{10} and P_{50} values of mice kept in 8% oxygen for 48 hours and then exposed to air for 24.5 hours did not differ significantly from that of air-breathing mice ($p > 0.2$) (Table 4.3).

4.4.6 ATP Levels of B16 Melanoma Cells.

ATP levels were measured in B16 melanoma cells treated according to the protocols detailed in section 3.4.1.2. The ATP levels are expressed as a percentage of that determined in B16 melanoma cells exposed to pH 7.2 - 7.4 and incubated under aerobic conditions. The mean ATP level of these control aerobic cells from 8 determinations was 2.98 +/- 0.03 mol ATP/cell.

Cellular ATP levels of B16 melanoma cells were reduced to 36% that of control levels after a 16 hour exposure to hypoxia at pH 6.25 (Figs.4.34 and 4.35).

When cells were made hypoxic for 16 hours and then incubated under aerobic conditions at pH 7.2 - 7.4, ATP levels were observed to increase as a function of the duration of incubation in air. The exposure of hypoxic cells to a 5 hour incubation in air yielded ATP levels that were not significantly different from that of aerobic cells ($p > 0.1$) (Fig.4.34).

When cells were made hypoxic for 16 hours, then incubated under aerobic conditions for 0.5 hour and then re-exposed to hypoxia and low pH, the ATP concentration was not significantly different from that of cells exposed to hypoxia for 16 hours only ($p > 0.3$) (Fig.4.35). There was, however, a significant difference between the ATP levels of hypoxic cells that were reoxygenated for 5 hours and hypoxic cells that were reoxygenated for 0.5 hour but then exposed to a further hypoxic episode for 4.5 hours ($p < 0.05$) (Figs.4.34 and 4.35).

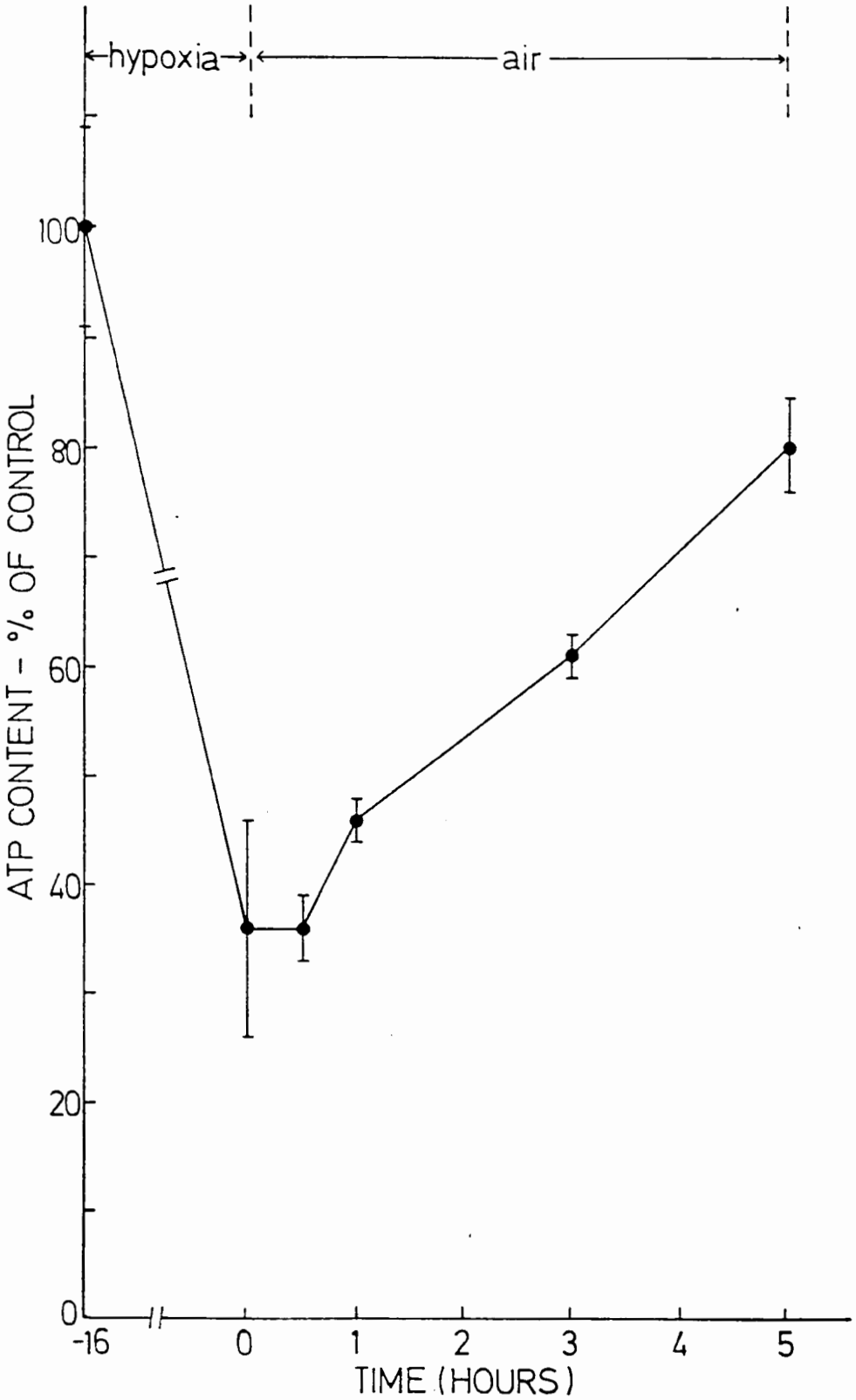


Fig.4.34 Relative concentrations of ATP in B16 melanoma cells incubated under the indicated conditions. ATP levels are expressed as percentage of controls. Each point is the mean of 5 values +/- standard deviation.

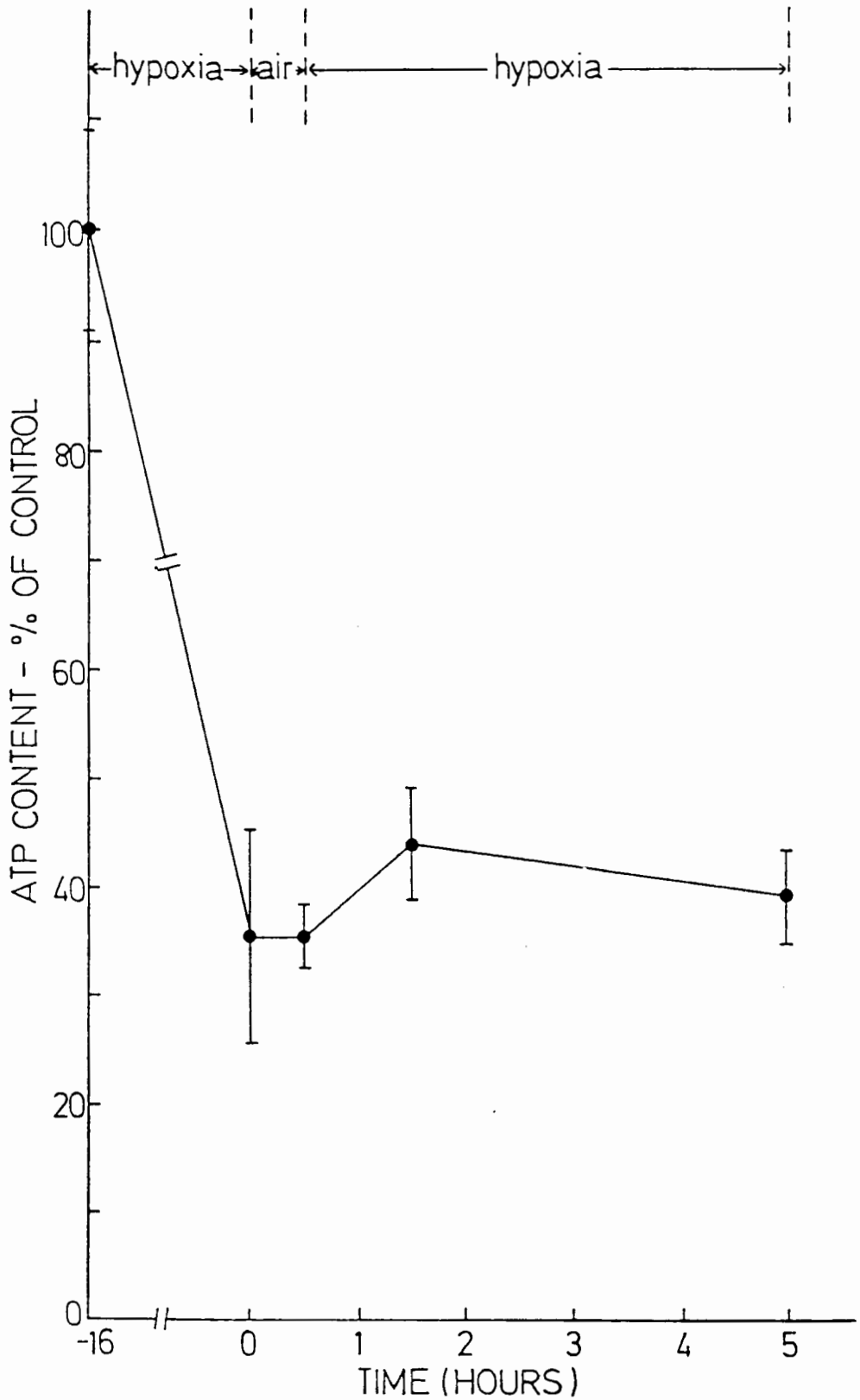


Fig.4.35 Relative concentrations of ATP in B16 melanoma cells incubated under the indicated conditions. ATP levels are expressed as percentage of controls. Each point is the mean of 5 values +/- standard deviation.

CHAPTER 5

ANALYSIS OF TWO FRACTION RADIATION STUDIES

It was perceived that in order to calculate the dose modifying factors and their errors for the various investigations involving two fractions of radiation as set out in Chapters 3 and 4, a fairly sophisticated method of analysis would be required. The development of this was kindly undertaken by Dr.R. Schall of the Institute for Biostatistics of the South African Medical Research Council.

The method represents a novel way of comparing the responses of tumour cells to different treatment regimens, and in view of this the full argument is set out in this Chapter.

Slope-Ratio versus Parallel Line Assays in Radiobiology.

A slope-ratio assay may be applied to estimate dose modifying factors in radiobiology.[154] The procedure adopted by Pike and Alper for calculating dose modifying factors involves fitting a regression line by least-squares to each set of observations, and then estimating the dose modifying factor by the ratio of the slopes of the regression lines. In a slope-ratio assay, the logarithm of the survival fraction (SF) is regressed against the dose of radiation. The survival curves are given by

$$\log SF = a + bD$$

where D is the dose of radiation, and a and b are parameters. When a dose modifying agent is present, the survival curves are described by

$$\log SF = a + pbD$$

where p is the dose modifying factor.

The standard assay for quantal responses, such as the survival or death of a cell under radiation, is the parallel line assay.[155] Here, the logit or some suitable function of the survival fraction (e.g. the probit) is regressed against the logarithm of the dose.

The Condition of Similarity.

If the response of any cell receiving a dose of radiation is u, then the mean or expected response may be written as $E(u) = U$. The mean response will depend on the dose through a regression function

$$(1) \qquad U = F(D)$$

For the purpose of this thesis, the response U will refer to the surviving fraction of a number of cells exposed to ionizing radiation. In attempting to determine the relative potency of a standard and a test procedure, where S is the standard and T is the test, the respective regression functions are given by

$$(2) \qquad U_S = F_S(D)$$

$$U_T = F_T(D)$$

For a given response U, doses D_S and D_T can be found such that the mean response to either is U. Thus $F_S(D_S) = F_T(D_T) = U$. The relative potency of D_T to D_S, at the level of response U, is then $p = D_S/D_T$. The ratio p is also called the dose modifying factor. At the level of response U, the effect of dose D under the test preparation is the same as that of dose pD under the standard preparation. If this relationship holds for all doses (at least over the range of practical interest), then

(3) $F_T(D) = F_S(pD)$

Equation (3) is the algebraic statement of the condition of similarity. When this condition is satisfied, then one can describe the relative effect of two stimuli by a single number, namely the relative potency p.

Slope-Ratio and Parallel Line Assays.

For many dose response relationships, the regression equation (1) can be written in the form

(4) $U = f(a + bD^c), \quad c > 0$

Here f is some known function and a, b, and c are parameters. For e -> 0, equation (4) becomes

(5) $U = f(a + b\log D)$

Setting $Y = f^{-1}(U)$, one can write

(6) $Y = a + bD^c, \quad c > 0$, or

$Y = a + b\log D, \quad$ for $c \to 0$.

Slope-Ratio Assay.

When c > 0 in equation (6), the regressions for the standard and test preparations are

(7) $Y_S = a_S + b_S D_S^c$

$Y_T = a_T = b_T D_T^c$

Under the condition of similarity, one has

(8) $Y = a_S + b_S D^c = a_T + b_T(pD)^c$

for all D. It follows that if a common intercept is assumed (three-parameter model[154]), then

(9) $a_S = a_T$ and

$b_S = p^c b_T$

The relative potency p is given by solving the equation

$p^c = b_S/b_T$

In the slope-ratio assay, the logarithm of the survival

fraction is regressed against dose. Thus $Y = f^{-1}(U) = \log(U)$, and $c = 1$, in that case.

Parallel Line Assay.

When $c \to 0$ in equation (6), the regressions for the standard and test preparations are

(10) $$Y_S = a_S + b_S \log D_S$$
$$Y_T = a_T + b_T \log D_T$$

Under the condition of similarity, one has

(11) $$Y = a_S + b_S \log D = a_T + b_T(\log p + \log D)$$

for all D. It follows that if a common slope is proposed, then

(12) $$b_S = b_T \quad \text{and}$$
$$a_S = a_T + b_T \log p$$

The two regression lines are parallel with slope $b = b_S = b_T$, and the relative potency is given by solving the equation

$$\log p = (a_S - a_T)/b$$

Thus, when regression is on the logarithm of the dose, then the condition of similarity leads necessarily to conditions (12). The linear regression of the logit of the survival fraction against the logarithm of the dose leads to a parallel line assay. Here $Y = f^{-1}(U) = \log(U/1-U)$, where $\log(U/1-U) = \text{logit } U$.

Advantages of the Parallel Line Assay for Quantal Responses.

The parallel line assay has two main advantages over the slope ratio assay for quantal responses.

Firstly, the logit of the survival fraction is unbounded below and above, so that the linear model in the parallel line assay is potentially applicable over all dose ranges.

For $0 \leq U \leq 1$ where U = survival fraction

Then $-\infty < \log U \leq 0$ (slope ratio assay)

Whereas $-\infty < \log(U/1-U) < \infty$ (parallel line assay)

The linear model in a slope ratio assay is only valid for fairly large doses. This implies that the extrapolation number in a slope ratio assay is not zero in practice. It should, however, be zero in theory because for zero dose one expects total survival.

Secondly, a regression of the logit of the survival fraction against the logarithm of the dose can theoretically be justified by the concept of the tolerance distribution.[156] An experimental unit such as a cell is assumed to have a certain tolerance, with the dose exceeding this tolerance leading to death. The distribution of tolerances in the population is called the tolerance distribution. In practice it is reasonable to assume a normal distribution for the log-doses,[157] as opposed to a normal distribution for the doses. Similarly, a logistic distribution for the log-doses is proposed, where a logistic tolerance distribution implies a regression involving the logit of the survival fraction against log-dose.

Table 5.1 compares the dose modifying factors obtained by

1) fitting lines through the data points by the method of least squares, then determining the dose modifying factor at different survival levels and calculating the mean dose modifying factor.

2) the slope ratio assay (Pike-Alper).

3) the parallel line assay (logit).

Table 5.1 Comparison of Dose Modifying Factors (DMF's) for two fraction irradiation regimens.

Control Treatment Scheme	Test Treatment Scheme	DMF (a)	DMF (b)	DMF (c)
(6)	(7)	1.17	1.14 (1.11-1.18)	1.19 (1.11-1.28)
(6)	(8)	1.21	1.18 (1.14-1.22)	1.23 (1.14-1.33)
(1)	(2)	1.14	1.12 (1.08-1.16)	1.11 (1.05-1.17)
(1)	(3)	1.32	1.27 (1.22-1.31)	1.34 (1.26-1.42)
(1)	(6)	1.11	1.10 (1.06-1.14)	1.10 (1.02-1.18)
(4)	(5)	1.04	1.02 (0.98-1.06)	1.05 (0.96-1.15)
(4)	(9)	1.13	1.08 (1.04-1.12)	1.14 (1.07-1.21)
(1)	(4)	1.26	1.26 (1.22-1.31)	1.23 (1.16-1.31)
(2)	(5)	1.15	1.15 (1.11-1.20)	1.18 (1.09-1.28)
(3)	(9)	1.09	1.10 (1.06-1.13)	1.08 (1.02-1.14)

(a) DMF was calculated at 16 survival levels and the mean DMF then determined.

(b) DMF was calculated by the Slope Ratio Assay.

(c) DMF was calculated by the Parallel Line Assay.

(See following page)

(1) Air irradiation, 48h-air pretreatment, 24h between fractions in air.

(2) Air irradiation, 48h-8% O_2 pretreatment, 24h between fractions in air.

(3) Air irradiation, 48h-8% O_2 pretreatment, 24h between fractions in 8% O_2.

(4) HBO irradiation, 48h-air pretreatment, 24h between fractions in air.

(5) HBO irradiation, 48h-8% O_2 pretreatment, 24h between fractions in air.

(6) Air irradiation, 48h-air pretreatment, 4h between fractions in air.

(7) Air irradiation, 48h-8% O_2 pretreatment, 4h between fractions in air.

(8) Air irradiation, 48h-8% O_2 pretreatment, 4h between fractions in 8% O_2.

(9) HBO irradiation, 48h-8% O_2 pretreatment, 24h between fractions in 8% O_2.

CHAPTER 6

DISCUSSION

There is little doubt that most tumours contain foci of hypoxic cells - cells which, although reproductively viable, may not be actively proliferating in their nutritionally deficient environment. It is also generally accepted that the radiocurability of tumours is limited by the increased resistance shown by these hypoxic cells to ionizing radiation.

