41

THE EFFECTS OF THE MODIFICATION OF ENERGY METABOLISM ON CELLULAR RESPONSE TO IONIZING RADIATION

Alistair John Hunter

Thesis presented for the Degree of
DOCTOR OF PHILOSOPHY
in the Department of Radiation Oncology
UNIVERSITY OF CAPE TOWN
1996

The University of Cape Town has been given the right to reproduce this thesis in whole or in part. Copyright is held by the author.

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

MT 612.01448 HUN
97/10315

ABSTRACT

It is generally accepted that energy is required for repair of radiation-induced damage in living cells. Some of this energy is probably provided by adenosine triphosphate (ATP), which is derived from energy substrates via energy metabolism. This dissertation follows two general avenues. The first explores the effect of radiation on ATP levels after irradiation of cells. The second investigates the effect of inhibitors of certain pathways associated with energy metabolism on radiation response.

It was proposed that ATP levels might be raised after irradiation in some systems and that this rise in ATP might be due to compensatory mechanisms related to repair. Experiments were conducted using B16 melanoma cells *in vitro* and using normal murine liver and CaNT tumours *in vivo*. ATP concentration was measured in extracts of these cells after irradiation using the luciferase-luciferin method. No major changes from unirradiated controls were found.

Several types of substrates exist from which cells can derive energy, including glucose and glutamine which are initially metabolised via glycolysis and glutaminolysis, respectively, before their products are further metabolised in respiration. Since energy is necessary for repair of radiation damage, it has been proposed that the inhibition of energy metabolism might alter the radiation response of cells.

An inhibitor of glycolysis, 2-deoxyglucose (2DG), and an inhibitor of glutaminolysis, aminooxyacetic acid (AOA), were administered to CHO cells *in vitro* to determine the effects of these substances on cellular radiosensitivity and repair. Repair was assessed by means of a split radiation dose experiment. The design of such an experiment

required that cells be exposed to inhibitory test media for different times between two fractions of radiation. Any changes in clonogenic survival with time between fractions could, therefore, be as a result of repair effects or as a result of changes in radiosensitivity. A method of estimating and subtracting the effects of radiosensitivity to make conclusions concerning repair is presented and discussed. Most combinations of 2DG, AOA, glucose omission and glutamine omission in culture media resulted in reductions in repair rate but the extent of repair was found to vary from one medium variation to the next.

In addition, the effects of various culture media on glycolysis/PPP (glycolysis/pentose phosphate pathway) and glutaminolysis were investigated by determining the production of CO_2 and lactate from radiolabelled-glucose and -glutamine substrates. It was apparent that the presence of either of the inhibitors, 2DG or AOA, could inhibit the activity of glutaminolysis and reduce oxygen consumption. 2DG was shown to inhibit glycolysis/PPP but AOA was shown to stimulate glycolysis/PPP, suggesting a regulatory link between glutaminolysis and glycolysis/PPP. The presence of either inhibitor resulted in a reduction in the rate of radiation damage repair. The medium which had the most significant effect in respect of repair inhibition and increased radiosensitivity was medium lacking both glucose and glutamine and containing both 2DG and AOA. This medium was shown to inhibit oxygen consumption and to result in a depression of both cellular glycolysis/PPP and glutaminolysis.

The effect of 2DG on the rate of growth and radiation induced growth delay of three murine tumours *in vivo* was assessed. 2DG alone inhibited the growth of B16 tumours. However, 2DG alone produced little if any change in the rates of growth of Fib/T tumours and rhabdomyosarcomas but the combination of 2DG and AOA produced an inhbition of growth in the Fib/T tumour. 2DG appeared to enhance the effects of radiation in the Fib/T and B16 tumours but not in the rhabdomyosarcoma, although, in the Fib/T, the combination of AOA, 2DG and radiation was less effective in inhibiting tumour growth than was radiation alone. The effects of radiation and 2DG did not appear

to be additive in the Fib/T tumour and the B16 tumours which may imply an influence of 2DG on repair or radiosensitivity.

This work suggests that the effects of radiation can be altered by manipulation of metabolic pathways associated with the supply of energy. However, a complex interaction of pathways is probably also involved and it is the detail of this interaction which may partially determine the severity of radiation response.

ACKNOWLEDGEMENTS

I am extremely grateful to my supervisor, Professor Gerry Blekkenhorst for his able direction and for the many suggestions which he has made during the execution of this work. I also acknowledge with gratitude the advice and comments which I have received from Dr Andre Hendrikse. In addition, I would like to thank Rodger Duffett, Fatima Mohamed and Kevin Goldberg and those already acknowledged, for their assistance in counting cells and also for their comments. I appreciate the efforts of Zwai Nongalaza and Enver Orgill in their care of the mice. I am also deeply indebted to Dr Anwar Mall and the Department of Surgery, for the kind use of their freeze-drier. My thanks are also given to Dr Egbert Hering for his advice and several fruitful discussions. I also give sincere thanks to Dr Karen Esler for her comments on the manuscript. I am grateful to Leroy Constance and Mervyn de Rock for their help with the electronics. My thanks are given to the Department of Radiation Oncology for putting their resources at my disposal and to the Department of Medical Physics for the use of their printing facilities. The continued support afforded by the Medical Research Council is also acknowledged with appreciation.

TABLE OF CONTENTS

ABSTRACT. iii

ACKNOWLEDGEMENTS. vii

TABLE OF CONTENTS. ix

ABBREVIATIONS. xvi

CHAPTER 1 **INTRODUCTION.** 1

CHAPTER 2 **SOME EXTREMES OF THE CELLULAR ENVIRONMENT.** 5

HYPOXIA. 5

Energy Supply in the Hypoxic Environment. 6

pH AND CELL DEATH. 9

HYPOXIA AND pH. 9

CHAPTER 3 **RADIATION DAMAGE AND REPAIR.** 11

INTRACELLULAR TARGETS. 11

MODELS OF RADIATION ACTION. 12

REPAIR OF RADIATION DAMAGE. 14

Sublethal Damage (SLD). 16

Potentially Lethal Damage (PLD). 16

MODELS OF REPAIR. 18

Saturation of Repair. 18

Split Dose Analysis of Repair. 20

Recovery Ratio (RR). 23

Repair Half-life ($t_{1/2}$). 25
Repair Time (t_r). 25
Mean Repair Time 26
THE ROLE OF ENERGY IN REPAIR. 27
The Facilitation of Repair by ATP. 28
ATP as a Source of Free Energy. 29
pH and Radiation Response. 30
Hyperthermia and pH. 30
Ionizing Radiation and pH. 30
Lactate and Repair. 31
Membrane Changes. 32
Hypoxia and Repair. 33
Glycolysis, Cell Proliferation and Repair of Radiation Damage. 35
Repair, Misrepair and Fixation. 35

CHAPTER 4 **ENERGY METABOLISM AND THE CHOICE OF SUBSTRATE.** 39

CARBOHYDRATES AS ENERGY SOURCES. 40
Glucose. 40
Other sources of energy. 42
PREFERENCE OF SUBSTRATE. 45
Glutamine. 46
PASSAGE OF GLUCOSE AND GLUTAMINE ACROSS THE CELL MEMBRANE. 48

CHAPTER 5 **NORMAL AND TUMOUR CELL ENERGY METABOLISM.** 51

HETEROGENEOUS MICROENVIRONMENTS AND CELL POPULATIONS. 51
THE PASTEUR EFFECT. 53
THE WARBURG EFFECT. 54
THE CRABTREE EFFECT. 56
GLUTAMINOLYSIS. 57

CELL PROLIFERATION AND ENERGY. 57
ATP and Cell Kinetics. 58
Energy status and Tumour Size. 59

CHAPTER 6 **INHIBITORS OF ENERGY METABOLISM.** 61

INHIBITORS OF GLYCOLYSIS. 61
2-Deoxy-D-glucose. 62
INHIBITORS OF GLUTAMINOLYSIS AND ANTAGONISTS OF GLUTAMINE. 65
Aminooxyacetic acid. 65

CHAPTER 7 **FORMULATION OF HYPOTHESES.** 67

CHAPTER 8 **THE EFFECT OF RADIATION ON ADENOSINE TRIPHOSPHATE LEVELS IN CELLS.** 71

INTRODUCTION. 74
METHODS AND MATERIALS. 74
Experimental Animals. 74
Tumour. 74
Cells *In vitro*. 75
Irradiation. 75
CELLS *IN VITRO*. 76
LIVERS IN VIVO. 76
100 KVp X-RAY IRRADIATION OF TUMOURS IN VIVO. 76
Reagents. 77
Extraction of ATP. 77
ATP EXTRACTION FROM LIVER AND TUMOUR. 77
ATP EXTRACTION FROM BLOOD. 78
ATP EXTRACTION FROM CELLS IN CULTURE. 78
ATP FROM CELL CULTURE MEDIUM. 79
Luciferase-Luciferin ATP assay. 79
EQUIPMENT. 80

PREPARATION OF REAGENTS. 80
PROCEDURE. 81
Standard Curve. 81
Test Samples. 82
RESULTS. 82
ATP Content of Unirradiated Controls. 82
Cells *In vitro*. 81
Confluent Cells, 100 KVp X-rays. 83
Confluent Cells, ^{60}Co γ-rays. 84
Cells in Exponential Growth, ^{60}Co γ-rays. 84
Liver. 86
Tumour. 86
DISCUSSION AND CONCLUSIONS. 89

CHAPTER 9 **INHIBITION OF ENERGY METABOLISM AND THE MODIFICATION OF RADIATION RESPONSE. 93**

INTRODUCTION. 93

THE EFFECT OF DEPLETED CULTURE MEDIUM AND INHIBITORS ON THE RESPONSE OF CULTURED CELLS TO IONIZING RADIATION. 96
METHODS AND MATERIALS. 97
Reagents. 97
Cells. 97
Radiation. 98
Repair Experiments. 98
Sensitivity Experiments. 99
Experimental Culture Medium. 99
INHIBITORS. 101
2-Deoxy-D-glucose. 101
Aminooxyacetic acid. 101
RESULTS. 102
DISCUSSION. 135

CHAPTER 10 THE EFFECT OF 2-DEOXY-D-GLUCOSE ON TUMOUR GROWTH IN VIVO. 144

METHODS AND MATERIALS. 144
Animals and Tumours. 144
2-Deoxy-D-glucose. 145
Aminooxyacetic acid. 145
Irradiation. 145
Tumour Growth Assessment. 146
RESULTS. 147
DISCUSSION. 150

CHAPTER 11 METABOLIC CONSEQUENCES OF 2DG AND AOA ADMINISTRATION IN CELLS. 155

THE EFFECT OF DEPLETING GLUCOSE AND GLUTAMINE AND THE ADDITION OF 2DG AND AOA ON LACTATE AND CO_2 PRODUCTION IN CULTURED CHINESE HAMSTER OVARY CELLS. 156
METHODS AND MATERIALS. 158
Apparatus. 158
Conway units. 158
Scintillation counter. 159
High pressure liquid chromatography (HPLC). 159
Reagents. 159
Procedure. 159
Preparation of cells and medium for lactate analysis. 160
Extractions. 161
Derivatization with bromoacetophenone. 161
High Performance Liquid Chromatography. 161
Lactate Standard Curve. 162
RESULTS. 163
Total lactate and radiolabelled lactate. 163
Production of radiolabelled CO_2 . 165

DISCUSSION. 168
Total lactate and labelled lactate. 171
Production of labelled CO_2. 173

THE EFFECT OF DEPLETING GLUCOSE AND GLUTAMINE AND THE ADDITION OF INHIBITORS OF GLYCOLYSIS AND GLUTAMINOLYSIS ON OXYGEN CONSUMPTION. 177

METHODS AND MATERIALS. 177
The electrode. 177
Polarographic Oxymetry of the bipolar electrode. 177
Oxygen Chamber. 179
Testing the system. 179
Experimental Procedure. 181
RESULTS. 182
DISCUSSION AND CONCLUSIONS. 182

CHAPTER 12 SUMMARY AND CONCLUSIONS. 185

APPENDIX STANDARD METHODS, REAGENTS AND MEDIA. 191
TISSUE CULTURE REAGENTS AND MEDIA. 191
McCoy's 5a Medium. 191
Trypsin 0.05%. 192
Ethylenediaminetetra-acetic acid (EDTA). 192
Hanks' Balanced Salt Solution (HBSS) (without glucose). 193
TISSUE CULTURE TECHNIQUES. 193
Trypsinization of cells *in vitro*. 193
Staining and assessment of clonogenic survival. 194
EXPERIMENTAL ANIMALS. 194
Passaging of tumours. 195
3-Methylcholanthrene-induced Rhabdomyosarcoma. 195
Fib/T Tumour. 195

CaNT Tumour. **195**
B16 Tumour. **196**
LIST OF REAGENTS **196**
REFERENCES 199

ABBREVIATIONS

ADP	Adenosine Diphosphate
AMP	Adenosine Monophosphate
AOA	Aminooxyacetic acid
ATP	Adenosine Triphosphate
ATPase	Adenosine Triphosphatase
CHO	Chinese Hamster Ovary
Cr	Creatine
CrP	Creatine phosphate
2DG	2-Deoxy-D-glucose
2,4 DNP	2,4-Dinitrophenol
EAT	Erhlich Ascites Tumour
$FADH_2$	Reduced Flavin Adenine Dinucleotide
HBSS	Hanks' buffered salt solution
I.P.	Intraperitoneal
KCN	Potassium Cyanide
LPL	Lethal Potentially Lethal
LQ	Linear Quadratic
NAD^+	Nicotinamide Adenine Dinucleotide
NADH	Reduced NAD^+
Na/K-ATPase	Sodium/Potassium ATPase
NTP	Nucleotide Triphosphate
PEP	Phosphoenolpyruvate
PFK	Phosphofructokinase
Pi	Inorganic phosphate
PK	Pyruvate kinase
PLD	Potentially Lethal Damage
PLDR	PLD repair
PPP	Pentose phosphate pathway
RMR	Repair Misrepair

RR	Recovery Ratio
sem	Standard error of the mean
SLD	Sublethal Damage
SLDR	SLD repair
SPF	Specific pathogen free
SSD	Source to surface distance
$t_{1/2}$	Repair half-life/half-time
U-^{14}C-	Uniformly labelled with ^{14}C (All carbon atoms in molecule are ^{14}C)

CHAPTER 1

INTRODUCTION

There is a multitude of points within a mammalian cell where manipulation of various processes may influence the function of that cell. Such interference may lead to alterations in the living cell or may cause or contribute to its death.

Radiation therapy of cancer deals with sterilization and ultimate killing of cells. The efficiency with which cells are killed and any differential between damage caused in tumour and normal tissue are important. It is, therefore, of great practical significance to discover new ways of manipulating cells and tissues so that they respond differently to radiation.

One aspect of cell physiology involves the ability of a cell to use fuel substrates from which it can derive energy and so power its activities. The role of adenosine triphosphate (ATP) has been well established as the energy currency of the cell (Mathews and van Holde, 1990). So, it is largely the supply of this molecule which can either directly or indirectly determine whether a cell can perform certain functions or not. The linking of an energetically favourable reaction such as the hydrolysis of ATP to energetically unfavourable reactions allows the cell to work. Hence, syntheses, active transport, cellular repair and certain enzyme mediated events, to name a few examples, are able to take place.

ATP is essentially provided by energy metabolism via a number of different substrates, for example, carbohydrates, fats and amino acids. For substrates to be used, they must first be internalised by the cell

and introduced to the various biochemical pathways which, through suitable generation of flux of material, provide energy intermediates for the cell.

If the sequence of events which begins with the entry of energy substrates into the cell is followed, a number of suitable places can be identified at which interference may lead to a potential modification in energy supply to the cell. Several factors which may affect energy supply include the ability of energy substrates and other substances to enter the cell, the type of substrate, the metabolic pathways involved, the inhibition of steps in such pathways and the initiation of events which lead to physiological modifications of the cell. For example, damage induced in a cell may result in malfunctions or compensatory modifications.

In order to achieve effective treatment of cancer, it is necessary to exploit natural differences between tumours and normal tissue. It may be possible to create a more severe response in malignant tissue than in healthy tissue. Many strategies exist for creating such a differential between tumour and normal tissues *in vivo*. Accurate radiation dosimetry and fractionation of the treatment dose have partially addressed the problem of lack of differential. The use of cytotoxic drugs allows systemic treatment of patients with cancer but toxic side effects are often a limiting factor. Certain cytotoxics may be used as cytotoxic agents alone or in combination with radiation to increase radiosensitivity as well. Ideally, a clinically useful radiosensitizer should have no toxicity of its own and affect only irradiated tumour cells.

Cells from different organs, sites, species and origins often have widely differing metabolisms but the machinery of many pathways is very similar and frequently functions according to identical principles. The addition of a biochemical modifier to two cell types with similar but different target pathways might, therefore, result in similar responses of different intensity, thereby creating a differential response. This approach could be applied to tumour and normal tissue.

It has been suggested that repair of radiation-induced damage is dependent on cellular energy supply and that if energy metabolism is inhibited, the response of cells to ionizing radiation might be altered (Matsudaira *et al.*, 1970; Jain *et al.* 1977a). If this were the case, then the approach could be applied to different tissue types, such as tumour and normal tissue, in an attempt to achieve a differential radiation response using inhibitors that inhibit energy metabolism more in tumours than normal tissue.

A general review of published work relating to energy metabolism and radiation damage repair as well as certain observations and comments by the author are presented in Chapters 2 to 7. In Chapters 8, 9, 10 and 11, the experimental work is presented. There are two general independent avenues which the experimental work in this thesis takes. The first attempts to evaluate further the presence of a compensatory increase in ATP content in tumour cells after radiation, which has been found to occur in certain mammalian systems (Szienfeld, 1987). It has been proposed that this phenomenon may be associated with repair processes after exposure to ionizing radiation. The second avenue examines the effect of glucose and glutamine, two established energy substrates, and inhibitors of glycolysis and glutaminolysis on the radiation response of cells *in vitro*. Of interest, is the interaction of glycolytic and glutaminolytic pathways as elucidated by metabolic studies.

CHAPTER 2

SOME EXTREMES OF THE CELLULAR ENVIRONMENT

There are several conditions which have effects on the state of cellular energy metabolism. Many of the mechanisms associated with these effects are common to several modifiers of energy status. A discussion of the results of exposure of cells to such modifiers is, therefore, pertinent to the understanding of changes in energy metabolism induced by other circumstances. The supply of oxygen, pH and the availability of fuel are known to be important factors in determining the response of cells to radiation and to each other. Energy metabolism is particularly susceptible to alterations of these factors and can have a significant effect on the outcome of cellular damage.

HYPOXIA

Terms like anoxic and hypoxic are often used to describe states (anoxia and hypoxia) of tissue or cells when they are far removed or cut off from oxygen (Guyton, 1986). The implications of a reduction of oxygen supply are many. If a state of anoxia exists, there is no oxygen present and processes which require oxygen in order to function will shut down. If a state of hypoxia exists, it means that there is an amount of oxygen present which is below that found in the normal, well oxygenated state. The definition of hypoxia can take on more specific meaning in radiobiology. Radiobiological hypoxia has been defined as when cells have a partial pressure of oxygen below 20-30 mm Hg (Hirst, 1986). Another term, ischaemia, refers to the situation when blood supply is witheld from a certain organ or site thus preventing supply of oxygen and nutrients and the removal of waste-products.

The importance of oxygen as a radiosensitizer is well established and its importance in radiotherapy has been dealt with extensively (Gray *et al.*, 1953; Gray, 1961; Hall, 1994). The presence of hypoxic cells in tumours may present problems in that they are radioresistant (Powers and Tolmach, 1963) and limit the effectiveness of radiation treatment. The process of reoxygenation between fractions during radiotherapy, as described by van Putten and Kallman (1968), is beneficial to the outcome of cancer treatment. Apart from the quantity of oxygen present in cells, which will determine the extent of radiation damage due to the interaction of free radicals (oxygen is thought to fix radiation damage), there are structural and functional consequences of low oxygen in cells which may determine the outcome of any treatments directed at the cells (Hall, 1994). These consequences will be considered later.

Hypoxia has been shown to modulate signal transduction and gene expression. In addition, hypoxic conditions have been shown to result in increased synthesis of specific proteins even though protein synthesis as a whole may be suppressed. These proteins have been termed hypoxic stress proteins (Sutherland, 1995). Previously, Sutherland (1986) and Sutherland *et al.* (1986) described the presence of oxygen related proteins (ORPs). It is thought that cells which are hypoxic, and also radioresistant, may make tumours more aggressive or more metastatic.

Different tissues have different sensitivities to hypoxia. Brain is especially susceptible to injury after exposure to reduced oxygen concentration while other tissues such as liver, kidney and muscle are more resistant (Robbins, 1974).

Energy Supply in the Hypoxic Environment

A primary effect of hypoxia is to inhibit aerobic respiration and thereby cause a serious reduction in the production of ATP (Gerweck *et al.*, 1993, Jennings and Reimer 1981). This may be true for both normal and tumour tissues. Tozer *et al.* (1987) showed that murine kidney and MCaIV tumour exhibited increases in anaerobic glycolysis as well as an increase in AMP and a decrease in ATP after occlusion of blood supply.

It appears that anoxia is not the only factor which determines energy status after oxygen depletion. The ATP content of cultured monkey kidney cells (LLC-MK2) has been shown to decrease under conditions of anoxia (Snowdowne *et al.*, 1985). Cells kept in medium lacking glucose showed both a drop in ATP and an increase in calcium efflux after an hour in anoxia. After reperfusion with oxygen and 5mM glucose, ATP and calcium levels returned to normal. However, in the presence of 20 mM glucose, anoxia did not affect ATP or calcium levels.

Eskey *et al.* (1993) investigated the relative importance of oxygen and glucose in energy metabolism in an *ex vivo* tumour model. The NTP/Pi ratio of tumours, which indicates the relative presence of high energy nucleotide phosphates to separated inorganic phosphates, did not change when oxygen was reduced but when glucose was removed or replaced with glutamine, the ratio dropped. In this case, at least, the energy supply in the tumour seemed to be predominantly glucose mediated. Tumour energy status was also shown to be dependent on changes in blood flow. Gerweck *et al.* (1993) showed a similar trend in CHO cells. Energy status was found to be much more sensitive to hypoxia when glucose was absent than when it was present.

Reperfusion of myocardial cells after ischaemia may result in the influx of calcium into the cell as a result of altered calcium homeostasis (Nayler, 1981). Excess calcium ions may affect mitochondrial function by activating phospholipases which degrade membrane phospholipids and disrupt membrane integrity. Some of the effects of hypoxia may, therefore, be reperfusion related.

Depletion of ATP alone is not the only factor which determines whether necrosis takes place if factors such as cell permeability are controlled in other ways. ATP (Jennings and Reimer, 1981) and creatine phosphate are decreased after ischaemia of heart muscle (Nayler, 1981). A drop in ATP levels has significant effects on ion exchange across the cell membrane (Jennings and Reimer, 1981). Permeability is increased dramatically when ATP drops below a threshold level. The sodium pump relies on ATP-dependent active transport. When the sodium pump is inhibited, sodium leaks into the cell followed by fluid which causes the

cell and organelles to swell (Robbins, 1974). This ultimately leads to the death of the cell. In dog ventricular muscle, when ATP drops below 2 μmol/g dry weight, cell membrane permeability is increased to such an extent that the cell is committed to undergo necrosis. Once this has happened, reperfusion with well-oxygenated fluid will not reverse the process. However, chlorpromazine, which blocks calcium flux across the cell membrane, protects cells against the lethal effects of ischaemia despite a low ATP level (Farber *et al.*, 1981).

Permeability of the cell membrane of rat liver cells is altered when the blood supply is withheld. Ischaemic cells lose phospholipids, notably phosphatidylcholine and phosphatidylethanolamine (Chien *et al.*, 1978). This is thought to be due to the action of phospholipases from lysosomes and cell membranes. Phospholipase activity has been linked to the entry of calcium into cells. Rupture of lysosomes leads to gross cellular damage and autolysis (Robbins, 1974). Injury to cell membranes may involve the endoplasmic reticulum which will affect protein synthesis. Villa-Trevino *et al.* (1966) showed in rat liver cells that a decrease in ATP induced by ethionine produced a corresponding decrease in RNA synthesis. If protein synthesis is disrupted by a low cellular ATP level, certain enzyme-mediated functions of the cell may be inhibited. Chlorpromazine also prevents the loss of phospholipids from ischaemic cells (Chien *et al.*, 1978).

So it would seem that hypoxia is only one factor which may result in an altered energy status. This is reinforced by Gerweck *et al.* (1992) who noted that two tumours *in vivo*, which had different hypoxic fractions, did not have matching differences in energy status. Also, it is possible that in some tissues, an altered energy status may influence the degree of hypoxia through adaptive physiological mechanisms which modify oxygen utilization. There is evidence obtained from the *in vitro* sandwich model (Hlatky *et al.*, 1986) that growth of cells in a low glucose concentration can reduce the hypoxic fraction (Hlatky *et al.*, 1989).

pH AND CELL DEATH

Definite, narrow ranges of pH within the cell are required for enzymes to function adequately (Newsholme and Leach, 1983). If the cell is exposed to an environment that is too far removed from that which is optimal, then the cell will not function correctly and with chronic exposure to a sub-optimal pH, will eventually die (Guyton, 1986). The pH of body fluids must be close to 7 for an animal to survive. However, cells taken from the body can tolerate much greater variations in pH. Generally, mammalian cells can withstand pHs ranging from 6.6 to 7.8 without serious consequences. Optimal growth usually occurs between pH 7.2 to 7.4. Most cells will die within 24 hours if they are exposed to pH less than 6.8 or greater than 7.8 (Paul, 1970).

Metabolic changes have been observed in cells exposed to extremes of biological pH. Several investigators have shown that the rate of glycolysis is maximal under basic pH conditions (pH 8) and that the rate decreases with increasing acidity (Halperin *et al.*, 1969; Rotin *et al.*, 1986).

HYPOXIA AND pH

It has been reported that in order for ATP levels to decrease during hypoxia, there must be an accompanying drop in alkalinity to at least pH 6. CHO cells exposed for 6 hours to hypoxia at pH 6 resulted in an 85% drop in ATP (Rotin *et al.*, 1986). All glycolytic enzyme activities have been shown to be raised during hypoxia whether the enzyme is rate-limiting or not (Robin *et al.*, 1984). When glycolysis is upregulated to compensate for a reduction in ATP, reduced pyruvate oxidation and the lactic acid build up may result in a decrease in pH.

Rotin *et al.* (1986) considered the effect of hypoxia on cell viability of CHO cells and the human bladder cancer cell-line, MGH-UI, kept in an environment at pH 7. In MGH-UI cells kept at pH 7, exposure to hypoxia for up to 6 hours had little effect on cell viability but exposure of aerobic cells to pH 6 was cytotoxic. The combination of hypoxia and an acidic pH resulted in a decreased plating efficiency. Although

hypoxia together with low pH resulted in a marked drop in cell survival which depended on the duration of exposure and pH, no effect on plating efficiency was noted in CHO cells exposed to hypoxia or low pH (pH 6-6.5) for up to 6 hours. In both the CHO and MGH-UI lines, glucose consumption and lactate production were inhibited by low pH and glycolysis was almost completely stopped at pH 6. Glucose consumption and lactate production by CHO cells was greater under hypoxic conditions than in air but MGH-UI cells had similar rates under both aerobic and hypoxic conditions. Net pyruvate removal from both cell-lines was not affected by pH and was slightly higher in air than in hypoxia (Rotin *et al.*, 1986).

Glucose depletion of CHO cells resulted in a gross reduction in ATP and energy charge compared to the values for hypoxic cells at pH 6. The greatest reduction in ATP levels and in cell viability took place under conditions in which pH was low (pH 6), cells were hypoxic and glucose was absent (Rotin *et al.*, 1986).