It is pertinent at this time to re-introduce and further elaborate on the concept of the "tumour cord". Tumour cords may assume two possible geometrical arrangements. In the first type, the tumour parenchyma grow as multi-cell-layer, cylindrical cuffs that separate central, rather long capillaries from areas of gross necrosis.[126,158,159,160,161] Tumour cords with this type of architecture receive their nutrients by an outward diffusion process from a central blood vessel towards the necrotic tissue at the periphery of the cylinder. Tumour cords might, on the other hand, present as spheroidal or rod-like structures with a central core of necrosis and a rim of viable tissue surrounded by, but not penetrated by, blood vessels.[24] With this type of architecture, nutrient metabolites diffuse in towards the centre of the cord.

Oxygen has to reach the cell and penetrate within it by diffusion. The oxygen concentration around any particular cell will depend on a number of factors, which have been enumerated by Churchill-Davidson et al[162] as follows:

1) The coefficient of diffusion.
2) The oxygen tension in that part of the capillary which

constitutes the principle source of oxygen to the cell in question.

3) The rate of oxygen consumption of the intervening tumour cells. A number of workers[163,164,165,166] have found a practically constant rate of oxygen consumption from an air-saturated suspension down to one in which the oxygen concentration was very low indeed. Since larger cells have many more mitochondria, the oxygen consumption rate per cell will vary as a function of cell volume.[167]

4) The distance of the cell from the capillary.

From the analysis of tumour cords in bronchial cell carcinomas, Thomlinson and Gray[24] inferred that, because of progressive oxygen depletion, radioresistant hypoxic cells exist at the boundary between apparently intact cells and gross necrotic regions. Tannock[168] derived an oxygen-diffusion equation which enabled him to estimate the radial distance from a blood vessel at which the oxygen tension would fall to zero. This equation yielded tumour cord radii that accorded reasonably well with the measured cord radii of Thomlinson and Gray.[24] It also lent support to the postulate that histologically-intact cells adjacent to necrotic regions would have reduced oxygen tensions which in turn would render them resistant to the sterilizing effect of ionizing radiation.

It is important to point out that although oxygen deficiency is the most probable factor in causing necrosis, other factors may play a role. The interrelationship between the effect of oxygen and glucose concentrations on cellular growth and metabolism and the development of necrosis has been extensively investigated using the multicellular spheroid model. From these studies it has been shown that necrosis may develop in spheroids despite a sufficient oxygen[169,170,171] and

glucose supply.[172] Factors other than oxygen and glucose depletion such as the accumulation of injurious concentrations of hydrogen ions, lactate and ammonia may play a significant role in the development of necrosis.[173] If cell death in tumours is the result of the interaction of several mechanisms, then the theory of limited oxygen diffusion postulated by Thomlinson and Gray[24] is probably too simplistic a model to account for this phenomenon. However, anoxia undoubtedly is a major factor in the causation of the necrotic regions present in tumour cords. (Strictly speaking, anoxia implies the absence of oxygen. However, for the purposes of this thesis, the term has been extended to include those oxygen tensions at which cell viability no longer can be sustained for a length of time, the duration of which is contingent on the type of cell). An explanation for the cell death observed under conditions of anoxia is that the cells die of energy deprivation.[174] During anaerobic metabolism, which occurs during anoxic conditions, hydrogen ions are no longer removed in the mitochondria by oxidation but continue to be generated by glycolysis for a period of time. As a consequence, intracellular pH decreases until glycolysis is inhibited in part by a significant reduction of phosphofructokinase activity near pH 6.0.[175] The resultant reduction in tissue ATP is thought to contribute to cell death.

As mentioned previously, the oxygen tensions within a tumour depend, among other factors, on the oxygen tension in the blood. A reduction in the oxygen partial pressures along the capillary subtending a tumour cord should theoretically result in a decrease in the oxygen tension of all cells supplied by that capillary. Consequently, the length of the oxygen diffusion paths from the capillary will be shortened.

As a result it can, therefore, be hypothesized that the hypoxic/necrotic cell boundary will be brought closer to the capillary, i.e. some of the most distant (originally) hypoxic cells from the blood supply will now be even further deprived of oxygen and thus die in anoxia. This is the so-called "reduced tumour cord radius" model[74] (Fig.6.1). It would also be expected that the oxic/hypoxic cell boundary will be moved closer to the blood supply. With subsequent restoration of the oxygen partial pressures along the capillary to previous normal levels, the oxic/hypoxic cell boundary will temporarily revert, until tumour reconstitution takes place, to the position it occupied before reduction of the oxygen tension in the blood was effected. The overall result should be a reduction at that time in the proportion of radioresistant hypoxic cells and, therefore, an increase in tumour sensitivity to ionizing radiation.

A reduction in the oxygen tension in the blood can be accomplished by decreasing the partial pressure of oxygen in the inspired gas. Ways in which this can be achieved are by reducing the percentage of oxygen breathed from the normal 21%, by decreasing the pressure of the gas mixture from the normal 760 mm Hg or by a combination of the two.

In considering the "reduced tumour cord radius" model, the assumption is made that there are cells in tumours which may die because of their severely hypoxic status. It is also postulated that this premise may be exploited therapeutically by further reducing the oxygen delivery to the tumour. It is, therefore, important that the following question be raised: For what length of time do cells actually survive under the severely hypoxic conditions which may prevail at some tumour sites? The survival time of cells in monolayer culture during

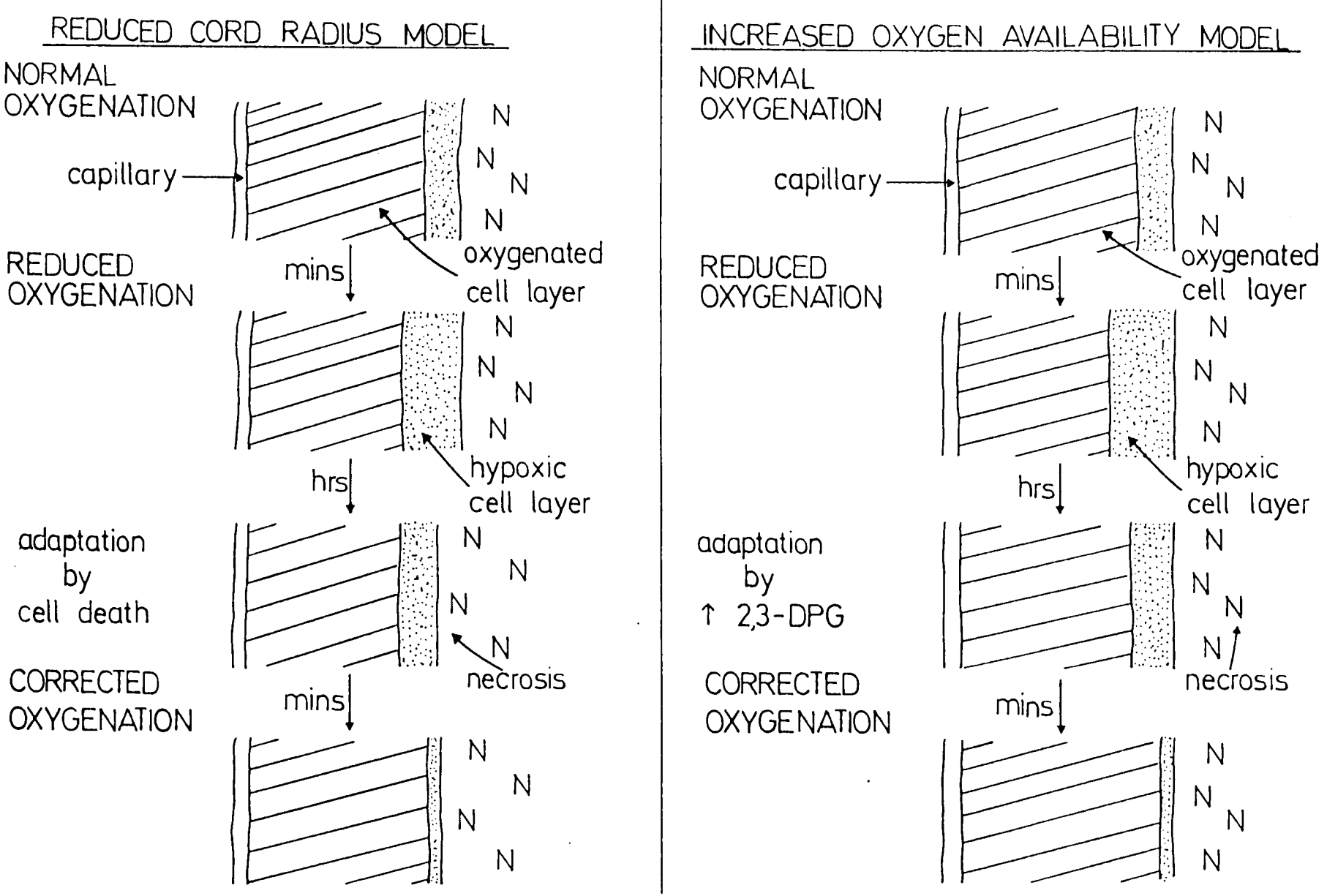


Fig.6.1 Illustration of the theoretical "reduced cord radius" and "increased oxygen availability" models.

exposure to extreme hypoxia has been studied extensively. Littbrand and Revesz[176] noted that ELD ascites tumour cells and Chinese hamster cells persisted in the absence of oxygen without suffering any lethal damage for 11 and 18 hours respectively. However, a gradual decrease in reproductive integrity started thereafter with less than 1% of both cell types surviving a 72 hour anoxic episode. Born et al[177] showed that a 48 hour exposure of B-14-FAF 28 Chinese hamster cells to hypoxia resulted in an 80 - 85% reduction in viability. Bedford and Mitchell[178] were able to demonstrate that less than 10% of the CHL-F strain of Chinese hamster cells survived a 52 hour exposure to hypoxia (70 ppm oxygen). The plating efficiency of HeLa cells was observed by Hall et al[179] to decrease by approximately 60% by storage for 60 hours under conditions of extreme hypoxia. In all these experiments the change in survival of hypoxic cells was based upon that of control cells kept for the same period of time in 95% air, 5% carbon dioxide. Franko and Sutherland[180] investigated the rate of death of hypoxic cells in the central regions of spheroids. From their results it was apparent that hypoxia caused rapid cell death in spheroids, with up to 32% of all cells becoming non-clonogenic within 6 hours.

As an exploratory experiment aimed at investigating the "reduced cord radius" hypothesis, mice were exposed to 8% oxygen for a period of 72 hours before receiving radiation. Any radiosensitizing effect that may be produced by this hypoxic pretreatment regimen was measured by an increased delay in tumour growth compared to that obtained when mice were kept in air prior to radiation. From the growth delays recorded in Figs.4.4 and 4.3, it is evident that an increased growth delay occurs in one tumour type (rhabdomyosarcoma in BALB/c mice),

but not in another (CaNT tumour in CBA mice) when radiation is preceded by exposing the host animals to a hypoxic environment for 72 hours. If the "tumour cord" model is invoked, then in the case of the rhabdomyosarcoma it seems plausible to postulate that the hypoxic pretreatment caused a reduction in the tumour's hypoxic cell fraction, which in turn resulted in an increase in tumour sensitivity to ionizing radiation, as reflected in the increased growth delay. One may ask why, if this postulate is correct, an improvement in the radiation response of the CaNT tumour was not observed in CBA mice pretreated with 8% oxygen for 72 hours. A possible explanation for the contrasting responses observed in the two murine tumours may lie in their different hypoxic cell fractions. For the rhabdomyosarcoma and the CaNT tumour, hypoxic cell fractions of 27% and 54% respectively were obtained using the clamped tumour growth delay method as the technique for measuring tumour hypoxic fractions. If the "tumour cord" model is considered, then pretreatment with 8% oxygen should also lead to a reduction in the fraction of hypoxic cells present in the CaNT tumour. It may be postulated that the absolute number of initially hypoxic cells dying in anoxia is similar in the two types of tumour. Since the CaNT tumour has a "large" initial proportion of hypoxic cells (54%), the death of some of these cells may not lead to a critical reduction in their proportion. One may expect (as in the rhabdomyosarcoma of the BALB/c mice) that this critical level is reached in a tumour where the initial hypoxic cell fraction is smaller, so that an enhanced radiation response will be observed. It might be felt that these observations should be treated with caution because, as pointed out by Moulder and Martin[137] and by Moulder and Rockwell,[181] the

techniques for measuring tumour hypoxic fractions require the use of several assumptions. Although these assumptions may lead to spurious hypoxic fraction estimations, they only really take on importance when citing absolute rather than relative hypoxic fraction values or when comparing the values derived by different hypoxic fraction assay techniques. It might, therefore, be fallacious to infer that the rhabdomyosarcoma in the BALB/c mouse has a hypoxic cell fraction of 27%, but quite valid to assert that the same tumour has 50% less hypoxic cells than the CaNT tumour in the CBA mouse.

It is noteworthy that 8% oxygen, as compared to air, had a significant inhibitory effect on the growth rate of both murine tumours over the 72 hour period that the mice were exposed to this hypoxic environment. One explanation consistent with this finding is that the breathing of 8% oxygen in nitrogen initially results in an increase in the proportion of hypoxic cells in the tumour. With time those hypoxic cells furthest from the blood supply die in anoxia. At this stage, although the thickness of the hypoxic cell component should be similar to that present in the tumours of air-breathing animals, the proportion of hypoxic to oxic cells will depend on the particular geometrical arrangement of the tumour cords. There is, however, little doubt that the number of oxic cells in each tumour cord is reduced by virtue of the oxic/hypoxic cell boundary being brought closer to the capillary. If the rate of cell proliferation is dependent on cellular PO_2 levels,[126,182,183] then a slower rate of tumour growth is to be expected when the host animal breathes 8% oxygen than when it breathes air.

When mice were returned to an air-breathing environment following a 72 hour 8% oxygen exposure, the time required for

their tumours to grow to four times their initial volume (initial volume being that measured immediately following removal of the mice from 8% oxygen) was only slightly longer than that recorded for mice exposed only to air. However, in view of this growth inhibitory effect produced by the hypoxic pretreatment, in all experiments care was taken to expose control mice to the same conditions of oxygen deprivation as the irradiated mice. Therefore, in the growth delay studies, any delay in tumour growth following radiation reflects solely the sterilizing effect of ionizing radiation on tumour cells and not any growth inhibitory effect resulting from the pretreatment conditions.

A striking feature of these investigations was the observation that no significant differences in growth delay were achieved in either of the murine tumours by the use of radiation under conditions of hyperbaric oxygen compared to radiation in air, regardless of the pretreatment. One explanation which may be put forward to account for this finding is that the vasoconstriction associated with hyperoxia causes a reduction in oxygen availability. Hence, the potential advantage of hyperbaric oxygenation, which is based on an increase in the oxygen carrying capacity of the blood in order to achieve increased tumour oxygenation, may be to a large degree negated. Hyperbaric oxygen has been shown to cause vasoconstriction in tumours,[184],[185] in retinal vessels,[186] as well as in brain[187] and to a lesser extent bowel.[188]

In the preceding discourse, the assumption has been made that the reduction in the fraction of hypoxic cells in a tumour resulting from a reduction in the oxygen tension in the blood is caused by the direct cytotoxic effect of anoxia. The

results from the growth delay studies employed in this study have been interpreted tentatively to support the "reduced cord radius" hypothesis.

An alternative explanation may be offered for the improved tumour response to radiation observed in animals exposed to a low oxygen environment. A number of adaptation mechanisms, such as an increase in the blood haemoglobin level and an increase in erythrocyte 2,3-DPG, become operative when animals are exposed to a reduced oxygen atmosphere. If these compensatory mechanisms are able to some extent to counter the reduction in oxygen delivery to the tumour caused by the lowered PaO_2, then an improvement in tumour oxygenation may be expected when the PaO_2 is restored to normal on returning the animal to an air-breathing environment ("increased oxygen availability" model[74] - see Fig.6.1). Consequently an improvement in the tumour response to ionizing radiation should occur. The effectiveness of this model is dependent on the proviso that adaptation mechanisms operating in animals exposed to low oxygen environments persist for some time when the animals are returned to an air-breathing environment. Siemann et al[3] have shown in mice that erythrocyte 2,3-DPG levels take 24 hours to return to normal on terminating the exposure of the animals to 12% oxygen. The time interval between restoration of the PaO_2 to normal and delivery of radiation, therefore, assumes critical importance. Furthermore, if the time interval is too long, other adaptation mechanisms such as an increase in tumour cell proliferation, which may occur in response to the improvement in oxygenation, could negate the effect of those adaptation mechanisms that were responsible for the improved oxygenation status of the tumour.[74]

As part of this study it was decided to investigate two physiological changes which allow animals to adapt to low oxygen environments, viz. changes in blood haemoglobin levels and changes in erythrocyte 2,3-DPG concentrations. As shown in Fig.4.31, it is particularly notable that the largest augmentation in 2,3-DPG occurred where mice were exposed to 8% oxygen as compared to 10% oxygen or 15% oxygen. It is also noteworthy that 2,3-DPG attained maximal levels following a 48 hour exposure to 8% oxygen. For each of the reduced oxygen environments investigated, haemoglobin levels were higher following a 72 hour rather than a 48 hour exposure period (Figs.4.26, 4.27 and 4.28). It is meaningful to point out that the time interval between terminating an exposure to a reduced oxygen environment and performing blood measurements for haemoglobin and 2,3-DPG was of a similar order as that allowed between ending a reduced oxygen exposure and commencingradiation.