Sutherland *et al.* (1986) showed how pO_2 decreases from the periphery to the centre of spheroids. The thickness of the viable rim of cells surrounding the necrotic centre of spheroids is related to the concentration of glucose in which the spheroids were cultured (Sutherland, 1986). Although a low oxygen content may result in the death of tumour cells which are far removed from their blood supply, low glucose and high lactic acid concentrations may also contribute appreciably. It is possible, in tumours with a poor blood supply, that a low oxygen level may often be associated with a low glucose level .

Finally, reduced extracellular pH delays the expression of cell death in hepatocytes depleted of ATP. In these cells which were exposed to KCN or iodoacetate to mimic hypoxia, a more alkaline pH (7.4) resulted in greater cell death than cells which were exposed to a more acidic pH (5.5-7). Inhibition of sodium-hydrogen exchange increased cell survival. Promotion of exchange of intracellular chloride ions for HCO_3^- increased cell killing. At pH 6.4 and pH 7.4, ATP levels after exposure of cells to KCN and iodoacetate were reduced to less than 5% of normal levels (Gores *et al.*, 1988).

CHAPTER 3

RADIATION DAMAGE AND REPAIR

INTRACELLULAR TARGETS

Damage induced by ionizing radiation is inflicted by radiation-induced free radicals which disrupt biologically important molecules (Hall, 1994). Radiation can cause many types of damage in the cell. Damage caused by radiation may result in reproductive death, interphase death, division delay, chromosomal aberrations, mutations and may transform cells (Hall, 1994; Scherer *et al.*, 1991; Cole *et al.*, 1980). Since radiation deposits its dose throughout the cell, every cell in an irradiated field will probably be damaged to some degree. The end result of such damage will, therefore, be determined by the nature and extent of the damage.

There are several radiation targets within the cell which are of differing relevance to cellular response in the therapeutic dose range. Enzymes and other proteins are thought to be fairly resistant to radiation. Cellular membranes have also been considered as radiation targets (Review by Edwards, 1984). The premier candidate as the critical target for radiation damage is, however, the nucleus, more specifically, DNA (Hall, 1994).

There are several types of recognised DNA damage which occur as a result of radiation including single strand breaks, double strand breaks, base damage, DNA-DNA and DNA-protein cross-links. These end points have been outlined in a review by Kelland and Steel (1988). There are also DNA-nuclear membrane attachment sites at which DNA is more

radiosensitive than at other locations in the nuclear DNA (Cole *et al.*, 1980).

Damage will depend on the pattern of ionization in an irradiated volume. For this reason, it is necessary to consider the deposition of energy and the number of ionizing events. Complex three-dimensional cluster damage within DNA has been modelled using Monte Carlo methods (Goodhead, 1995a). It was reported by Goodhead (1995b) that the majority of DNA double strand breaks are complex and that very few are simple. This has relevance when considering the repairability of DNA damage.

MODELS OF RADIATION ACTION

A number of models have been proposed by several authors to describe radiation responses in cells. Some of the better known models are briefly considered so that a familiarity with the approaches and possible mechanisms can be gained.

1) Target theory: single hit, single-hit multi-target (Zimmer, 1961).
2) Dual radiation action models of Kellerer and Rossi (1972, 1978).
3) Linear-Quadratic (LQ) model of Chadwick and Leenhouts (1981).
4) Cybernetic model of Kappos and Pohlit (1972).
5) Lethal potentially-lethal (LPL) model of Curtis (1986).
6) Repair-misrepair (RMR) model of Tobias (1985).
7) Inducible repair - a model by Joiner and Marples (1992) to explain the initial low dose region of the survival curve.

Target theory was postulated to describe the way in which radiation acts on certain undefined critical targets in the cell thereby inactivating them. This inactivation would lead to changes in cellular function including cell death. The critical target can either be hit resulting in critical damage or missed resulting in no effect. This single-hit kinetics yields a simple exponential type dose response relationship, $S=e^{(-\lambda D)}$ (where S is surviving fraction, D is dose and λ is the rate constant), which is simplistic and does not take repair of radiation damage into account. An extension of this theory is the single-hit multitarget theory

in which several targets must be inactivated before a critical event occurs. This roughly describes how sublethal events must be accumulated before critical events occur. This is illustrated by the response of the radiation survival curve which exibits an insensitive shoulder region followed by a steeper more dose-responsive region.

The dual radiation action theories of Kellerer and Rossi (1972, 1978) introduced the concept of the interaction of pairs of sublethal lesions which were able to interact to form lethal lesions. From this, an expression was derived to describe radiation response which included a linear and a quadratic component: $S=e^{-(aD+bD^2)}$. Repair is taken into account in the exponential function in the b term.

The so called Linear-Quadratic (LQ) model of Chadwick and Leenhouts (1981) is an extension of Kellerer and Rossi's model (1978) and uses the same mathematical equation to describe radiation response. The theory, however, is more mechanistic in that it relates all damage to single and double strand breaks of DNA and the equation is usually written with the coefficients α and β substituted for a and b in the Kellerer and Rossi model. The α term relates to damage from the path of one ionizing particle track causing a critical DNA double strand break which is considered to be irreparable. The β term relates to damage caused by two separate ionizing particle tracks which cause two sublethal single strand breaks in close proximity to interact, thereby resulting in a critical double strand break. The model postulates that some sublethal single strand damage can be repaired before the second sublethal event and thus prevent a lethal event from occurring. There is, therefore, a time dependence on repair of DNA.

The Cybernetic model of Kappos and Pohlit (1972) has been modified by Curtis (1986) to create the Lethal-Potentially-Lethal model. Both models describe the presence of an intermediate stage of damage (B), as shown in Figure 3.1, which can either be repaired, in which case a sublesion is reversed (B$\rightarrow$A), or not, in which case it may progress to an irreparable, critical lesion (B$\rightarrow$C). The models also postulate the production of irrepairable damage (A$\rightarrow$C) which has no intermediate step. Irradiated cells could, therefore, be divided into three groups,

those which incur damage which has no bearing on their survival, those which incur damage which may cause cells to die if it is not repaired and those which incur damage which will result in their death because of irreparable damage (Figure 3.1).

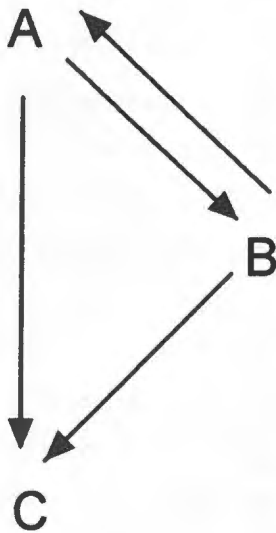


Figure 3.1: Schematic diagram showing three states of irradiated cells. A: No damage, B: Repairable damage, C: Irrepairable damage.

The Repair-Misrepair model of Tobias (1985) postulates no irrepairable damage. All damage, which is characterised by U-lesions (uncommited lesions), is either repaired or misrepaired. Lesions, as before, can interact and their existence is time dependent. Linear repair and quadratic misrepair processes are postulated to compete with one another and therefore determine survival.

The radiation response of many cell-lines including human fibroblasts and human tumour cell lines is well fitted by the Linear-Quadratic and other models above. However, with more accurate analysis using flow cytometry and dynamic image processing scanning (DMIPS), closer study of the low dose region of the survival curve (<0.5 Gy) has been possible. In many cell-lines, a low dose hypersensitivity reaction is shown to occur followed by a decrease in radiosensitivity at higher doses. This may be due to induced repair. If induced radioresistance is the case, then in a split dose experiment, the first dose should initiate an adaptive response that provides protection against a subsequent exposure (Joiner and Marples, 1992; Marples and Joiner, 1993).

REPAIR OF RADIATION DAMAGE

Repair of radiation damage has already been introduced in the preceding section because it forms an integral part of any model used to describe radiation response. Cells which are damaged by radiation can repair themselves to different extents. The critical form of radiation lesion is generally accepted to be some form of DNA damage but this is not to say that other less apparent forms of damage do not influence the fate of

cells as well. Repair of DNA damage is generally accepted as being enzyme mediated.

Base excision repair or nucleotide excision repair may take place depending on the nature of the DNA damage (Friedberg *et al.*, 1995). Base excision repair (XRCC1 gene mediated) occurs in response to alterations or modifications of bases. Base damage is recognised by DNA glycosylases which catalyse the hydrolysis of N-glycosylic bonds which link the altered bases to the deoxyribose backbone. The altered base is thus excised and the deficient sites in the DNA attacked by endonucleases which cause a strand break with a 5' terminal deoxyribose phosphate moiety. This is excised by DNA deoxyribophosphodiesterase. The single nucleotide void is then made good by repair synthesis and DNA ligase. (XRCC1 protein stimulates ligase III). Nucleotide excision repair results from the presence of consequences of damage such as pyrimidine dimers which are recognised by damage specific endonucleases which nick the DNA strand on each side of the lesion to free an oligonucleotide fragment which is excised. Repair synthesis followed by DNA ligase activity follows. Nucleotide excision repair is mediated by the Excision Repair Cross Complementing genes (ERCC 1-9). This system is important under conditions of hypoxia. In addition, proteins such as Ku protein and DNA-dependent protein kinase (XRCC 5-7 mediated system) have been found to be involved in the repair of DNA double strand breaks indicating links between DNA repair and other cellular processes. (Friedberg *et al.*, 1995).

There is a distinction to be made between recovery and repair although the two terms are often interchanged. Repair occurs when damage is reversed whereas recovery will include any factor which results in an improvement in the status of a population of damaged cells. Improvement may result from factors not directly associated with repair, for example, proliferation. Often, conclusions are drawn concerning repair from experiments which use cell surviving fraction as an end point. For example, in a clonogenic assay used to assess the effect of radiation on cell survival, proliferation of cells prior to plating will result in spuriously high surviving fraction values. If cells divide before plating, the number of viable cells plated to form colonies would be

high, not because a greater proportion of cells survives a dose of radiation, but because of a proliferation artifact. Although surviving fraction as an end point gives information concerning the outcome of damage and repair processes after radiation, it is, nevertheless, an indirect method which should be combined with more direct studies of damage and repair of selected critical targets.

Repair of radiation damage is often described by the type of experiment used to detect the presence of such damage. Sublethal damage and potentially lethal damage, which are determined from clonogenic assays, are briefly described in the following section.

Sublethal Damage (SLD)

Sublethal damage is damage which is not sufficient to kill a cell. This damage must be accumulated in order to become lethal. Repair of SLD is often investigated by means of split dose experiments as first conducted by Elkind and Sutton (1959). Cells are able to repair SLD even after several doses of radiation. Radiation therapy is often administered in multiple fractions to allow SLD to repair between dose fractions and thereby to increase the tolerance of normal tissue. Both normal and tumour cells can repair SLD. Less SLD is able to accumulate after exposure to high LET radiations such as neutrons than after exposure to lower LET radiations such as X-rays (Hall, 1994).

Potentially Lethal Damage (PLD)

The concept of potentially lethal damage was described by Phillips and Tolmach (1966). PLD is damage which will only be expressed if the post irradiation conditions allow it. A frequently used example of potentially lethal damage is when cells are irradiated and plated out immediately compared to when they are plated some hours later. The survival of cells is greater when the plating of the cells is delayed. Potentially lethal damage repair (PLDR) is said to have occurred. The expression of such damage may depend on the presence of a drug or other set of circumstances which affect post-irradiation cell conditions. Post-irradiation conditions which are sub-optimal for cell growth often allow

repair of PLD to occur. The term, liquid holding recovery, is usually applied to irradiated diploid yeasts which are kept in nutrient-free liquid, such as water or saline, prior to plating (Alper, 1979). Under such conditions, PLD repair is able to take place. Some mammalian cells have also been shown to recover more effectively if held in balanced salt solution for a time prior to plating in nutrient medium (Alper, 1979).

Raaphorst and Dewey (1979) showed that hypo- or hypertonic salt solutions could be used to fix, that is, make permanent PLD. PLD fixation is thought to be due to factors which change the conformation of chromatin (Iliakis, 1988). Certain post-irradiation conditions such as cell cycle progression may also fix radiation damage. Reddy *et al.* (1989), however, showed that increased survival due to delayed plating can be partly explained by effects such as trypsinization and not because of cell division itself.

At least two different types of PLD have been shown: a fast type (within 1 hour) which can be fixed by 0.5 M NaCl and a slower type which relates to progression through the cell cycle (2-6 hours) (Kimura *et al.*, 1995). It was shown by Marchese *et al.* (1987) in three human cell lines that the extent of PLD repair is greater in plateau phase cells than in exponential phase cells.

Little (1971) showed that survival curves of exponentially growing cells kept in conditioned medium after irradiation were shifted to the right of those for cells kept in fresh medium after irradiation. V79 cells *in vitro* have been reported to have a higher degree of survival after radiation when plating is delayed in conditioned medium than in growth medium. PLD remained unrepaired during delayed recovery in growth medium but could be repaired when medium was later changed to conditioned medium (Lange *et al.*, 1995). Repair phenomena which are related to nutrient supply in the post-irradiation environment, may be determined by events which occur when cells are allowed to repair under energetically favourable conditions.

Sublethal damage and potentially lethal damage are non-specific terms and may even be part of the same type of damage. The exact mechanisms

of such damage are unknown. Some workers prefer to steer clear of the above terminology and refer rather to terms such as repairable damage and non-repairable damage (Pohlit and Heyder, 1981).

MODELS OF REPAIR

Saturation of Repair

Repair is an enzymatically controlled process and so has been described in a similar way to Michaelis-Menten enzyme kinetics (Thames, 1989). Repair terms can be applied to a Michaelis-Menten type equation:

$$dN/dt=VN/(K+N) \qquad (1)$$

where dN/dt is the rate of change of number of potentially lethal lesions; N=number of potentially lethal lesions; t=time; V=Maximum rate of recovery and K=Number of lesions at V/2.

At small radiation doses, the number of radiation induced lesions, N is small. When N is small K+N approximately equals K and the rate of repair, dN/dt is approximately (V/K)N, that is, repair is exponential with rate constant=V/K. When dose is large, N is large and K+N approximately equals N. dN/dt is therefore equal to V, a constant, that is, repair rate saturates at V.

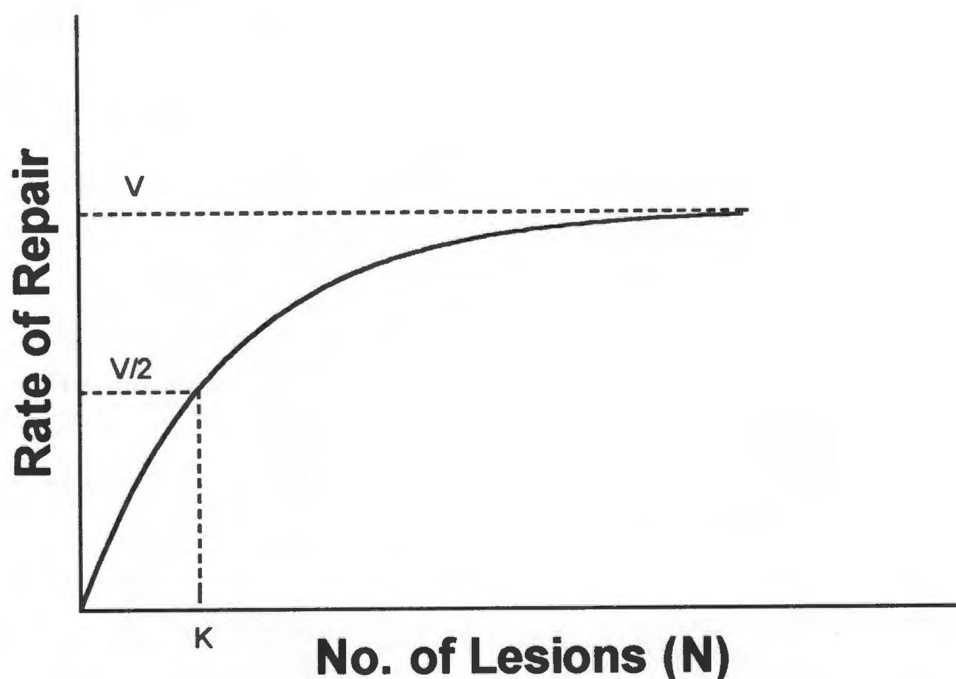


Figure 3.2: The saturable kinetics of the repair mechanism.

Under unsaturated conditions, the number of lesions is less than the number of units of repair machinery. When saturation of the repair machinery takes place, the rate of repair cannot be increased any further and from this point, a constant number of lesions are repaired per unit time (Wheeler, 1987). Pohlit and Heyder (1981) reported from PLD studies using EAT cells, that PLD is repaired for about an hour after radiation by a saturated repair mechanism which removes about one lesion every 15 minutes per cell. They also discussed the presence of a slower unsaturated process.

Under unsaturated conditions, the half time for repair ($t_{1/2}$) is the same for all doses (Wheeler and Nelson, 1987). This occurs in the relatively low dose region. At higher doses, when saturation is reached, $t_{1/2}$ increases with dose as repairable damage accumulates. Repairable lesions occur in a dose-dependent manner so, once saturation has occured, repairable lesions may accumulate and interact to form irrepairable lethal lesions.

Wheeler (1987) has suggested that cells are killed as a result of post-irradiation metabolic processes competing with one another. Cells may

be committed to die at a time which depends on the rate of repair of critical damage and rate of cellular metabolism that creates a need for normal functioning of the critical targets. Cells in which cellular targets are not repaired by a certain time after radiation are committed to die irrespective of the amount of damage at the time of death. The probability of cell survival is, therefore, dependent on the rate of production of DNA lesions, the rate of removal of DNA lesions and the rate of metabolic processes required for maintaining cellular integrity. The Accumulation-Interaction model of Reddy *et al.* (1990) postulates that competition between the rate of damage production and rate of repair will determine the kinetics of repair, that is, whether it is saturated or unsaturated, and that this competition occurs only during irradiation, when damage is interacting and accumulating.

A certain amount of confusion has arisen concerning the relationship between radiation dose and repair rate. Repair rate may be affected by the size of the dose of radiation but the relationship may differ depending on the tissue or system investigated. In mouse skin, a faster rate of recovery was found after 4.4 Gy fractions than 10.5 Gy fractions. However, a slower rate of recovery was noted for kidney after 2 Gy than after 7 Gy fractions (Rojas and Joiner, 1989). Reddy *et al.* (1990) showed a linear relationship between repair time, the time for cells to recover and reach maximum possible survival levels after irradiation, and dose in V79 cells. A linear relationship was also found between repair half time, the time to reach half maximum survival levels after radiation, and dose. Log of recovery ratio, the survival of cells given time to repair relative to the survival of cells given no time to repair, was shown to vary in a linear-quadratic way with dose.

Split Dose Analysis of Repair

Several models exist which describe repair kinetics in terms of cell survival. Such models can be applied to split-dose repair data *in vitro* to provide useful answers concerning rates and other characteristics of repair in different systems and under different repair conditions. A mono-exponential model may be used to describe repair. Such a model implies that the repair rate is proportional to the number of radiation

induced lesions and that an equal proportion of lesions are reversed for a given increment in time. Repair may follow a pattern similar to that of Michaelis-Menten enzyme kinetics where the number of lesions decreases in an exponential manner. A mono-exponential model often used to describe split-dose repair is the Incomplete Repair model of Thames (1985).

The linear-Quadratic model (Chadwick and Leenhouts, 1981) describes radiation response in the relatively low dose region. The description of repair in terms of linear quadratic parameters by Chadwick and Leenhouts (1981) and the later description of the incomplete repair model of Thames (1985) essentially describe the same analysis for repair between two fractions of radiation.

The equation :

$$N=\alpha D + \beta D^2 \qquad (2)$$

represents the relationship between the number of critical radiation induced lesions (N) and dose (D). α and β are constants.

If there is no time between 2 fractions (D_1 and D_2) of radiation i.e. there is no time between two doses and two fractions are considered as a single dose,

$$N=\alpha(D_1 + D_2)+\beta(D_1+ D_2)^2 \qquad (3)$$

$$=\alpha(D_1 + D_2)+\beta(D_1^2+2D_1D_2+ D_2^2) \qquad (4)$$

When there is enough time for complete repair to occur, the effects of the two fractions are additive.

$$N=\alpha D_1 +\beta D_1^2 +\alpha D_2 +\beta D_2^2 \qquad (5)$$

Repair is a function of time which is described by an exponential function: $f(t)= e^{-\lambda t}$, where λ is the rate constant and t is time.

In equation (4), the additional number of lesions present as a result of incomplete repair is given by the term $\beta(2D_1D_2)$.

Hence, including the time function,

$$N=\alpha\ (D_1 + D_2)+ \beta\ (D_1^2+2D_1D_2.f(t)+ D_2^2), \qquad (6)$$

For doses of equal magnitude: $D_1 = D_2 = d$ and

$$N=2\alpha d + 2\beta d^2 + 2\beta d^2\ e^{-\lambda t} \qquad (7)$$

Cell survival, S is related to the number of lesions, N by the following formula:

$$\ln S = -pN \qquad (8)$$

where, according to the LQ model, each induced double strand break has a probability, p, of causing cell death.

Substituting equation (7) into equation (8),

$$\ln S = -2\ p\alpha d - 2p\beta d^2 - 2p\beta d^2\ e^{-\lambda t} \qquad (9)$$

Mono-exponential repair models may be simplistic and give only an approximate fit to repair data. Bi-exponential and multi-exponential models also exist to describe repair. In the case of a bi-exponential process, repair is modelled to contain two separate repair components. For example, initially, a rapid exponential process may occur followed by a second slower exponential process. A bi-exponential equation in the above format might then appear as:

$$\ln S = -2\ p\alpha d - 2p\beta d^2 - 2\ p\beta_1 d^2\ e^{-\lambda t}-2\ p\beta_2 d^2\ e^{-\mu t} \qquad (10)$$

where $p\beta= p\beta_1+ p\beta_2$

Dikomey and Franzke (1986) showed three components of DNA rejoining for CHO cells after irradiation. Half times for repair were 0.03 hours, 0.3 hours, and 2.8 hours respectively.

From the above discussion, therefore, equation (9) describes the mechanistic Linear-Quadratic model of cell killing after two fractions of radiation and assumes exponential repair kinetics. Mathematically similar phenomenological models can also be used to describe repair data.

Recovery Ratio (RR)

In the case of a split dose experiment, recovery ratio is the surviving fraction of cells, after a split dose of radiation, which are given time to repair between fractions, relative to the surviving fraction of cells given no time to repair between fractions.

$$RR=S_t/S_o \quad (11)$$

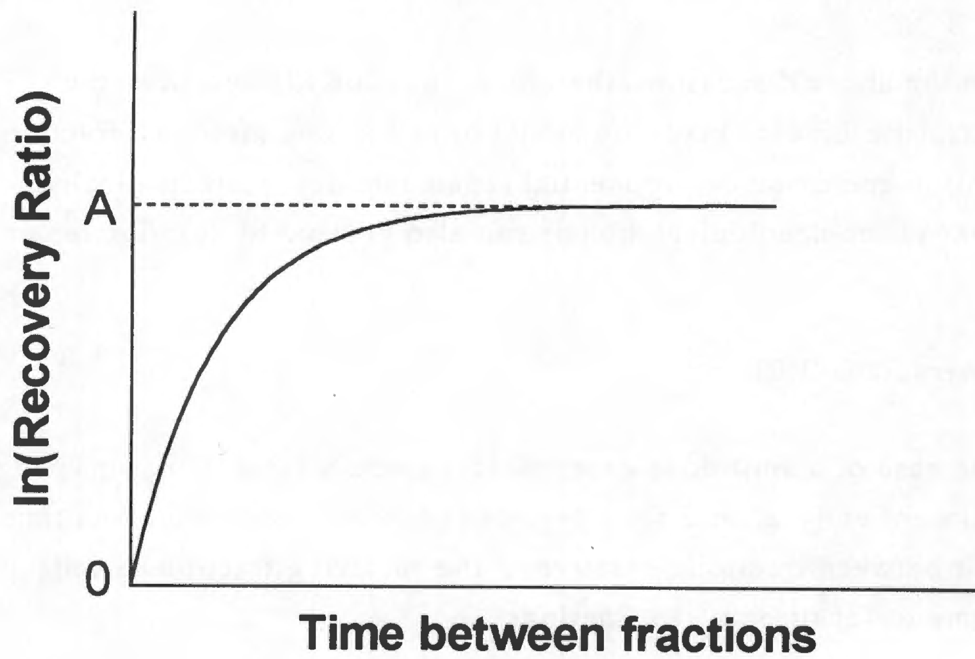
where S_t represents the survival of cells after two doses of radiation separated by a time t and S_o represents the survival after two doses given together with no time between them. Recovery ratio is, therefore, proportional to S_t.

S_o is often assumed to be the level of survival when no repair takes place but some repair will probably take place during irradiation unless cells are irradiated under conditions which prevent repair, such as when cells are kept on ice.

The equation,

$$\ln RR=A(1-e^{-\lambda t}) \quad (12)$$

can be used to describe the upper curve shown in Figure 3.3. RR is Recovery Ratio and A is Amplitude, which represents lnRR once the maximum amount of repair has taken place. λ represents the rate at which lnRR changes and so is indicative of the relative rate of change in survival with time.



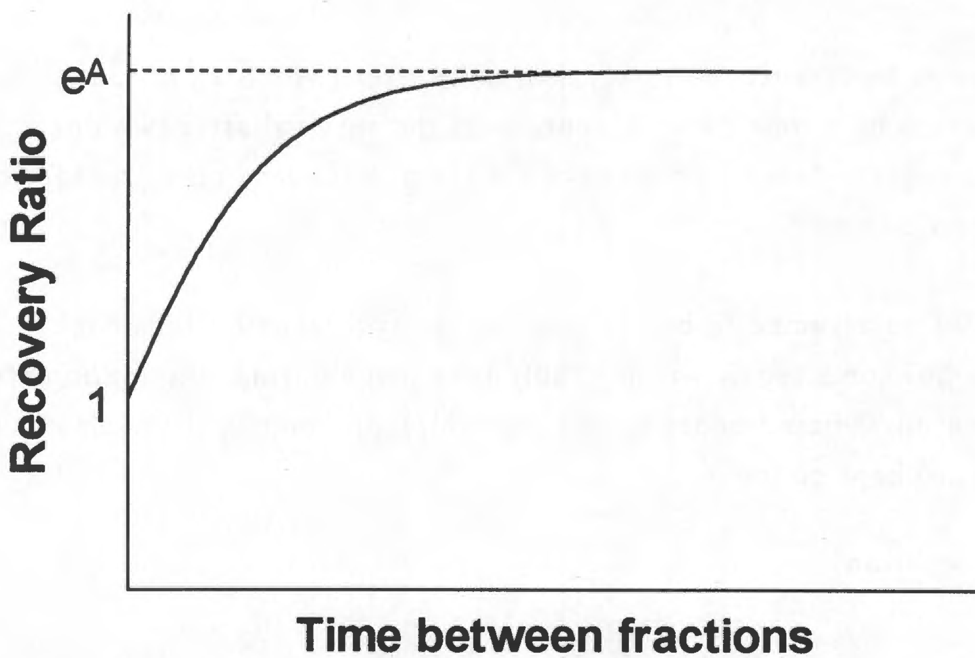


Figure 3.3: Two ways in which Recovery Ratio can be represented as a function of time between two fractions of radiation.

This formula (12) can be written in the form,

$$RR = e^{A(1-e^{-\lambda t})} \quad (13)$$

which describes the lower curve shown in Figure 3.3. When the time between fractions is zero, that is, no time is allowed for recovery, then the RR is equal to 1. When recovery ratio saturates, RR will be e^A, and the maximum extent of repair is proportional to the maximum change in RR, which is given by e^A-1.

Split dose curves are sometimes modelled to include bi-exponential rather than mono-exponential repair kinetics. A bi-exponential form of equation (12) above would appear as:

$$\ln RR = A(1-e^{-\lambda t}) + B(1-e^{-\mu t}) \qquad (14)$$

Repair Half-life($t_{1/2}$)

Repair half-life is the time taken to repair half the repairable damage as interpreted from the split-dose curve. This will appear as the time it takes for cell survival or recovery ratio to reach half its maximal level, where the starting value is that obtained for zero time between fractions. $t_{1/2}$ can be mathematically derived from the recovery rate constant, λ.

$$t_{1/2} = \ln 2/\lambda$$

Repair Time (t_r)

Other parameters have been presented to describe repair, such as Repair Time (t_r), which is the time between fractions necessary to reach the plateau region of a split-dose curve, as shown in Figure 3.4. In the case of experiments in which damage is fixed at different times after irradiation by treatments such as exposure to hypertonic salt solution (Raaphorst and Dewey, 1979), t_r is the time after irradiation before cell survival reaches a maximum plateau level (Reddy *et al.*, 1990).

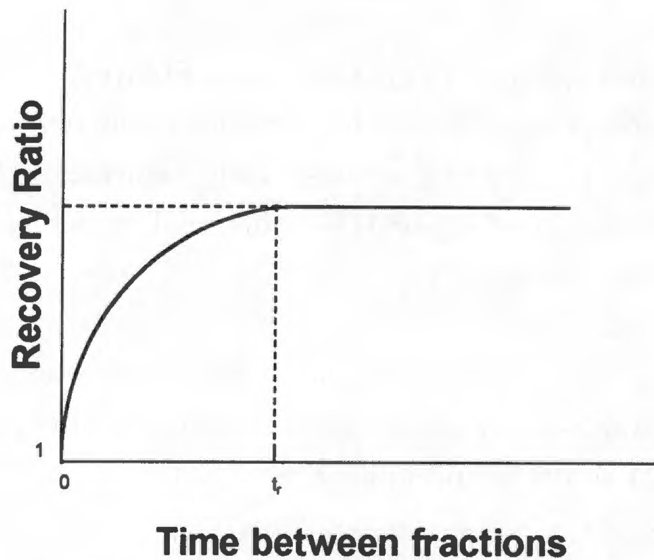


Figure 3.4: Split dose recovery curve showing a plateau response region from time t_r onwards.

For split doses, once the repair time is reached, cells surviving the first fraction cease to be as susceptible to a second fraction of radiation as they are for shorter interfraction intervals. This implies that damage repair, possibly repair of DNA, in cells which survive the first fraction, is complete before the second fraction is given, once an interfraction interval of t_r has elapsed. However, it is often difficult to decide where the plateau region of a repair curve begins because of uncertainties associated with experimental data.

Mean Repair Time (τ)

Equation (8) was used to describe the relationship between cell survival (S) and number of lesions (N). Equation (2) illustrated the link between N and radiation dose (D). Hence,

$$S = e^{\alpha D + \beta D^2}$$

This equation can be written in the form

$$S = e^{-CD + 1/2\ AD^2}$$

where D is dose and both C and 1/2 A are constants analogous to α and β.

If there is complete repair between two doses of radiation, then survival is equal to the product of the survivals of after each dose separately, that is, $S = S_1 S_2$.

If less than complete repair takes place and assuming that the rate of repair is proportional to the to the amount of damage present, the amount of damage after a time, t, after the first dose will be proportional to $e^{-t/\tau}$, where τ is the mean time required for repair of a sublethal lesion. Essentially, τ is the reciprocal of the rate constant for repair and, therefore, also the time required to reduce the damage to a level of 1/e (37%) of the initial amount of damage.

S for two doses becomes $S = S_1 S_2 e^{-AD1D2\, e^{-t/\tau}}$ (with a repair function) and $\ln(S_1 S_2/S) = AD1D2\, e^{-t/\tau}$

Because this equation is linear with respect to time, it is possible to determine τ from the slope of a regression line of $\ln(S_1S_2/S)$ plotted against t. (Nelson *et al.*, 1990).

THE ROLE OF ENERGY IN REPAIR

Patrick and Haynes (1964) proposed that repair of radiation damage in yeasts is an energy requiring enzymatic process. Recovery after irradiation can be inhibited by witholding oxygen from cells or by inhibiting oxidative metabolism with agents such as KCN, sodium azide or 2,4-dinitrophenol. Recovery can be enhanced by the addition of ATP to the system.

ATP has been shown to be required for repair of DNA and cellular recovery from radiation damage (Matsudaira *et al.*, 1970; Verma *et al.*, 1982). ATP has been shown to be important in DNA repair of isolated hepatic cell nuclei (Kaufmann *et al.*, 1982). DNA repair is largely enzyme mediated and it has been established that some of the enzymes involved require ATP in order to function. Any interference with the production of ATP, therefore, may have the potential to affect radiation response.

Studies have shown that the glucose analogue, 2-deoxy-D-glucose (2DG) can inhibit repair processes in cellular systems which are dependent on glycolysis. Jain *et al.* (1982) showed in respiratory deficient yeast that PLD repair could be inhibited by 2DG which reduces the content of ATP through the inhibition of glycolysis. 2DG inhibits respiration only slightly in wild type yeasts, which have respiratory metabolism. Further discussions concerning the use and effects of 2DG will be found in Chapters 6, 9, 10 and 11. Holahan *et al.* (1988) found in irradiated yeasts that when glucose (100 mM) was added to recovery medium, a greater liquid holding recovery (PLDR) could be achieved. In the presence of 5 mM 2DG, liquid holding recovery was totally inhibited. ATP was detectable only when glucose was present in the recovery medium. Dalrymple *et al.* (1969), however, presented evidence that ATP may not be required for SLD repair to take place. Treatment with 2,4-DNP, which inhibits oxidative metabolism, did not result in a decrease in SLD repair.

Spiro *et al.* (1985) showed that SLD repair of V79 cells after irradiation could be inhibited by allowing cells to repair in Hanks' buffered salt solution (HBSS) under anaerobic conditions. However, recovery was able to take place under anaerobic conditions if glucose was added or if culture medium was substituted for HBSS, although a greater amount of recovery occurred when the cells were allowed to recover in HBSS in air.

The Facilitation of Repair by ATP

Adenosine triphosphate's function as an energy carrier facilitates repair of cellular damage. In order to understand the importance of ATP as an energy carrier, it is necessary to consider the thermodynamics of reactions. The amount of free energy in a molecule depends on the atoms within it and the arrangement of these atoms. When chemical bonds are rearranged, that is, broken and reformed, energy is redistributed. Every chemical reaction has a free energy change which depends on the difference between the reactants and the products (Mathews and van Holde, 1990). Chemical reactions with a negative free energy change are said to be exergonic (free energy of final state <

initial state) while reactions with a positive free energy are said to be endergonic (free energy of final state > initial state). An endergonic reaction requires an input of energy for the reaction to take place and so is energetically unfavourable (Mathews and van Holde, 1990).

ATP as a Source of Free Energy

The reaction: ATP→ADP + Pi is an exergonic reaction and accordingly does not require an input of energy, apart from the initial activation energy. Free energy released by ATP can then be harnessed by endergonic reactions which require an energy input. This is what happens, for example, in the synthesis of macromolecules which rely on the presence of ATP. Chemical energy is given to ADP to form ATP which then loses its terminal phosphate to an acceptor molecule which is thus given free energy (This process is not peculiar to phosphate groups or ATP). ATP is the link between mostly exergonic reactions and chemical work in the cell (McGilvery and Goldstein, 1983).

Consider the repair of radiation damage to be represented by an endergonic reaction in which substrate A is metabolised to product B, that is, A → B. Because the reaction is endergonic, it will not occur spontaneously and requires an input of energy. This reaction can be coupled to the hydrolysis of ATP which is an exergonic, free energy yielding reaction.

A → B (endergonic)
ATP → ADP + Pi (exergonic)

A + ATP → B + ADP + Pi (exergonic)

The combined reaction is, thus, an exergonic reaction and the process of repair is facilitated.

Some extremes of the cellular environment which were discussed in Chapter 2 have been shown to be capable of modifying aspects of energy metabolism in cells. These extremes have also been shown to modify

radiation response under certain circumstances, as discussed below, possibly because of these energy modifying effects.

pH and Radiation Response

A factor of importance with regard to enzyme activity and functional ability of the cell is pH. If the pH of the enzyme environment is too far removed from that which is optimal, then enzymes will not function.

pH may affect the way in which cells respond to different types of radiation, both ionizing and non-ionizing. Although the main consideration is with the effects of ionizing radiation, a brief mention is made of hyperthermia and how cellular response to hyperthermia is modified by pH.

Hyperthermia and pH

The acidity of the cellular environment has a bearing on the cell's ability to withstand hyperthermic treatment (Freeman *et al.*, 1980). Sensitivity of CHO cells to hyperthermia increases as environmental pH drops from about 7.4 to 6.6. However, pH dependent changes in thermal sensitivity do not seem to occur secondarily to energy depletion. Extracellular pH ranging from 6 to 9 had little effect on the energy status of CHO cells but creatine phosphate was decreased at the low pH end of the scale (Fellenz and Gerweck, 1988). However, Kim *et al.* (1988) reported that a deprivation of energy can increase hyperthermic cell kill. When glycolysis is inhibited or cells are deprived of glucose, hypoxic cells become more sensitive to heat.

Ionizing Radiation and pH

The pH of the cellular environment can affect the radiosensitivity of cells to ionizing radiation. In general, a more acidic environment may induce radioresistance in cells as seen by a right shift of the radiation cell survival curve. Holahan *et al.* (1982) and Freeman and Sierra (1984) found that an acidic environment after irradiation resulted in less radiation damage being expressed. An acidic environment during or after

irradiation seemed to allow PLDR to occur. Freeman *et al.* (1981) reported that when the pH of the medium above exponentially growing CHO cells was acidic (pH 6.75), cells were more resistant to the effects of radiation than when the medium was more alkaline (pH 7.45). This phenomenon was also reported by Raaphorst *et al.* (1988) who also linked this apparent resistance to an increased PLD repair after irradiation.

It is uncertain exactly how pH exerts its effect on radiation response but it is possible that an acidic environment may reduce the fixation of radiation induced damage. This may be partially dependent upon factors associated with cell division because a decrease in pH results in a right shift in the radiation survival curve of exponentially growing CHO cells but not of those in plateau phase. The rate of glycolysis is maximal under basic pH conditions (pH 8) and decreases with increasing acidity (Ibsen *et al.*, 1960; Halperin *et al.*, 1969). The apparent equilibrium constant, K'=[ADP][PCr]/[ATP][Cr], for the reaction catalysed by creatine phosphokinase, ATP+Cr→ADP+PCr, increases with increasing pH (Noda *et al.*, 1954). It might be speculated that if there is a tendency for ATP energy to be stored in the form of phosphocreatine, that energy may not be available for repair and that this may explain why cells at a higher pH are more radiosensitive than those at a lower pH.

Lactate and Repair

It was thought that lactate production by cells might alter radiation response. Seymour and Mothersill (1981) examined the effect of lactate on the radiation response of cells in culture. They were able to show that physiological levels of lactate can enhance the response of CHO-K1 cells to radiation.

Glucose breakdown and net lactate accumulation were also inhibited by the presence of lactate (Mothersill and Seymour, 1986). Pretreatment of cells with 10 mM lactate for 18 hours resulted in a greater degree of split dose recovery after radiation than non-pretreated cells. However, survival after a single dose was reduced. When lactate was added immediately before radiation, there was a protective effect but no effect

on recovery. It is possible that any reduction in pH caused by the presence of lactate may have contributed to the changes in radiation response.

Membrane Changes

Cells may be vulnerable to events which affect the integrity of the cell membrane because it is intimately involved with cellular function. The cell membrane has been proposed as a critical target for radiation damage and it has been shown that radiation can cause alterations in transmembrane ion transport. The ionic constituents of the cell are dependent upon the passage of substances across the cell membrane so if the membrane becomes damaged, homeostasis can be disturbed. Irradiation of murine spleen lymphocytes has been shown to cause an increase in calcium uptake immediately after irradiation (up to 50 Gy and beyond). This was observed in both oxic and anoxic states (Kale and Samuel, 1987).

Mechanisms of cell transport, namely passive diffusion, facilitated diffusion and active transport are crucial to the functioning of the cell (Guyton, 1986). Active transport mechanisms maintain ionic gradients across cell membranes. An example of an active transport mechanism is the sodium pump whose function it is to maintain a low sodium: potassium ratio within the cell. Outside the cell in the intercellular fluid, the sodium:potassium ratio is very high. Low calcium levels within the cell are also maintained by such an active transport mechanism. If K^+ could pass freely accross the membrane, then a situation would develop where the ratio of Na^+ to K^+ becomes the same on both sides of the membrane. Ion gradients are created by using free energy liberated by the hydrolysis of ATP. This process is made possible by a protein known as the Na^+K^+-adenosine triphosphatase (Na/K-ATPase) , which spans the cell membrane. Gradients of ions represent a store of free energy. (McGilvery and Goldstein, 1983).

Active transport requires a large energy input in the form of ATP. This energy input is far greater than that required for biosynthetic processes. If the energy supply of the cell is halted, then active transport will be

shut down. This would have dire consequences on ion exchange homeostasis and would eventually result in cell death.

It is tempting to speculate about the possible ramifications of energy limitation on active transport. If ATP supply was reduced, active transport might be slightly affected which would result in slight disruption of ion homeostasis. Such effects, due to uncontrolled entry and exit of substances, could affect many aspects of the cell interior including pH and thus affect amongst other things, DNA repair enzymes within the nucleus and protein synthesis. It is known that a high intracellular K^+ is required for protein synthesis and is also required by glycolysis for pyruvate kinase to act maximally (Lehninger, 1970). Therefore, a reduction in ATP could have a modifying effect on radiation response. It is conceivable that both slight damage to DNA and changes in membrane permeability could even be thought of as sub-lesions which have the potential to interact and accumulate.

The cardiac glycoside, ouabain, affects several cellular functions including volume regulation, ion transport and protein synthesis. Ouabain inhibits the action of the sodium-potassium pump in the plasma membrane (Lawrence, 1988). This drug has been shown to sensitize tumour cells but not normal cells to radiation. A reduction in the shoulder of the tumour radiation survival curve was reported which may indicate a decrease in sublethal repair (Lawrence, 1988).

The activity of the Na/K pump and the Na/K-ATPase are often increased in tumour and transformed cells compared to normal cells. Lawrence (1988) has compared the differences between NaK pumps of normal and tumour cells with a view to exploiting these differences.

Hypoxia and Repair

It is well known that cells irradiated under hypoxic conditions are more radioresistant than those irradiated under well-oxygenated conditions. However, the post-irradiation oxygen status can also influence the radiation response considerably. It was shown by Gupta *et al.* (1986) that cells irradiated in hypoxia and kept under well oxygenated

conditions after irradiation were more radioresistant than cells which were irradiated under conditions of hypoxia and kept hypoxic after irradiation. Cells which were irradiated under oxic conditions and were kept under hypoxia after irradiation showed the same radiosensitivity as normal oxic cells. This phenomenon may be partly because of a reduced energy supply due to being kept in hypoxia for an extended period. Hendrikse (1989) showed in B16 cells *in vitro* that ATP levels dropped in hypoxia but recovered progressively over the subsequent few hours if returned to an aerobic environment. This highlights the importance of the post-irradiation environment with respect to the expression of radiation damage.

Ling *et al.* (1988) showed in V79 cells that ATP and energy charge were reduced when cells were exposed to hypoxia and deprived of glucose. Hypoxic cells in medium containing glucose, however, failed to show a decrease in ATP and energy charge. Aerated cells deprived of glucose also failed to show a decrease in energy status. Hypoxic cells without glucose seemed to be more radiosensitive than euoxic cells with glucose. Ling *et al.* (1988) suggested a relationship between energy status and sub-lethal damage (SLD). It would seem that under low energy conditions, SLD may not be repaired.

SLD repair appears to be dependent on cellular energy status which may only partly depend on oxygen status. There are, however, conflicting results concerning the ability of hypoxic cells to repair SLD. Hypoxia has been shown in some instances to inhibit SLD repair (Hall, 1972). Other workers show partial reduction in repair (Nias *et al.*, 1973). Others showed that hypoxia had no effect on split-dose repair (Elkind *et al.*, 1965). Cellular diversity, which implies variability in energy metabolism between cells and the ability to interfere with repair, could very well be responsible for the conflicting results.

DNA strand break studies in euoxic cells with metabolic inhibitors support the idea that hypoxia-induced inhibition of SLD repair may be associated with an alteration in cellular energy status. Hypoxia can cause an increase in yield of unrepaired DNA strand breaks after radiation (Koch *et al.*, 1980). However, Nagle *et al.* (1980) found that

rejoining of breaks in hypoxic cells was as effective as in aerobic cells. A glycolytic inhibitor, 5-thio-D-glucose (5-TDG), inhibited strand repair in hypoxic cells. If 5-TDG was combined with an uncoupler of oxidative phosphorylation instead of hypoxia, then a similar inhibition of repair occurred.

A substantial decrease in the SLD repair of hypoxic V79 cells has been noted but a complete inhibition of SLD repair and strand break repair was found when glucose was omitted as well (Spiro *et al.*, 1985). The loss of repair capacity was correlated with reduced ATP. The omission of glucose had no effect on well aerated cells.

Glycolysis, Cell Proliferation and Repair of Radiation Damage

Jain *et al.* (1985) showed in HeLa cells how both cell proliferation and repair of DNA damage after radiation increased as the rate of glycolysis increased from an inhibited state (inhibited by 2DG) to one approaching control levels. It was shown that DNA repair was more dependent on glycolysis than cell proliferation. However, micronuclei frequency of energy depleted, irradiated cells, which was initially greater than that of controls, decreased to about 60% of control values as the rate of glycolysis increased to about 80% of the control rate. Micronuclei frequency then increased to control levels as glycolysis reached the activity of controls (Jain *et al.*, 1985).

Repair, Misrepair and Fixation

A summary of the involvement of energy supply in radiation response was presented by Jain *et al.* (1985) and is represented in Figure 3.5.

DNA damage can be repaired by error-free repair (excision repair) which results in complete reversal of radiation induced damage. DNA damage can also be repaired by error-prone repair (misrepair) which may result in survival of cells with altered genetic components. In addition, if DNA damage undergoes fixation, it may become stable and irrepairable. Error-free repair occurs most readily during the pre-replication period and is supported by conditions which are unfavorable for cell growth.

Misrepair and fixation of damage become apparent during and after DNA replication.

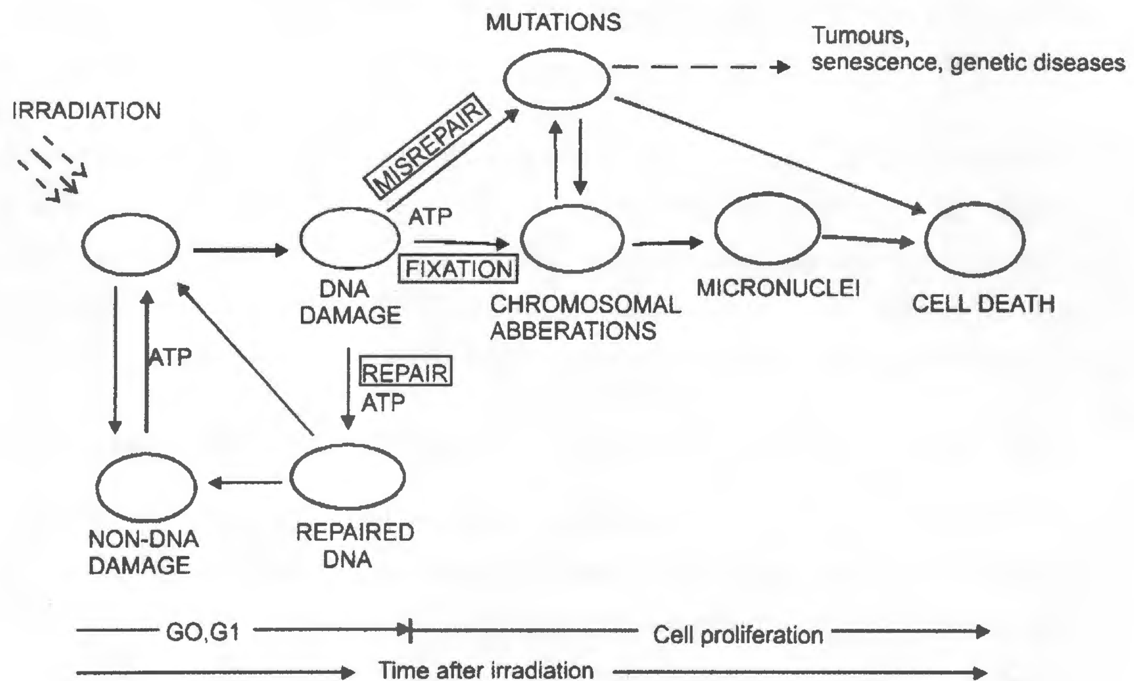


Figure 3.5: The points at which ATP may modify radiation responses in cells. Adapted from Jain *et al.*, (1985)

Figure 3.6 shows how a reduced glycolytic rate may affect micronuclei frequency. If glycolysis is slightly depressed (region C in Figure 3.6), error-free repair is unchanged but the rates of cell proliferation and consequently the rates of misrepair and fixation are reduced. The number of cells undergoing error-free repair, therefore, increases and the micronuclei frequency is decreased. If glycolysis is depressed further (region B in Figure 3.6), the rate of DNA repair decreases and the frequency of cells with micronuclei increases. As glycolysis is reduced further still (region A of Figure 3.6), the DNA repair rate becomes severely depressed. As the glycolytic rate decreases, DNA repair is reduced more rapidly than the rate of proliferation which determines misrepair and fixation of damage and, therefore, the micronuclei frequency becomes raised above that of controls. The rate of glycolysis may thus determine radiation response.

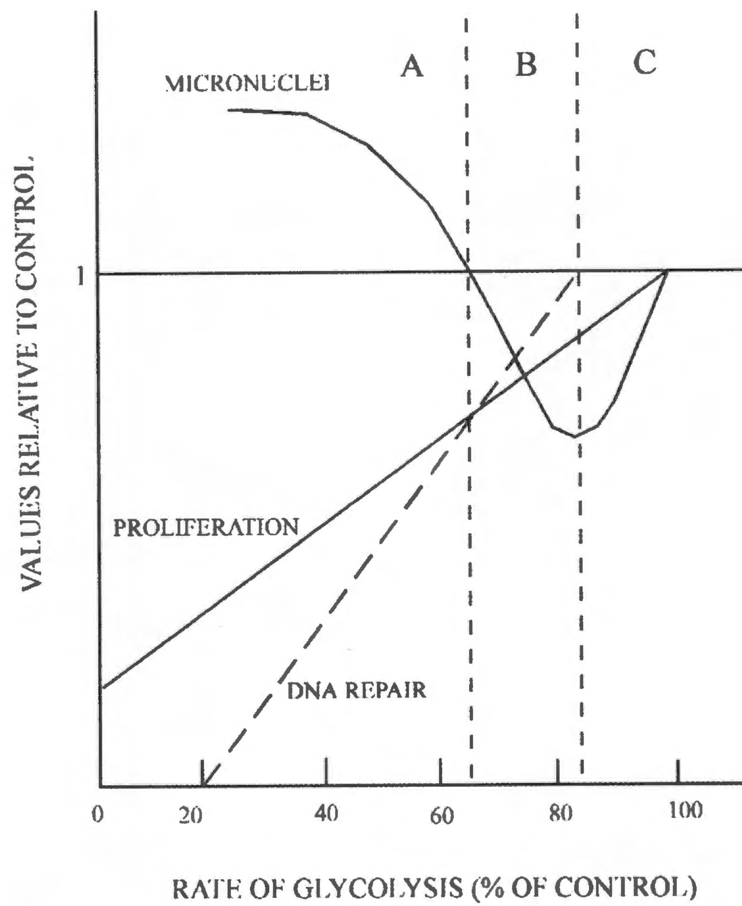


Figure 3.6: Micronuclei frequency, cell proliferation and DNA repair relative to the rate of glycolysis in HeLa cells. Adapted from Jain *et al.*, (1985).

CHAPTER 4

ENERGY METABOLISM AND THE CHOICE OF SUBSTRATE

This subject has been well described in numerous text books of biochemistry and physiology and, therefore, does not require a detailed description in this thesis. However, there are some important points which require brief discussion.

There are many different substrates from which cellular energy may be derived including sugars, fatty acids, ketone bodies and amino acids. The yield of ATP from some fuels under aerobic and anaerobic conditions is shown in Table 4.1.

Table 4.1: ATP yield from some energy substrates (Modified from Newsholme and Leach, 1983).

FUEL	CONDITIONS	ATP YIELD (MOLES) PER MOLE FUEL UTILIZED
glucose	aerobic, complete oxidation	38
glucose	anaerobic, conversion to lactate	2
glycogen	aerobic, complete oxidation	39
glycogen	anaerobic, conversion to lactate	3
palmitate	aerobic, complete oxidation	129
acetoacetate	aerobic, complete oxidation	24
acetyl-CoA	aerobic, complete oxidation	12

A certain amount of interconversion between carbohydrates, fats and proteins is possible. For example, glycerol from fats can be converted to dihydroxyacetone phosphate and certain amino acids can be converted to intermediates of the Emden-Myerhof pathway (glycolysis) and citric acid cycle by deamination. When energy levels are high and ATP is being produced in abundance, gluconeogenesis can take place. This is essentially a process whereby non-carbohydrate sources can be converted to glucose. Several organs require glucose as their prime energy source, for example, brain and nervous system, kidney medulla, testes and erythrocytes and so it may on occasion be necessary to provide glucose from other sources (Ganong, 1987; Mathews and van Holde, 1990). Glucose can be converted to fats via acetyl CoA. However, fats cannot produce glucose via this pathway because the reaction, pyruvate to acetyl CoA, is irreversible (Ganong, 1987).

CARBOHYDRATES AS ENERGY SOURCES

Much emphasis has been placed on carbohydrates and predominantly glucose as major sources of energy in the cell. Disaccharides and polysaccharides generally are not absorbed from the intestine and so are not found extensively in the bloodstream. Glycogen, the storage form of glucose in animal cells, is found in most cell types but predominantly in liver and skeletal muscle (Guyton, 1986).

Glucose

Glucose is a major constituent of most cell culture media because of its importance as a source of energy (Freshney, 1987). It is metabolised mainly by glycolysis to form pyruvate which can be converted to lactate or can enter the citric acid cycle to be processed by oxidative metabolism. Glucose provides 2 moles of ATP under anaerobic conditions and 38 moles under well-oxygenated conditions in average normal cells (See Table 4.1). Cultured animal cells, which often have a high rate of glycolysis, may convert a large proportion of glucose to lactate and a lesser proportion to CO_2 (Paul, 1970).

An alternative pathway for glucose oxidation, which functions to a variable extent in different tissues, is the pentose phosphate pathway (Mathews and van Holde, 1990). Glucose can be oxidized to CO_2 and water via this pathway. The pentose phosphate pathway can produce energy independently of the citric acid cycle when NADPH is being synthesised. In cases of energy demand, it is possible for glyceraldehyde-3-phosphate, which is a common intermediate of both the pentose phosphate cycle and glycolysis, to be metabolised via glycolysis and the products to enter the citric acid cycle.