In an attempt to analyse the response of the Fib/T tumour to ionizing radiation following exposure of WHT mice to different oxygen environments for varying time periods, an excision and in vitro colony forming assay was used. The Fib/T tumour arose spontaneously in a WHT mouse in 1964 in Dr.H.B. Hewitt's animal colony and has been maintained since then in the same inbred strain of mice.[189,190] The cells of the Fib/T tumour have adapted to in vitro growth and the composition of the tissue culture medium used is well established. Excision assays have a number of advantages over the tumour growth delay assay. One of these involves the ability to measure cell survival directly. Growth delay, though often taken to reflect the magnitude of clonogenic survival, is usually the result of this component and of a

component reflecting altered regrowth kinetics of surviving tumour cells. Other advantages include the greater accuracy of the excision assay which allows lesser amounts of damage to be assessed as well as giving finer resolution between treatments.[191]

There are, however, a number of factors that must be taken into account when carrying out excision assays,[192] factors which may reduce the validity of one's results. It is important that the cell suspension obtained by the dispersion technique provides an adequate random sample of the cells initially present in the tumour. Also as high a plating efficiency as possible is desirable (this may be promoted by the addition of trypan blue to cell suspensions prior to counting, which allows cells damaged by the disaggregation procedure with trypsin to be excluded from the count). In all excision assays carried out in these investigations, the plating efficiency of the cells from the untreated control Fib/T tumours varied between 43% and 61%. Another question to be considered is whether all colonies are of tumour cell origin. Stephens et al[193] have provided evidence that clonogenic host cells like macrophage progenitor cells can be grown from disaggregated animal tumours. I sought to avert this problem by including only those cells which on microscopic observation were of similar size and appearance.[194] It should be stated that all colonies from the Fib/T tumour excision assays appeared to be of a similar "compact" cellular nature, i.e. colonies consisted of tightly packed cells. Moreover, macrophages are supposedly clonable in agar only. It is, therefore, unlikely that macrophages would influence the colony count in the case of the Fib/T tumour, which is grown in monolayer. A further problem arises where excision assays are

used to assess cell survival following protracted treatments such as fractionated or low-dose-rateradiation. This will be dealt with in more detail later.

Using an excision assay, the response of Fib/T tumours in WHT mice exposed to different low oxygen environments for varying times prior to receiving single doseradiation in air was compared to that obtained where mice were kept in an air-breathing environment before and duringradiation (Figs.4.5, 4.6 and 4.7). It is revealing to correlate the observed DMF with the erythrocyte 2,3-DPG value for each pretreatment regimen. As shown in Fig.6.2, it can be seen that there is a trend for the DMF to increase as the 2,3-DPG levels increase. This finding is consistent with the notion that an increase in the erythrocyte 2,3-DPG concentration will cause haemoglobin to release extra quantities of oxygen to the tissues. Thus on restoring the PaO_2 to normal by returning the animal to an air-breathing environment, the oxygen diffusion paths from the tumour capillaries will increase in length. Oxygenation of some of the hypoxic cells in the tumour should take place and the reduction in the proportion of radioresistant hypoxic cells will be reflected by an increased sensitivity to ionizing radiation. The "increased oxygen availability" model appears to conform with the results obtained from the Fib/T studies. A shift of the oxygen-haemoglobin dissociation curve to the right was confirmed by a 19.4% increase in the P_{50} value obtained where WHT mice were pretreated with 8% oxygen for 48 hours (Siemann et al[195] observed a +/- 30% increase in the P_{50} value after a 48 hour exposure of C3H mice to 12% oxygen). Slowing of the tumour capillary blood flow as a result of vascular obstructions and the release of oxygen to other tissues may reduce the oxygen tension at the venous end of the

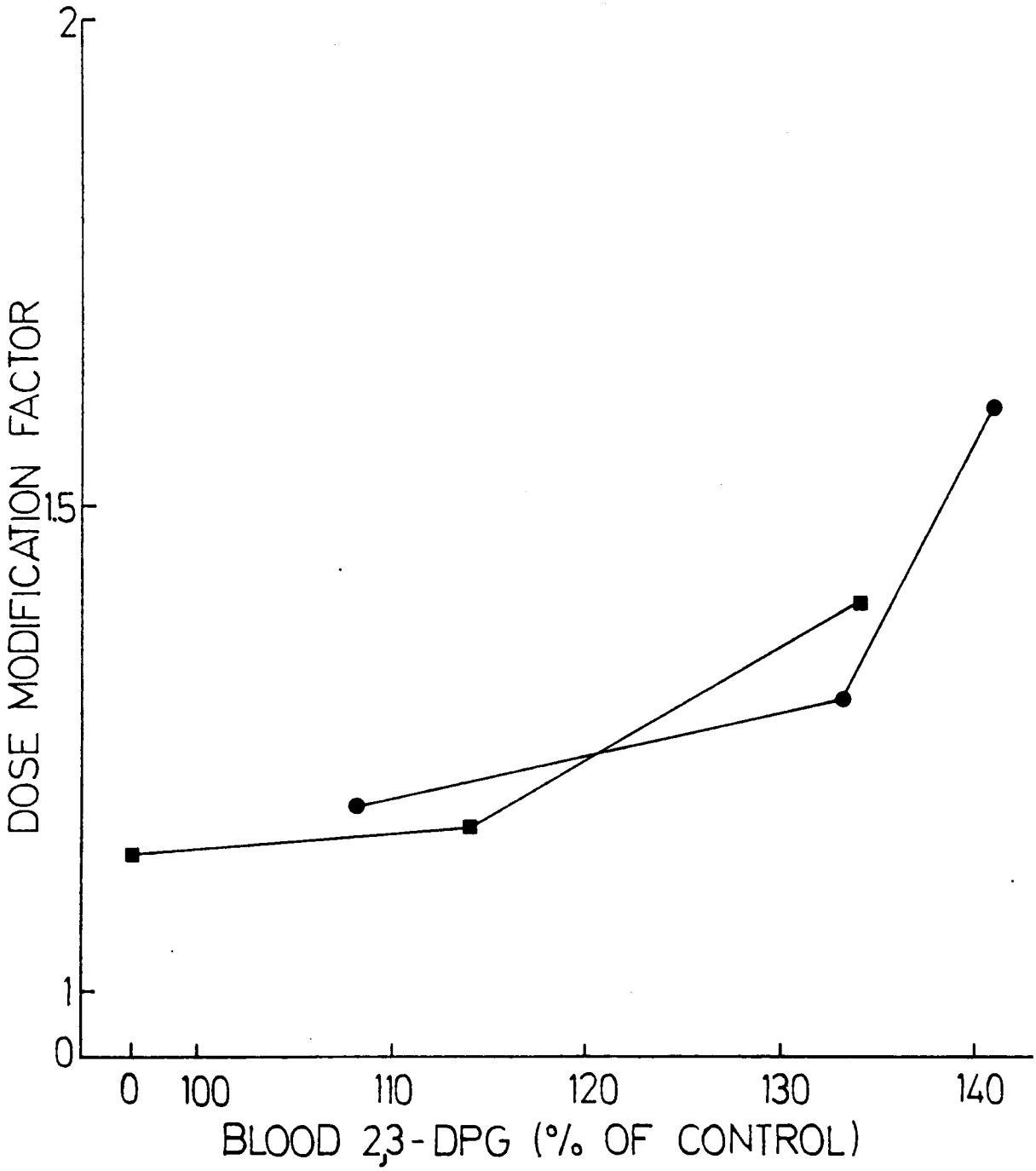


Fig.6.2 Change in DMF with 2,3-DPG levels. The 2,3-DPG levels (expressed as percentage of controls) were obtained from data relating to the 48h and 72h exposures of WHT mice to hypoxia, as shown in Fig.4.31.

capillary to far below the normal P_{50} value,[23] which in WHT mice was 34 mm Hg. It may, therefore, be more appropriate to assess shifts of the oxygen-haemoglobin dissociation curve by measuring changes in the P_{10} value. The P_{10} value was noted to increase by 70% after a 48 hour exposure of WHT mice to 8% oxygen.

It is tempting to draw the conclusion that the improved radiation response in the case of the Fib/T tumour studies is a consequence of improved oxygenation of the tumour which results solely from physiological adaptations occurring during exposure of the tumour-bearing mice to low oxygen environments. However, I am of the opinion that the findings presented in this study do not exclude the "reduced cord radius" model and consider that this mechanism probably also plays a role in the improved radiation response observed. The "reduced cord radius" model requires that during the period of low oxygen exposure, because of a reduction in the oxygen tension in the blood, some originally hypoxic cells furthest from the capillary will die in anoxia. It might be argued that adaptation mechanisms such as an increase in erythrocyte 2,3-DPG, which occurs rapidly (Lenfant et al[83] have shown that 50% of the increase in 2,3-DPG occurs within 15 hours upon ascent to high altitude), by making more oxygen available will restore the oxygenation status of the tumour back to normal. Siemann et al[196] have investigated the tumour response to radiation of mice in which the PaO_2 was chronically reduced by exposing the animals to a 12% oxygen environment. They found that, even if kept at low PaO_2 duringradiation, these animals exhibited the same tumour growth delay as air-breathing animals not exposed to the low oxygen environment. The authors conclude that by increasing their haemoglobin concentrations, the mice adapted

sufficiently to the low oxygen environment so that the effective oxygenation of their tumours was the same as that which would occur if the PaO_2 had been normal. It should, however, be pointed out that the low oxygen exposure times used by these investigators varied from 10 to 16 days. There is, on the other hand, evidence to suggest that adaptation mechanisms active during the period of a hypoxic exposure may not be capable of preventing a reduction in cell viability. Heath and Williams in their book "Man at high altitude"[81] state that with the drop in blood PO_2 levels that occurs at high altitude, the PO_2 in the tissues would be expected to fall below the critical level at which the mitochondria can function efficiently. This would occur in spite of the various mechanisms of adaptation discussed in Chapter 2. As a result, the survival of cells at the periphery of a cylinder of tissue being provided with oxygen from a capillary would seriously be endangered unless further factors facilitating diffusion to the mitochondria operated. Factors such as an increase in capillary density and an increased amount of myoglobin in the tissues represent long-term acclimitization mechanisms taking weeks to evolve and, therefore, will not play a role in the scenario where experimental animals are exposed to low oxygen environments for periods up to 72 hours. Tannock and Steel[126] demonstrated significant reductions in the mean distance separating blood vessels from regions of necrosis in mouse mammary tumours where the animals were kept in a 10% oxygen environment for periods up to two weeks. They conclude that hypoxia is a significant factor in the causation of necrosis. Further evidence to support the "reduced cord radius" model emerges from the plating efficiency experiments, the results of which are best interpreted by reference to Table 4.1. The

plating efficiencies of tumours from mice exposed to 8% oxygen for 24 or 48 hours were significantly less than that from mice exposed only to air. It seems reasonable to postulate that these observations are indicative of a progressive increase in tumour cell death occuring with time of exposure to 8% oxygen. It is possible that some of the cells that succumb to the conditions of extreme oxygen deprivation will become pyknotic and lyze before they can be counted in the haemocytometer. This will have the effect of spuriously elevating the plating efficiency - an effect which assumes greater significance as the time interval in hypoxia is increased. Where mice are kept in 8% oxygen for 48 hours, the difference between the actual plating efficiency and the measured plating efficiency may be greater than in the case where mice are exposed to 8% oxygen for 24 hours. The difference in the measured tumour plating efficiencies between mice pretreated with 8% oxygen for 24 hours and mice pretreated with 8% oxygen for 48 hours may, therefore, be larger than that noted in Table 4.1.

The proportion of anoxic cells that actually die during the period of exposure to 8% oxygen may, in fact, be small. There is evidence to show that a substantial degree of tissue damage resulting from anoxia occurs during the period of reoxygenation.[197,198] The reperfusion of anoxic cells with molecular oxygen results in their damage and much of this injury is thought to be mediated through the superoxide radical.

In summarising the relative roles played by the "reduced cord radius" and "increased oxygen availability" models, I feel that both models are important in improving the radiation response of tumours in animals given a low oxygen pretreatment. During the low oxygen pretreatment, the "reduced cord radius"

model predominates resulting in the death of some of the originally hypoxic cells furthest from the capillary, despite those physiological adaptation mechanisms aimed at improving tissue oxygenation. When the animal is returned to an air-breathing environment and the PaO_2 restored to normal, then adaptation mechanisms (e.g. increased 2,3-DPG) will have the effect of increasing the oxygen diffusion distance thus oxygenating some of the originally hypoxic cells closest to the capillary. The net result is an overall reduction, perhaps even elimination, of the chronically hypoxic cells in the tumour.

The temporary closure of blood vessels in tumours can produce acutely hypoxic cells.[199] These cells may be found in the well vascularized rim of tumours and consequently not be amenable to methods based either on the direct killing of chronically hypoxic cells, or on attempts to increase the oxygen diffusion distance from blood vessels.[200]

Hyperbaric oxygen has been used in an attempt to improve tumour oxygenation in both experimental animal tumours[201] as well as in clinical radiation therapy.[202,203,204,205,206] Suit and Maeda[207] have shown that hyperbaric oxygen is a potent modifier of the response of small tumours (0.6 mm^3 and less) to single-dose radiation, but not of large tumours. Hewitt[208] has brought evidence to suggest that the accessibility of oxygen to tumour cells is normally very restricted even in the presence of hyperbaric oxygen and pointed out that the classic paper[209] showing benefit from radiation in hyperbaric oxygen was concerned with a tumour having a small fraction of hypoxic cells. Fischer et al,[210] on

the basis of theoretical considerations, feel that hyperbaric oxygen, as generally used in radiation therapy, is unlikely to provide more than a marginal improvement in tumour oxygenation. Therefore, the expected benefits of hyperbaric oxygen may only be reaped whereradiation is delivered to tumours containing a small hypoxic cell fraction.

The response of the Fib/T tumour in mice pretreated with air was improved when theradiation was delivered in hyperbaric oxygen rather than in air. It is significant that where mice were irradiated in hyperbaric oxygen, pretreatment with 8% oxygen for 48 hours had little effect in improving the tumour response compared to a 48 hour 21% oxygen pretreatment. From Figs.4.10 and 4.5, it is apparent that hyperbaric oxygen (mice pretreated with air) is at least as efficient as a 48 hour 8% oxygen pretreatment (mice irradiated in air) in modifying the response of the Fib/T tumour to ionizing radiation. If the "increased oxygen availability" hypothesis is applied, then one can postulate that the fraction of hypoxic cells oxygenated as a result of physiological adaptations to the 8% oxygen pretreatment is similar to that oxygenated as a result of the increased blood PO2 induced by the inhalation of oxygen under high pressure. It may also be proposed that the hypoxic cells that respond to an 8% oxygen pretreatment occupy the same location within the tumour cord as the hypoxic cells that respond to hyperbaric oxygen - i.e. that region of the hypoxic zone closest to the blood supply. This would explain why a 48 hour pretreatment with 8% oxygen did not increase the sensitivity of tumours to radiation delivered while the animals breathed hyperbaric oxygen. From the Fib/T results, it seems as though a similar mechanism is involved in the case of both hyperbaric oxygen therapy and a hypoxic pretreatment in

improving the response of tumours to radiation, and that tumours with a "smaller" fraction of hypoxic cells should respond to either procedure. It might be suggested that if this is true, then hyperbaric oxygen, being the less time consuming, would be the treatment of choice. It must, however, be pointed out that the 3-MC-induced rhabdomyosarcoma in BALB/c mice, although responding to a hypoxic pretreatment, showed no increased sensitivity to ionizing radiation delivered while the animals breathed hyperbaric oxygen. If the response of tumours to a hypoxic pretreatment or to hyperbaric oxygen is dependent on the tumour hypoxic cell fraction, then it would be expected that the 3-MC-induced rhabdomyosarcoma, which has a smaller fraction of hypoxic cells than the Fib/T tumour, should be rendered more sensitive to radiation under conditions of hyperbaric oxygen than air. It is possible that other factors, as yet undetermined, might have a bearing on whether the sensitivity of tumours to radiation will be modified by hyperbaric oxygen.

A way of overcoming the problem of hypoxic cells in tumours is to use high linear energy transfer (LET) radiations. The application of fast neutrons in radiation therapy is based largely on the fact that the oxygen enhancement ratio (OER) is smaller for neutrons than for X-rays.[211] Although there is a tendency for the OER to rise slowly, from 1.6 to 1.7,[212,213] with increasing neutron energy, there is evidence that a significant reduction in OER occurs as the mean neutron energy is increased above 25 MeV. The OER of neutrons from 101 MeV protons on beryllium has been found to be 1.1 by Harrison et al.[214] The observation that OER varies inversely with LET can be explained on the basis of radiation chemistry.[215]

When one refers to radiation-chemical yields of primary species, what is meant is the number of free radicals, G_R (where R is H, OH, or e-aq) or molecules, G_M (where M is H_2O_2 or H_2), per 100 eV absorbed, present during the chemical stage of water radiolysis. Although these products are found in irradiated water irrespective of the type and energy of radiation, their amounts per 100 eV absorbed depend on the LET of the radiation. An increase of LET leads to an increase in the density of primary events per unit space and unit time. This favours reactions of recombinations of primary species. The value of G_M will, therefore, be expected to increase and G_R to decrease.[216] Oxygen chemically acts as a radiosensitizer only in the presence of free radicals. Because the radiochemical yield of radical products in the radiolysis of water decreases with increasing LET, one would predict that fast neutrons should be less affected by oxygen. It is, therefore, not surprising that the sensitizing efficiency of any procedure aimed at reducing the importance of hypoxic cells in tumours is partially vitiated by the use of fast neutron therapy.

Reference has already been made to some of the disadvantages associated with excision assays. The use of excision assays to assess survival following fractionated treatments deserves special consideration. A proportion of cells sterilized by the treatment may be lost before the excision assay is performed. As these cells would not contribute to the count of a cell suspension prepared from the treated tumours, an artificially increased surviving fraction would result thus making the treatment appear to have been less effective than it was. Cell proliferation between radiation fractions will further increase the value of the surviving

fraction, but this may be regarded as an expected consequence of fractionation and will influence the comparison of single dose treatments with multi-dose treatments regardless of the assay used.[191]

In all fractionated studies carried out with the Fib/T tumour, the time interval between delivery of the first dose of radiation and initiation of the excision assay was never allowed to exceed 24 - 25 hours. During the 24 hour post-radiation period, no change in cell recovery was observed by Hill[217] in the mouse KHT sarcoma. However, Jung et al[218] have shown that the number of sterilized tumour cells in the R1H rhabdomyosarcoma decreased immediately following a dose of X-radiation and that the decrease was exponential with a halving time of about 3.5 days. Interestingly, these investigators did not find evidence of a fraction of non-clonogenic tumour cells which were capable of dividing a few times before being eliminated. It should be kept in mind that even if the cell loss factor is small in the Fib/T tumour during the 24 hour period between radiation fractions, this phenomenon may argue against comparisons being made between surviving fractions of split-dose studies and surviving fractions of single-dose irradiations.