Under anaerobic conditions, glucose undergoes anaerobic glycolysis. The citric acid cycle does not function when oxygen is absent and there may be a buildup of lactic acid because of the reduced capacity to remove pyruvate. Anaerobic glycolysis is important in tissues when no oxygen is present and in tissues lacking mitochondria. In addition, several tissues and cells normally rely extensively on glycolysis, including erythrocytes, white blood cells, kidney medulla cells, tissues of the eye and skin (Newsholme and Leach, 1983).

Carbohydrate substrates other than glucose may be used by cells for energy (Mathews and van Holde, 1990), for example, fructose, galactose and mannose which can be metabolised by glycolysis, as shown in Figure 4.1. Polysaccharides and oligosaccharides may be used but must first be broken down to monosaccharides. Lactate and pyruvate can also be used as sources of energy by the cell in the presence of oxygen (Paul, 1970). Yushok (1964) showed that respiration is inhibited by the presence of both glycolysable sugars and non-glycolysable sugars such as 2-DG.

Zwartouw and Westwood (1958) reported that, *in vitro*, cell division was dependent upon a good supply of glucose and that without glucose, cells began to degenerate. This may not be true in all situations. Wice *et al.* (1981) found that medium deficient in glucose resulted in cell disintegration and reduced cell growth but could support growth to the same extent as glucose-containing medium if ribosides were added instead.

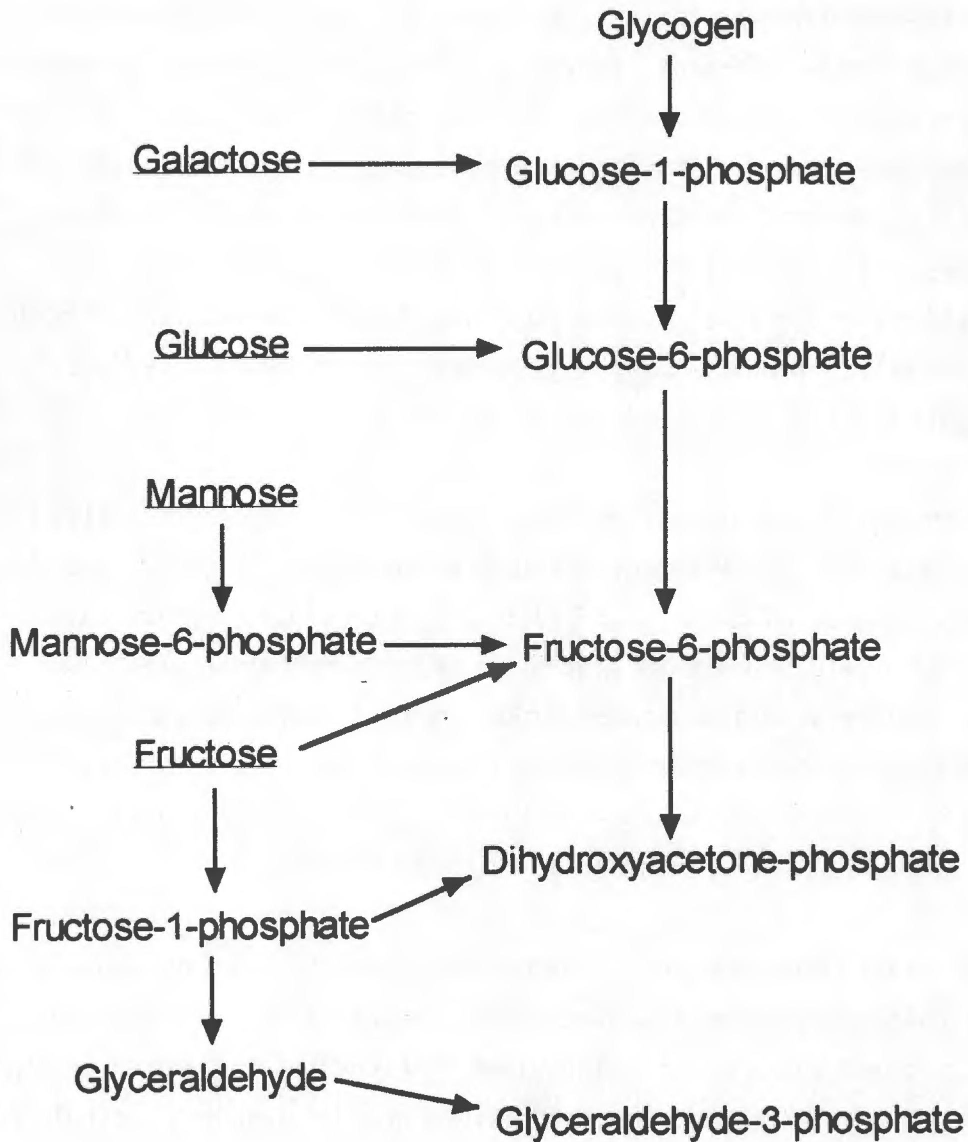


Figure 4.1: Paths showing the utilization of monosaccharides other than glucose in glycolysis. (Adapted from Mathews and van Holde, 1990.)

Other sources of energy

Different cell types and organs differ with respect to function and to the levels of certain enzymes. Consequently they put different emphasis on the use of different substrates. Under conditions of starvation or when certain substrates are unavailable, cells make use of different energy substrates to different extents. Both fats and proteins can be used as energy sources under the appropriate conditions.

Fatty acids can be broken down via β-oxidation to acetyl CoA which can be metabolised by the citric acid cycle to yield appreciable amounts of

ATP (Guyton, 1986). Lipids are more efficient stores of energy than carbohydrates. This means that a fatty acid molecule can provide more ATP than a molecule of glucose. For example, palmitate, a bigger molecule than glucose, can provide 129 molecules of ATP compared to just 38 for glucose (See Table 4.1) (Newsholme and Leach, 1983).

When carbohydrates are present in abundance, energy supply from fats is suppressed and carbohydrates are preferentially used. There are two main reasons for this fat-sparing effect. First, fats exist in two major forms: as triglycerides and as small amounts of free fatty acids. If the quantity of free fatty acids is kept low through the equilibrium between the triglycerides and fatty acids being in favour of the triglycerides, then the energy supply from fats will be low. This situation prevails when α-glycerophosphate is present in excess. This is a product of glucose metabolism, so, provided there is glucose being metabolised, free fatty acids will not be used. The second reason for suppression of energy supply from lipids is that when carbohydrates are being used extensively, there tends to be a large amount of acetyl-CoA present, which means that the equilibrium is shifted towards storage of free fatty acids in the adipose tissue or other fat stores. Fatty acids also tend to be stored due to the presence of acetyl-CoA carboxylase, the enzyme which is rate limiting for fatty acid synthesis and whose activity is stimulated by intermediates of the citric acid cycle (Guyton, 1986).

When carbohydrates are not present, fat-sparing effects are lost and fat is mobilised and used for energy. A lack of carbohydrate in the body will also result in a reduction in insulin secretion which will tend to prevent fat synthesis (Ganong, 1987; Guyton, 1986).

Under conditions of starvation, proteins may be catabolised to provide energy. This process can be prevented by small quantities of glucose, that is, there is also a protein-sparing effect in the presence of carbohydrate.

Amino acids can be used as a source of fuel for ATP production. Two routes of amino acid degradation by which amino acids can provide energy intermediates, are deamination and transamination. Deamination

involves the loss of ammonia in an oxidation reaction catalysed by an enzyme such as glutamate dehydrogenase (Newsholme and Leach, 1983).

Glutamate + $NAD(P)^+$ + $H_2O \rightarrow \alpha$-ketoglutarate + NAD(P)H + NH_4^+ + H^+

Amino acids can be degraded by transamination. In this reversible process, an amino group is lost from an amino acid and transfered to α-ketoglutarate by aminotranferases (transaminases) to yield glutamate and the α-keto acid (Newsholme and Leach, 1983).

α-ketoglutarate + amino acid $\leftrightarrow$ glutamate + α-keto acid

The α-keto acids are then further metabolised to various citric acid cycle intermediates depending on the keto acid which is formed. These intermediates can be metabolised further and glutamate can be deaminated again.

Glutamine, for example, can react with α-ketoglutarate via transamination to yield glutamate and the α-keto acid, α-ketoglutaramate, which is then converted to the citric acid cycle intermediate, α-ketoglutarate.

Glutamine can also be converted to glutamate by glutaminase. The carbon skeleton of glutamine, therefore, enters the citric acid cycle via α-ketoglutarate. Whittaker and Danks (1978) reported that, in most cell types, glutamine enters the citric acid cycle by first being converted to glutamate and then undergoing transamination with an α-keto acid to yield α-ketoglutarate. However, it has been stressed by Newsholme and Leach (1983) that, although production of citric acid cycle intermediates from amino acids will raise the concentrations of such intermediates, to achieve complete oxidation of the amino acid carbon skeletons, the intermediates must first be converted to oxaloacetate. Oxaloacetate is then converted to acetyl-CoA via phosphoenol pyruvate (catalysed by phosphoenolpyruvate carboxykinase) and the products are then oxidized via the citric acid cycle. (Newsholme and Leach, 1983).

A composite picture of glutamine usage by the citric acid cycle is presented in Figure 4.2. Different organs and tissues may make use of different branches of the pathway to different extents.

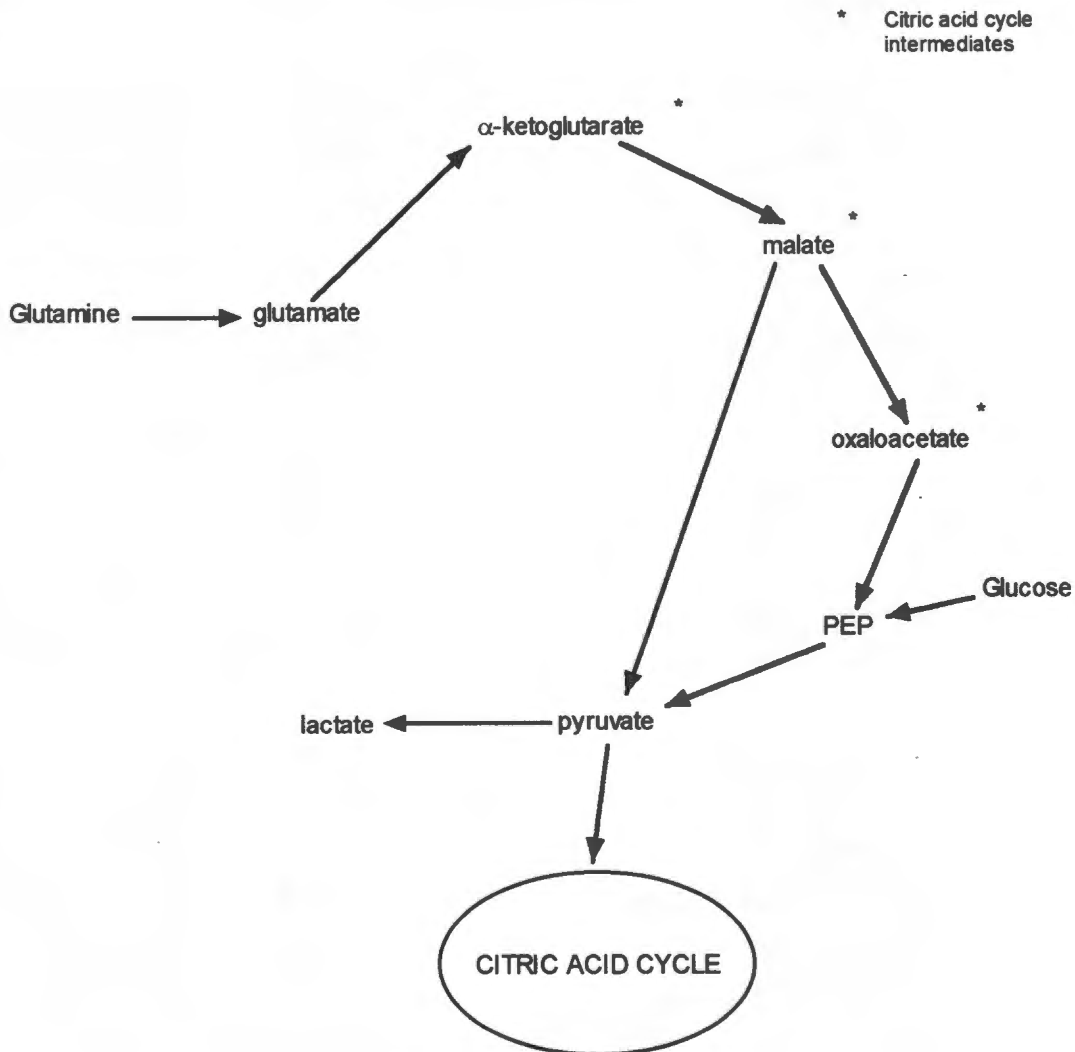


Figure 4.2: The convergence of glutaminolysis and glycolysis. Adapted from Newsholme and Leach, 1983.

PREFERENCE OF SUBSTRATE

Sumbilla *et al.* (1981) found that human fibroblasts, *in vitro*, had preferences for certain energy substrates. These fibroblasts oxidise primarily glutamine and small quantities of glucose, fatty acids and ketone bodies. Glucose was found to inhibit the oxidation of glutamine by about 85% but the other fuel substrates did not.

Table 4.2 shows the preferences of some different tissue types for different energy substrates under different conditions.

Table 4.2: Energy substrate usage in some tissue types (Adapted from Mathews and van Holde, 1990.)

TISSUE	*FUEL STORE*	*PREFERRED FUELS*	*EXPORTED FUEL SOURCES*
Liver	Glycogen, triacylglycerol	Amino acids, glucose, fatty acids	Fatty acids, glucose, ketone bodies
Adipose tissue	Triacylglycerol	Fatty acids	Fatty acids, glycerol
Skeletal muscle	Glycogen	Fatty acids (resting), Glucose (exertion)	Lactate (exertion)
Heart	Glycogen	Fatty acids	None
Brain	None	Glucose, ketone bodies during starvation	None

Glutamine

Both glucose and glutamine have been shown to be important sources of energy of mammalian cells *in vitro*. (Zielke *et al.*, 1978; McKeehan, 1982; Glacken, 1988). A lack of glutamine can inhibit growth of cells in culture (Tannock *et al.*, 1986). Glutaminolysis, which is especially important in providing energy under conditions of low glucose concentration, involves the passage of products originating from glutamine into the citric acid cycle via α-ketoglutarate.

It was shown by Zielke *et al.* (1976) that cultured human diploid fibroblasts could grow effectively without using glucose. The most probable alternative substrate was thought to be glutamine. Zielke *et al.* (1978) stated that these cells use both glucose and glutamine as energy sources. The presence of each substrate regulates the use of the other. Both low (70 μM) and high (5.5 mM) concentrations of glucose in Eagle's medium supported identical growth rates but glutamine utilization was shown to increase when the low glucose concentration was used. Glutamine usage tended to increase glucose utilization via the

pentose phosphate pathway but inhibited glucose metabolism via the citric acid cycle.

Reitzer *et al.* (1979) found that cultured HeLa cells grow at a similar rate whether the sugar source is glucose, galactose or fructose. When the cells were incubated in the absence of sugar, intracellular ATP levels remained constant but were slightly reduced. This implies that an alternative energy source was available. When, in addition, oxygen was reduced, ATP levels dropped significantly. These investigators suggested that, in normoxic cells, glutamine was able to provide sufficient energy from citric acid cycle metabolism to maintain ATP levels. Glutamine may provide more than half the cellular energy when glucose is present in high concentrations but when fructose or galactose is substituted for glucose, glutamine may provide more than 98% of the energy.

It was postulated that the lesser activity of glutamine usage when glucose was present in significant amounts, relative to the glutamine usage when glucose was absent, might be due to inhibition of glutamine usage by high lactate production. This, however, does not seem to be the case. When lactate was added to cells metabolising fructose instead of glucose, no decrease in the rate of glutamine usage could be determined (Reitzer *et al.*, 1979). Glacken (1988) reported that the presence of glucose may suppress glutamine usage because of low intracellular Pi. The presence of a high glucose concentration would cause an increase in the rate of glycolysis which would lead to an increase in ATP synthesis and a decreased Pi. This would inhibit phosphate activated glutaminase and result in a decrease in glutamine utilization. Zielke *et al.* (1984) concluded that, for tissue culture medium containing both glucose and glutamine, glutamine contributes between 30 and 50% of the energy supply of cultured mammalian cells. As glucose concentration drops, glutamine becomes more important and may become the sole energy source.

Glutamine has also been shown to be an important energy source of tumour cells *in vivo* (Sauer *et al.*, 1982). Kvamme and Svenneby (1961) found that Ehrlich ascites tumour cells metabolised glutamine extremely

rapidly. When these cells were allowed to undergo glycolysis, they were shown to inhibit glutamine uptake indicating a possible interaction of these processes. Medina *et al.* (1988) found in Ehrlich ascites cells that glucose was the preferred energy source but that glutamine was still used. The presence of glucose resulted in a reduced utilization of glutamine suggesting that a higher rate of glycolysis inhibited glutaminolysis. However, a higher rate of glutaminolysis did not seem to inhibit glycolysis. This is in disagreement with the results of Gonzalez-Mateos *et al.* (1993) who found that the presence of glutamine did result in a depression of glycolytic flux in ascites tumour cells. Kovacevic and Morris (1972) showed a good correlation between mitochondrial glutaminase activity and the rate of respiration of Morris hepatoma tumour mitochondria in the presence of glutamine. They concluded that glutamine was an important energy substrate in the energy metabolism of malignant cells *in vivo*.

Several of the aforementioned studies have alluded to the existence of some sort of reciprocal regulation between glucose using pathways and glutamine using pathways. Generally, it would seem that the rate of glutaminolysis is inversely proportional to the sum of energy production from glycolysis and pyruvate oxidation.

PASSAGE OF GLUCOSE AND GLUTAMINE ACROSS THE CELL MEMBRANE

A regulated passage of molecules across the cell membrane is important because it maintains not only the correct amounts of vital substances for optimal functioning of the cell but also prevents adverse osmotic effects such as swelling, bursting or shrinkage. There are several kinds of transmembrane transport, namely, passive diffusion, facilitated diffusion and active transport (Guyton, 1986).

The glucose molecule is not lipid soluble and is too big to pass through pores in the cell membrane to any great extent. It, therefore, cannot effectively enter the cell by passive diffusion. Nevertheless, glucose readily enters the cell by facilitated diffusion using carrier proteins which transport glucose molecules down the concentration gradient (Newsholme and Leach, 1983).

Glucose and amino acids are absorbed through the gastrointestinal membrane or the epithelium of renal tubules by means of sodium dependent glucose transport. This is an active transport process requiring an energy input to allow the substrate to be absorbed against a concentration gradient. This latter process is reserved for cell types responsible for absorption (Guyton, 1986).

The normal route of entry of glucose into most cells, as mentioned above, is via facilitated diffusion. Facilitated diffusion is used by many constituents of blood such as sugars, amino-acids, purines and glycerol. The gradient is maintained by keeping the concentration of specific intracellular constituents relatively low by constant use of these substances by the cell. Facilitated diffusion follows kinetics which are similar to Michaelis-Menten enzyme kinetics (Guyton, 1986).

Glucose transport is dependent on several factors.Uptake can be enhanced by insulin (Guyton, 1986) and other growth factors. Uptake of the glucose analogue 2DG is also insulin dependent (Nakada and Wick, 1956). Transformed cells often take up increased amounts of glucose. Hatanaka (1974) described how the transport of some sugars (glucose, mannose, galactose and glucosamine) is increased in some tumour cell lines. Glucose starvation can result in enhanced glucose uptake. This may be due to the production of increased amounts of glucose transporter protein (Haspel *et al.*, 1986). Competition for entry into the cell by, for example, glucose analogues such as 2-deoxy-D-glucose can limit the amount of glucose taken up. The passage of substances across the cell membrane is also greatly influenced by pH. At pH less than 7, the permeability of cell membranes decreases dramatically.

The details concerning the passage of glutamine across the cell membrane is less clear. Nevertheless, glutamine is known to be able to cross plasma membranes readily (McGilvery and Goldstein, 1983). Intracellular concentrations of glutamine are often higher than those in the bloodstream and consequently an active transport process may be required for its uptake (Newsholme and Leach, 1983).

CHAPTER 5

NORMAL AND TUMOUR CELL ENERGY METABOLISM

Metabolic patterns of different normal tissues vary considerably because they perform a wide range of specialised functions. Tumours have many metabolic characteristics which are different to those of normal tissues. Many characteristics of tumours can be related to their growth and to the inherent differences in the tumour cells which make them behave differently to normal cells.

The degree of vascularization of tumours will determine the relative supply of nutrients and oxygen to the tumour's cells. Tumours often develop more rapidly than the blood vessels which supply them resulting in tumours outstripping their blood supply. This may result in some tumour cells becoming severely depleted of nutrients and oxygen and in the death of some of these cells (Hall, 1994). The depletion of oxygen, because of this vascular insufficiency, may, therefore, also result in hypoxic regions in tumours. Although hypoxic cells, which have reduced oxygen contents, may be metabolically inhibited, transient continually changing hypoxia within a tumour may allow tumour cells in nutrient deprived states to respire intermittently during pulses of oxygen.

HETEROGENEOUS MICROENVIRONMENTS AND CELL POPULATIONS

Vaupel (1995) has examined blood supply of solid tumours in humans. Heterogeneous distribution of tumour vasculature was reported to be an important factor in the resistance of tumours to radiation and chemotherapy. He also reported that there was great intra- and inter-

tumoral heterogeneity with respect to oxygenation and bioenergetic status.

Because of the physical arrangement of cells relative to their blood supply, there are likely to be gradients of critical metabolites and products of metabolism, for example, low concentrations of glucose may be found in regions far removed from the blood supply. Spheroids are spherical clumps of cells grown in culture which are used to model the multicellular, three dimensional structure of tumours (Hall, 1994). Sutherland (1986) presented a model of tumour microregions based on spheroids, which shows how gradients of oxygen and nutrient supply may exist in a tumour. Mueller-Klieser (1995) reported certain properties of spheroids which may be common to the tumour systems they model. Spheroids have been shown to consist of heterogeneous populations of cells and it has been found that concentric layers of these cells have properties which correlate with their radial positions in a spheroid. This is apparent from the radial variation in sensitivity to drugs and radiation which may be why gradients of oxygen and nutrients in tumours have been shown to be determinants of resistance to cancer therapy. Radiosensitivity in some spheroid systems has been linked to the prevalence of more glycolytic as opposed to more oxidative metabolism in cells. Oxygen content, in such cases, is often of lesser consequence (Meuller-Klieser, 1995).

Vaupel *et al.* (1981) showed heterogeneous oxygen partial pressure and pH distribution in the C3H mouse mammary adenocarcinoma. Metabolic imaging of microregions with bioluminescence has indicated that the distributions of lactate, ATP and especially glucose within tumour tissue are much more heterogeneous than within normal tissue. (Meuller-Klieser *et al.*, 1988). Tamulevicius and Streffer (1995) demonstrated similar phenomena with their work on metabolic imaging in frozen sections of human tumours which they also imaged using bioluminescence. All tumours showed a high degree of intra- and inter-tumoral heterogeneity. It was shown in a slowly growing head and neck carcinoma and a colorectal carcinoma that glucose and ATP were confined to the peripheral regions of the tumour and that there was a central necrotic area devoid of metabolites. Metabolites were more

evenly distributed in tumours such as melanoma and adenocarcinoma. Lactate was found to be distributed evenly over tissue sections in all the tumours studied.

There are other factors which may have effects on tumour metabolism, for example, tumour size. Tumours tend to grow rapidly when they are small and slow down when they are larger probably due to vascular restrictions, cell-loss, metastasis and host factors, such as immune response (Kallman, 1987).

For detailed descriptions of the carbohydrate metabolism of tumours, a number of reviews have been published including those by Bodansky (1975), Pedersen (1978) and Eigenbrodt *et al.* (1985).

THE PASTEUR EFFECT

In 1861, Pasteur noticed that yeasts used less glucose under oxic conditions than under anoxic conditions (Reported by Lehninger, 1970; Krebs, 1972). This was due to a down regulation of glycolysis when oxidative metabolism takes place - the so-called Pasteur effect. The rate of glycolysis is normally limited by the presence of oxygen which facilitates the production of ATP via oxidative phosphorylation. The concentration of ATP is important because ATP has allosteric effects on certain enzymes which regulate glycolysis (Mathews and van Holde, 1990). It follows, therefore, that glucose utilization by glycolysis in normal cells is greater under anaerobic conditions than under aerobic conditions, so that the energy requirements of the cell can still be met when respiration is prevented.

Regulation of the glycolytic pathway takes place by allosteric regulation of the activities of certain enzymes, namely, phophofructokinase (PFK), pyruvate kinase (PK) and hexokinase. Hexokinase is regulated by negative feedback by the presence of its product glucose-6-phosphate. If glycolysis or glycogen production is blocked, entry of more glucose into the pathway is prevented. PFK is activated by high concentrations of substances which indicate a low energy charge, namely AMP and ADP, and by fructose-1,6-bisphosphate. Inhibition of PFK occurs when ATP

or citrate are present in high concentration. Similarly, PK is inhibited by ATP. PK also has feed-forward activation by high fructose-1,6-bisphosphate and negative feedback by high acetyl-CoA concentration. (Mathews and van Holde, 1990).

It has been reported that most cells in culture have a marked Pasteur effect (Paul, 1970). In some tumours, the Pasteur effect may be absent and glycolysis may continue unchecked regardless of the oxygen concentration. However, many tumours have been found to have a normal Pasteur effect (Evans, 1991).

THE WARBURG EFFECT

Normal cells have a tendency to respire anaerobically under conditions of low oxygen. When this happens, oxidative metabolism shuts down and glycolysis persists with lactate as an end product. Tumour tissue, however, often has an elevated rate of glycolysis even under aerobic conditions (Krebs, 1972; Bodansky, 1975; Mangiardi and Yodice, 1990). This is known as the Warburg effect and was originally shown by Warburg in the 1920's with slices of tumour tissue *in vitro* (Reported by Bodansky, 1975; Racker, 1976; Murray, 1987). Warburg showed that tumour tissue could produce increased amounts of lactic acid relative to normal tissue when it was incubated aerobically in a solution containing glucose. A high lactate production may be responsible for acidity in some tumours. For example, Gerweck *et al.* (1991) showed that the extracellular and intracellular pH of murine FSA-II tumours *in vivo* decreased after administration of 5 g/kg I.P. glucose while very little change in pH was found in normal muscle.

Not all normal tissue types produce raised lactate only under anaerobic conditions; proliferating normal tissues and several quiescent normal tissues, for example, intestinal mucosa, renal medulla and retina have elevated rates of glycolysis under aerobic conditions which may result in significant lactate production (Krebs, 1972; Eigenbrodt *et al.*, 1985). Indeed, not all tumours have high rates of glycolysis (Bodansky, 1975; Wenner and Tomei, 1981). For example, some liver tumours such as

hepatoma 5123 have glycolytic rates similar to those of normal liver (Murray, 1987).

The Warburg effect was explained originally as being due to irreversible damage of the respiratory system by carcinogenesis (Racker, 1976; Murray, 1987). The build-up of lactate may be due to a number of factors which will be outlined below. Cells may have an inherently elevated rate of glycolysis which results in the accumulation of pyruvate. This pyruvate may not be able to be oxidized rapidly enough by the citric acid cycle and is consequently redirected to lactate production.

A reason for high rates of glycolysis in some tumour cells is the presence of altered enzyme characteristics compared to those in normal cells (Bodansky, 1975). Induction and repression of enzymes are often abnormal in tumours (Murray, 1987). Tumour cells may exhibit the induction of isoenzymes many of which may not be subjected to the same degree of regulation as normal cells (Eigenbrodt *et al.*, 1985). Mitochondrial hexokinase (Type II), found in some tumour types, is not inhibited by the presence of glucose-6-phosphate, as is the case for hexokinase in normal cells. Tumoural phosphofructokinases (Type L, F and M) may have similar characteristics to the normal enzyme. Tumoural pyruvate kinase (Type M2) has been shown to be inhibited to a lesser extent by phosphoenol pyruvate in some tumours than in normal tissue. These enzymes are all points at which regulation can be altered in some tumours so that glycolysis may be induced to operate more rapidly (Eigenbrodt at al., 1985).