It is, however, suggested that an alternative approach be adopted for more protracted treatments. This involves expressing cell counts in terms of the number of cells per tumour instead of per ml of suspension. At the start of treatment the number of clonogenic cells per tumour is obtained from appropriate control tumours and after treatment the number of surviving clonogenic cells per tumour is obtained from treated tumours. The ratio of these two numbers is then the "tumour surviving fraction".[191,219,220]

Fig.4.12 illustrates that the pretreatment of mice with 8% oxygen for 48 hours increased the sensitivity of their tumours to a split-dose radiation regime (spaced by 24 hours) compared to where mice were kept in air prior to the first fraction of radiation. A likely explanation for this finding is that the hypoxic pretreatment, by reducing the fraction of hypoxic cells in the tumour, rendered the tumour more sensitive to the first dose of radiation. The question arises as to whether the sensitizing effect of the hypoxic pretreatment was retained for the 24 hour period during which the tumour-bearing animals were kept in air so that the second fraction of radiation was still delivered to a more sensitive tumour. From Fig.4.8 it is at once clear that some degree of improved radiosensitization is apparent in the tumours of mice which were exposed to 8% oxygen for 48 hours followed by air for 24 hours as compared to mice kept only in air. This finding cannot be ascribed to increases in either haemoglobin concentrations or 2,3-DPG levels which would result in an improved tumour oxygenation at the time of delivery of the second dose of radiation. The haemoglobin concentrations and 2,3-DPG levels of mice kept in an 8% oxygen environment for 48 hours and then in air for 24 hours were not significantly different to those of mice exposed only to an air-breathing environment (Figs.4.29 and 4.32).

However, it is of interest to discuss these findings in relation to the "reduced cord radius" hypothesis. An improvement in tumour radiosensitivity may result from a reduction in the tumour hypoxic cell fraction brought about by the direct cytotoxic effect of anoxia. As mentioned before, tumour cell proliferation that occurs after a low oxygen treatment will with time restore the hypoxic fraction of a

tumour to its original value. The possibility presents itself that at 24 hours after completion of a 48 hour 8% oxygen treatment, the tumour hypoxic cell fraction is still less than that of tumours in mice exposed only to air. Furthermore, on the basis of this explanation and according to the haemoglobin and 2,3-DPG data, it is to be expected that tumours would be less sensitve to the second fraction of radiation than to the first.

It is evident that for mice pretreated with 8% oxygen for 48 hours, returning them to this environment for the 24 hour period between radiation fractions resulted in a significant increase in radiation damage when compared to where mice were kept in air between radiation fractions (Fig.4.12). There are four possible ways of explaining this finding:

1) Enhanced tumour sensitivity to second dose of radiation.

When mice are kept in an 8% oxygen environment between radiation fractions, it is likely that the tumours were rendered more sensitive to the second dose of radiation when compared to the tumours of mice which were kept in air between fractions. From Fig.4.32 it is apparent that the 2,3-DPG levels of mice, which were returned to the 8% oxygen environment for a 24 hour period after spending 48 hours in such an environment with a half hour in air inbetween, were significantly higher than the 2,3-DPG levels of mice kept in air for 24 hours following a 48 hour exposure to 8% oxygen. The same can be said for the haemoglobin concentrations (Fig.4.29). Furthermore, the difference in 2,3-DPG levels was substantiated by significant differences in both the P_{50} and P_{10} values being observed between the two treatment regimens (Table 4.3).

2) Inhibition of repopulation.

Tumour cell repopulation entails the division and regrowth of cells which, after a treatment such as radiation, have retained their capacity for unlimited proliferation.[221] A number of studies have documented repopulation following radiation of experimental tumours.[222,223,224,225] The postulate that maintaining mice in 8% oxygen between radiation fractions inhibits the repopulation of cells that survive the first dose of radiation provides a possible explanation for the improved response to ionizing radiation observed in the tumours of this group of animals. This postulate is based on the premise that hypoxia inhibits cell proliferation. The rate of cell proliferation has been shown to be dependent on cellular PO_2 levels.[182,183] To test this hypothesis it was decided, following a 48 hour 8% oxygen pretreatment, to keep mice in air between radiation fractions but to reduce this radiation-free interval to 4 hours. The principle behind this reasoning is that little repopulation would occur in the shorter time period between the first radiation and the excision assay. Fig.4.15 reveals that in the case of mice pretreated with 8% oxygen for 48 hours, a 24 hour interval in 8% oxygen between radiation fractions had a similar effect to a 4 hour interval in air between radiation fractions. If it is accepted that the number of cells lost over a 24 hour period will exceed the number lost over 4 hours, then a misleadingly higher surviving fraction might result where the two radiation fractions are spaced by a time interval of 24 hours as compared to 4 hours. Thus the actual difference between the two, almost identical curves shown in Fig.4.15 may, in fact, be larger than indicated.

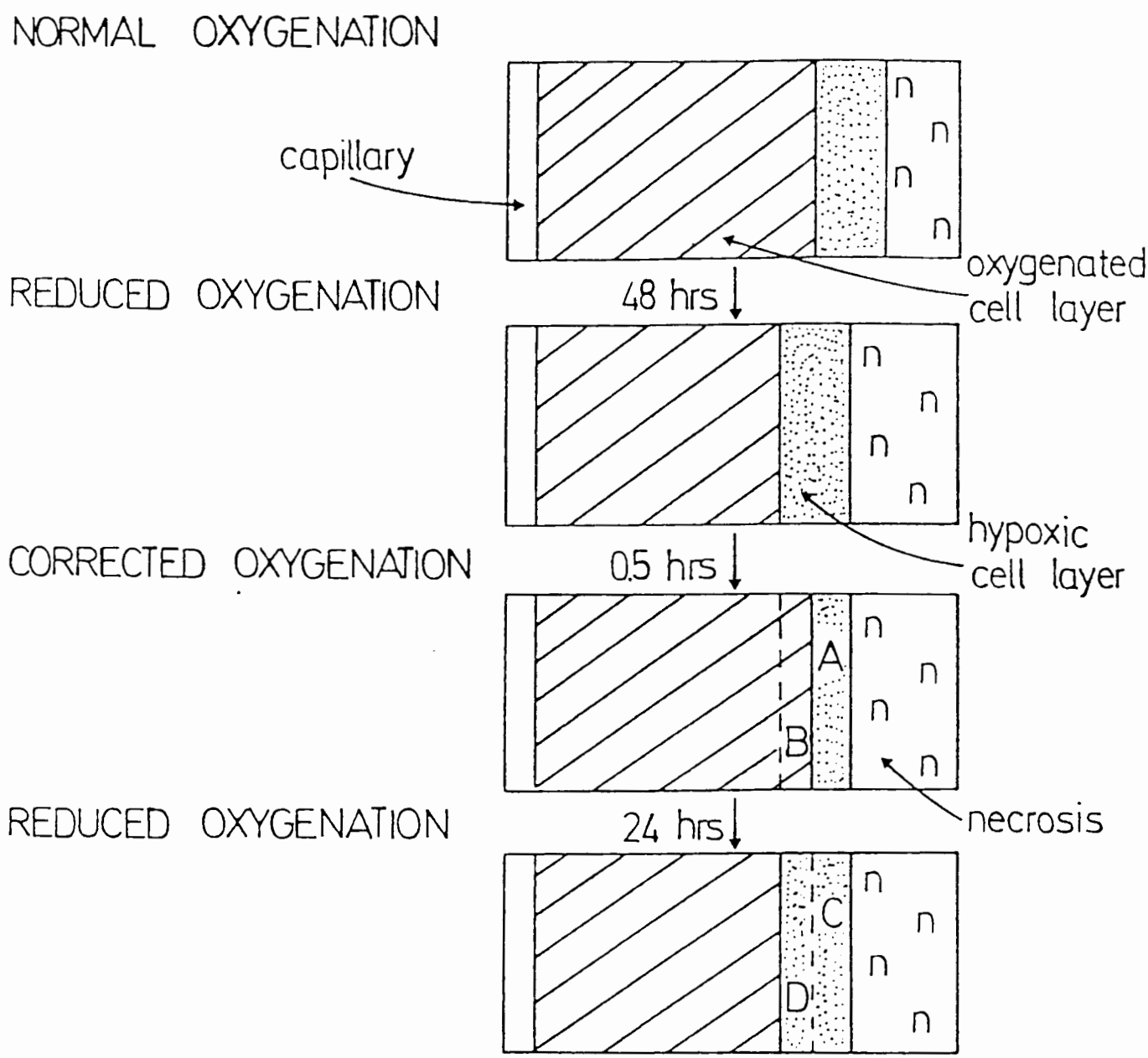


Fig.6.3 Proposed oxygenation status of "tumour cords" as a result of changes in the oxygen concentration breathed by host animals
A and C: Cells which were hypoxic at all times.
B: Cells which were oxic during and after delivery of the first fraction of irradiation. These cells were hypoxic before irradiation.
D: Cells which were oxic during delivery of the first fraction of irradiation, but were made hypoxic immediately following irradiation. These cells were hypoxic before irradiation.

It is possible that the 4 hour interval in air between radiation fractions results in the inhibition of a larger sub-population of repopulating cells than the 24 hour period in 8% oxygen. This can be more easily elucidated by reference to Fig.6.3, which is a schematic representation of a capillary and adjacent cell layers. I would like to postulate that, in the case of mice kept in 8% oxygen for 24 hours between radiation fractions, any inhibition of repopulation is probably limited to cell layers "C" and "D". Layer "C" consists of cells which were hypoxic before and after the first fraction of radiation and were also hypoxic during the time of delivery of the first fraction of radiation. A corresponding layer of cells ("A") does exist in the tumours of mice that were kept in air for the 24 hour period between radiation fractions. Although there is a gradient of oxygen tensions across both cell layers "A" and "C", one might expect that the mean PO_2 will be lower in layer "C". However, at the time of delivery of the first fraction of radiation, the oxygenation status of layers "A" and "C" is the same and, therefore, the same proportions of cells in layers "A" and "C" should survive. Following the first fraction of radiation, because of the proposed lower oxygen tensions existing in layer "C", it can be hypothesized that a greater inhibition of recruitment of surviving hypoxic cells from the quiescent into the proliferative pool will occur when compared to layer "A". Layer "D" consists of cells which, although being oxic at the time of delivery of the first fraction of radiation, were made hypoxic immediately following radiation. The low PO_2's existing here may slow down the movement of cells, which survive the radiation, through the cell cycle and thus inhibit some repopulation that may have occurred if the cells had higher oxygen tensions during the 24

hour period between radiation fractions. I am of the opinion that a reduction in the interval between fractions to 4 hours in air has a similar effect on the repopulation kinetics of cell layers "C" and "D" as a 24 hour interval in 8% oxygen. Cells that were chronically hypoxic during radiation (layer "C") or just before radiation (layer "D") would not be expected to progress from their position in Go (Go cells are resting cells that can be recruited into proliferation[226]) or early G_1 round the cell cycle and divide within 4 hours (Mc Nally[227] has estimated the cell number doubling time of Fib/T cells during exponential growth in cell culture to be 12-14 hours). Siemann and Keng[228] using fluorescence activated cell sorting, based on Hoechst 33342 stain diffusion, to characterize hypoxic cells have shown that chronically hypoxic cells situated near necrotic regions distant from blood vessels are in the resting phase of the cell cycle. Reducing the interval between radiation fractions to 4 hours may, in addition, inhibit the repopulation of some cells that survive the first dose of radiation but exist outside of layers "C" and "D". As a result of transient fluctuations in blood perfusion some cells, which are to be found in the well-vascularized rim of the tumour, may be acutely hypoxic and thus resistant to the sterilizing effect of ionizing radiation. A number of cells may survive a dose of radiation, not because of any lack of oxygen but because they occupy a more resistant phase in the cell cycle, e.g. the latter part of the S phase.[229]

3) Inhibition of repair.

The improved response of the tumours in mice that were replaced in 8% oxygen between radiation fractions may be due to hypoxia associated inhibition of repair of radiation damage. Is there evidence to suggest that the repair of radiation

damage is inhibited by hypoxia? A number of investigators have studied the effects of hypoxia on the cellular repair of radiation damage using both in vivo animal systems[230,231,232] as well as cells in culture.[233,234,235] A general finding was that repair was inhibited if the hypoxic conditions were maintained both during and after radiation. This may account for the experimental finding that chronically hypoxic cells are more radiosensitive than acutely hypoxic cells.[236] It has also been shown that for hypoxia to effectively inhibit repair, stringent levels (<16 ppm of oxygen) of hypoxia are necessary.[234]

If the role of extreme, prolonged hypoxia in inhibiting repair is considered, then any inhibition of repair that may occur in the tumours of mice kept in 8% oxygen between radiation fractions will be restricted to cell layer "D", and possibly "C", depicted in Fig.6.3. Because the mean oxygen tension would be lower in "C" than "A", a greater inhibition of repair may occur in "C", although the cells of both layers are chronically hypoxic. However, it is suggested that the greater differential in repair inhibition is to be found between cell layers "B" and "D". Anaerobic glycolysis, which occurs in hypoxic cells, leads to the accumulation of lactic acid with a resultant decrease in pH. As hypoxic regions of tumours may have pH values in the range of 6.0 - 6.5,[237,238] an attempt was made to reproduce these conditions in vitro[174] (See section 3.4.1.2.1). The rationale of these experiments was to measure cellular ATP, which may be related to repair, in hypoxic cells that were subsequently oxygenated (cell layer "B") or in hypoxic cells, which after being oxygenated, were re-exposed to hypoxia (cell layer "D"). A number of authors have intimated that ATP is implicated in the repair of

radiation-induced damage.[239,240,241,242] If ATP is involved in the repair of radiation damage, then the lower ATP levels, which were determined in hypoxic cells that were re-exposed to hypoxia after a brief period of oxygenation (Fig.4.35) as compared to hypoxic cells that were subsequently re-oxygenated (Fig.4.34), could account for the greater repair inhibition that is proposed to occur in the tumours of those mice kept in 8% oxygen between radiation fractions.

The validity of this proposal was further tested on mice pretreated with 8% oxygen for 48 hours by reducing the time interval between radiation fractions to 4 hours and comparing the tumour response to radiation of mice kept in 8% oxygen between fractions with that of mice kept in air between fractions (Fig.4.11). If it is assumed that little, if any, repopulation will occur in this 4 hour period, then it follows that the increase in radiation damage resulting from retaining the 8% oxygen environment between radiation fractions cannot be due to an inhibition of repopulation. Furthermore, the increase in radiation damage to the tumours of mice kept between radiation fractions in 8% oxygen instead of air cannot be explained on the basis of increases in either haemoglobin concentrations (Fig.4.30) or 2,3-DPG levels (Fig.4.33). It is, therefore, possible to conclude that the left shift of the cell survival curve to lower doses in the case of mice kept in 8% oxygen for 4 hours between fractions may be due to the inhibition of repair of radiation damage.

This small shift may imply that the inhibition of repair is of lesser importance in accounting for the improved tumour response observed in mice which, after being pretreated with 8% oxygen for 48 hours, were kept for 24 hours between radiation fractions in 8% oxygen rather than in air. However, this may

not necessarily be true - for two reasons:

i) It must be borne in mind that in the case where the two fractions of radiation are delivered spaced by a time interval of 4 hours, any inhibition of repair by the hypoxia associated with the 8% oxygen environment will be limited to radiation-induced damage that normally would be repaired within 4 hours. There is evidence that not all repair of radiation damage is complete within 4 hours. Current radiobiology teaches that there are two kinds of radiation damage that can be repaired, viz. sublethal and potentially lethal damage. However, the terms "sublethal" and "potentially lethal", although being convenient operational definitions, may or may not refer to different types of repair mechanisms or different types of lesions. The time period required for the repair of sublethal damage is of the order of 1-2 hours.[243],[244] The time period required for the repair of potentially lethal damage is longer than for sublethal damage. Hahn et al[245] have estimated the half-life for repair of potentially lethal lesions in the NCTC 2472 fibrosarcoma and EMT6 mammary sarcoma after a dose of 20 Gy X-rays to be 2 to 4 hours. Repair was complete within about 5 hours. The repair of potentially lethal damage in Lewis lung carcinoma cells has been shown to be nearly complete in 8 hours after radiation with ^{60}Co gamma rays.[246] Chang liver cells, irradiated in vitro, required 3-6 hours to repair potentially lethal damage.[247] Marchese et al[248] investigated potentially lethal damage repair in three malignant tumour cell lines (breast, colon, neuroblastoma) and two normal diploid lines (lung and fibroblasts). The results indicated that potentially lethal damage repair was complete within 6 hours.

ii) When an asynchronous population of cells is exposed to a dose of radiation, cells in the most sensitive phases of the

cell cycle are reduced to a lower surviving fraction than cells which at the time of radiation are in a relatively resistant phase of the cell cycle. The surviving population of cells, therefore, tends to be partly synchronized.[249] Where mice are exposed to two doses of radiation separated by an interval of 4 hours, following the first dose the cohort of surviving cells may progress around the cell cycle and may be exposed to the second dose while in a sensitive period of the cell cycle - that is if the mice are kept in air between radiation fractions. It is possible that where mice are kept in 8% oxygen between radiation fractions that this age response distribution of cells is altered by the period of hypoxia. As a result the cells may be in a more resistant phase of the cell cycle when the second dose of radiation is delivered than where mice are kept in air between fractions. This, in the case of the split dose experiment spaced by 4 hours, may partially oppose the tumour-sensitizing effect that the hypoxia, by inhibiting repair of radiation damage, is purported to produce.

4) Inhibition of cell loss.

A proportion of cells that are sterilized by the first dose of radiation may be lost during the 24-25 hour period before the tumours are excised. This will have the effect of spuriously elevating the surviving fraction. When mice are retained in 8% oxygen for the 24 hour interval between fractions instead of in air, an inhibition of cell loss may occur. This then may contribute to the finding that there was an increase in radiation damage to the tumours of mice which, following a 48 hour pretreatment with 8% oxygen, were kept in 8% oxygen during the 24 hour interval between radiation fractions instead of in air. It has been proposed that the cell loss factor for hypoxic cells, because of their

nutritionally deprived state, will be expected to be greater than for oxic cells.[217]

In the case of mice receiving two fractions of radiation in hyperbaric oxygen, spaced by an interval of 24 hours, the proportion of tumour cells surviving after radiation was markedly reduced when compared to the proportion of cells surviving after radiation in air, irrespective of whether the pretreatment and inter-fraction environments were 8% or 21% oxygen (Figs.4.17, 4.18 and 4.19). It was interesting to observe that if mice received two fractions of radiation, both delivered under conditions of hyperbaric oxygen, a 48 hour hypoxic pretreatment did not significantly alter the tumour response to radiation when compared to a 48 hour pretreatment with air (Fig.4.16). However, where mice were pretreated with 8% oxygen for 48 hours and then returned to this environment for the 24 hour interval between radiation fractions, a significant increase in tumour cell kill was noted (Fig.4.16). It is proposed that this result may be explained on the basis of the 24 hour hypoxic interval producing an inhibition of repair, repopulation and cell loss, as discussed before. If it is accepted that hyperbaric oxygen is a more efficient sensitizer than an 8% oxygen treatment, then it is unlikely that the exposure of mice to 8% oxygen for the 24 hour period between radiation fractions, as compared to air, will increase the sensitivity of the tumours to the second dose of radiation, where the second dose of radiation is delivered in hyperbaric oxygen.