An elevated rate of glycolysis in tumours may also be due to an increased demand for energy by various processes. Racker (1976) suggested that the increased rate of glycolysis in tumour cells may be caused by the activation of ATPases which may be mitochondrial, in the plasma membrane or viral. Inhibitors of certain ATPases resulted in a decrease in aerobic lactate formation in tumours. For example, rutamycin was used to inhibit mitochondrial ATPase and ouabain was used to inhibit plasma membrane ATPases. The responses of different tumour cells to the inhibitors were, however, varied. A certain amount

of confusion arose as glycolytic upregulation was, consequently, attributed to different ATPases depending on the type of tumour observed.

In a review, Pedersen (1978) concluded that many tumours with high glycolytic rates had reduced mitochondrial contents compared to that of normal tissue. Reduced mitochondrial capacity may result in upregulation of pathways such as glycolysis.

In tumours, *in vivo*, a build-up of lactic acid may be partially caused by vascular insufficiency. Reduced oxygen supply may cause tumour cells to become hypoxic and force them to respire anaerobically. Under such conditions, oxidative phosphorylation and also pyruvate oxidation is prevented. If pyruvate oxidation is prevented, then flux of material may be diverted to lactate, as one might expect for hypoxic normal cells.

Tumours are thought to undergo natural selection in the hostile host evironment (Fidler and Hart, 1982). Tumours would survive better in an anaerobic environment if they could provide themselves with energy without respiring. An elevated rate of glycolysis would go a long way towards achieving this end. Changes in genetic composition as a result of genetic instability amongst tumour cells have been recorded. Selection of cells with suitable characteristics for survival would allow those better-adapted cells to prevail while those less able to cope would perish.

THE CRABTREE EFFECT

The Crabtree effect is characterised by the inhibition of respiration by glycolytic activity (Racker, 1965). It is a phenomenon which occurs in tissues which have a high glycolytic rate such as many tumours and renal medulla, leukocytes and cartilage (Krebs, 1972). It may also be initiated by the presence of sugars such as fructose and mannose. It has also been described as a reverse Pasteur effect (Racker, 1965).

GLUTAMINOLYSIS

As discussed above, some tumours have high rates of lactate production as a result of elevated glycolytic rates. However, excessive lactate production may occur under aerobic conditions in cells whose glycolytic rates are not elevated. Lactate is also a product of other pathways such as glutaminolysis, as discussed in Chapter 4. When the carbohydrate supply is limited, most of the lactate may arise from glutamine metabolism (Eigenbrodt *et al.*, 1985).

Isoenzymes of normal glycolytic enzymes exist in some tumours, as discussed in the previous section. Similarly, some tumour cells and proliferating normal cells have been shown to possess isoenzymes of enzymes found in glutaminolysis. Enzymes in this category include glutaminase and malic enzyme (Eigenbrodt *et al.*, 1985). Malic enzyme in normal proliferating and tumour cells uses NAD^+ or $NADP^+$ to catalyse the decarboxylation of malate to pyruvate. However, other isoenzymes of malic enzyme cannot use NAD^+. The NAD^+- associated isoenzyme may be regulatory in nature because it is activated by succinate, fumarate and isocitrate while ATP and ADP are competitive inhibitors of malate (Eigenbrodt *et al.*, 1985).

From preceding discussions, therefore, it would seem that some tumours may have elevated rates of aerobic glycolysis, some may have increased glucose uptake, some an increased glutaminolysis and some a lower sensitivity to oxygen. There may also exist tumours which possess all of these properties.

CELL PROLIFERATION AND ENERGY

A sufficient rate of energy metabolism is necessary for the cell to perform functions such as cell proliferation. Pohlit and Heyder (1981) found that the ATP content of exponentially dividing Ehrlich Ascites cells dropped from about 5 pg/cell to about 2.5 pg/cell as cells reached plateau phase. It has been proposed that a high rate of glycolysis is necessary for cell division. This may be supported by the demonstration in which the addition of serum to resting cells was shown to stimulate

the formation of lactate from glucose (Eigenbrodt *et al.*, 1985). An increased flux through the glycolytic pathway is, however, not thought to be an absolute requirement for cell division. Similarly, an increased rate of glutaminolysis is probably not a prerequisite for cell proliferation either (Eigenbrodt *et al.*, 1985).

Several intermediates of energy metabolism have been implicated in the control of cell proliferation. If glucose concentration is reduced to less than 40μM, human diploid fibroblasts *in vitro* stop dividing. The addition of serine derivatives restarts division at approximately the same rate (Zielke *et al.*, 1976). Glycerol-3-phosphate regulates triglyceride and phospholipid synthesis (Eigenbrodt *et al.*, 1985). Addition of serum to resting G_o cells results in 5 to 10 fold increases in the levels of fructose-1,6-bisphosphate. Fructose-1,6-bisphosphate promotes its own synthesis by acting on PFK and promotes its own degradation by acting on pyruvate kinase in dividing normal and tumour cells. It also stimulates protein synthesis. There is evidence that increasing NADH/NAD ratio is a signal which induces cells to progress from G_o to G_1 (Eigenbrodt *et al.*, 1985).

ATP and Cell Kinetics

It was shown by Chapman *et al.* (1971), in synchronous Chinese Hamster V79-379A cells, that the cellular ATP content varied through the cell cycle. A steady increase was noted from G_1 to late G_2 with levels peaking just prior to mitosis. This ATP increase was paralleled by an increased mean cell volume. Alterations in ATP content per cell may, therefore, in certain circumstances, be expected not because of factors associated with up or down regulation of energy metabolism but rather due to physical factors such as a change in cell volume. A similar finding concerning cell size was made by Skog and Tribukait (1986) who reported that Ehrlich Ascites cell volume increases as cells pass from G_1 to S to G_2+M.

Energy status and tumour size

It was demonstrated in murine FSa-II fibrosarcomas by Gerweck *et al.* (1989) that the levels of various energy intermediates changed with tumour size. Both ATP and creatine phosphate decreased as tumour size increased while AMP and ADP were shown to increase. The overall adenylate energy charge decreased and the sum of ATP, ADP and AMP remained constant with tumour size. The decrease in energy status was correlated with an increase in hypoxic cell fraction which may have been responsible for the increase in TCD_{50} (the dose required to locally control 50% of a group of tumours), which was found with increasing tumour volume. Okunieff *et al.* (1986) found that as tumour volume increased, the ratio of CrP/Pi dropped as did the nucleotide triphosphate/Pi level. Rofstadt *et al.* (1988) also showed a decreased bioenergetic status in KHT, RIF-1 and MLS tumours with increasing tumour volume.

CHAPTER 6

INHIBITORS OF ENERGY METABOLISM

Many inhibitors of energy metabolism have been identified. Some inhibitors decrease the Pasteur effect, which has been discussed in Chapter 5. These inhibitors include imidazole and uncouplers of oxidative phosphorylation, for example KCN, which increase aerobic glycolysis, and ethylmaleimide or iodoacetate, which decrease aerobic glycolysis. Others, such as ferricyanide or nucleotides may increase the Pasteur effect (Racker, 1965). Two inhibitors detailed in the present work are 2-deoxy-D-glucose (2DG) and aminooxyacetic acid (AOA), which inhibit glycolysis and glutaminolysis respectively.

Some inhibitors of energy metabolism have been found to modify radiation response (Seymour and Mothersill, 1987; Jain *et al.*, 1977a-c, 1982). 2DG has been found to enhance the effects of both ionizing radiation and hyperthermia (Gridley *et al.*, 1985). More about the effects of 2DG on radiation response will be presented in subsequent chapters.

INHIBITORS OF GLYCOLYSIS

Ouabain, an inhibitor of active transport, can inhibit glycolysis in Ehrlich ascites tumour cells (Racker *et al.*, 1983). This suggests that efficient transmembrane transport may be required for effective glycolytic function.

Glycolysis may also be inhibited by means of glucose antimetabolites (Woodward and Hudson, 1954; Wick *et al.*, 1957). Substances of this type are similar in structure to glucose and compete with glucose for uptake by cells and binding to enzymes and so effectively block glucose

metabolism. Two well known glucose antimetabolites are 2DG and 5-thio-D-glucose.

2-Deoxy-D-glucose

Woodward and Hudson (1954) showed that 2DG could inhibit both anaerobic and aerobic glycolysis in tumour slices and anaerobic glycolysis of certain normal tissues. However, no major effects on respiration were noted. Similarly, Barban and Schultze (1961) showed in HeLa cells and human embryonic intestinal cells that 2DG could inhibit glycolysis and cell growth at concentrations equal to those of glucose in growth medium. This inhibition could be reversed by the addition of glucose or mannose. Dwarkanath and Jain (1989) have shown also that 2DG can inhibit glycolysis in human cerebral glioma cells *in vitro*.

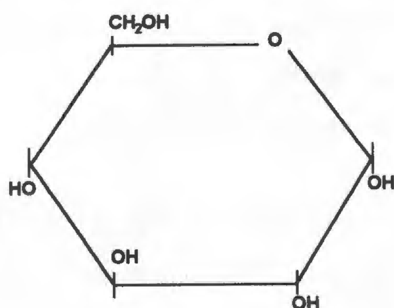


Figure 6.1: D-glucose

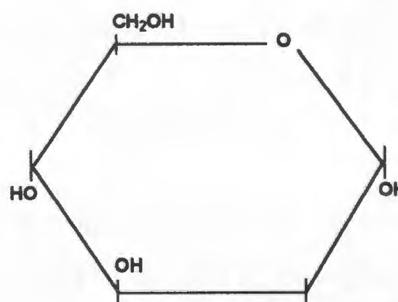


Figure 6.2: 2-deoxy-D-glucose

2-Deoxyglucose is a structural analogue of glucose. As shown in Figures 6.1 and 6.2, the 2DG molecule is similar in structure to the glucose molecule except that 2DG lacks one oxygen atom at the second carbon position (Newsholme and Leach, 1983). This alteration prevents 2DG from being metabolised by cells in the same way as glucose. Wick *et al.* (1957) reported that 2DG was not readily oxidized to CO_2 when administered to eviscerated rabbits, thus indicating a block in metabolism. However, 2DG did not affect the oxidation of acetate, which suggested that the block was in glycolysis. From *in vitro* studies, it was determined that 2DG is phosphorylated by hexokinase to 2-deoxyglucose-6-phosphate in the same way that glucose is phophorylated to glucose-6-phosphate. However, there is competition

between 2-deoxyglucose-6-phosphate and glucose-6-phosphate for binding with the enzyme, glucose phosphate isomerase. Glucose phosphate isomerase is unable to produce 2-deoxyfructose-6-phosphate from 2-deoxyglucose-6-phosphate, thus resulting in a competitive block in the production of fructose-6-phosphate from glucose-6-phosphate, and so, a block in glycolysis (Wick *et al.*, 1957).

It was proposed that 2DG might indirectly inhibit glycolysis at the hexokinase level in some systems. However, hexokinase activity in muscle cells has been shown not to be altered as a result of either 2-deoxyglucose-6-phosphate accumulation or reduced ATP levels (Jacobs *et al.*, 1990). In cellular homogenates of cultured human muscle cells, hexokinase showed a greater affinity for glucose than for 2DG. Intact cells preferentially phosphorylated glucose than 2DG implying that transport into the cell rather than phosphorylation may be rate limiting with respect to uptake of glucose (Jacobs *et al.*, 1990).

Inhibitors of glycolysis might be expected to inhibit energy production in systems which rely on glucose as an energy source. It is well established that ATP production can be inhibited by 2DG in several systems including Krebs-2 ascites tumour cells, yeasts, cultured human muscle cells and HeLa cells (Yushok, 1964; McComb and Yushok, 1964; Van Steveninck, 1968; Jain *et al.*, 1982; Jacobs *et al.*, 1990; Hamilton *et al.*, 1995). However, Purohit and Pohlit (1983) were able to show a substantial decrease in ATP in EAT cells after 2DG treatment only in hypoxic cells but not in well oxygenated cells.

Purohit and Pohlit (1982) showed that the presence of 2DG inhibited the division of EAT cells *in vitro*. This inhibition depended on the molar ratio of 2DG to glucose. At a molar ratio of 1, cells were 80% inhibited. This was found to be reversible if 2DG was removed within 48 hours. The reproductive viability of cells could be more effectively reduced when cells were kept hypoxic than when they were kept euoxic.

Work by Sridhar *et al.* (1978) has shown how the reproductive viability of V79 cells from spheroids treated with 2DG was reduced, possibly due to the presence of hypoxic cells. In addition, hypoxic monolayers of

cells were also found to be sensitive to the cytotoxic effects of 2DG but aerobic monolayers were found to be more resistant. Hypoxic cells are more sensitive to the cytotoxic effects of 2DG than aerobic cells because oxidative phosphorylation in hypoxic cells is inhibited. It is possible that administration of 2DG might be an effective way of treating tumours which contain radioresistant hypoxic cells that may limit the success of radiotherapy.

2DG has been found to be effective as an antitumour agent alone or in combination with other agents. 2DG has been shown to inhibit growth of liver tumours in rats (Cay *et al.*, 1992). Because of the ability of 2DG to interfere with energy supply, it is often used in combination with other agents such as radiation (Jain *et al.*, 1977a; Jain *et al.*, 1977b; Purohit and Pohlit, 1982; Jain *et al.*, 1985) and cytotoxic drugs (Hamilton *et al.*, 1995) in attempts to modify responses of tumours to these agents by, for example, interfering with cellular recovery processes. Further discussions concerning 2DG combined with radiation will be presented in Chapters 9 and 10 .

The administration of 2DG in human subjects has been limited. However, a number of studies have been undertaken. For example, Laszlo (1961) administered 60 mg/kg 2DG intravenously to fasting, rested volunteers. Plasma free fatty acid, glucose and lactate levels were shown to rise after administration of 2DG. Administration of insulin, glucose and fructose reversed the rise in free fatty acids. Insulin also reversed the rise in glucose but not the clinical side effects of drowsiness, hunger, sweating and nausea, normally indications of hypoglycaemia. More recently, Mohanti *et al.* (1996) presented results from 20 patients with cerebral gliomas (grade 3/4) who received four weekly fractions of oral 2DG (200 mg/kg body weight) administered before 5 Gy whole brain irradiation, that is, 20 Gy in 4 fractions. This was followed two weeks later by 14Gy given in 7 fractions, at 5 fractions a week, to the target volume. Blood glucose was found to rise after 2DG administration. Some vomitting was observed in 50% of patients on the days when 2DG and radiotherapy were combined. Some drowsines and giddiness were also reported. No late radiation effects in the normal brain, as assessed clinically and by magnetic resonance

imaging, were seen in surviving patients at 18 up to 63 months. Mean survival time of patients was comparable to that shown in other published results.

INHIBITORS OF GLUTAMINOLYSIS AND ANTAGONISTS OF GLUTAMINE

Interference with glutamine utilization may be achieved in several ways. Glutamine usage may be prevented by inhibiting glutaminolysis with, for example, aminooxyacetic acid, which will be discussed below. Other methods of preventing the use of glutamine include the use of glutamine antimetabolites such as 6-diazo-5-oxo-l-norleucine (DON), acivicin and Azaserine (Huber, 1988; Hanka, 1979). A method of essentially removing glutamine by converting it to glutamate is also discussed below.

As was discussed in previous chapters, glutamine may be an important source of energy in the cell. Glutaminase, an enzyme which normally catalyses the conversion of glutamine to glutamate in glutaminolysis, has been used to reduce blood glutamine in mice with experimental tumours. This resulted in an increased growth delay after radiation in KHT tumours. Glutaminase was also shown to reduce survival of CHO cells exposed to cell culture medium lacking glutamine but had little effect in medium containing glutamine (Tannock *et al.*, 1986).

Aminooxyacetic acid

Aminooxyacetic acid is an inhibitor of the aminotransferases and acts by competing with amino acid substrates (Hopper and Segal, 1962). Glutaminolysis, which relies on aminotransferases, is, therefore, also inhibited by AOA. Because AOA is an inhibitor of aminotransferase reactions and not of glutamate dehydrogenase reactions, cell types which rely on the latter path for metabolism of glutamine, will still be able to use glutamine in the presence of the inhibitor.

The inhibition of the aminotransferase reaction by AOA is highly pH dependent. Hopper and Segal (1962) showed that the activity of

glutamic-alanine transaminase changed as pH was increased. The activity of the enzyme approximately doubled, as the pH increased from pH 6 to pH 7.5, and then decreased to the initial pH 6 value at pH 9. In addition, the extent by which the enzyme was inhibited by AOA was also found to be pH dependent. A decrease in inhibition of the glutamic-alanine transaminase by AOA from about 75%, at pH 7, to about 10%, at pH 8, was recorded. Therefore, in order to maintain consistent AOA activity in experiments conducted *in vitro*, it is necessary to ensure sufficient buffering of culture media.

Hornsby and Gill (1981) showed that AOA can inhibit the oxidation of glutamine to CO_2 in cultured adrenocortical cells. They found that AOA could prevent cell proliferation at a 2 mM concentration (Hornsby and Gill, 1981; Hornsby, 1982). This effect could be reversed by the addition of glutamine, although the concentration of glutamine needed to be at least 25 mM. However, 100 mM glutamine seemed to have a negative effect on cell growth (Hornsby and Gill, 1981). In addition, Hornsby (1982) found that AOA toxicity can be prevented by the presence of 100 nM vitamin E and various other substances such as other tocopherols, antioxidants and ubiquinones. Vitamin E may prevent side reactions relating to lipid peroxidation and allow the cell to function even at a reduced level of glutamine oxidation.

Gonzalez-Mateos *et al.* (1993) have shown that the glutamine induced inhibition of glycolysis, which they found to be the case in ascites tumour cells, could be prevented by the administration of AOA. It would seem, therefore, that inhibitors such as AOA may also have effects on the reciprocal regulation of other pathways, such as glycolysis, as mentioned in Chapter 4.

CHAPTER 7

FORMULATION OF HYPOTHESES

As suggested in Chapters 1 and 3, the repair of repairable cellular damage inflicted by ionizing radiation is energy dependent. The aim of the present work was to investigate whether radiation response was dependent on energy supply in the systems tested and whether energy supply could be altered in such a way as to induce changes in radiation response. A number of hypotheses were tested, as outlined below. Experiments conducted to test these hypotheses and detailed discussions are presented in Chapters 8, 9, 10 and 11.

Firstly, the idea that ATP levels may be altered in irradiated cells was explored. It has been proposed that ATP levels may be raised after radiation as a compensatory response which may facilitate cellular repair mechanisms (Szeinfeld, 1987). This notion was explored further in experiments with cultured cells and in murine tumour as well as normal tissue. Hence, the first hypothesis that ***there is a change in ATP levels in cells after irradiation*** was proposed. Szienfeld (1987) found that ATP levels after irradiation depended on the energy of the beam. This lead to the proposal of a secondary hypothesis which was dependent on the first hypothesis not being found false: ***any change in cellular ATP content after irradiation of cells is beam energy dependent.***

Secondly, instead of focusing on radiation induced changes in energy metabolism, inhibitors of energy metabolism were tested ***in vitro*** to see whether cellular radiation response could be altered by the action of such inhibitors. The inhibitors tested in the present work were 2-deoxyglucose and aminooxyacetic acid which have been found to be able to inhibit glycolysis and glutaminolysis respectively. The first general hypothesis was, therefore, that ***inhibitors of energy metabolism alter the***

radiation response of cells. Radiation response is a non-specific term and had to be defined more specifically. This lead to more focused hypotheses. The first, *inhibitors of energy metabolism inhibit repair of radiation damage*, can be broken down to refer directly to the specific inhibitors tested and thus includes the hypotheses, *2-deoxyglucose inhibits repair of radiation damage* and *aminoxyacetic acid inhibits repair of radiation damage.*

Split-radiation-dose experiments were used to assess the level of repair in a cultured cell system in which the culture medium was varied in respect of the presence of inhibitors. Another component of radiation response involves cellular radiosensitivity. A change in radiosensitivity may lead to ambiguity of results of split dose experiments if it is not taken into account. Split dose experiments of this sort involve the exposure of cells to culture medium containing inhibitors for different times between radiation fractions before the second dose and assessment of survival. If the radiosensitivity of cells is altered by increasing times of exposure to an inhibitory medium, then cells might have an altered response to a second radiation dose. Therefore, it might not be possible to ascribe any change in radiation response to a change in repair alone. The possibility that inhibitory medium might affect radiosensitivity lead to the hypothesis that *inhibitors of energy metabolism increase the radiosensitivity of cells.* When relating this to specific inhibitors, the hypotheses were that *2-deoxyglucose increases the radiosensitivity of cells* and *aminooxyacetic acid increases the radiosensitivity of cells.*

Although the hypotheses above have been stated in terms of 2DG and AOA individually, combinations of the two were also tested to determine their combined effects on radiation response. Hence the hypothesis, *the effects of 2-deoxyglucose and aminooxyacetic acid interact with one another*, was also proposed.

If the presence of 2DG or AOA were found to alter the radiation response of cells, it would be of interest to determine whether this effect might be due to their effect on energy metabolism. Hence, the hypothesis that *the alteration of radiation response of cells exposed to 2-deoxyglucose and aminooxyacetic acid is due to the effects of these*

substances on energy metabolism was presented. An hypothesis was that *2-deoxyglucose and aminooxyacetic acid alter the activities of glycolysis and glutaminolysis respectively*. The activities of glycolysis and glutaminolysis were assessed in cells in media containing inhibitors, as for the radiation experiments.

As presented in Chapter 4, there are several energy substrates other than glucose and glutamine that can be used by cells. The supply of energy from these substrates under aerobic conditions is often dependent on oxidative phosphorylation. If it were able to be shown that glycolysis or glutaminolysis were inhibited but that oxidative phosphorylation was unaffected by the presence of these inhibitors, then it would be unlikely that energy supplies in the cell would be seriously depleted. This might, therefore, suggest that any alterations in radiation response as a result of inhibition of glycolysis and glutaminolysis were not due to energy depletion. The hypothesis that *2-deoxyglucose and aminooxyacetic acid alter the activity of oxidative metabolism* was, therefore, also proposed.

CHAPTER 8

THE EFFECT OF RADIATION ON ADENOSINE TRIPHOSPHATE LEVELS IN CELLS

INTRODUCTION

The aim of the experiment presented in this chapter was to determine the effect of radiation on ATP levels in B16 murine melanoma cells *in vitro*, CaNT murine tumours and murine livers.

In Chapter 3, the role of cellular energy in radiation repair was discussed. ATP is one of the main energy carriers in the cell, and thus, it is reasonable to assume that it will be involved in repair of radiation induced damage. Indeed, many authors have indicated that ATP is involved in radiation damage repair (Patrick and Haynes, 1964; Matsudaira *et al.*, 1970; Jain *et al.*, 1977a; Verma *et al.*, 1982; Jain *et al.*, 1982; Kaufmann *et al.*, 1982; Jain *et al.*, 1985). Radiation has the potential to influence energy metabolism in several ways. First, radiation may cause damage to the machinery of energy metabolism. This may result in a reduction in the efficiency of energy supplying pathways. However, radiation damage severe enough to inactivate enzymes generally occurs at doses well above those used clinically. Secondly, radiation may result in cellular damage which rapidly uses available ATP in the cell thus causing an ATP deficit, which may be detected as a decrease in ATP content in cells immediately after irradiation. Similarly, other ATP-using processes initiated by radiation may reduce cellular ATP levels. Thirdly, radiation damage may stimulate repair mechanisms and also compensatory mechanisms which will allow the cell to increase cellular activity, for example, ATP production may be increased.

Glycolysis and respiration are thought to be relatively resistant to the effects of radiation (Altman *et al.*, 1970). However, as detailed below, irradiation of cells can result in altered cellular ATP content in some cases. Investigators, using various systems, have shown conflicting results regarding changes in ATP levels after irradiation. Early workers often investigated effects after radiation doses exceeding those used clinically. Crabtree (1935) found that the administration of 100 Gy γ-radiation to tissue slices of Jensen rat sarcomas resulted in a reduction in the rate of respiration three hours after irradiation but had little effect on glycolysis. Betel (1967) showed a decrease in ATP levels in rat thymocytes after 4.8 Gy whole body irradiation. This was thought to have resulted from a reduction in oxidative phosphorylation. Klouwen and Appelman (1967) found similar decreases in ATP levels and depression of ATP synthesis in thymus, lymphoma and myeloid leukaemia cells after doses of 9 Gy up to 100 Gy whole body irradiation in mice. Retelewska and Leyko (1978) observed cyclical changes in adenine nucleotides in pig lymphocytes irradiated in saline after different doses of γ-rays. Decreases in ATP, AMP and ADP levels were found within 1 hour after 5 to 10 Gy of radiation but the levels of these adenine nucleotides increased to levels higher than control levels after 30 Gy and decreased to below control levels after 40 Gy. A decrease in NAD was also shown after 5 and 20 Gy as was a drop in the energy charge. Lymphocytes are generally considered to be relatively radiosensitive (Scherer *et al.*, 1991) and are prone to interphase death or mitotic death after irradiation. It can be speculated that certain radiation effects seen in lymphocytes at relatively low doses may occur in other more resistant cell types but at higher doses.

There is some evidence that ATP levels may be lowered after irradiation as a result of increased permeability of cell membranes. Billen *et al.* (1953) showed that radiation caused membranes of E.Coli to become excessively permeable to ATP resulting in a reduced intracellular ATP content. However, the structure of the bacterial cell membrane is different to that of mammalian cells, so the response of mammalian cells may be different.

When CHO cells were given a lethal dose of x-rays, they were able to maintain a normal rate of glycolysis for 24 hours and a normal energy charge for 6 hours afterwards. Radiation also did not affect lactate production. (Rotin *et al.*, 1986). Similarly, doses up to 80 Gy failed to alter oxygen consumption or glucose consumption in Ehrlich ascites tumour cells irradiated *in vitro* (Coe *et al.*, 1963). Lactate accumulation was unchanged for doses up to 40 Gy but was slightly decreased at 80 Gy. ATP levels were shown to increase after doses ranging from 5 Gy to 80 Gy radiation. Changes in ATP after irradiation do not necessarily indicate direct physical effects of radiation on the mechanism of ATP supply but possibly indirect effects initiated by radiation damage to other targets. Coe *et al.* (1963) suggested that an increase in ATP after radiation might be due to a reduction in activity of those systems in the cell that use ATP, which may result in a surplus of ATP.

Clement *et al.* (1978) showed increases in oxygen consumption rate per cell relative to unirradiated controls in mouse mastocytoma cells 3 to 12 hours after doses up to 20 Gy. This reflected changes in respiration rate and was thought to represent an increase in metabolic activity in response to radiation damage. However, some cells were shown to be killed by radiation, which would result in a decrease in oxygen consumption in those cells. Other cells, which survived radiation, were able to proliferate, which resulted in more cells and thus an increase in oxygen consumption by these cells. Thus, the viable cell fraction, which depended on the dose of radiation given and the extent of cell division, changed with time after radiation. Oxygen consumption of the total cell population, calculated from the product of the viable cell fraction and cellular oxygen consumption rate, was also found to be raised after 2.5 Gy and 5 Gy, remained unchanged after 10 Gy and declined steadily after 20 Gy.

Szeinfeld (1987) showed in murine CaNT mammary adenocarcinomas, that ATP levels increased after irradiation relative to unirradiated controls and reached a maximum at 2.5 hours after irradiation. This increase was shown to depend on dose, time after irradiation and beam energy, and it was proposed that the rise in ATP may have occurred in response to radiation in order to facilitate repair of radiation damage.