An unexpected finding was that the tumours of mice that were pretreated with air but exposed to 8% oxygen for the 24 hour period between radiation fractions did not show an

improved response to radiation when compared to the tumours of mice that were similarly pretreated and then kept in air between fractions. This result was independent of whether radiation was delivered in air or in hyperbaric oxygen. One would envisage, where mice were kept in 8% oxygen for 24 hours between radiation fractions, that an increase in tumour cell kill would occur as a result of a hypoxia-associated inhibition of repair, repopulation and cell loss. Furthermore, it is to be expected that the 24 hour inter-fraction hypoxic exposure would render tumours more sensitive to the second dose of radiation. This expectation was supported by the finding that where mice were either pretreated for 24 hours with air or with 8% oxygen, the 8% oxygen exposure caused a left shift of the cell survival curve to lower doses, such that the DMF was 1.36 (Fig.4.5).

It is not possible to offer a definite explanation for the finding that, following a 48 hour pretreatment with air, the exposure of mice to 8% oxygen for the 24 hour interval between radiation fractions did not increase tumour radiation damage as compared to where mice were kept in air between fractions. The reason for this can only be surmised and it may be related to tumour reoxygenation.[250] In a two-fraction radiation therapy regimen, the first dose of radiation will be expected to eliminate preferentially the well-oxygenated cells from the tumour. Consequently, there is a sharp increase in the hypoxic fraction of the tumour. It has, however, been demonstrated in a number of animal tumours that the hypoxic fraction then falls again as a result of reoxygenation returning to nearly pre-irradiated levels.[251,252,253] Reoxygenation, therefore, enables those cells that survived the first dose of radiation because they were hypoxic at the time

of exposure to be more radiosensitve at the time of delivery of the second dose of radiation. If it is hypothesized that reoxygenation is impeded by exposing mice to an 8% oxygen environment following delivery of the first fraction of radiation, then it follows that this may be responsible for negating any increase in tumour cell kill that would be expected to result from keeping mice in 8% oxygen for 24 hours between radiation fractions. It is reasonable to assume that where mice were pretreated with 8% oxygen for 48 hours, which results in a reduction of the tumour hypoxic cell fraction, reoxygenation would be of lesser importance following the first dose of radiation than where pretreatment was with air. As a consequence, if mice pretreated with 8% oxygen for 48 hours are then retained in this hypoxic environment for the 24 hour period between radiation fractions, the increase in tumour cell kill resulting from the inter-fraction hypoxic exposure would be expected to exceed any radioprotective effect which the hypoxic environment, by inhibiting reoxygenation, may induce.

This thesis also included studies of the effects of hypoxic treatments of host WHT mice bearing the Fib/T tumour on various substances and enzymes related to the control of oxygen toxicity. Radiation-induced radicals produced in critical molecules like DNA undergo competitive reactions either with oxidizing species such as oxygen or with reducing species such as non-protein thiols. Reaction with oxygen leads to damage "fixation" whereas reaction with non-protein thiols leads to damage "repair" followed by continued cell viability. Hypoxic protection is, therefore, the result of a reaction between a short-lived radiation-induced radical and a sulfhydryl compound in which the sulfhydryl donates a hydrogen atom.[254,255] The

cellular non-protein thiol glutathione has been regarded as the main endogenous reducing agent responsible for restoration of radiation-induced lesions by hydrogen donation.[256] Another approach to increasing the sensitivity of hypoxic tumour cells to radiation would be to decrease their endogenous radioprotective capacity.[257]

Glutathione may be depleted as a result of inhibiting its biosynthesis using buthionine sulfoxamine, a selective inhibitor of glutamylcysteine synthetase.[258] Diethyl maleate is able to deplete glutathione levels via a glutathione-transferase catalysed covalent binding with glutathione as well as by spontaneous conjugation with glutathione.[259] There have been numerous demonstrations that glutathione depletion results in dose-modifying sensitization to radiation under hypoxic conditions.[260,261,262]

It appears that glutathione reacts about thirty times more slowly than oxygen with radiation-induced radicals. This is supported by the finding that oxygen can compete with a concentration of glutathione thirty times as large.[255] The difference in rate constants between fixation of radical damage by oxygen and repair by glutathione predicts that glutathione will have no effect on aerobic cell sensitivity to ionizing radiation. However, Russo et al[263] have suggested that glutathione may contribute to the aerobic radiation response through its link with glutathione peroxidase in the detoxification of hydrogen peroxide and organic hydroperoxides produced by radiation. Under aerobic conditions, oxygen forms peroxy radicals to "fix" the damage produced by radiation. Biaglow et al[264] have proposed that the peroxy radicals are not always lethal to the cell and that glutathione can chemically reduce these radicals by hydrogen donation to produce

hydroperoxides which then may be further reduced by glutathione in the reaction catalysed by glutathione peroxidase. Aerobic radiosensitization has been observed for cells depleted of glutathione by buthionine sulfoxamine or by diethyl maleate.[265,266,267]

One of the means by which radiation may produce damage to biologically important molecules is by producing peroxides. Hydrogen peroxide is produced in a reaction catalysed by superoxide dismutase, with the superoxide radical as substrate. Catalase has long been considered to be the primary scavenger of intracellular hydrogen peroxide but it is now accepted that glutathione peroxidase is as, if not more, efficient.[268] In intact cells the activity of glutathione peroxidase is sustained by the continuous reduction of oxidized glutathione by glutathione reductase. Glutathione peroxidase not only reduces hydrogen peroxide as fast as catalase but other hydroperoxides in addition. Hydroperoxide intermediates are known to be toxic and may lead to malondialdehyde which is toxic and mutagenic. Enzymes interacting with peroxides may, therefore, decrease the incidence of mutagenetic events. Thymine hydroperoxide has been shown to be mutagenic and is a substrate of glutathione peroxidase.[269] Glutathione peroxidase is not only involved in protection against radiation mutagenesis but also to whole body radiation.[270]

If a decrease in the activity of the scavenger enzymes, catalase and glutathione peroxidase, or in the content of glutathione was determined where mice were pretreated with 8% oxygen for 48 hours, it may have been possible to explain the improved tumour response to radiation observed in this group of animals on the basis of increased levels of radiation-induced radicals or hydroperoxides. This, however, proved to be not

the case. It should be noted that biochemical measurements were made on the whole tumour, yet in radiobiological terms only the hypoxic sub-population is being modified. Clearly, biochemical changes occurring in this small sub-population may well be masked by the otherwise unaffected aerobic cells. It is interesting to note that some facultative anaerobes such as the eukaryote Saccharomyces cerevisiae and the Escherichia coli show higher catalase levels when grown under aerobic conditions.[271] An enhanced turnover of glutathione peroxidase in rat liver and lung has been demonstrated to be caused by hyperbaric oxygen (4.1 atmospheres oxygen for 15 minutes).[272]

CONCLUSION

This study consisted essentially of four parts:

Part 1) The effect of a 72 hour 8% oxygen pretreatment on the response of tumours to single dose radiation was investigated in two types of murine tumours, viz. the 3-MC-induced rhabdomyosarcoma and the CaNT tumour. The response of the 3-MC-induced rhabdomyosarcoma to X-radiation was significantly increased if the tumour-bearing mice were exposed to the reduced oxygen environment. The 72 hour 8% oxygen pretreatment, however, had no effect in increasing the sensitivity of the CaNT tumour to X-radiation. The explanation for these results involves the "reduced cord radius" hypothesis. Here it is proposed that the hypoxic pretreatment causes a drop in the tumour capillary PO_2. This, in turn, will cause some of the tumour's hypoxic cells to die as a result of oxygen starvation with a consequential reduction in the tumour's hypoxic cell fraction. It is proposed that, because the 3-MC-induced rhabdomyosarcoma has a "small" initial proportion of hypoxic cells, the death of some of these hypoxic cells resulting from the hypoxic pretreatment will lead to a critical reduction in their proportion. It is suggested, however, that this critical level is not reached in the CaNT tumour where the initial hypoxic cell fraction is larger.

An interesting finding was that neither the CaNT tumour nor the 3-MC-induced rhabdomyosarcoma was rendered more sensitive to X-radiation delivered while the host animals breathed 100% oxygen under pressure as opposed to air. This observation was independent of whether pretreatment was with 8% oxygen or with air. It is proposed that any increase in

tumour oxygenation is negated by hyperoxic-associated vasoconstriction.

Part 2) The response of the Fib/T tumour to single dose gamma radiation, delivered in air, was investigated where host WHT mice were pretreated with different hypoxic gas mixtures (8%, 10% or 15% oxygen) for varying times (48 or 72 hours), or with air. A maximal reduction in tumour cell survival was obtained in the case of animals that were pretreated with 8% oxygen for 48 hours. It is proposed that adaptation mechanisms, such as an increase in the blood haemoglobin level and an increase in erythrocyte 2,3-DPG, by making more oxygen available, oxygenate some of the tumour's hypoxic cells. This "increased oxygen availability" hypothesis, thus, provides an additional explanation for why a hypoxic pretreatment may sensitize tumours to radiation. It is noteworthy that the largest augmentation in erythrocyte 2,3-DPG was measured in mice which were kept in an 8% oxygen environment for 48 hours.

It is concluded that the "reduced cord radius" and "increased oxygen availability" hypotheses are not mutually exclusive in providing a possible explanation for the radiation sensitization effect induced by a hypoxic pretreatment.

The Fib/T tumour was observed to show a greater sensitivity to ionizing radiation if the host WHT mice breathed hyperbaric oxygen, rather than air, during radiation (this was especially significant where pretreatment was with air). In this respect the Fib/T tumour differed from both the CaNT tumour and the 3-MC-induced rhabdomyosarcoma. Some investigators[207] have shown that smaller tumours (thus, possibly, containing fewer hypoxic cells) are more sensitive to radiation delivered under conditions of hyperbaric oxygen than air. However, as the Fib/T tumour has been shown to have a

larger hypoxic cell fraction than the 3-MC-induced rhabdomyosarcoma, the difference in response of these two tumours to radiation in hyperbaric oxygen cannot be elucidated on the basis of their differing hypoxic cell fractions.

Part 3) The pretreatment of WHT mice with 8% oxygen for 48 hours was found to increase tumour cell kill where the host animals received two fractions of radiation, both delivered in air. Tumour cell kill was further and significantly increased where mice were retained in the 8% oxygen environment for the 24 hour interval between radiation fractions. Four possible reasons for this finding are proposed:

1). The inter-fraction hypoxia maintains elevated levels of both haemoglobin and erythrocyte 2,3-DPG and, thus, enhances tumour sensitivity to the second fraction of radiation.

2) The inter-fraction hypoxia inhibits tumour cell repopulation.

3) The inter-fraction hypoxia inhibits the repair of radiation-induced damage.

4) The inter-fraction hypoxia inhibits the loss of those tumour cells killed by the first fraction of radiation.

It was particularly notable, where WHT mice were given two fractions of radiation both delivered in hyperbaric oxygen, that a significant increase in tumour cell kill was observed compared to where both fractions of radiation were delivered in air (this was more significant in the case where mice were pretreated with air rather than with 8% oxygen).

Mice pretreated with 8% oxygen and then retained in this hypoxic environment between radiation fractions showed an increased tumour cell kill compared to mice similarly pretreated but kept in air between fractions. This finding is compatible with, and strengthens, a clinical finding. Dische

and Hewitt[273] treated a severely anaemic patient, who refused transfusions, with fractionated radiation. It is possible that the improved local tumour control observed in this patient was due to the low level of haemoglobin between fractions maintaining tumour hypoxia which in turn caused an inhibition of repopulation and/or repair.

Part 4) No changes in the activity of the anti-oxidant enzymes, catalase and glutathione peroxidase, nor in the levels of glutathione were established in the tumours of mice that were given a low oxygen treatment. It is, therefore, inferred that it is not possible to explain the increased response to radiation observed in these animals on the basis of elevated levels of radiation-induced radicals or hydroperoxides.

The breathing of hypoxic gas mixtures may afford an approach that is applicable to the treatment of primary tumours in patients. It is clear, in view of the differing responses shown by the three mouse tumours investigated in this study to hypoxic treatments followed by radiation in air or in hyperbaric oxygen, that an uniform approach to all tumour types in the clinical situation may not be possible. There are other viable alternatives to the normobaric breathing of low oxygen concentration gas mixtures, including the induction of anaemia (see Chapter 2) or hypobaria. It is suggested that the normobaric breathing of hypoxic gas mixtures would be a safer, and therefore more practicable, therapeutic procedure in that it could be easily and expeditiously reversed. It would, of couse, be imperative to ensure that the selected oxygen concentration and pretreatment duration will not cause any functional impairments to the patient.

It would be of interest to extend these studies experimentally using, for example, a five-fractionated radiation regimen. The efficacy of introducing a drug (e.g. methotrexate) into such a treatment protocol could also be investigated.

Whether a hypoxic pretreatment is capable of making tumours more sensitive to the cytotoxic effect of a drug, such as methotrexate, also deserves consideration and possibly investigation. It might be possible to optimize the cytotoxic effect of a bioreductively-activated drug like misonidazole[274] by administering this agent when the tumour's hypoxic cell fraction would be expected to be larger, i.e. during the exposure period of the tumour-bearing host animal to the low oxygen concentration gas mixture.

APPENDIX A

Methods pertaining to the calculations of hypoxic cell fractions are described in this appendix.

A.1 Clamped Tumour Growth Delay Method.

In the clamped tumour growth delay method, it is assumed that growth delay is determined solely by the time required to rebuild the tumour cell population. The growth delay (T) occurring after survival is reduced to level F is

(1) $T = (-T_d)(\log F)/\text{Log } 2$

where T_d is the volume doubling time for treated tumours. The survival of hypoxic cells (F_h) and the survival of a population containing a mixture of both aerobic and hypoxic cells (F_m) can be determined from equation (4):

(2) $F_h = 0.5^{Tc/Td}$

(3) $F_m = 0.5^{Ta/Td}$

where T_c and T_a are the growth delays for clamped and normally-aerated tumours.

Equations (2) and (3) can be substituted in equations (6) and (7) and the hypoxic fraction, f, can be calculated:

$$f = 0.5^{(Ta-Tc)/Td}$$

The error on f, Sf, = $Sy.d/dy.(0.5^y)$ where $y = (T_a-T_c)/T_d$.
The error on $y = \{[S(T_a-T_c)]^2 - (ST_d)^2\}^{1/2}$,
where the error on $(T_a-T_c) = \{(ST_a)^2 - (ST_c)^2\}^{1/2}$.

A.2 Paired Survival Curve Method.

In the paired survival curve method, the assumption is made that tumours contain only two cell populations: an aerobic component with maximal radiosensitivity and a hypoxic

component with full radioresistance. The fraction of cells surviving a dose (D) of irradiation in such a mixed population (F_m) is

(4) $F_m = fF_h + (1-f)\ F_o$

where f is the fraction of hypoxic cells, and F_h and F_o are the proportions of hypoxic and oxic cells respectively which survive dose D. When D is large, the survival of oxic cells is negligible and equation (4) can be rearranged to

(5) $\log(f) = \log(F_m) - \log(F_h)$

If it is assumed that the survival of naturally hypoxic cells (F_h) and the survival of cells in clamped, artificially - induced hypoxic tumours (F_c) are the same, the aerobic and clamped survival curves can be fitted (at high doses) with parallel curves:

(6) $\log(F_m) = \log(n_m) - (\ln/D_o)D$

(7) $\log(F_h) = \log(F_c) = \log(n_c) - (\ln/D_o)D$

where D_o describes the slope of both lines, and n_m and n_c are the extrapolation numbers for the parallel lines. Equations (6) and (7) can be substituted into equation (5) to yield

(8) $\log(f) = \log(n_m) - \log(n_c)$

The error on log(f), i.e. Sf, is given by $(Sn_m^2 + Sn_c^2)^{1/2}$

where Sn_m and Sn_c are the standard deviation of n_m and n_c respectively. If $K = C(1/n^{1/2})Sf$, where C can be obtained from tables and n refers to the number of sample points, then the 95% Confidence Interval is

$$(\log(f) - K;\ \log(f) + K)$$

APPENDIX B

Calculation of Enzyme Activities.

B.1. Catalase.

umole H_2O_2 degraded/minute/mg protein

$$\Delta A/0.0436 \times a/b \times 1/c$$

where ΔA = change in absorbance per minute at 240 nm.

a = total cuvette volume in ml.

b = volume of tumour sample in cuvette in ml.

c = protein concentration per ml of sample.

B.2 Glutathione Reductase and Glutathione Peroxidase.

umole GSSG degraded/minute/mg protein

$$\Delta A/6.22 \times a/b \times 1/c$$

where ΔA = change in absorbance per minute at 340 nm.

a = total cuvette volume in ml.

b = volume of tumour sample in cuvette in ml.

c = protein concentration per ml of sample.

APPENDIX C

Statistical Formulae used in this Thesis.

C.1 Mean (x) is given by

$$x = (\Sigma x)/n$$

where Σx = sum of all observations

n = number of observations

C.2 Standard Deviation (S) is given by

$$S = [(\Sigma x^2-(\Sigma x)^2/n)/n-1]^{1/2}$$

where Σx^2 = the sum of the square of all observations

$(\Sigma x)^2$ = the square of the sum of all observations

n = number of observations

C.3 The significance of difference between two mean values was determined using Student's two-tailed t-test, with n^1+n^2-2 degrees of freedom.

$$t = (x_1-x_2)/(S_1^2/n_1 + S_2^2/n_2)^{1/2}$$

where x_1 = mean value of observations in first sample population

x_2 = mean value of observations in second sample population

S_1 = standard deviation for first sample population

S_2 = standard deviation for second sample population

n_1 = number of observations in first sample population

n_2 = number of observations in second sample population.

The value of p was ascertained from tables.

C.4 The coefficients of regression (a and b) in the regression line whose equation is $y = a + bx$, and the correlation coefficient (r^2) are given by

$$b = (\Sigma xy-(\Sigma x)(\Sigma y)/n)/(\Sigma x^2-(\Sigma x)^2/n)$$

$$a = (\Sigma y)/n-b(\Sigma x/n)$$

$$r^2 = (\Sigma xy-(\Sigma x\Sigma y)/n)^2/[(\Sigma x^2-(\Sigma x)^2/n)(\Sigma y^2-(\Sigma y)^2/n)]$$

REFERENCES

1) Ellis, F.: The use of hypoxia in radiotherapy. Br. J. Radiol. 35: 506-507, 1962.

2) Thomlinson, R.H.: A comparison of fast neutrons and X-rays in relation to the "oxygen effect" in experimental tumours in rats. Br. J. Radiol. 36: 89-91, 1963.

3) Siemann, D.W., Hill, R.P., Bush, R.S., Chhabra, P.: The in vivo radiation response of an experimental tumour: The effect of exposing tumor-bearing mice to a reduced oxygen environment prior to but not during irradiation. Int. J. Radiat. Oncol. Biol. Phys. 5: 61-68, 1979.

4) Holthusen, H.: Beitrage zur biologie der strahlenwirkung. Untersuchungen an Askarideneiern. Pflugers Arch. 187: 1, 1921.

5) Mottram, J.C.: On the skin reactions to radium exposure and their avoidance in therapy; An experimental investigation. Br. J. Radiol. 29: 174-180, 1924.

6) Mottram, J.C.: A factor of importance in the radiosensitivity of tumours. Br. J. Radiol. 9: 606-614, 1936.

7) Evans, T.C., Goodrich, J.P., Slaughter, J.C.: Temperature and radiosensitivity of skin of newborn rats. Effects of decreased circulation and breathing during radiation. Radiology 38: 201-206, 1942.

8) Dowdy, A.H., Bennett, L.R., Chastain, S.M.: Protective action of anoxic anoxia against total body roentgen irradiation of mammals. Radiology 55: 879-885, 1950.

9) Read, J.: The effect of ionizing radiations on the broad bean root. Part 10: The dependence of the X-ray sensitivity on dissolved oxygen. Br. J. Radiol. 25: 89-99 and 154-160, 1952.

10) Gray, L.H., Conger, A.D., Ebert, M., Hornsey, S., Scott, O.C.A.: The concentration of oxygen dissolved in tissues at the time of irradiation as a factor in radiotherapy. Br. J. Radiol. 26: 638-648, 1953.

11) Deschner, E.E., Gray, L.H.: Influence of oxygen tension on X-ray-induced chromosomal damage in Ehrlich ascites tumour cells. Radiat. Res. 11: 115-146, 1959.

12) Hewitt, H.B., Wison, C.W.: The effect of tissue oxygen tension on the radiosensitivity of leukemia cells irradiated in situ in the livers of leukemic mice. Br. J. Cancer 13: 675-684, 1959.

13) Dewey, D.L.: Effect of oxygen and nitric oxide on the radiosensitivity of human cells in tissue culture. Nature 186: 780-782, 1960.

14) Alper, T., Howard-Flanders, P.: Role of oxygen in modifying the radiosensitivity of E. Coli B. Nature 178: 978-979, 1956.

15) Giles, N.H., Beatty, A.V.: The effect of X-irradiation in oxygen and in hydrogen at normal and positive pressure on chromosome aberration-frequency in Tradescantia Microspores. Science 112: 643-645, 1950.

16) Gray, L.H.: Radiobiologic basis of oxygen as a modifying factor in radiation therapy. Amer. J. Roentgenol. 85(5): 803-815, 1961.

17) Halliwell, B., Gutteridge, J.M.C.: The chemistry of oxygen radicals and other oxygen-derived species. In Free radicals in biolgy and medicine. Clarendon Press, Oxford, 1985, pp 20-64.

18) Haber, F., Weiss, J.: Catalytic decomposition of hydrogen peroxide by iron salts. Proc. R. Soc. London 147: 332-351, 1934.

19) Samuni, A., Chevion, M., Halpern, Y.S., Ilan, Y.A., Czapski, G.: Radiation-induced damage in T4 Bacteriophage: The effect of superoxide radicals and molecular oxygen. Radiat. Res. 75: 489-496, 1978.

20) Srivastava, B.S.: Irradiation and DNA breaks. Nature 259: 425-426, 1976.

21) Dean, C.J., Ormerod, M.G., Serianni, R.W., Alexander, P.: DNA strand breakage in cells irradiated with X-rays. Nature 222: 1042-1044, 1969.

22) Churchill-Davidson, I.: The oxygen effect in radiotherapy. Historical review. In Hyperbaric oxygen and radiation therapy of cancer. (Proceedings - 1st Annual San Francisco Cancer Symposium) Edit. by J.M.Vaeth, 1965.

23) Thomlinson, R.H.: Radiation and the vascularity of tumours. Br. Med. Bulletin 29: 29-32, 1973.

24) Thomlinson, R.H., Gray, L.H.: The histological structure of some human lung cancers and the possible implications for radiotherapy. Br. J. Cancer 9: 539-549, 1955.

25) Cater, D.B., Silver, I.A.: Measurement of oxygen tension in tumours. Br. J. Radiol. 31: 340, 1958.

26) Cater, D.B., Silver, I.A.: Quantitative measurements of oxygen tension in normal tissues and in the tumours of patients before and after radiotherapy. Acta Radiol. 53: 233-256, 1960.

27) Vaupel, P., Manz, R., Mueller-Klieser, W., Grunewald, W.A.: Intracapillary HbO_2 saturation in malignant tumour during normoxia and hyperoxia. Microvascular Res. 17: 181-191, 1979.

28) Chapman, J.D., Franko, A.J., Sharplin, J.: A marker for hypoxic cells in tumours with potential clinical applicability. Br. J. Cancer 43: 546-550, 1981.

29) Chapman, J.D.: The detection and measurement of hypoxic cells in solid tumours. Cancer, 54: 2441-2449, 1984.

30) Churchill-Davidson, I., Sanger, C., Thomlinson, R.: High-pressure oxygen and radiotherapy. Lancet 1: 1091-1095, 1955.

31) Thomlinson, R.H.: Experimental method for comparing treatments of intact malignant tumours in animals and its application to use of oxygen in radiotherapy. Br. J. Cancer 14: 555-576, 1960.

32) Du Sault, L.A., Eyler, W.R., Dobben, G.D.: Combination of oxygen and optimum fractionation in radiation therapy of adenocarcinoma. Amer. J. Roentgenol. Rad. Therapy and Nuclear Med. 82: 688-692, 1959.

33) Cohen, A., Cohen, L.: Estimation of cellular lethal dose and critical cell number for C3H mouse mammary carcinoma from radiosensitivity studies in vivo. Nature 185: 262-263, 1960.

34) Powers, W.E., Tolmach, L.J.: A multicomponent X-ray survival curve for mouse lymphosarcoma cells irradiated in vivo. Nature 197: 710-711, 1963.

35) Hirst, D.G.: Oxygen delivery to tumours. Int. J. Radiat. Oncol. Biol. Phys. 12: 1271-1277, 1986.

36) Wright, E.A.: Some potential applications of the "oxygen effect" to radiotherapy. Amer. J. Roentgenol. 96: 755-759, 1966.

37) Wright, E.A., Hahn, G.M., Steele, R.E.: Towards the ideal use of the "oxygen effect" in radiotherapy. Amer. J. Roentgenol. 96: 749-754, 1966.

38) Curtis, S.B., Tenforde, T.S., Afgal, S.M.: Hypoxic cell sensitizers and heavy charged particle beams may play complementary roles in killing hypoxic tumour cells. Int. J. Radiat. Oncol. Biol. Phys. 10: 1203-1205, 1984.

39) Hall, E.J., Cavanagh, J.: The oxygen effect for acute and protracted radiation exposures measured with seedlings of Vicia faba. Br. J. Radiol. 40: 128-133, 1967.

40) Berry, R.J.: Hypoxic protection and recovery in tumour cells irradiated at low dose-rates and assessed in vivo. Br. J. Radiol. 41: 921-926, 1968.

41) Yarmonenko, S.P., Wainson, A.A., Bereznova, L.I., Krimker, V.M., Ovakimov, V.G., Petrosia, E.P., Shmakova, N.L.: The selective protection of normal tissues by irradiation of tumour-bearing mice in hypoxic hypoxia. Radiat. Res. 67: 447-458, 1976.

42) Mallams, J.T., Balla, G.A., Finney, J.W.: Regional oxygenation and radiation therapy: current status. Amer. J. Roentgenol. 93: 160-169, 1965.

43) Biaglow, J.E., Durand, R.E.: The effects of nitrobenzene derivatives on oxygen utilization and radiation response of an in vitro tumour model. Radiat. Res. 65: 529-539, 1976.

44) Bloch, M., Bloom, H.J.G., Penman, J., Walsh, L.: Irradiation of cerebral astrocytomata under whole-body hypothermia. Lancet 2: 906-909, 1961.

45) Geyer, R.P., Monroe, R.C., Taylor, L.: Survival of rats having red cells totally replaced with emulsified fluorocarbon. Fed. Proc. 27: 384, 1968.

46) Teicher, B.A., Rose, C.M.: Perfluorochemical emulsions can increase tumour radiosensitivity. Sci. 223: 934-936, 1984.

47) Teicher, B.A., Rose, C.M.: Oxygen-carrying perfluorochemical emulsion as an adjuvant to radiation therapy in mice. Cancer Res. 44: 4285-4288, 1984.

48) Rockwell, S.: Use of a perfluorochemical emulsion to improve oxygenation in a solid tumour. Int. J. Radiat. Oncol. Biol. Phys. 11: 97-103, 1985.

49) Du Boulay, C.H., Adams, G.E., Stratford, I.J., Butler, S., Nolan, J.: Induction of necrosis in an experimental mouse lymphoma using modifiers of tumour oxygenation and a bioreductive drug. Proc. 8th. Int. Congress Radiat. Res. 1: 246, 1987.

50) Cole, S., Robbins, L., Nolan, J.: Radiobiology and experimental therapy of a human colon carcinoma xenograft in nude mice. Proc. 8th. Int. Congress Radiat. Res. 1: 292, 1987.

51) Fitzharris, P., Mc Lean, A.E.M., Sparks, R.G., Weatherley, B.C., White, R.D., Wootton, R.: The effects in volunteers of BW12C, a compound designed to left-shift the blood-oxygen saturation curve. Br. J. Clin. Pharmac. 19: 471-481, 1985.

52) Adams, G.E., Barnes, D.W.H.: Induction of hypoxia in normal and malignant tissues by changing the oxygen affinity of haemoglobin - Implications for therapy. Int. J. Radiat. Oncol. Biol. Phys. 12: 1299-1302, 1986.

53) Forster, R.E.: Exchange of gases between alveolar air and pulmonary capillary blood: pulmonary diffusing capacity. Physiol. Rev. 37: 391-405, 1957.

54) Roughton, F.J.W., Forster, R.E.: Relative importance of diffusion and chemical reaction rates in determining rate of exchange of gases in the human lung, with special reference to true diffusing capacity of pulmonary membrane and volume of blood in the lung capillaries. J. Appl. Physiol. 11: 290-302, 1957.

55) Slonim, N.B., Hamilton, L.H.: Blood-gas transport. In Respiratory physiology (2nd Edit.) C.V. Mosby Company, 1971, pp.76-89.

56) Muirhead, H., Cox, J.M., Mazzarella, L., Perutz, M.F.: Structure and function of haemoglobin. 3. A three-dimensional fourier synthesis of human deoxyhaemoglobin at 5.5 Angstrom resolution. J. Mol. Biol. 28: 117-156, 1967.

57) Perutz, M.F.: Stereochemistry of co-operative effects in haemoglobin. Nature 228: 726-739, 1970.

58) Murray, J.F.: Diffusion of gases, oxyhaemoglobin equilibrium, and carbon dioxide equilibrium. In The normal lung (2nd Edit.) W.B.Saunders Company. Philadelphia, London, Toronto, Mexico City, 1986, pp 163-182.

59) Ganong, W.F.: Respiratory adjustments in health and disease. In Review of medical physiology (12th Edit.) Lange Medical Publications, 1985, pp.558-571.

60) Lynch, E.C., Alfrey, C.P., Hettig, R.A.: Rational treatment of anaemias in malignant disease. Tex. Med. 67: 67-74, 1971.

61) Hyman, G.A.: Anaemia in malignant neoplstic disease. J. Chron. Dis. 16: 645-666, 1963.

62) Greenfield, R.E., Godfrey, J.E., Price, V.E.: Studies on the anaemia of tumour-bearing animals. Distribution of radio-iron following the injection of labelled erythrocytes. J. Natl. Cancer Inst. 21: 641-656, 1958.

63) Mottram, J.C., Eidinow, A.: On the effect of anaemia on the reactions of the skin and of tumours to radium exposure. Br. J. Surg. 19: 481-487, 1932.

64) Evans, J.C., Bergsjo, P.: Influence of anaemia on the results of radiotherapy in carcinoma of the cervix. Radiology 84: 709-716, 1965.

65) Vigario, G., Kurohara, S.S., George, F.W.: Association of haemoglobin levels before and during radiotherapy with prognosis in uterine cervix cancer. Radiology 106: 649-652, 1973.

66) Hierlihy, P., Jenkin, R.D.T., Stryker, J.A.: Anaemia as a prognostic factor in cancer of the cervix. Canad. Med. Assoc. J. 10: 1100-1102, 1969.

67) Schreiner, P., Siracka, E., Siracky. J., Manka, I.: The effect of anaemia on the radiotherapy results of uterine cervix cancer. Neoplasma 22: 655-660, 1975.

68) Rustowski, J., Kupsc, W.: Factors influencing the results of radiotherapy in cases of inoperable endometrial cancer. Gynaecol. Oncol. 14: 185-193, 1982.

69) Overgaard, J., Hansen,H.S., Jorgensen,K., Hanses,M.H.: Primary radiotherapy of larynx and pharynx carcinoma - analysis of some factors influencing local control and survival. Int. J. Radiat. Oncol. Biol. Phys. 12: 515-521, 1986.

70) Quilty, P.M., Duncan, W.: The influence of haemoglobin level on the regression and long term local control of transitional cell carcinoma of the bladder following photon irradiation. Int. J. Radiat. Oncol. Biol. Phys. 12: 1735-1742, 1986.

71) Hill, R.P., Bush, R.S., Yeung, P.: The effect of anaemia on the fraction of hypoxic cells in an experimental tumour. Br. J. Radiol. 44: 299-304, 1971.

72) Hewitt, H.B., Blake, E.R.: Effect of induced host anaemia on the viability and radiosensitivity of murine malignant cells in vivo. Br. J. Cancer 25: 323-336, 1971.

73) Hirst, D.G., Hazlehurst, J.L., Brown, J.M.: The effect of alterations in haematocrit on tumour sensitivity to X-rays. Int. J. Radiat. Biol. 46: 345-354, 1984.

74) Hirst, D.G.: Anaemia: a problem or an opportunity in radiotherapy? Int. J. Radiat. Oncol. Biol. Phys. 12: 2009-2017, 1986.

75) Bush, R.S., Jenkin, R.D.T., Allt, W.E.C., Beale, F.A., Bean, H., Dembo, A.J., Pringle, J.F.: Definitive evidence for hypoxic cells influencing cure in cancer therapy. Br. J. Cancer (Suppl.3) 37: 302-306, 1978.

76) Bush, R.S.: The significance of anaemia in clinical radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 12: 2047-2050, 1986.

77) Rojas, A., Stewart, F.A., Smith, K.A., Soranson, J.A., Randhawa, V.S., Stratford, M.R.L., Denekamp. J.: Effect of anaemia on tumour radiosensitivity under normo and hyperbaric conditions. Int. J. Radiat. Oncol. Biol. Phys. 13: 1681-1689, 1987.

78) Dische, S., Anderson, P.J., Sealy, R., Watson, E.R.: Carcinoma of the cervix - Anaemia, radiotherapy and hyperbaric oxygen. Br. J. Radiol. 56: 251-255, 1983.

79) Hurtado, A.: The influence of high altitude on physiology. In High altitude physiology: Cardiac and respiratory aspects. Edited by R. Porter and J. Knight. Churchill Livingstone; Edinburgh and London, 1971, pp 3-13.

80) Chance, B., Jamieson, D., Williamson, J.R.: Control of the oxidation reduction state of reduced pyridine nucleotides in vivo. In Hyperbaric medicine, Proc. 3rd Int. Conf. Washington DC. Natl. Acad. Sci., Natl. Research Council Publ. No. 1404, 1966, pp 15-41.

81) Heath, D., Williams, D.R.: Tissue diffusion. In Man at high altitude. Churchill Livingstone; Edinburgh, London, Melbourne and New York, 1981, pp 67-72.

82) Lenfant, C., Sullivan, K.: Adaptation to high altitude. New Eng. J. Med. 284: 1298-1309, 1971.

83) Lenfant, C., Torrance, J.D., Reynafarje, C.: Shift of the O_2-Hb dissociation curve at altitude: mechanism and effect. J. Appl. Physiol. 30: 625-631, 1971.

84) Cumming, G., Semple, S.J.: In Disorders of the respiratory system. Oxford: Blackwell Scientific Publ. 1973, pp 236.

85) Arias-Stella, J., Bustos, F.: Chronic hypoxia and chemodectomas in bovines at high altitude. Arch Pathol. Lab. Med. 100: 636-639, 1976.

86) Hurtado, A., Merino, C., Delgado, E.: Influence of anoxemia on the hemopoietic activity. Arch Intern. Med. 75: 284-323, 1945.

87) Lord, B.I., Murphy, M.J.: Hematopoietic stem cell regulation. Chronic effects of hypoxic-hypoxia on CFU kinetics. Blood 42: 89-98, 1973.

88) Hurtado, A.: Some physiologic and clinical aspects of life at high altitude. In Aging of the lung (10th Hahnemann Symposium). Edited by L. Cander and J.H. Moyer. New York: Grune and Stratton, 1964, pp 257.