From the work of various researchers reported above, it would appear that the response of energy metabolism to radiation is variable, being both dose and system dependent. It was, however, proposed that the phenomena pertaining to possible compensatory increases in ATP after irradiation, reported by Szeinfeld (1987), be investigated further in tumour and normal tissue *in vivo* and in cell culture systems. Experiments, in which ATP levels of cellular extracts are determined after irradiation of these systems, using a luciferase-luciferin method, are presented by the author in this chapter.

METHODS AND MATERIALS

Cellular ATP content was determined after irradiation of murine tumours and livers *in vivo* and cells grown *in vitro*. Having been irradiated, tissues and cultured cells were ruptured and cellular extracts assayed for ATP using the luciferase-luciferin assay. Two different radiation types of different energy were used to determine whether beam energy could influence any potential change in ATP levels after irradiation. In addition, the ATP content of blood from mice whose tumours had been irradiated and culture medium from irradiated cells, were assessed.

Experimental Animals

The animals used in the experiments were male CBA mice obtained from specific pathogen free stock. At the time of ATP determination, all animals used were between 7 and 9 weeks old. Food and water were given *ad libitum* (Mouse pellets were supplied by Specialist Animal Feeds, Delft, South Africa). Separate animals were used for tumour and liver studies.

Tumour

The tumour used was the CaNT neck tumour, a murine mammary carcinoma, which was inoculated subcutaneously into the chests of the CBA mice and was maintained by serial passage *in vivo*, as described in

the Appendix. The volume of tumours used in experiments was 0.2 cm^3. This was a convenient size for excision and assay. The volume of tumours was determined by the following formula, assuming tumours to be spherical:

$$v=4/3\ \pi\ r^3 \quad \text{(volume of a sphere)}$$

where $r=(L+H+B)/6$ and L, H and B are Length, Height and Breadth respectively of a tumour from measurements made with Vernier calipers.

Cells *In vitro*

B16 murine melanoma cells were cultured in McCoy's 5a medium (Highveld Biological) containing 10% foetal calf (v/v) serum and antibiotics (50 mg/l penicillin, 50 mg/l neomycin, 50 mg/l streptomycin) according to standard techniques which are described in the Appendix. Cells grown in tissue culture flasks were passaged and periodically plated into 30 mm diameter tissue culture dishes (Sterilin) and allowed to grow to the desired cell density before treatment and determination of ATP. Each culture dish contained 2 ml medium. To obtain confluence, 400 000 cells were plated into each culture dish and allowed to grow for 72 hours. Confluence was verified by observation under the microscope. To obtain cells growing in the exponential phase, 400 000 cells were plated and allowed to grow for 24 hours. Cells were observed to be sparsely plated after this time had elapsed. Both sparsely plated and confluent cells were investigated to overcome any potential proliferation artifacts.

Irradiation

100 KVp X-rays were produced by a 100 KVp Philips RT100 X-ray unit and ^{60}Co γ-rays (1.17 and 1.33 MeV) were administered with an Eldorado 6 Cobalt unit modified with a fixed lead collimator.

CELLS *IN VITRO*

In the case of 100 KVp X-rays, cells were irradiated in the culture dishes from below using a 20 cm diameter perspex (lucite) applicator (SSD=30 cm,dose rate=0.83 Gy min^{-1}). The centres of the culture dishes were arranged on the applicator face along a circle of 5 cm radius with its centre on the central axis of the beam.

In the case of ^{60}Co γ-rays, cells were irradiated from above with full backscatter in a 20 cm x 20 cm field (SSD=80 cm, dose rate=0.65 Gy min^{-1}). Full buildup was achieved by having 0.5 cm depth of cell culture medium above the cells.

LIVERS IN VIVO

In the case of 100 KVp X-rays, mice were restrained in a prone position by taping their feet to a perspex (lucite) plate so that a cut-away portion of the plate was positioned at the region of the liver. Livers were irradiated through the cut-away window from below using a circular 2.5cm diameter applicator (SSD=10 cm, dose-rate=7.5 Gy min^{-1}).

For ^{60}Co γ rays, mice were irradiated from above with full backscatter in a 20 cm x 20 cm field (SSD=80 cm, dose rate=0.65 Gy min^{-1}). The anterior and posterior sections of the mouse outside the field of interest were shielded with 10 cm thick lead blocks so that only the mid-sections of the mice were irradiated. Mice were restrained as for the 100 KVp X-irradiation of livers. No additional build-up was used.

100 KVp X-RAY IRRADIATION OF TUMOURS IN VIVO

Mice were restrained in the same way as for liver irradiation with the tumours protruding through the cut-away window in the perspex (lucite) plate. Tumours were irradiated from the side using the 2.5 cm diameter applicator, thereby avoiding exposure to the bodies of the mice. Half of the dose was administered from one side and half from the opposite side to ensure homogeneous dose distribution (SSD=10 cm, dose rate= 7.5 Gy min^{-1}).

Reagents

Reagents are listed and their sources recorded in the Appendix. Preparation of individual reagents is described in the individual method sections below.

Extraction of ATP

ATP EXTRACTION FROM LIVER AND TUMOUR

For livers, extractions were conducted 1, 3 and 5 hours after 5, 10 and 15 Gy ^{60}Co γ-irradiation. After 5 and 15 Gy 100 KVp X-rays, extractions were conducted 1, 3 and 5 hours after irradiation and after 10 Gy, extractions took place 1, 2, 3, 4 and 5 hours after irradiation. For tumours, extractions took place 2.5 hours after 10 Gy 100 KVp X-rays or in the case of the stress experiments, 2.5 hours after sham irradiation. All control mice were sham-irradiated and extractions performed at the same time as for the irradiated mice.

Mice were anaesthetized with ether and a lobe of liver or section of tumour was excised with clean instruments. The tissue was immediately placed into liquid nitrogen (0.1-0.3 g per sample). The sample was quickly weighed in a clean Petri dish before being transferred to a stainless steel mortar containing enough liquid nitrogen to cover the tissue. One ml of 6% (v/v) perchloric acid was added to the liquid nitrogen and, together with the tissue, was crushed to a fine powder using a pestle. A sufficient level of liquid nitrogen was maintained during crushing. Once the remaining liquid nitrogen had evaporated, the powder could be scraped with the aid of a spatula into a glass homogenizer. Once thawed, the material was homogenized. The homogenate was then decanted into a tube. The homogenizer was rinsed with a further 1 ml of perchloric acid and the liquid added to the centrifuge tube. The contents were centrifuged at at 17000 x g for 25 minutes at 4°C to remove the cell debris. The supernatant was removed and 0.5 ml 6% (v/v) perchloric acid added to the debris pellet. The mixture was vortexed to wash out remaining ATP and recentrifuged. The supernatant was removed and added to the previous supernatant. Plastic

freezing tubes containing this supernatant could then be stored in liquid nitrogen until the day of assay.

In a solution of which the pH is close to 2, ATP does not hydrolyse to ADP and AMP to any great extent. This is because adenylate kinase, which is responsible for the hydrolysis reaction, is denatured and, therefore, cannot function. The low temperature of the liquid nitrogen also slows metabolism to very low rates thus effectively preventing changes in biochemical components of the samples.

Immediately prior to assay, samples were thawed and adjusted to pH 7.5 with 5 M K_2CO_3,which was added dropwise, taking care that the CO_2 liberated during the reaction did not cause the contents of the vials to bubble out. Samples were centrifuged for 25 minutes at 4°C at 17000 x g to remove the resulting perchlorate precipitate. The supernatant was removed and its volume measured.

ATP EXTRACTION FROM BLOOD

Two and a half hours after irradiation of tumours, mice were anaesthetized with ether. Appropriate dissection was performed to expose the inferior vena cava. A fine syringe needle was inserted into this vessel and a minimum of 0.2 ml blood was drawn. 0.2 ml blood was added to a 7 ml polypropylene test tube containing 0.8 ml 6% (v/v) perchloric acid. Samples were stored in liquid nitrogen until just prior to assay when samples were thawed and the pH adjusted to pH 7.5 with 5M K_2CO_3. Tubes were centrifuged for 25 minutes at 4°C at 17000 x g to remove cell debris and perchlorate precipitate. Supernatants were taken and volumes recorded.

ATP EXTRACTION FROM CELLS IN CULTURE

Cells in Petri dishes were grown either to confluence or to less than confluence, as described above. Cells were rinsed with 1 ml cold sterile phosphate buffered saline. Cell membranes were ruptured *in situ* by adding cold (4°C) 6% (v/v) perchloric acid. Cells were dislodged with an adjustable Gilson pipette by scraping and drawing up and expelling the liquid several times. The contents of the Petri dishes were

transferred to plastic freezing tubes and the dishes rinsed with a further 0.5 ml of the perchloric acid which was then also added to the tubes. The tubes were stored in liquid nitrogen until required.

Samples were thawed and adjusted to pH 7.5 immediately prior to assay as for the solid tissue extraction method. Tube contents were centrifuged once at 17000 x g for 25 minutes at 4°C to remove perchlorate precipitate and cell debris. The supernatant was removed and its volume measured.

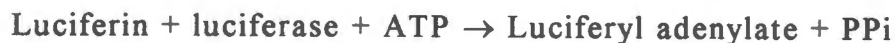
ATP FROM CELL CULTURE MEDIUM

Medium (0.2 ml) was taken from medium above cells and added to 7 ml polypropylene test tubes containing 0.8 ml 6% (v/v) perchloric acid. Samples could then be stored in liquid nitrogen until the day of assay when samples were thawed and the pH of samples adjusted to pH 7.5 with 5 M K_2CO_3. Tubes were centrifuged at 4°C for 25 minutes at 17000 x g, the supernatant was removed and its volume measured.

Luciferase-Luciferin ATP assay
(Modified from the method of Stanley and Williams, 1969)

ATP contents of the supernatant samples produced by the extractions described above were determined using the luciferase-luciferin method as described below.

Luciferase is an enzyme extracted from the American firefly, *Photinus Pyralis*, and is responsible for the characteristic glow which is observed in these insects when it is dark. The enzyme catalyses the reactions:


Luciferin + luciferase + ATP $\rightarrow$ Luciferyl adenylate + PPi

Luciferyl adenylate + O_2 + H_2O $\rightarrow$ Light + Reaction products

Luciferin is activated by adenylation. Luciferyl adenylate is then oxidised. On decarboxylation, oxyluciferin is formed in the excited state which then returns to the ground state by the release of a photon. ATP

can thus be measured by detecting photons of light using a scintillation counter set to the single photon mode of detection.

EQUIPMENT

A Beckman LS1801 scintillation counter was used to detect photon emission in these experiments.

All glassware, plastic vials, pipette-tips and other disposables were soaked in 2N HCl for at least 24 hours and rinsed thoroughly with deionized water and dried before use to eliminate any trace amounts of ATP which may have contaminated the equipment through the presence of incidental micro-organisms or from handling. These items were stored wrapped in aluminium foil prior to use.

PREPARATION OF REAGENTS

Luciferase-luciferin

Two and a half millilitres of luciferase-luciferin solution was made up in water with a concentration of 40 mg/ml. The solution was stored in the dark in liquid nitrogen and removed 7 days before the day of assay to stabilize at 0 to 4°C in the dark. The luciferase-luciferin was allowed to reach room temperature (23°C) in a dark room before commencement of the assay.

Tris(Hydroxymethyl)aminomethane (Tris) Buffer

100 ml 0.25 M aqueous Tris buffer was made up and adjusted to pH 7.5 with glacial acetic acid on the day of assay.

ATP standard

An ATP stock solution was made up in water (27.56 mg ATP/100 ml). A standard solution of ATP was made up by making a 1 in 10 000 dilution of the stock solution in water.

Fresh quantities of Tris buffer, ATP stock and standard solutions were made up on the day of assay.

PROCEDURE

10 µl luciferase-luciferin solution was added to 480 µl of buffer in a 8ml plastic scintillation vial (Beckman) five minutes before the addition of 10 µl ATP sample. During this period, the ATPase present in the luciferase-luciferin solution had time to deplete the vial of any incidental traces of ATP prior to the addition of the ATP sample. The procedure was performed under subdued lighting conditions at room temperature (23°C).

After the addition of each constituent, the vial was swirled gently to ensure adequate mixing. The sample was held firmly in the hand to prevent light from activating the luciferase-luciferin which will cause it to emit an excessive number of photons. The vial was then transported to the scintillation counter while enclosed in the hand, placed into the counting rack under the cover of a black plastic bag and read. Each sample was mixed and read individually rather than in a batch.

Standard curve

10 µl of luciferase-luciferin reagent was added to suitable amounts of ATP standard solution and Tris buffer to make up a final volume of 0.5 ml in scintillation vials. Samples were then read in the scintillation counter.

A new standard curve was determined for each assay session. Light emission, expressed as counts per minute, was plotted against moles of ATP and the quantities of ATP contained in the test samples were determined from the plot according to their counts. The standard curves were found to be consistent from one session to the next.

Test samples

Test samples were read as for standard samples. Appropriate dilutions with buffer were made so that the counts fell within the range of the standard curve. Different volumes of sample made up to 0.5 ml in the scintillation vial were found to yield similar results once dilution had been taken into account.

For the solid tissues assayed, ATP was determined as moles per gram of tissue. For cultured cells, ATP was determined as moles per Petri dish. The number of cells per dish was found to be consistent for the same time allowed for proliferation.

RESULTS

Results shown in Figures 8.1 to 8.9 show the changes in ATP levels expressed as ATP content relative to unirradiated control values after either 100 KV X-rays or ^{60}Co γ rays for different systems. These systems are: confluent and exponentially growing B16 cells *in vitro*, culture medium from irradiated cells, liver, CaNT tumour and blood from mice with irradiated tumours. ATP content of each group of data was compared to control groups and deemed to be significantly different if p values were less than 0.05 in a Student's t-test.

ATP content of unirradiated controls

Mean ATP values in unirradiated controls for confluent cells were 1.02×10^{-8} mol/dish with a standard deviation of $\pm 2.62 \times 10^{-9}$ mol/dish. For exponentially growing cells, the unirradiated control ATP values were 8.42×10^{-9} mol/dish with a standard deviation of $\pm 3.10 \times 10^{-10}$ mol/dish. Mean control liver ATP values were 2.07×10^{-6} mol/g tissue with a standard deviation of $\pm 7.45 \times 10^{-7}$ mol/g tissue.

Cells *in vitro*

Confluent Cells, 100 KVp X-rays

As shown in Figure 8.1, a depression of ATP after 3, 6 and 15 Gy but not after 9 Gy was found 2 hours after irradiation with 100 KVp X-rays followed by a return to control levels at 4 hours. However, in all cases, these decreases were not statistically significant (p>0.05).

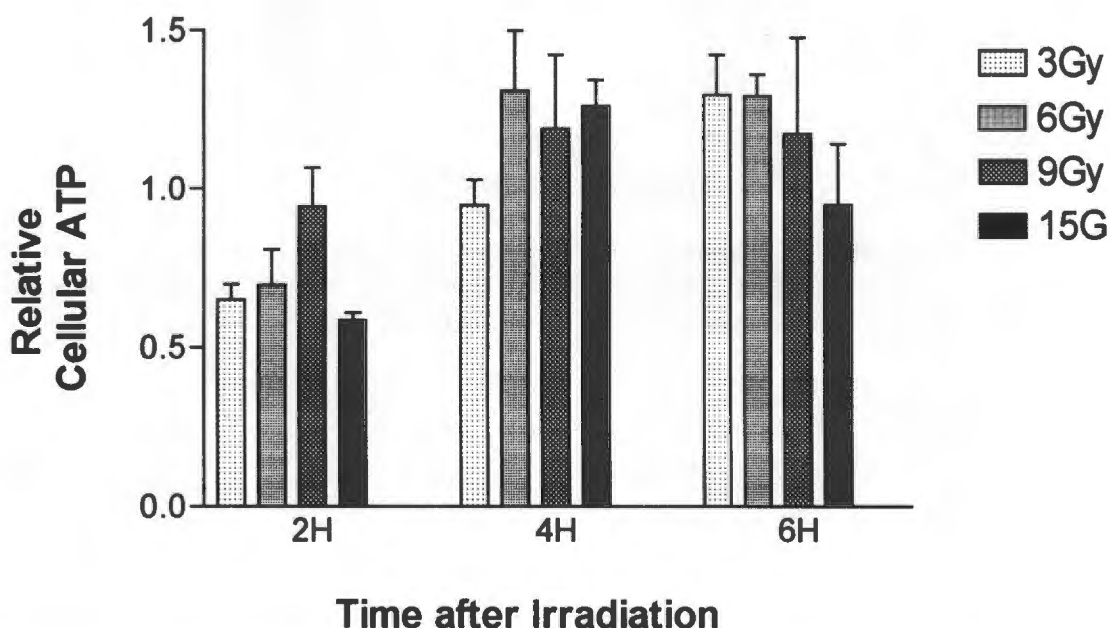


Figure 8.1: ATP content relative to unirradiated controls of confluent B16 melanoma cells at different times after doses of up to 15 Gy 100KVp X-rays. Controls are assigned an arbitrary value of 1. Heights of bars represent means ± standard deviations. n=4.

Confluent Cells, ^{60}Co γ rays

As shown in Figure 8.2, ATP levels remained unchanged from those of unirradiated controls for doses up to 15 Gy and for times up to 6 hours after radiation with the exception of a statistically significant increase at 2 hours after 12 Gy ($p<0.05$).

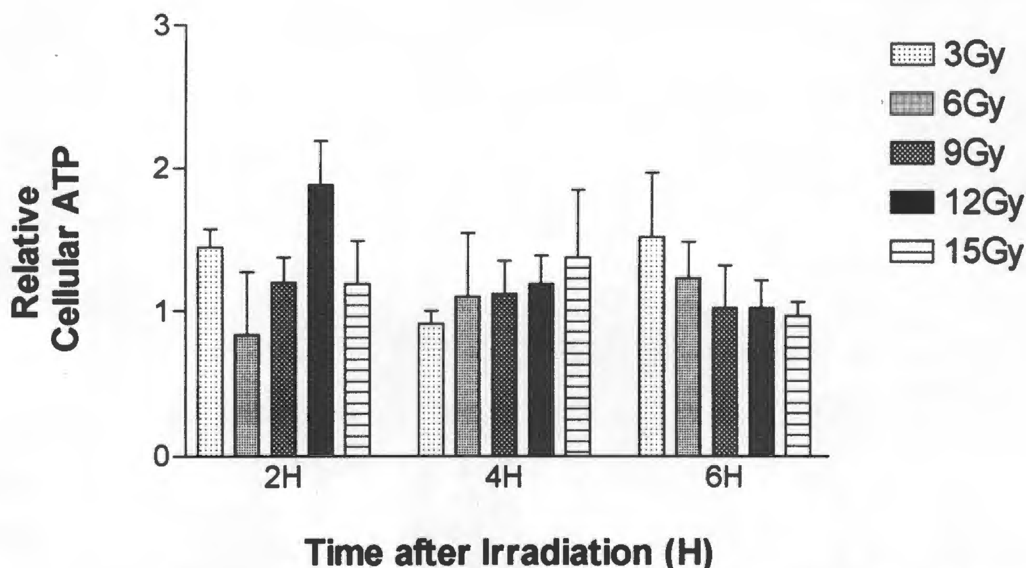


Figure 8.2: ATP content relative to unirradiated controls of confluent B16 melanoma cells at different times after doses of up to 15 Gy ^{60}Co γ-rays. Controls are assigned an arbitrary value of 1. Heights of bars represent means ± standard deviations. n=4.

Cells in exponential growth, ^{60}Co γ rays

No significant difference in ATP levels from unirradiated controls could be detected 3 hours after irradiation for doses up to 15 Gy in exponentially growing cells, as demonstrated in Figure 8.3.

It was postulated that a failure to detect major changes in ATP levels in cells was due to an interchange of cellular constituents with the cell culture medium. This, however, did not appear to be the case with ATP, as the ATP content of the culture medium above irradiated cells was not

significantly different from that of unirradiated control cells ($p>0.05$), as shown in Figure 8.4.

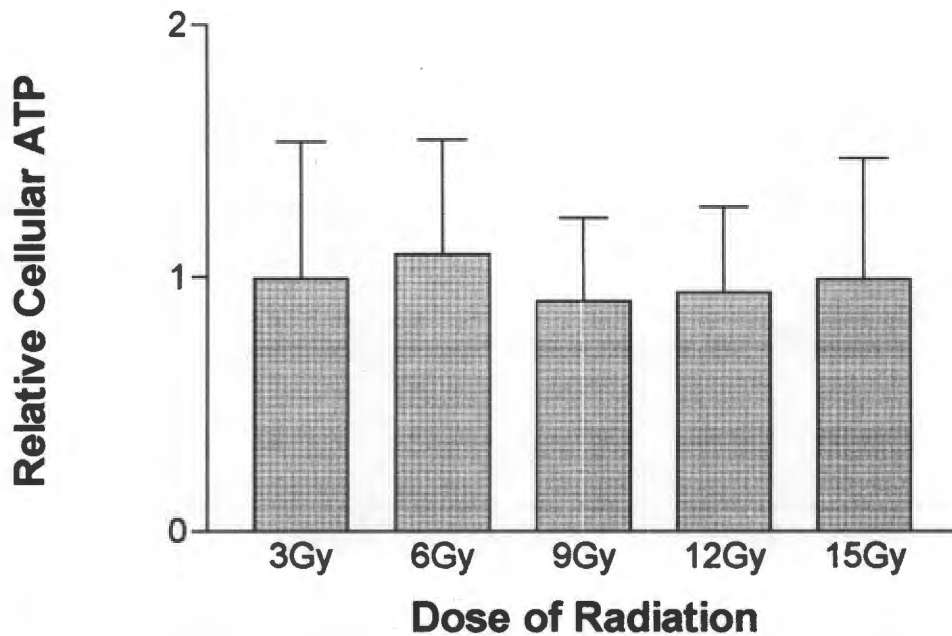


Figure 8.3: ATP content relative to unirradiated controls of B16 melanoma cells 3 hours after doses up to 15 Gy ^{60}Co γ-rays. Controls are assigned an arbitrary value of 1. Heights of bars represent means ± standard deviations. n=4.

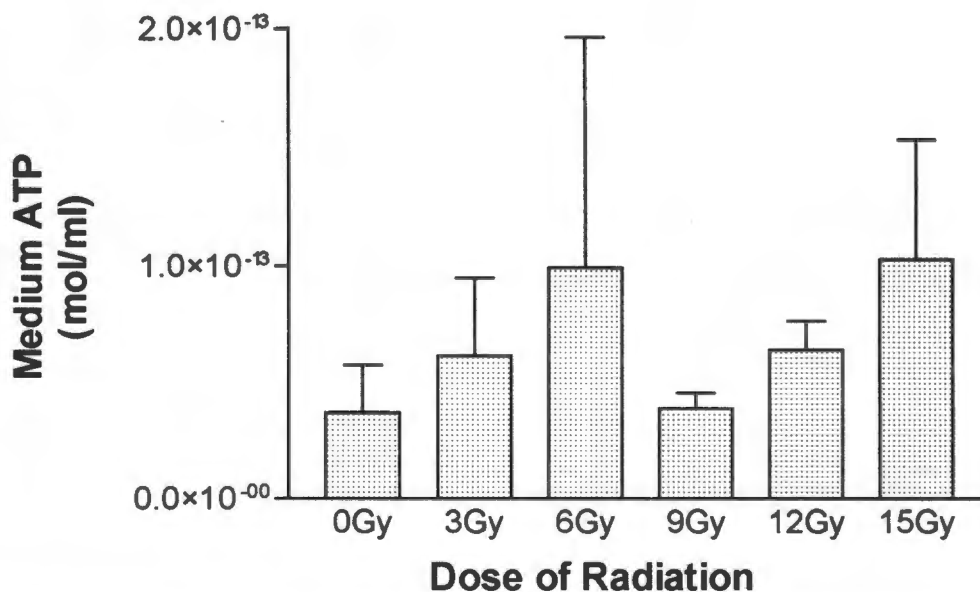


Figure 8.4: ATP content of McCoy's medium above exponentially growing B16 melanoma cells irradiated 3 hours after doses up to 15 Gy 100KVp X-rays. Heights of bars represent means ± standard deviations. n=4.

Liver

No statistically significant difference in liver ATP levels from that of unirradiated controls could be detected for times up to 5 hours after irradiation and for doses up to 15 Gy for either ^{60}Co γ-rays (Figure 8.5) or 100 KVp X-rays (Figure 8.6). However, there seemed to be a trend for ATP levels to be lowered 1 hour after ^{60}Co γ-irradiation, as shown in Figure 8.5.

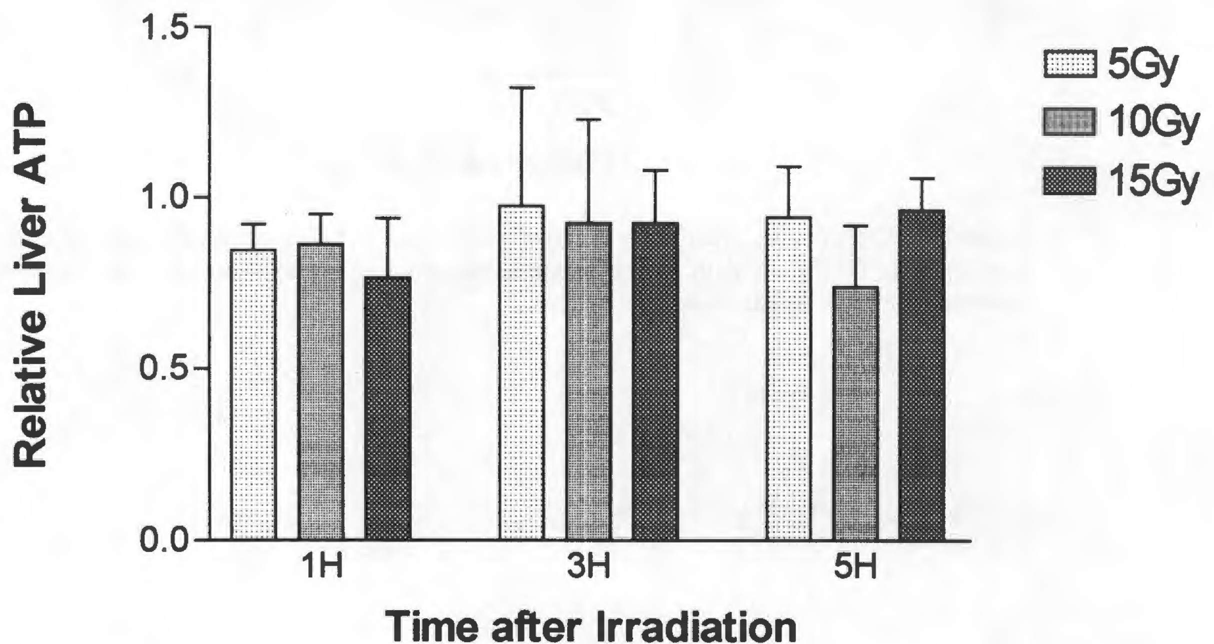


Figure 8.5: ATP content of CBA mouse liver relative to unirradiated controls at different times after doses up to 15 Gy ^{60}Co γ-rays administered in vivo. Controls are assigned an arbitrary value of 1. Heights of bars represent means ± standard deviations. n=4.

Tumour

No significant difference in ATP levels from controls was found for CaNT tumours *in vivo* after 10 Gy 100 KVp X-rays, as is shown in Figure 8.7.