89) Reynafarje, C.: Physiological patterns: hematological aspects. In Life at high altitudes. Scient. Publ. No.140. Washington: Pan American Health Organization, 1966, pp 32.

90) Reynafarje, C.: Bone marrow studies in the new born infant at high altitude. J. Pediatr. 54: 154-161, 1959.

91) Carmena, A.O., De Testa, N.G., Segade, A., Frias, F.L.: Erythropoiesis-stimulating activity in plasmas of young men living permanently at high altitude. Acta Physiol. Lat. Amer. 17: 145-148, 1967.

92) Siri, W.F., Vandyke, D.C., Winchell, H.S., Pollcove, M., Parker, H.G., Cleveland, A.S.: Early erythropoietin, blood and physiological responses to severe hypoxia in man. J. Appl. Physiol. 21: 78-80, 1966.

93) Consolazio, C.F., Matoush, L.O., Johnson, H.L., Daws, T.A.: Protein and water balances of young adults during prolonged exposure to high altitude (4,300 m). Am. J. Clin. Nutr. 21: 154-161, 1968.

94) Hannon, J.P., Shields, J.L., Harris, C.W.: Effects of altitude acclimatization on blood composition of women. J. Appl. Physiol. 26: 540-547, 1969.

95) Aste-Salazar, J., Hurtado, A.: The affinity of haemoglobin for oxygen at sea level and at high altitude. Amer. J. Physiol. 142: 733-743, 1944.

96) Eaton, J.W., Brewer, G.J., Grover, R.F.: Role of red cell, 2,3-diphosphoglycerate in the adaptation of man to altitude. J. Lab. Clin. Med. 4: 603-609, 1969.

97) Lenfant, C., Torrance, J.D., Finch, C.A.: The regulation of haemoglobin affinity for oxygen in man. Trans. Assoc. Amer. Physicians 82: 1969.

98) Chanutin, A., Curnish, R.R.: Effect of organic and inorganic phosphates on the oxygen equilibrium of human erythrocytes. Arch. Biochem. 121: 96-102, 1967.

99) Benesch, R., Benesch, R.E.: The effect of organic phosphates from the human erythrocytes on the allosteric properties of haemoglobin. Biochem. Biophys. Res. Commun. 26: 162-167, 1967.

100) Rapoport, S., Luebering, J.: The formation of 2,3-diphosphoglycerate in rabbit erythrocytes: the existence of a diphosphoglycerate mutase. J. Biol. Chem. 183: 507-516, 1950.

101) Rose, Z.: Purification and properties of DPG mutase from human erythrocytes. J. Biol. Chem. 243: 4810-4820, 1968.

102) Torrance, J.D.: Clinical implications of the interaction between 2,3-DPG and haemoglobin. S.A.J.Med. Sci. 39: 33-61, 1974.

103) Arnone, A.: X-ray diffraction study of binding of 2,3-diphosphoglycerate to human deoxyhaemoglobin. Nature (London) 237: 146-149, 1972.

104) Lenfant, C., Torrance, J., English, E., Finch, C.A., Reynafarje, C., Ramos, J., Faura, J.: Effect of altitude on oxygen binding by haemoglobin and on organic phosphate levels. J. Clin. Invest. 47: 2652-2656, 1968.

105) Mulhausen, R.O., Astrup, P., Mellemgaard, K.: Oxygen affinity and acid-base status of human blood during exposure to hypoxia and carbon monoxide. Scand. J. Clin. Lab Invest. 22 (Suppl 103) 9-15, 1968.

106) Klausen, K.: Cardiac output in man in rest and work during and after acclimatization to 3,800 m. J. Appl. Physiol. 21: 609-616, 1966.

107) Vogel, J.A., Harris, C.W.: Cardiopulmonary responses of resting man during early exposure to high altitude. J. Appl. Physiol. 22: 1124-1128, 1967.

108) Lenfant, C., Torrance, J.D., Reynafarje, C.: Shift of the O_2-Hb dissociation curve at altitude: mechanism and effect. J. Appl. Physiol. 30: 625-631, 1971.

109) Cunningham, W.L., Becker, E.J., Kreuzer, F.: Catecholamines in plasma and urine at high altitude. J. Appl. Physiol. 20: 607-610, 1965.

110) Hoon, R.S., Balasubramanian, V., Mathew, O.P., Tiwari, S.C., Sharma, S.C., Chadha, K.S.: Effect of high-altitude exposure for 10 days on stroke volume and cardiac output. J. Appl. Physiol. 42(5): 722-727, 1977.

111) Stefansson, V.: In Cancer, disease of civilization. An anthropological and historical study. New York: Hill and Wang, 1960, pp 147.

112) Loewy, A., Wittkower,E.: In The pathology of high altitude climate; with contributions to the climatology of highland regions and to the constitution of high altitude inhabitants. London, England, Oxford University Press, 1937, pp 40-61.

113) Hever Kruger, P., Javier Arias-Stella.: Malignant tumours in high-altitude people. Cancer 17: 1340-1347, 1964.

114) Mori-Chavez, P,. Hellriegel, K.O.: A survey of human cancer at high altitude in the Peruvian Andes. Natl. Cancer Inst. Monogr. 14: 333-337, 1964.

115) Eckhoff, N.D., Shutes, J.K., Clark, R.W., Ramer, E.R.: Correlation of leukemia mortality rates with altitude in the United States. Health Phys. 27: 377-380, 1974.

116) Mason, T.J., Miller, R.W.: Cosmic radiation at high altitudes and U.S. cancer mortality, 1950-1969. Radiat. Res. 60: 302-306, 1974.

117) Amsel, J., Waterbor, J.W., Oler, J., Rosenwaike, I, Marshall, K.: Relationship of site-specific cancer mortality rates to altitude. Carcinogenesis 3(5): 461-465, 1982.

118) Mori-Chavez, P.: Development of spontaneous pulmonary tumours at high altitude in strain A mice. J. Natl. Cancer Inst. 28: 55-73, 1962.

119) Mori-Chavez, P., Upton, A.C., Salazar, M., Conklin, J.W.: Influence of transitory, as compared with permanent,

high altitude exposure on the pathogenesis of spontaneous and X-ray induced neoplasms in RF/Un mice. Cancer Res. 34: 328-336, 1974.

120) Mori-Chavez, P.: Spontaneous leukemia at high altitude in C58 mice. J. Natl. Cancer Inst. 21: 985-997, 1958.

121) Blatteis, C.M.: Depressed growth of Morris hepatomas in altitude and heat-stressed but not in cold-stressed Buffalo rats. J. Natl. Cancer Inst. 64: 1451-1458, 1980.

122) Blatteis, C.M., Filkins, J.P., Fuste, F.J., Morris, H.P.: Depressed growth of Morris hepatomas and altered lysosomal hydrolases during altitudinal hypoxia. Proc. Soc. Exp. Biol. Med. 146: 786-794, 1974.

123) Eagle, H.: The effect of environmental pH on the growth of normal and malignant cells. J. Cell. Physiol. 82: 1-8, 1973.

124) Burton, A.C.: Cancer and altitude. Does intracellular pH regulate cell division. Eur. J. Cancer 11: 365-371, 1975.

125) Turek, Z., Franz, A., Kreuzer, F.: Hypoxic pulmonary steady-state diffusing capacity for CO_2 and alveolar-arterial O_2 pressure differences in growing rats after adaptation to a simulated altitude of 3,500 m. Pflugers Arch. 335: 1-9, 1972.

126) Tannock, I.F., Steel, G.G.: Tumour growth and cell kinetics in chronically hypoxic cells. J. Natl. Cancer Ins. 45: 123-131, 1970.

127) Jennings, W.A., Harrison, R.M.: X-rays: Half-value layer range 0.01-8.0 mm Al (approx. 6-150 kV peak potential difference). Br. J. Radiol. (Suppl.) 17: 1-22, 1983.

128) Holt, J.A.G.: The principle of hyperbaric and anoxic radiotherapy. Br. J. Radiol. 48: 819-826, 1975

129) Puck, T.T., Marcus, P.I.: Action of X-rays on mammalian cells. J. Exp. Med. 103: 655-666, 1956.

130) Thomlinson, R.H., Craddock, E.A.: The gross response of an experimental tumour to single doses of X-rays. Br. J. Cancer 21: 108-123, 1967.

131) Hawke, M.J., Hill, R.P., Lindop, P.J., Ellis, R.E., Rotblat, J.: The response of C3H mammary tumours to irradiation in single and fractionated doses. Br. J. Radiol. 41: 134-141, 1968.

132) Reinhold, H.S.: A cell dispersion technique for use in quantitative transplantation studies with solid tumours. Eur. J. Cancer 1: 67-71, 1965.

133) Kallman, R.F., Silini, G., van Putten, L.M.: Factors influencing the quantitative estimation of the in vivo survival of cells from solid tumours. JNCI 39: 539-549, 1967.

134) Twentyman, P.R., Martin Brown, J., Gray, J.W., Franko, A.J., Scoles, M.A., Kallman, R.F.: A new mouse tumour model system (RIF-1) for comparison of end-point studies. JNCI 64: 595-604, 1980.

135) Puck, T.T., Marcus, P.I.: Rapid method for viable cell titration and clone production with HeLa cells in tissue culture. Proc. Nat. Acad. Sci. 41: 432, 1955.

136) Chadwick, K.H., Leenhouts, H.P.: The molecular model for cell survival following radiation. In The molecular theory of radiation biology. Edited by R. Frankel. Springer-Verlag; Berlin, Heidelberg, New York, 1981, pp 25-50.

137) Moulder, J.E., Martin, D.F.: Hypoxic fraction determinations with the BA.1112 rat sarcoma: Variation within and among assay techniques. Radiat. Res. 98: 536-548, 1984.

138) Moulder, J.E., Rockwell, S.: Hypoxic fractions of solid tumours: Experimental techniques, methods of analysis and a survey of existing data. Int. J. Radiat. Oncol. Biol. Phys. 10: 695-712, 1984.

139) Sumner, J.B.: The chemical nature of catalase. Advances in Enzymology 1: 163-176, 1941.

140) Beers, R.F., Sizer, I.W.: A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J. Biol. Chem. 195: 133-141, 1952.

141) Worthington, D.J., Rosemeyer, M.A.: Human glutathione reductase: Purification of the crystalline enzyme from erythrocytes. Eur. J. Biochem. 48: 167-177, 1974.

142) Mills, G.C.: Haemoglobin catabolism. Glutathione peroxidase, an erythrocyte enzyme which protects haemoglobin from oxidative breakdown. J. Biol. Chem. 229: 189-197, 1957.

143) Rotruck, J.T., Pope, A.L., Ganther, H.E., Swanson, A.B., Hafeman, D.G., Hoekstra, W.G.: Selenium: Biochemical role as a component of glutathione peroxidase. Science 179: 588-590, 1973.

144) Little, C., O'Brien, P.J.: An intracellular GSH-peroxidase with a lipid peroxide substrate. Biochem. Biophys. Res. Commun. 31: 145-150, 1968.

145) Lawrence, R.A., Burk, R.F.: Glutathione peroxidase activity in selenium-deficient rat liver. Biochem. Biophys. Res. Commun. 71: 952-958, 1976.

146) Paglia, D.E., Valentine, W.N.: Studies on the quantitative and qualitative characterization of erythrocyte peroxidase. J. Lab. Clin. Med. 70: 158-169, 1967.

147) Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the folin phenol reagent. J. Biol. Chem. 193: 265-275, 1951.

148) Tietze, F.: Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione. Anal. Biochem. 27: 502-522, 1969.

149) Drabkin, D.L., Austin, J.H.: Spectrophotometric studies: spectrophotometric constants for haemoglobin derivatives in human, dog and rabbit blood. J. Biol. Chem. 98: 719, 1932.

150) Lowry, O.H., Passonneau, J.V., Hasselberger, F.X., Schulz, D.W.: Effect of ischaemia on known substrates and cofactors of the glycolytic pathway in brain. J. Biol. Chem. 239: 18-30, 1964.

151) Keitt, A.S.: Pyruvate kinase deficiency and related disorders of red cell glycolysis. Amer. J. Med. 41: 762-785, 1966.

152) Eaton, J.W., Brewer, G.J.: The relationship between red cell 2,3-diphosphoglycerate and levels of haemoglobin in the human. Proc. Natl. Acad. Sci. 61: 756, 1968.

153) Dacie, J.V., Lewis, S.M.: The oxygen dissociation curve. In Practical haematology (6th Edit.) Churchill Livingstone, Edingurgh, London, Melbourne and New York, 1984, pp 172-176.

154) Pike, M.C., Alper, T.A.: A method for determining dose-modification factors. Br. J. Radiol. 37: 458-462, 1964.

155) Finney, D.J.: Quantitative dose-response relationships. In Statistical method in biological assay. Griffin, London, 1964, pp 58-96.

156) Finney, D.J.: Quantal responses and the tolerance distribution. In Statistical method in biological assay. Griffin London, 1964, pp 441-443.

157) Finney, D.J.: Dilution assays. In Statistical method in biological assay. Griffin London, 1964, pp 38-39.

158) Tannock, I.F.: The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. Br. J. Cancer 22: 258-279, 1968.

159) Hug, O., von Szezepanski, L.: Cell proliferation and radiosensitivity of transplantable animal tumours. In Radiation-induced cancer, 1969, pp 85-95.

160) Hirst, D.G., Denekamp, J.: Tumour cell proliferation in relation to the vasculature. Cell Tissue Kinet. 12: 31-42, 1979.

161) Hirst, D.G., Denekamp, J., Hobson, B.: Proliferation kinetics of endothelial and tumour cells in three mouse mammary carcinomas. Cell Tissue Kinet. 15: 251-261, 1982.

162) Churchill-Davidson, I., Sanger, C., Thomlinson, R.H.: Oxygenation in radiotherapy. II. Clinical application. Br. J. Radiol. 30: 406-422, 1957.

163) Hill, D.K.: Oxygen tension and the respiration of resting frog's muscle. J. Physiol. 107: 479-495, 1948.

164) Longmuir, I.S.: Respiration rate of bacteria as a function of oxygen concentration. Biochem. J. 57: 81-87, 1954.

165) Longmuir, I.S.: Respiration rate of rat-liver cells at low oxygen concentration. Biochem. J. 65: 378-382, 1957.

166) Longmuir, I.S., Pistel, J., Magnus, I.A.: Respiration of epidermal cells. Biochim. biophys. Acta 57: 509-519, 1962.

167) Boag, J.W.: Cell respiration as a function of oxygen tension. Int. J. Radiat. Biol. 18: 475-478, 1970.

168) Tannock, I.F.: Oxygen diffusion and the distribution of cellular radiosensitivity in tumours. Br. J. Radiol. 45: 515-524, 1972.

169) Carlsson, J., Stalnacke, C.G., Acker, H., Haji-Karim, M., Nilsson, S., Larsson, B.: The influence of oxygen on viability and proliferation in cellular spheroids. Int. J. Radiat. Oncol. Biol. Phys. 5: 2011-2020, 1979.

170) Mueller-Klieser, W.F., Sutherland, R.M.: Influence of convection in the growth medium on oxygen tensions in multicellular tumour spheroids. Cancer Res. 42: 237-242, 1982.

171) Mueller-Klieser, W.F., Sutherland, R.M.: Oxygen tensions in multicell spheroids of two cell lines. Br. J. Cancer 45: 256-264, 1982.

172) Mueller-Klieser, W.F., Freyer, J.P., Sutherland, R.M.: Influence of glucose and oxygen supply conditions on the oxygenation of multicellular spheroids. Br. J. Cancer 53: 345-353, 1986.

173) Gullino, P.M., Grantham, F.H., Courtney, A.H.: Glucose consumption by transplanted tumours in vivo. Cancer Res. 27: 1031-1040, 1967.

174) Rotin, D., Robinson, B., Tannock, I.F.: Influence of hypoxia and an acidic environment on the metabolism and viability of cultured cells: Potential implications for cell death in tumours. Cancer Res. 46: 2821-2826, 1986.

175) Trivedi, B., Danforth, W.H.: Effect of pH on the kinetics of frog muscle phosphofructokinase. J. Biol. Chem. 241: 4110-4114, 1966.

176) Littbrand, B., Revesz, L: Survival of cells in anoxia. Br. J. Radiol. 41: 479-480, 1968.

177) Born, R., Hug,O., Trott, K: The effect of prolonged hypoxia on growth and viability of Chinese hamster cells. Int. J. Radiat. Oncol. Biol. Phys. 1: 687-697, 1976.

178) Bedford, J.S., Mitchell, J.B.: The effect of hypoxia on the growth and radiation response of mammalian cells in culture. Br. J. Radiol. 47: 687-696, 1974.

179) Hall, E.J., Bedford, J.S., Oliver, R.: Extreme hypoxia; its effect on the survival of mammalian cells irradiated at high and low dose-rates. Br. J. Radiol. 39: 302-307, 1966.

180) Franko, A.J., Sutherland, R.M.: Rate of death of hypoxic cells in multicell spheroids. Radiat. Res. 76: 561-572, 1978.

181) Moulder, J.E., Rockwell, S.: Comparison of tumour assay methods. In Rodent tumour models in experimental cancer therapy. Pergamon Books, 1987, pp 272-278.

182) Kligerman, M.M., Heidenreich, W.F., Greene, S.: Distribution of tritiated thymidine about a capillary in a transplanted mouse tumour. Nature 196: 282-283, 1962.

183) Johnson, H.A., Bond, V.P.: A method of labelling tissues with tritiated thymidine in vitro and its use in comparing rates of cell proliferation in duct epithelium, fibroadenoma, and carcinoma of human breast. Cancer 14: 639-643, 1961.

184) Johnson, R.J.R.: Changes in tissue circulation and oxygenation with alteration in arterial gas tension. In Proceedings of the Fourth International Congress on Hyperbaric Medicine, Japan, 1970, pp 37-42.

185) Johnson, R.J.R., Wiseman, N., Wilson, L.: The vascular response of tumour and normal tissue to hyperbaric oxygen. Lahey Clinic Bull. 21: 128-135, 1972.

186) Saltzman, H.A., Hart, L., Sieker, H.O., Duffy, E.J.: Retinal vascular response to hyperbaric oxygenation. JAMA 191: 114-116, 1965.

187) Lambertson, C.J., Kough, R.H., Cooper, D.Y., Loeschke, H.H., Schmidt, C.F.: Effects in man of oxygen inhalation at 1 and 3.5 atmospheres upon blood gas transport, cerebral circulation and cerebral metabolism. J. Appl. Physiol. 5: 471-486, 1953.

188) Bergofsky, E.H., Bertun, P.: Response of regional circulations to hyperoxia. J. Appl. Physiol. 21: 567-572, 1966.

189) Mc Nally, N.J., Sheldon, P.W.: The effect of radiation on tumour growth delay, cell survival and cure of the animal using a single tumour system. Br. J. Radiol. 50: 321-328, 1977.

190) Mc Nally, N.J., Denekamp, J., Sheldon, P., Flockhart, I.R., Stewart. F.A.: Radiosensitization by Misonidazole (Ro 07-0582). The importance of timing and

tumour concentration of sensitizer. Radiat. Res. 73: 568-580, 1978.

191) Hill, R.P.: Excision assays. In Rodent tumour models in experimental cancer therapy. Pergamon Press, New York, 1987, pp 67-75.

192) Barendsen, C.W.: Analysis of tumour responses by excision and in vitro assay of cellular clonogenic capacity. Br. J. Cancer (Suppl.4) 41: 209-216, 1980.

193) Stephens, T.C., Currie, G.A., Peacock, J.H.: Repopulation of gamma irradiated Lewis lung carcinoma by malignant cells and host macrophage progenitors. Br. J. Cancer 38: 573-582, 1978.

194) Bleehen, N.M., Stephens, T.C.: Chairman's summary of Section 5; Clonogenic cell survival after tumour excision. Br. J. Cancer (Suppl.4) 41: 257-258, 1980.

195) Siemann, D.W., Macler, L.M.: Tumour radiosensitization through reductions in haemoglobin affinity. Int. J. Radiat. Oncol. Biol. Phys. 12: 1295-1297, 1986.

196) Siemann, D.W., Hill, R.P., Bush, R.S.: The effect of chronic reductions in the arterial partial pressure of oxygen on the radiation response of an experimental tumour. Br. J. Radiol. 51: 992-996, 1978.

197) Manson, P.N., Anthenelli, R.M., Im, M.J., Bulkley, G.B., Hoopes, J.E.: The role of oxygen-free radicals in

ischaemic tissue injury in island skin flaps. Ann. Surg. 198: 87-90, 1983.

198) Dalsing, M.C., Grosfeld, J.L., Shiffler, M.A., Vane, D.W., Hull, M., Baehner, R.L., Weber, T.R.: Superoxide Dismutase: A cellular protective enzyme in bowel ischaemia. J. Surg. Res. 34: 589-596, 1983.

199) Brown, J.M.: Evidence for acutely hypoxic cells in mouse tumours, and a possible mechanism of reoxygenation. Br. J. Radiol. 52: 650-656, 1979.

200) Chaplin, D.J., Durand, R.E., Olive, P.L.: Acute hypoxia in tumours: implications for modifiers of radiation effects. Int. J. Radiat. Oncol. Biol. Phys. 12: 1279-1282, 1986.

201) DuSault, L.A.: The effect of oxygen on the response of spontaneous tumours in mice to radiotherapy. Br. J. Radiol. 36: 749-754, 1963.

202) Atkins, H.L., Seaman, W.B., Jacox, H.W., Matteo, R.S.: Experience with hyperbaric oxygenation in clinical radiotherapy. Amer. J. Roentgenol. 93: 651-663, 1965.

203) Bates, T.D.: The treatment of stage 3 carcinoma of the cervix by external radiotherapy and high pressure oxygen. Br. J. Radiol. 42: 266-269, 1969.

204) Chang, C.H., Conley, J.J., Herbert, C.: Radiotherapy of advanced carcinoma of the oropharyngeal region

under hyperbaric oxygen. Amer. J. Roentgenol. 117: 509-516, 1973.

205) Cade, I.S., Mc Ewen, J.B.: Clinical trials of radiotherapy in hyperbaric oxygen at Portsmouth, 1964-1976. Clin. Radiol. 29: 333-338, 1978.

206) Berry, G.H., Dixon, B., Ward, A.J.: The Leeds results for radiotherapy in HBO for carcinoma of the head and neck. Clin. Radiol. 30: 591-592, 1979.

207) Suit, H.D., Maeda, M.: Hyperbaric oxygen and radiobiology of a C3H mouse mammary carcinoma. J. Natl. Canc. Inst. 39: 639-650, 1967.

208) Hewitt, H.B.: The effect on cell survival of inhalation of oxygen under high pressure during irradiation in vivo of a solid mouse sarcoma. Br. J. Radiol. 39: 19-24, 1966.

209) Powers, W.E., Tolmach, L.J.: Demonstration of an anoxic component in a mouse tumour-cell population by in vivo assay of survival following irradiation. Radiology, 83: 328-336, 1964.

210) Fischer, J.J., Rockwell, S., Martin, D.F.: Perfluorochemicals and hyperbaric oxygen in radiation therapy. Int. J. Radiat. Oncol. Biol. Phys. 12: 95-102, 1986.

211) Fowler, J.F., Morgan, R.L., Wood, C.A.P.: The biological and physical advantages and problems of neutron therapy. Br. J. Radiol. 36: 77-80, 1963.

212) Hall, E.J.: Radiobiological intercomparisons in vivo and in vitro. Int. J. Radiat. Oncol. Biol. Phys. 3: 195-201, 1977.

213) Hall, E.J., Kellerer, A.M., Friede, H.: Dependence on neutron energy of the OER and RBE. Int. J. Radiat. Oncol. Biol. Phys. 8: 1567-1572, 1982.

214) Harrison, G.H., Kubiczek, E.B., Robinson, J.E.: OER reduction with high-energy neutrons. Br. J. Radiol. 49: 733, 1976.

215) Finkelstein, E., Glatstein, E.: Seduced by oxygen. Int. J. Radiat. Oncol. Biol. Phys. 14: 205-207, 1988.

216) Draganic, I.G., Draganic, Z.D.: Radiation-chemical yields of the primary products of water radiolysis and their dependence on various factors. In The radiation chemistry of water. Academic Press, New York and London, 1971, pp 123-162.

217) Hill, R.P.: An appraisal of in vivo assays of excised tumours. Br. J. Cancer (Suppl.4) 41: 230-239, 1980.

218) Jung, H., Beck, H.-P., Brammer, I., Zywietz, F: Depopulation and repopulation of the R1H rhabdomyosarcoma of the rat after X-irradiation. Europ. J. Cancer 17: 375-386, 1981.

219) Stephens, T.C.: Measurement of tumour cell surviving fraction and absolute numbers of clonogens per tumour in excision assays. In Rodent tumour models in experimental cancer therapy. Pergamon Press, New York, 1987, pp 90-94.

220) Beck-Bornholdt, H-P., Peacock, J.H., Stephens, T.C.: Kinetics of cellular inactivation by fractionated and hyperfractionated irradiation in Lewis lung carcinoma. Int. J. Radiat. Oncol. Biol. Phys. 11: 1171-1177, 1985.

221) Hall, E.J.: Time, dose, and fractionation in radiotherapy. In Radiobiology for the radiobiologist. Harper and Row, Publishers, Hagerstown, Maryland, New York, Evanstown, San Francisco, and London, 1978, pp 273-290.

222) Barendsen, G.W., Broerse, J.J.: Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. I) Effects of single exposures. Europ. J. Cancer, 5: 373-391, 1969.

223) Barendsen, G.W., Broerse, J.J.: Experimental radiotherapy of a rat rhabdomyosarcoma with 15 MeV neutrons and 300 kV X-rays. II) Effects of fractionated treatments, applied five times a week for several weeks. Europ. J. Cancer, 6: 89-109, 1970.

224) Hermens, A.F., Barendsen, G.W.: The proliferative status and clonogenic capacity of tumour cells in a transplantable rhabdomyosarcoma of the rat before and after

irradiation with 800 rad of X-rays. Cell Tissue Kinet. 11: 83-100, 1978.

225) Tubiana, M.: Cell kinetics and radiation oncology. Int. J. Radiat. Oncol. Biol. Phys. 8: 1471-1489, 1982.

226) Steel, G.G.: Resting cells in tumours. In Growth kinetics of tumours. Clarendon Press: Oxford, 1977, pp 181-184.

227) Mc Nally, N.J.: personal communication as quoted by Rockwell, S.: In vivo-in vitro tumour systems: New models for studying the response of tumours to therapy. Lab. Animal Sci. 27(5): 831-851, 1977.

228) Siemann, D.W., Keng, P.C.: Characterization of radiation resistant hypoxic cell subpopulations in KHT sarcomas. ii) Cell sorting. Br. J. Cancer 58: 296-300, 1988.

229) Sinclair, W.K.: Cyclic X-ray responses in mammalian cells in vitro. Radiat. Res. 33: 620-643, 1968.

230) Belli, J.A., Bonte, F.J., Rose, M.S.: Radiation recovery response of mammalian tumour cells in vivo. Nature 211: 662-663, 1966.

231) Belli, J.A., Dicus, G.J., Bonte, F.J.: Radiation response of mammalian tumour cells. I) Repair of sublethal damage in vivo. J. Natl. Cancer Inst. 38: 673-682, 1967.

232) Suit, H., Urano, M.: Repair of sublethal radiation injury in hypoxic cells of a C3H mouse mammary carcinoma. Radiat. Res. 37: 423-434, 1969.

233) Howard, A.: The oxygen requirement in split-dose experiments with Oedogonium. Int. J. Radiat. Biol. 14: 341-350, 1968.

234) Hall, E.J., Cavanagh, J.: The effect of hypoxia on recovery of sublethal radiation damage in Vicia seedlings. Br. J. Radiol. 42: 270-277, 1969.

235) Hall, E.J.: The effect of hypoxia on the repair of sublethal radiation damage in cultured mammalian cells. Radiat. Res. 49: 405-415, 1972.

236) Shrieve, D.C., Harris, J.W.: The in vitro sensitivity of chronically hypoxic EMT6/SF cells to X-radiation and hypoxic cell radiosensitizers. Int. J. Radiat. Biol. 48: 127-138, 1985.

237) Eden, M., Haines, B., Kahler, H.: The pH of rat tumours measured in vivo. J. Natl. Cancer Inst. 16: 541-556, 1955.

238) Jahde, E., Rajewsky, M.F.: Tumour-selective modification of cellular microenvironment in vivo: effect of glucose infusion on the pH in normal and malignant rat tissues. Cancer Res. 42: 1505-1512, 1982.

239) Benova, D., Baev, I.: Protection of the mouse from genetic radiation damage by an optimal-dose-ratio combination of ATP, AET and serotonin. Experientia 34: 876-877, 1978.

240) Kaufmann, W.K., Kaufman, D.G., Stenstrom, M., Grisham, J.W.: Requirements for ATP in DNA repair of isolated hepatic nuclei. Biochem. Biophys. Res. Comm. 108: 1040-1047, 1982.

241) Tikhomirova, M.V., Iashkin, P.N., Fedorenko, B.S., Chertkov, K.S.: Radiation-protective effectiveness of ATP and adenosine against high-energy protons. Kosm. Biol. Aviakosm. Med. 18: 75-77, 1984.

242) Szeinfeld, D., Blekkenhorst, G.: Effect of X-irradiation on adenosine triphosphate and glucose-6-phosphate dehydrogenase in the CaNT mouse tumour. Radiat. Res. 110: 305-307, 1987.

243) Elkind, M.M., Sutton, H.: X-ray damage and recovery in mammalian cells in culture. Nature 184: 1293-1295, 1959.

244) Elkind, M.M., Sutton, H.: Radiation response of mammalian cells grown in culture. I). Repair of X-ray damage in surviving Chinese hamster cells. Radiat. Res. 13: 556-593, 1960.

245) Hahn, G.M.,Rockwell, S., Kallman, R.F., Gordon, L.F., Frindel, E.: Repair of potentially lethal damage in vivo

in solid tumour cells after X-irradiation. Cancer Res. 34: 351-354, 1974.

246) Shipley, W.U., Stanley, J.A., Courtenay, D.V., Field, S.B. Repair of radiation damage in Lewis lung carcinoma cells following in situ treatment with fast neutrons and gamma-rays. Cancer Res. 35: 932-938, 1975.

247) Little, J.B.: Repair of sublethal and potentially lethal radiation damage in plateau phase cultures of human cells. Nature 224: 804-806, 1969.

248) Marchese, M.J., Zaider, M., Hall, E.J.: Potentially lethal damage repair in human cells. Radiother. Oncol. 9: 57-65, 1987.

249) Hall, E.J.: Lethal, potentially lethal, and sublethal damage, and the dose-rate effect. In Radiobiology for the radiobiologist. Harper and Row, Publishers, Hagerstown, Maryland, New York, Evanstown, San Francisco, and London, 1978, pp 129-171.

250) Van Putten, L.M., Kallman, R.F.: Oxygenation status of a transplantable tumour during fractionated radiation therapy. J. Natl. Cancer Inst. 40: 441-451, 1968.

251) Thomlinson, R.H.: The effect of fractionated irradiation on the proportion of anoxic cells in an intact experimental tumour. Br. J. Radiol. 39: 158, 1966.

252) Howes, A.E.: An estimation of changes in the proportions and absolute numbers of hypoxic cells after irradiation of transplanted C3H mouse mammary tumours. Br. J. Radiol. 42: 441-447, 1969.

253) Kallman, R.F.: The phenomenon of reoxygenation and its implications for fractionated radiotherapy. Radiology 105: 135-142, 1972.

254) Howard-Flanders, P.: Effect of oxygen on the radiosensitivity of bacteriophage in the presence of sulfhydryl compounds. Nature 186: 485-487, 1960.

255) Howard-Flanders, P., Levin, J., Theriot, L.: Reactions of deoxyribonucleic acid radicals with sulfhydryl compounds in X-irradiated bacteriophage systems. Radiat. Res. 18: 593-606, 1963.

256) Brown, M.J.: The mechanism of cytotoxicity and chemosensitization by misonidazole and other nitroimadazoles. Int. J. Radiat. Oncol. Biol. Phys. 8: 675-682, 1982.

257) Bump, E.A., Yu, N.Y., Martin Brown, J.: Radiosensitization of hypoxic tumour cells by depletion of intracellular glutathione. Science 217: 544-545, 1982.

258) Griffith, O.W., Meister, A.: Potent and specific inhibition of glutathione synthesis by buthionine sulfoxamine (S-n-butyl homocysteine sulfoxamine). J. Biol. Chem. 254: 7558-7560, 1979.

259) Boyland, E., Chasseaud, L.F.: Enzyme-catalysed conjugations of glutathione with unsaturated compounds. Biochem. J. 104: 95-102, 1967.

260) Bump, E.A., Yu, N.Y., Brown, M.J.: The use of drugs which deplete intracellular glutathione in hypoxic cell radiosensitization. Int. J. Radiat. Oncol. Biol. Phys. 8: 439-442, 1982.

261) Clark, E.P., Epp, E.R., Biaglow, J.E., Morse-Gaudio, M., Zachgo, E.: Glutathione depletion, radiosensitization, and misonidazole potentiation in hypoxic Chinese hamster ovary cells by buthionine sulfoxamine. Radiat. Res. 98: 370-380, 1984.

262) Guichard, M., Jensen, G., Meister, A., Malaise, E.P.: Depletion of glutathione synthesis by buthionine sulfoxamine decreases the oxygen enhancement ratio of V79 cells. Radiat. Res. 94: 613 (Ec-10), 1983.

263) Russo, A., Mitchell, J.B., Finkelstein, E., DeGraff, W.G., Spiro, I.J., Gamson, J.: The effects of cellular glutathione elevation on the oxygen enhancement ratio. Radiat. Res. 103: 232-239, 1985.

264) Biaglow, J.E., Varnes, M.E., Clark, E.P., Epp, E.R.: The role of thiols in cellular response to radiation and drugs. Radiat. Res. 95: 437-455, 1983.

265) Mitchell, J.B., Russo, A., Biaglow, J.E., McPherson, S.: Cellular glutathione depletion by diethyl

maleate or buthionine sulfoxamine: No effect of glutathione depletion on the oxygen enhancement ratio. Radiat. Res. 96: 422-428, 1983.

266) Biaglow, J.E., Varnes, M.E., Epp, E.R., Clark, E.P., Astor, M.: Factors involved in depletion of glutathione from A549 human lung carcinoma cells: implications for radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 10: 1221-1227, 1984.

267) Biaglow, J.E., Varnes, M.E., Tuttle, S.W., Oleinick, N.L., Glazier, K., Clark, E.P., Epp, E.R., Dethlefsen, L.A.: The effect of L-buthionine sulfoxamine on the aerobic radiation response of A549 human lung carcinoma cells. Int. J. Radiat. Oncol. Biol. Phys. 12: 1143-1147, 1986.

268) Cohen, G., Hochstein, P.: Glutathione peroxidase: The primary agent for the elimination of hydrogen peroxide in erythrocytes. Biochem. 2: 1420-1428, 1963.

269) Thomas, H.F., Herriott, R.M., Hahn, B.S., Wang, S.Y.: Thymine hydroperoxide as a mediator in ionizing radiation mutagenesis. Nature (London) 259: 341-343, 1976.

270) Meyers, C.F., Katki, A., Travis, E.: Effect of tocopherol and selenium on defences against reactive oxygen species and their effect on radiation sensitivity. NY Acad. Sci. 329: 419-426, 1982.

271) Chance, B., Sies, H., Boveris, A.: Hydroperoxide metabolism in mammalian organs. Physiol. Rev. 59: 527-605, 1979.

272) Nishiki, K., Oshino, N., Jamieson, D., Chance, B.: Oxygen toxicity in the perfused rat liver and lung under hyperbaric conditions. Biochem. J. 160: 343-355, 1976.

273) Dische, S., Hewitt, H.B.: Carcinoma of cervix with severe anaemia: treatment by radiotherapy without blood transfusion using hyperbaric oxygen. Br. J. Radiol. 45: 848-850, 1972.

274) Adams, G.E.: New approaches for chemical radiation sensitization. Proc. 7th Annual Meeting ESTRO. 267, 1988.

275) Palcic, B., Brosing, J.W., Skarsgard, L.D.: Survival measurements at low doses: Oxygen enhancement ratio. Br. J. Cancer 46: 980-984, 1982.