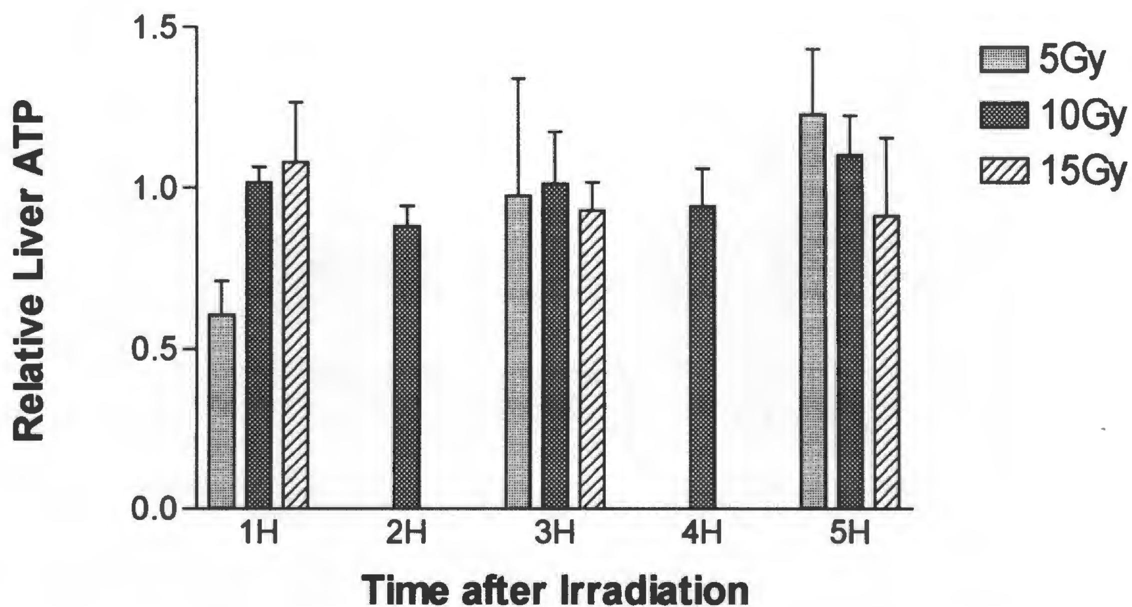


Figure 8.6: ATP content of CBA mouse liver at different times after doses up to 15 Gy 100 KVp X-rays administered in vivo. Heights of bars represent means ± standard deviations. n=4.

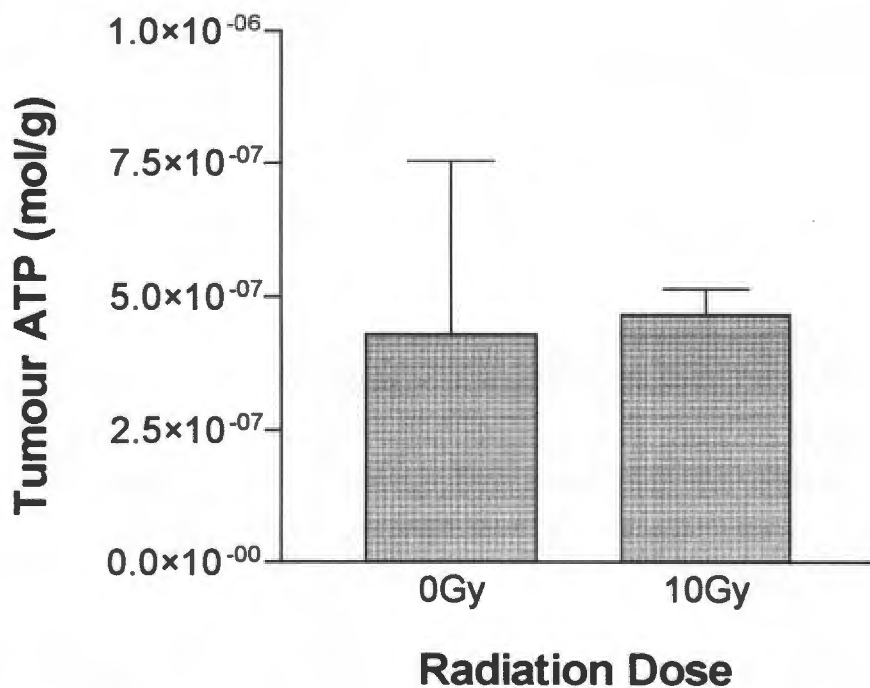


Figure 8.7: ATP content of CaNT tumours irradiated 2.5 hours after 10 Gy 100 KVp X-rays administered in vivo. Heights of bars represent means ± standard deviation. n=4.

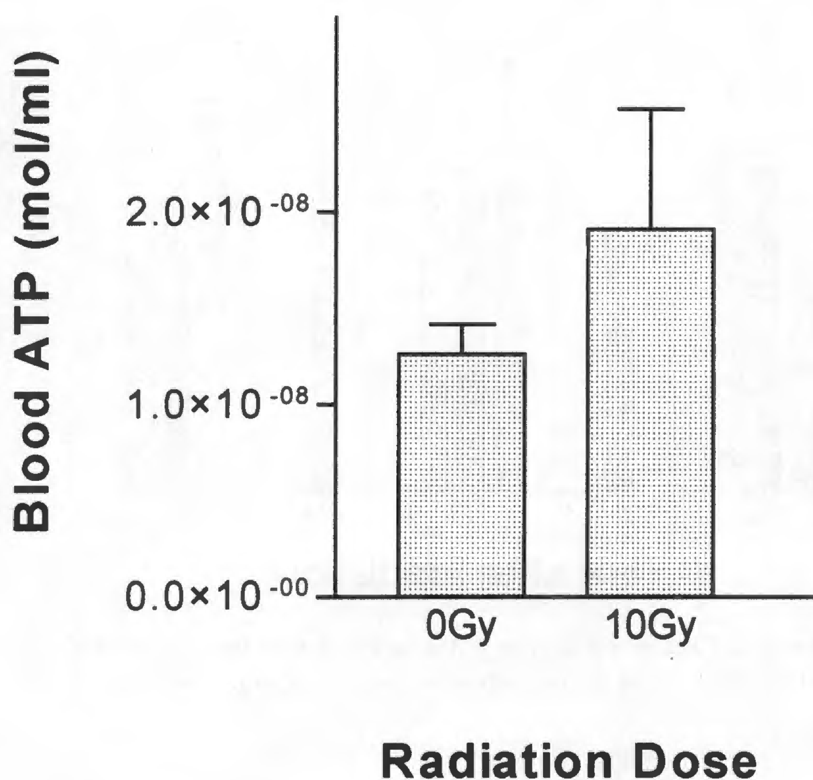


Figure 8.8: ATP content of blood taken from the inferior venae cavae of CaNT tumour bearing mice whose tumours were irradiated with 10Gy 100 KVp X-rays. Heights of bars represent means ± standard deviations. n=4.

As shown in Figure 8.8, the ATP content of blood taken from the inferior vena cavae of mice whose tumours had received 10Gy was compared to that of blood taken from mice with unirradiated tumours. Although an increase in blood ATP is noted after irradiation, the increase is not statistically significant ($p>0.05$).

It has been postulated that stress of animals may have confounding effects on the determination of energy metabolites from *in vivo* systems. Figure 8.9 indicates how elevated ATP levels in experimental tumours growing in mice may also be related to stress. A separate experiment was conducted in which mice of the stressed group were

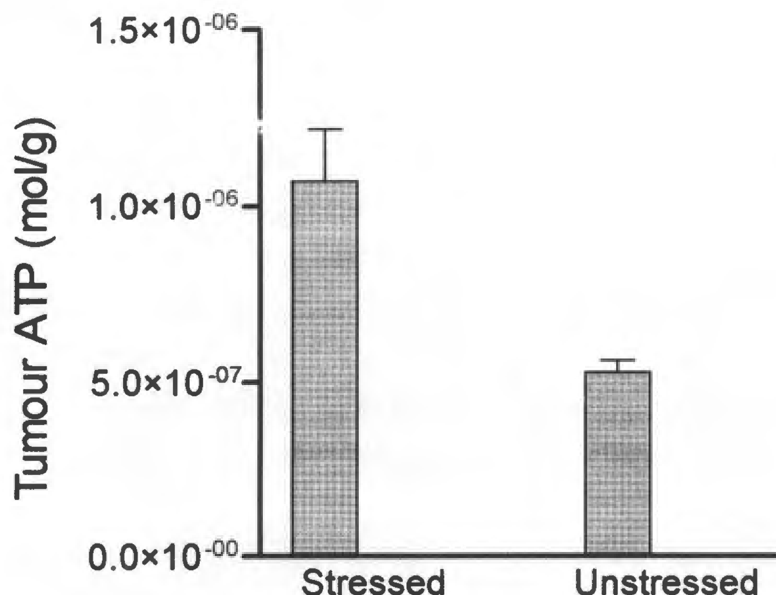


Figure 8.9: ATP content of mice 2.5 hours after mice subjected to immobilisation, sham irradiation and transportation in the open to and from the site of irradiation verses no imobilisation or transportation. Heights of bars represent means ± standard deviations. n=4.

restrained with tape and then carried uncovered on a jig to the site of irradiation, sham irradiated and transported back. Unstressed mice were not restrained or carried to the radiation unit. In the previous experiments, mice were transported in a covered box having been restrained. A statistically significant increase in ATP content of tumours from stressed mice was noted relative to the ATP content of tumours from unstressed mice ($p<0.05$), as illustrated in Figure 8.9.

DISCUSSION AND CONCLUSIONS

The results for B16 cells *in vitro* and CaNT tumours *in vivo* presented above are in disagreement with the results obtained by Szeinfeld (1987), who showed large increases in ATP content after irradiation and a relationship to beam energy in these cell and tumour lines. The solitary point in Figure 8.2 which shows an increase in ATP levels in confluent B16 cells 2 hours after ^{60}Co γ-irradiation, although statistically significantly different from unirradiated controls, may be incidental since it is not corroborated in other data presented. In addition, no significant

relationship between beam energy and post-irradiation ATP content could be shown in the present work. Other workers have not found changes in ATP levels after radiation. Tozer *et al.* (1987) could also not detect any changes in ATP in murine mammary adenocarcinoma after irradiation. Nevertheless, increases in ATP in EAT cells after doses in the 5-80 Gy range have been reported by workers such as Coe *et al.* (1963). Merchant *et al.* (1995) showed an increase in phosphocreatine and ATP relative to Pi 24 hours after irradiation of a murine mammary carcinoma *in vivo*. These increases were linked to reduction in hypoxic cell fraction which would result in an improved energy status.

A number of factors related to tumour vasculature may determine levels of ATP in tumour tissue and in blood. It has been proposed that excessive ATP in irradiated tumours might enter the blood and may be trapped within blood vessels of poorly perfused tumours, which would be detected as an increase in ATP within tumours after irradiation. If blood flow away from the tumour is more efficient, ATP may more freely enter the general circulation and reduce the perceived tumoural ATP content to that of unirradiated tumours. It has been shown by several investigators that radiation can increase tumour blood flow (Tozer *et al.*, 1991; Spence *et al.*, 1988) so it is possible that any trapped ATP could be dispersed as a result of irradiation. Results shown in Figure 8.8 indicate that blood ATP in mice whose tumours had been irradiated was raised relative to that in mice whose tumours had not been irradiated, but this increase was not statistically significant. This rise in ATP is consistent with a release of ATP from the tumour vasculature in response to radiation.

Another factor regarding tumour vasculature, which may affect tumour ATP levels and blood ATP levels after radiation, is vascular wall permeability. Vascular permeability may be increased in tissues after irradiation (Scherer *et al.*, 1991) and consequently ATP may be released by irradiated tumours into the blood stream.

A potential critisism of the results presented for ATP in tumours is that the viable cell fraction in tumours was not taken into account. ATP was presented as moles per gram of tissue. This implies a value per gram of

living tissue but the tumour consists partly of non-viable cells. Variability of the non-viable component will affect the value of ATP per gram of tissue but not ATP per gram of living tissue.

Hendrikse (1989) determined the hypoxic fraction of CaNT tumours to have a mean value of 54%, as determined by the clamped tumour growth delay method, with 95% confidence intervals of 43%-65%. A substantial proportion of cells in tumours of this type might, therefore, be relatively poorly supplied with oxygen. Because of a large variability in hypoxic fraction between tumour groups, it is possible that misleading values of ATP per gram of tissue might be obtained due to variable degrees of suppression of oxygen dependent respiration rather than as a result of irradiation.

A reduction in cellular ATP after irradiation, as shown in Figure 8.1, although not statistically significant, is not without precedent. As was discussed in the introduction of this Chapter, decreases in ATP after irradiation have been detected in lymphocytes and certain lymphocytic malignancies (Betel, 1967; Klouwen and Appelman, 1967; Retelewska and Leyko, 1978). Skog *et al.* (1983), using ascites tumour cells grown in mice, showed decreases in ATP levels up to 24 hours after 4 to 5 Gy and 9 Gy of whole body radiation without any appreciable change in lactate, pyruvate or lactate/pyruvate ratio. ATP dropped 20 to 24 hours after 5 Gy and then normalised 48 hours after irradiation. Skog *et al.* (1986) reported that such a decrease in ATP could be returned to normal by the addition of glucose to the ascites cell suspension when the ATP levels were down. Dwarkanath and Jain (1989) showed a rapid fall in ATP and NAD in a human glioma cell line 30 to 60 minutes after 20 Gy with a recovery over the next 3 hours. Levels of nucleotides could be lowered further with the addition of inhibitors of energy metabolism such as 2DG or KCN. In addition, recovery of ATP levels after irradiation could be prevented when both 2DG and KCN were added. A result which may have relevance to repair processes, if protein synthesis is considered to be relevant to the induction and synthesis of repair enzymes, was reported by Skog *et al.* (1982). They showed that both protein synthesis and ATP were reduced by a similar amount 24 hours after irradiation. Skog *et al.* (1983) reported that decreases in ATP after

irradiation may be due to interference with the electron transport process of respiration.

As shown in Figure 8.9, the ATP content of tumours in mice, which were considered to be stressed, was found to be significantly raised 2.5 hours after the stressful events relative to the tumour ATP content in mice which were deliberately not stressed. Mice which were considered to be significantly stressed, were those which were subjected to immobilisation, transportation in the open from the laboratory to the source of radiation and back and sham irradiation. Release of catecholamines are associated with stress in animals. Restraint can raise the levels of catecholamine levels in urine and can affect blood pressure, cardiac output and possibly tumour blood flow (Zanelli and Lucas, 1976) which may have effects on intratumoural metabolism and ATP.

ATP levels of of sham irradiated tumours in mice transported in a covered box, used as controls in the experiment whose results are shown in Figure 8.7, compare favourably with tumour ATP levels of deliberately unstressed mice shown in Figure 8.9. This would seem to suggest that mice in this group were minimally stressed and that their ATP levels were not altered due to a stress artifact. However, stress is a subjective concept which has not been quantified but rather inferred and consequently the existence of stress in supposedly stressed animals can be questioned.

The notion that altered ATP levels after irradiation are indicative of repair of radiation induced damage may be erroneous. It is possible that the rate at which ATP is produced by cells may be countered by the demand. Any increased demand for ATP, from repair processes being activated, would prevent any increase in production of ATP from being apparent. Similarly, it is possible that the effectiveness of systems which use ATP in the cell may be damaged (Coe *et al.*, 1963) and that ATP levels reflect a symptom rather than a compensatory mechanism. In addition, repair mechanisms may be compartmentalised and so involve only certain sections of the cell's machinery. ATP measured as total cellular quantities may, therefore, not be appropriate if changes occur in subcellular compartments.

CHAPTER 9
INHIBITION OF ENERGY METABOLISM AND THE MODIFICATION OF RADIATION RESPONSE

INTRODUCTION

It was explained in Chapter 6 how 2DG and AOA might be used as inhibitors of pathways that produce energy in mammalian cells. Since a supply of cellular energy is required for repair of radiation induced damage, it was proposed that the presence of these inhibitors might inhibit such repair. In this Chapter, experiments are presented which have attempted to determine whether the administration of 2DG and AOA can modify radiation response in cultured CHO cells and in murine tumours. As described below, previous workers have shown that the glycolytic inhibitor, 2DG, can modify radiation response in several systems. AOA, an inhibitor of glutaminolysis, had not been used to attempt to modify radiation response before the present work was undertaken.

A number of experiments have been carried out by investigators who attempted to modify radiation response by inhibiting energy supply. Sodium azide, an inhibitor of cytochrome a_3 of the electron transport chain of respiration (Mathews and van Holde, 1990), and iodoacetamide, an alkylating agent which inhibits enzymes by reacting with essential sulphydryl groups (Lehninger,1970), have been shown to inhibit recovery of diploid yeasts between split doses of ultraviolet radiation or electrons (Keifer, 1971). Reinhard and Pohlit (1977) showed in yeasts that liquid holding repair, which is an ATP dependent process, could be inhibited by 2DG. Jain *et al.* (1977a) also showed that repair of radiation induced DNA damage could be inhibited in yeasts by 2DG. Similarly, Jain *et al.* (1977c) showed a similar phenomenon in Ehrlich ascites cells. Complete inhibition of repair was achieved when the mol

ratio of 2DG:glucose was 1. The effects of radiation have also been shown to be increased by 2DG in solid tumours. Jain *et al.* (1977b) showed that radiation induced growth delay of sarcoma-180 tumours after 20 Gy was increased by 2DG relative to that for radiation alone. However, little growth delay could be seen when 2DG alone was administered. In addition to the increased growth delay in those tumour bearing mice treated with 2DG, the percentage survival of mice determined over a period of up to 40 days after treatment was also increased.

Purohit and Pohlit (1982) found that 2DG augmented the effects of radiation *in vitro* in Ehrlich ascites cells. A more than additive effect was shown, that is, a greater cell kill was obtained for the combined treatment of 2DG and radiation than that obtained for the sum of both treatments given separately. A greater than additive response was also shown in solid Ehrlich ascites tumours. This supra-additivity suggests that 2DG may act, to some extent, as a radiosensitizer. However, effects seen in ascites tumour cells may be different to those seen in solid tumours which contain hypoxic cells. In the case of solid tumours, this apparent sensitizing effect of 2DG may also arise partly from the effect of 2DG on hypoxic cells in which repair may be inhibited due to severe energy depletion.

Although 2DG has been shown to enhance the effects of radiation in certain tumour cells and yeasts, 2DG may protect normal tissue against the effects of radiation. Kalia *et al.* (1982) presented data from human leukocytes which showed that 2DG could reduce radiation induced chromosomal damage. Mice administered 2DG just prior to whole body irradiation were shown to have fewer chromosomal aberrations and an improved mouse survival after irradiation compared to the extent of these effects after radiation alone. Jain *et al.* (1985) found the reverse of the protective effect of 2DG found in leukocytes when they showed an increased radiation effect in human HeLa tumour cells after the administration of 5mM 2DG.

As discussed in Chapter 3, Jain *et al.* (1985) showed that the extent of DNA damage in HeLa cells after radiation, as determined from

micronuclei frequency, was dependent on the concentration of 2DG, which also determined the extent of glycolysis. At high concentrations of 2DG (>2.5 mM), DNA repair could be inhibited and micronuclei frequency was increased; at low concentrations (<2.5 mM), DNA repair was not inhibited and the micronuclei frequency was decreased.

Dwarakanath *et al.* (1995) showed in the BMG-1 human glioma cell-line that 5 mM 2DG increased micronuclei frequency after irradiation in exponentially growing but not plateau phase cells compared to that of irradiated cells which were not exposed to 2DG. When respiration was also inhibited with KCN, the micronuclei frequency could be increased in plateau phase cells as well. In addition, Dwarakanath *et al.* (1995) conducted experiments with the haematoporphyrin derivative, Photosan-III (PS-III), which inhibits respiratory metabolism by inhibiting cytochrome c-oxidase, and increases the rate of glycolysis. ATP/Pi ratios of EAT cells could be reduced by the administration of 2DG but levels returned to normal within 9 hours after its removal. However, when PS-III and 2DG were given, ATP/Pi ratios failed to return to normal levels even after their removal. It was found that PS-III can enhance the radiomodifying effect of 2DG by inhibiting DNA repair after irradiation which leads to greater cell damage expressed as increased micronuclei formation and both increased mitotic and interphase cell death.

THE EFFECT OF DEPLETED CULTURE MEDIUM AND INHIBITORS ON THE RESPONSE OF CULTURED CELLS TO IONIZING RADIATION.

The experiments presented in this section had several aims. The first aim was to determine the effect of inhibitors of energy metabolism, namely 2DG and AOA, on repair kinetics in CHO cells. The second aim was to determine the effect of these inhibitors on the radiosensitivity of CHO cells after different times of exposure to the inhibitors. The third aim was to determine whether there was any interaction between effects caused by inhibitors when administered together, in respect of radiation response. Where the term 'repair' is used below it should perhaps be interpreted as 'recovery' since cell survival is an indirect measure of cellular damage.

As mentioned in previous sections, it has been shown that ATP is required for repair of radiation damage to occur. In Chapter 4, it was explained that energy which is carried by ATP can be derived from energy substrates such as glucose and glutamine via the pathways of glycolysis, glutaminolysis and respiration. In Chapter 6, it was explained how glycolysis and glutaminolysis can be blocked by 2DG and AOA respectively. Bearing this in mind, it is possible that there might be an additional radiation response by cells if energy supply via these important pathways is prevented. It is possible that a cellular environment depleted of glucose and glutamine with the addition of 2DG and AOA might inhibit repair and result in the flattening of a split radiation dose repair curve, as described in Chapter 3.

Experiments were designed to determine the effect of depressed energy supply on split radiation dose cell survival and so to detect any effects on radiation repair. However, it was suspected that inhibitors of energy metabolism might also affect the radiosensitivity of the cells. As explained in Chapter 3, a split-dose recovery ratio that does not increase appreciably above a value of 1 with time between fractions, would seem to indicate that there is no interfraction repair. However, the recovery ratio after irradiation may be affected by a change in radiosensitivity

with time in the recovery culture medium. An attempt was made to extract the portion of effect due to a change in radiosensitivity, in order to examine repair kinetics without the confounding influence of altered sensitivity. Recovery curves were then reconstructed by transforming cell survival data according to the degree of sensitivity change and consequently recalculating recovery ratios.

METHODS AND MATERIALS

Experiments to determine radiation repair kinetics and radiosensitivity changes, using variations of McCoy's 5a medium, were conducted with clonogenic cell survival as an end point. Cells were deemed to have survived if they formed colonies of at least 50 cells after treatment. For each experiment, 3 flasks were irradiated per experimental point and then cells from each flask plated into 4 flasks. Experiments were repeated at least twice.

Reagents

Reagents are listed and their sources recorded in the Appendix. The contents of basic McCoy's 5a medium are also listed in the Appendix. Preparation of 2DG and AOA is described below.

Cells

Chinese Hamster Ovary (CHO) cells were grown in Hams F12 culture medium containing 10% (v/v) foetal calf serum and antibiotics (50 mg/l penicillin, 50 mg/l neomycin, 50 mg/l streptomycin) in 150 ml plastic tissue culture flasks (Sterilin). Cells were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C in a water-jacketed incubator.

Cells were harvested according to standard trypsinization techniques (see Appendix), plated and grown to confluence in 50 ml Sterilin plastic tissue culture flasks for experiments. Cells were plated at a density of 500 000 cells per 5 ml medium and were found to be confluent after 3 days. At this time, the medium was discarded and the cells were rinsed 3

times with Hanks' balanced salt solution. McCoy's medium (5 ml) containing the appropriate complements of glucose, glutamine and inhibitors, was then added to each flask of cells for the purposes of repair or sensitivity testing, as outlined below.

Radiation

Radiation (^{60}Co γ-rays, 1.17 and 1.33 MeV) was administered by means of an Eldorado 6 Cobalt unit with a fixed lead collimator. Flasks of cells were irradiated in a 20 cm x 20 cm field with full backscatter at 80 cm SSD with 0.5 cm depth of medium above the cells to act as build-up. The average dose rate was 0.78 Gy min^{-1}. For repair experiments, two 5 Gy doses separated by an interval of up to 5 hours were administered. For sensitivity experiments, a single 10 Gy dose was given after times up to 8 hours of exposure to the test culture medium.

Repair Experiments

Radiation repair kinetics were examined by means of a split radiation dose experiment in which clonogenic cell survival was taken as an end point. Confluent cells were exposed to variations of McCoy's medium, as listed below, for 3 hours prior to the first of two 5 Gy fraction of radiation so that energy supply was adequately inhibited from the commencement of the interfraction interval. During the pre-irradiation period, cells were kept in the incubator at 37°C. Cells were irradiated at room temperature (23°C) and returned to the incubator (37°C) for an interval of up to 5 hours before the second 5 Gy fraction was given. After the second fraction, cells were immediately trypsinized (described in Appendix), counted using a haemocytometer and plated into 50 ml tissue culture flasks in Hams F12 medium containing 10% (v/v) foetal calf serum and antibiotics (50 mg/l penicillin, 50 mg/l neomycin, 50 mg/l streptomycin) and returned to the incubator at 37°C. After 7 days, cells were stained and colonies, consisting of more than 50 cells, counted to assess cell survival (described in Appendix).

Sensitivity Experiments

For sensitivity testing, confluent cells were exposed to variations of McCoy's medium for times up to 8 hours prior to a single 10 Gy dose of radiation. Cells were incubated at 37°C prior to irradiation and irradiated at room temperature. Cells were then trypsinized, plated and assessed for survival, as outlined above under **Repair Experiments**.

For both repair and sensitivity experiments, any change in plating efficiency as a result of exposure to a particular medium was taken into account by the inclusion of unirradiated controls at each time point. In this way, any additional cell kill due to the effects of medium alone did not affect the calculated cell surviving fraction. Cell viability was assessed by uptake of trypan blue stain. Trypan blue was mixed 1:1 (v/v) with medium containing cells. Cells taking up or excluding the stain were counted using a haemocytometer and the percentage of dead cells determined. Intermitotic death could thus also be taken into account when plating cells for assessment of clonogenic cell survival. When plating out cells for clonogenic assays, numbers of cells plated were determined only from those cells which excluded trypan blue, that is, viable cells.

Experimental Culture Medium

McCoy's 5a medium was the basic medium to which cells were exposed in the determination of repair kinetics and radiosensitivity, as outlined above. Different modifications of McCoy's medium were made in which glucose or glutamine or both were omitted and/or 2DG or AOA or both were added. These combinations are listed below in Table 9.1. The concentrations of 2DG and AOA in media were both 10 mM, as described below.

All media listed above contained 50 mM HEPES buffer but contained no foetal calf serum or antibiotics. Confluent cells that are metabolising in poorly buffered medium can result in the medium becoming acidic. HEPES was, therefore, added to prevent significant changes in pH, which could otherwise affect the rate of metabolism, radiosensitivity and

cell viability by different amounts in different medium variations. Buffer salts in the McCoys medium, although adequate to maintain physiological conditions, were found to be insufficient to keep each medium used in the same narrow band of pH. Fresh media were prepared immediately prior to each experiment. This is important because glutamine, which is a constituent of some media, is unstable and has a half life in medium of 3 weeks at 4°C and 1 week at 36.5°C (Freshney, 1987).

Table 9.1: Variations of McCoy's 5a medium used in experiments to determine the effect of such media on repair and radiosensitivity of cells *in vitro*. The concentration of 2DG in the media above was 10 mM. The concentration of AOA was also 10 mM.

A:	Normal McCoy's (Full amounts of glucose and glutamine)
B:	Normal McCoy's + 2DG
C:	Normal McCoy's + AOA
D:	Normal McCoy's + 2DG + AOA
E:	McCoy's without glucose or glutamine
F:	McCoy's without glucose or glutamine + 2DG
G:	McCoy's without glucose or glutamine + AOA
H:	McCoy's without glucose or glutamine + 2DG + AOA
I:	McCoy's without glucose but with glutamine
J:	McCoy's without glucose but with glutamine + 2DG
K:	McCoy's without glucose but with glutamine + AOA
L:	McCoy's without glucose but with glutamine + 2DG + AOA
M:	McCoy's with glucose but without glutamine
N:	McCoy's with glucose but without glutamine + 2DG
0:	McCoy's with glucose but without glutamine + AOA
P:	McCoy's with glucose but without glutamine + 2DG + AOA

INHIBITORS

In both descriptions of the addition of inhibitors to medium below, McCoy's medium refers to McCoy's with the appropriate contents of glucose and glutamine for the particular medium alternative in question, as indicated in Table 9.1.

2-Deoxy-D-glucose

To make a 10 mM final concentration of 2DG in McCoy's medium, 400 mg 2DG was dissolved in 4 ml McCoy's medium and filtered through a 0.2 µm pore size syringe filter (Schleicher and Schuell) to ensure sterility; 0.8744 ml of this solution was made up to 80 ml with McCoy's medium, AOA solution, when applicable, and 1M HEPES buffer, so that the final HEPES concentration was 50 mM.

Aminooxyacetic acid

To make a 10 mM final concentration of AOA in McCoy's medium, 400 mg AOA was dissolved in 4 ml McCoy's medium and filtered through a 0.2 µm pore size syringe filter (Schleicher and Schuell) to ensure sterility; 1.3136 ml of this solution was made up to 80 ml with McCoy's medium, 2DG solution, when applicable, and 1M HEPES buffer so that the final HEPES concentration was 50 mM.

Addition of AOA to medium, as described above, resulted in a reduction in the pH of the medium. A concentrated solution of NaOH (4 g/5 ml McCoy's medium) was made up in McCoy's medium, filtered through a 0.2 µm pore size syringe filter (Schleicher and Schuell) and added dropwise until pH of the medium was 7.4.

The osmolality of McCoy's medium containing 10 mM 2DG and 10 mM AOA was measured with a Gonotec freezing point depression osmometer and was found to be 307 mOsm/Kg compared to a value of 288 mOsm/Kg for normal McCoy's medium. Most cells can tolerate a range of osmolalities from 260 mOsm/Kg to 320 mOsm/Kg (Freshney, 1987)

RESULTS

Figures 9.1 to 9.16 show cell survival after 10 Gy irradiation of CHO cells which had been exposed to different variations of McCoy's medium (media A to P in Table 9.1) for different times prior to irradiation. The cell survival is given relative to that where cells received 10 Gy, but were not exposed to the test medium prior to irradiation. Cells which were said to have spent no time in test medium before irradiation, were, however, irradiated in the test medium, which was added to the cells immediately before irradiation. The data was fitted by linear regression and the regression lines were forced through a relative survival of 1 at zero hours exposure to medium. This was done to determine any trends in radiosensitivity change based on visual inspection of the data to obtain a reasonable fit for most groups of data. Slopes of the regression lines and correlation coefficients are shown in the Figure legends. In general, correlation coefficients were considerably less than 1 indicating poor fits. To determine whether the slopes of the regression lines were significantly different from zero, an F test was applied. Significant difference from zero slope was considered to be when $p < 0.05$. The level of surviving fraction for cells in normal McCoys medium exposed to 10 Gy was 0.017.

Time in normal McCoy's (medium A in Table 9.1) or media P, I and G from Table 9.1 prior to irradiation, produced no significant change in relative survival after 10 Gy, as represented in Figures 9.1, 9.5, 9.7 and 9.12 respectively. However, most versions of McCoy's medium produced significant changes in relative survival after 10 Gy with time in each medium but varied with respect to their capacity to increase or decrease relative survival. Significant increases in relative survival were found for media M, K and C from Table 9.1 as shown in Figures 9.3, 9.9 and 9.16 respectively. Significant decreases in relative survival were found for the media E, N, O, J, L, F, H, D and B as shown in Figures 9.2, 9.4, 9.6, 9.8, 9.10, 9.11, 9.13, 9.14 and 9.15 respectively. The most dramatic decrease in relative survival with time found for the media tested, is shown in Figure 9.13 which shows the data for the McCoy's medium without glucose or glutamine with the addition of both 2DG and AOA (medium H in Table 9.1).

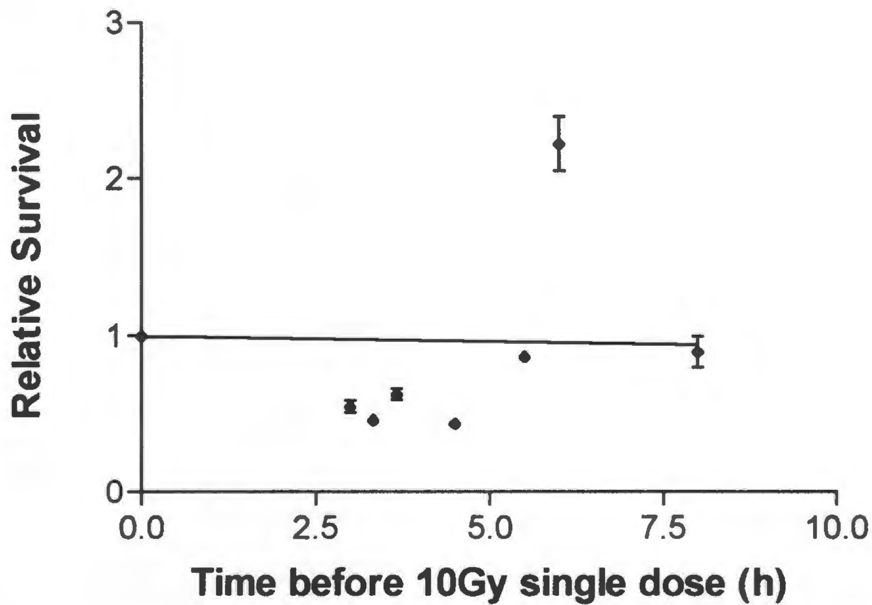


Figure 9.1: Relative survival of CHO cells incubated in McCoy's medium containing glucose and glutamine (medium A in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0072 ± 0.0437 and R^2=0.00316. Slope is not significantly non-zero (p=0.874). Each point represents mean ± sem.

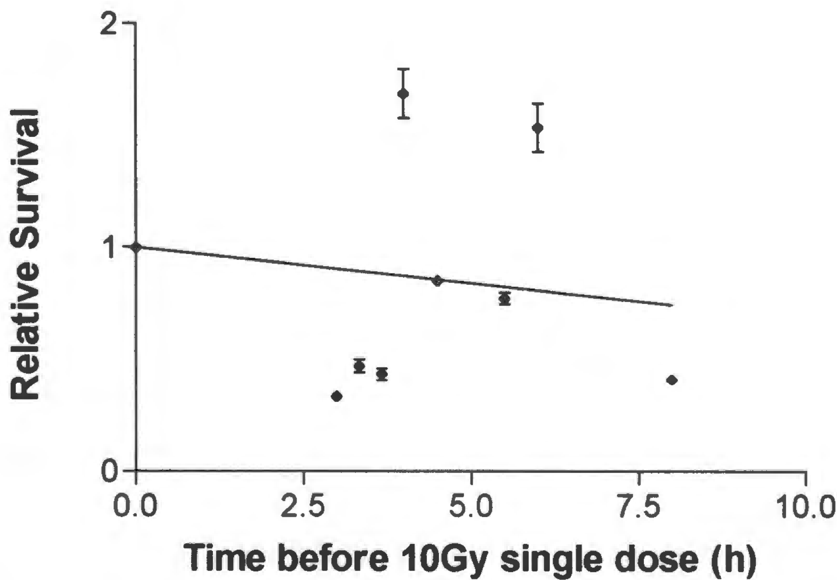


Figure 9.2: Relative survival of CHO cells incubated in McCoy's medium lacking glucose and glutamine (medium E in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0323 ± 0.0354 and R^2=0.0933. Slope is not significantly non-zero (p=0.388). Each point represents mean ± sem.

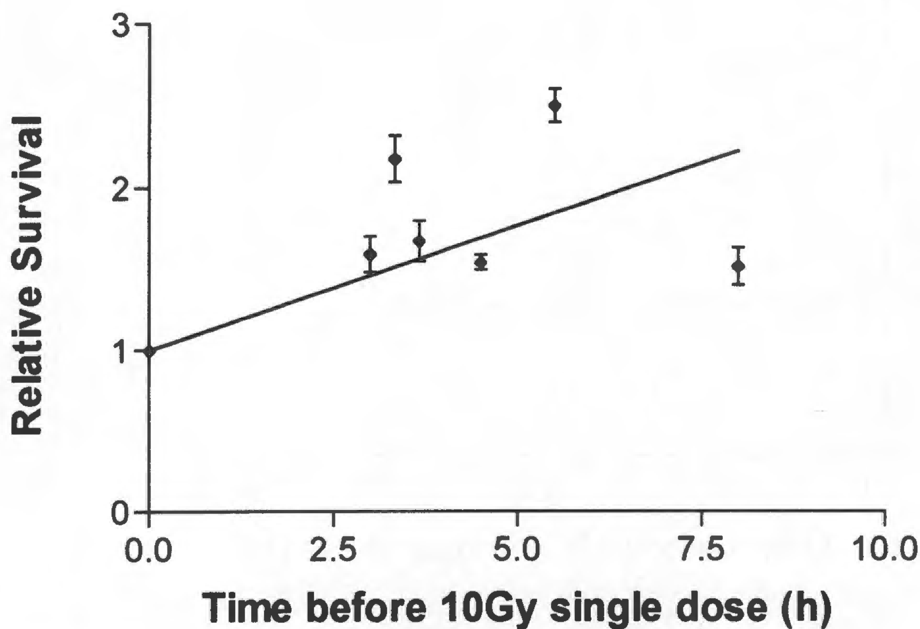


Figure 9.3: Relative survival of CHO cells incubated in McCoy's medium without glutamine (medium M in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = 0.156 ± 0.0401 and R^2 =0.711. Slope is significantly non-zero (p=0.0082). Each point represents mean ± sem.

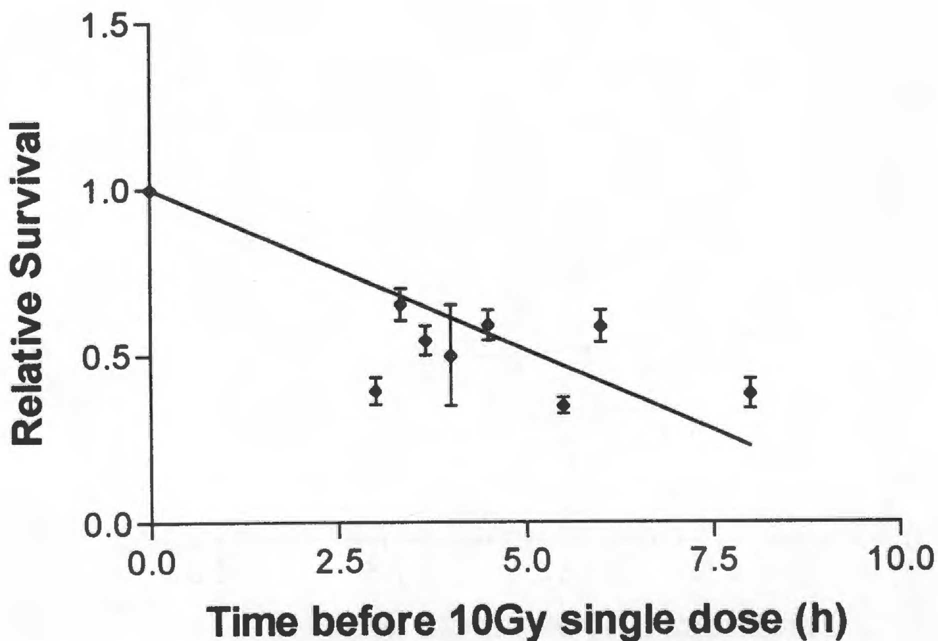


Figure 9.4: Relative survival of CHO cells incubated in McCoy's medium without glutamine with 2DG (medium N in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0971± 0.0109 and R^2 = 0.907. Slope is significantly non-zero (p=0.0148). Each point represents mean ± sem.

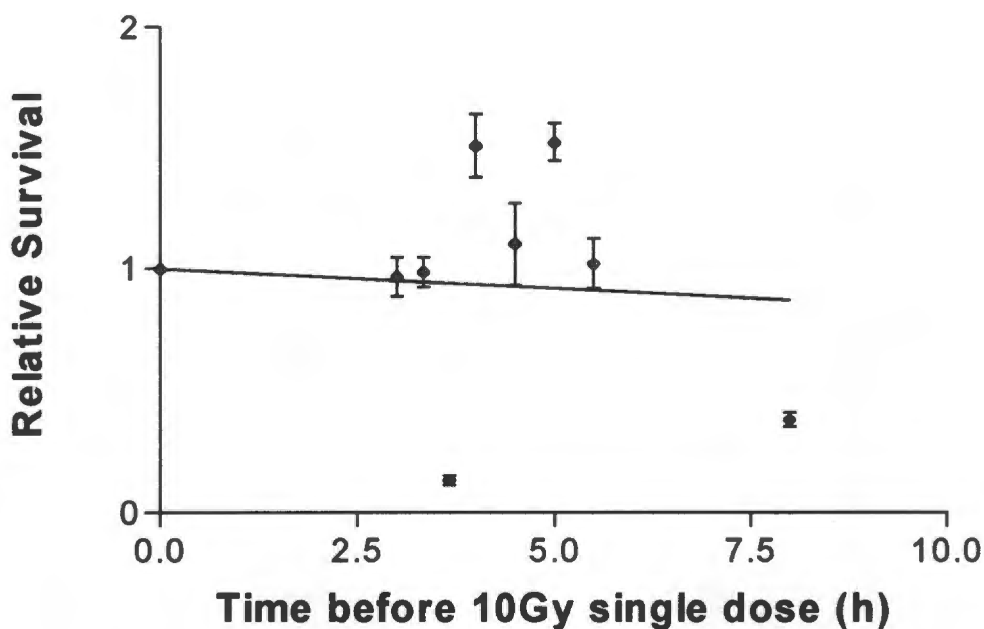


Figure 9.5: Relative survival of CHO cells incubated in McCoy's medium without glutamine with 2DG and AOA (Medium P in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0163 ± 0.0327 and R^2 = 0.0301. Slope is not significantly non-zero (p=0.631). Each point represents mean ± sem.

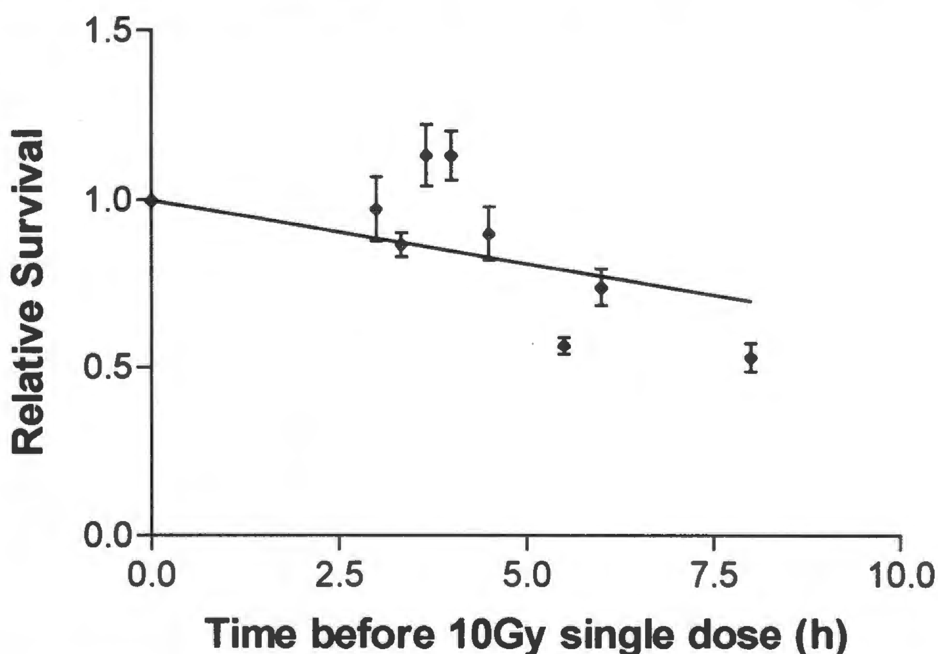


Figure 9.6: Relative survival of CHO cells incubated in McCoy's medium without glutamine with AOA (Medium O in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0385 ± 0.0125 and R^2= 0.543. Slope is significantly non-zero (p=0.0148). Each point represents mean ± sem.

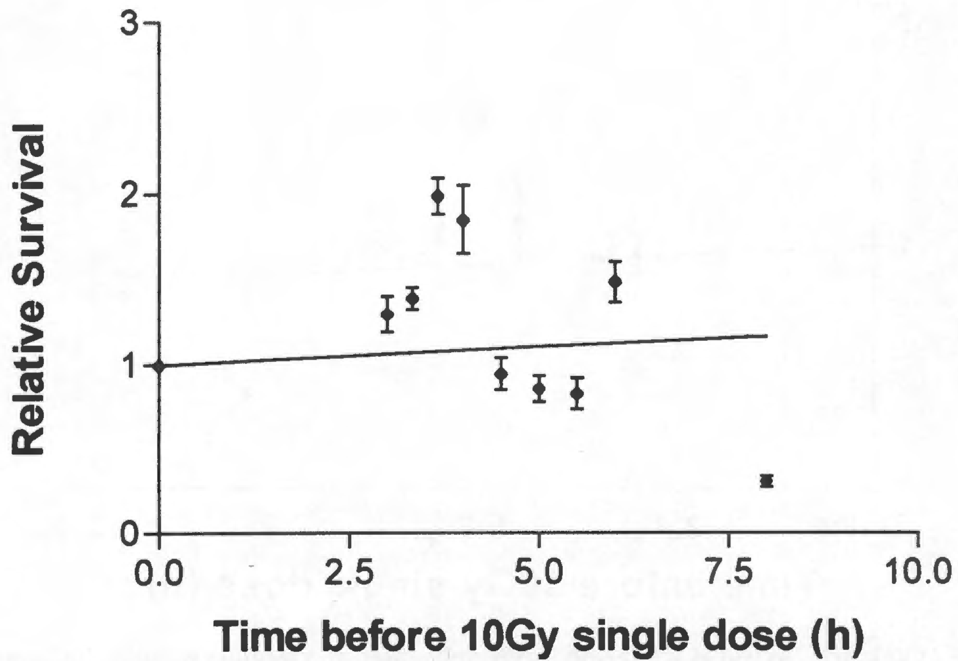


Figure 9.7: Relative survival of CHO cells incubated in McCoy's medium without glucose (Medium I in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = 0.021 ± 0.0359 and R^2 = 0.0358. Slope is not significantly non-zero (p=0.570). Each point represents mean ± sem.

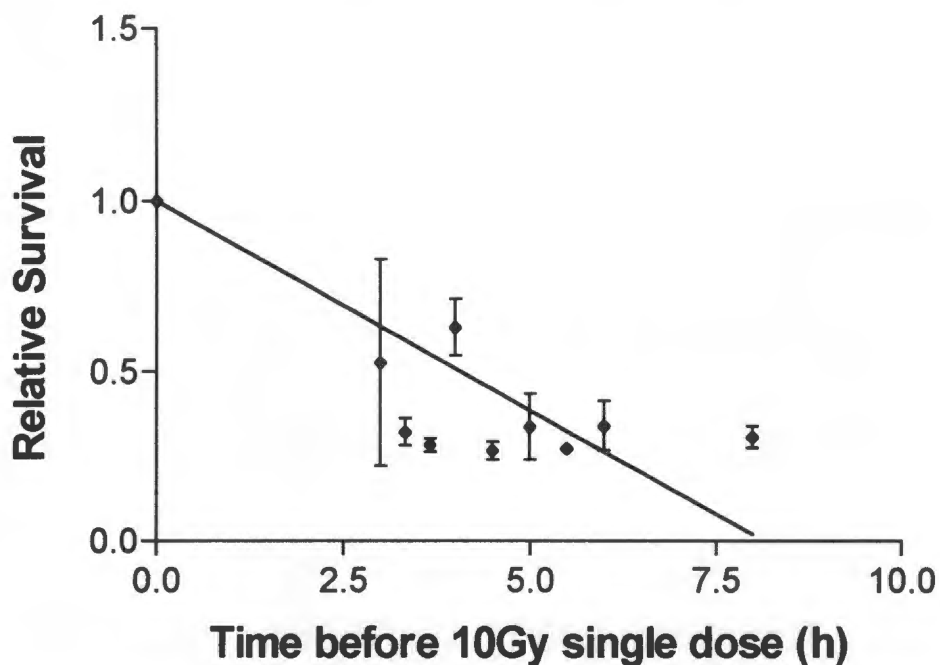


Figure 9.8: Relative survival of CHO cells incubated in McCoy's medium without glucose with 2DG (Medium J in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.124 ± 0.0121 and $R^2 = 0.920$. Slope is significantly non-zero (p=0.0001). Each point represents mean ± sem.

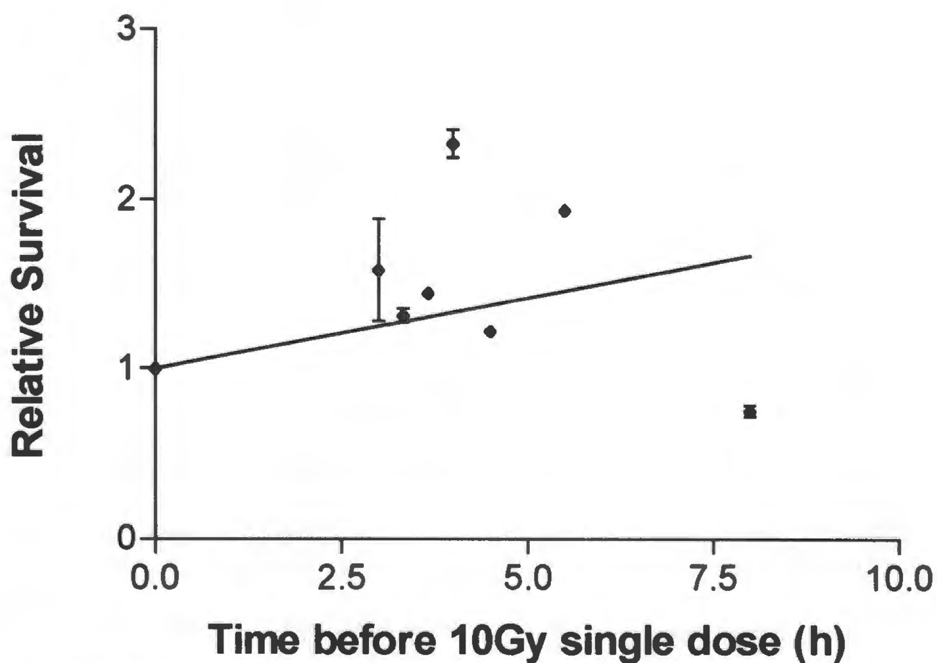


Figure 9.9: Relative survival of CHO cells incubated in McCoy's medium without glucose with AOA (Medium K in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = 0.0846 ± 0.0439 and $R^2 = 0.342$. Slope is not significantly non-zero (p=0.0951). Each point represents mean ± sem.

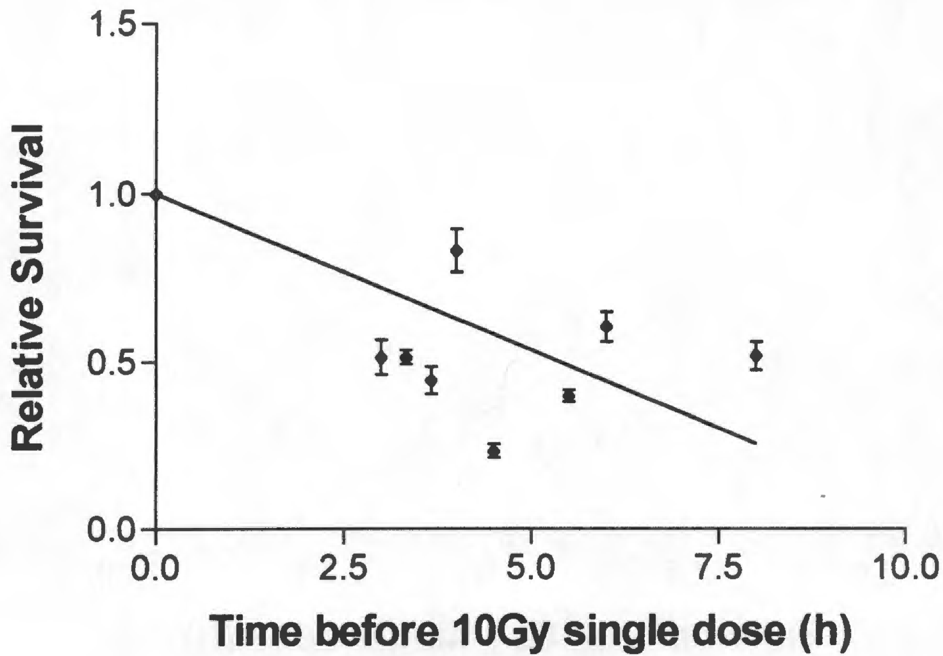


Figure 9.10: Relative survival of CHO cells incubated in McCoy's medium without glucose with 2DG and AOA (Medium L in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0936 ± 0.0155 and R^2 = 0..819. Slope is significantly non-zero (p=0.0003). Each point represents mean ± sem.

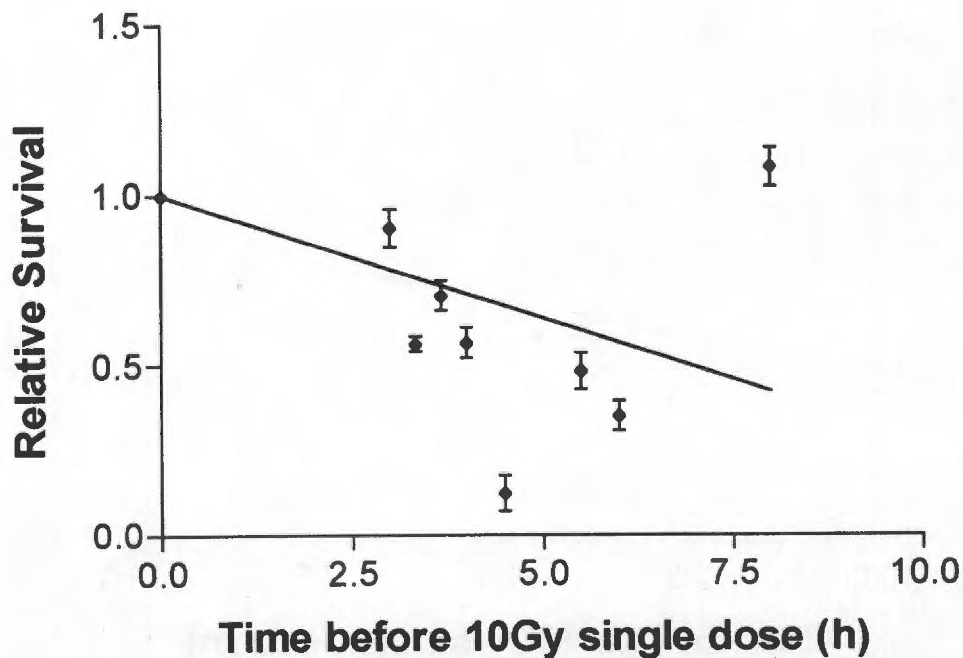


Figure 9.11: Relative survival of CHO cells incubated in McCoy's medium without glucose or glutamine with 2DG (Medium F in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0723 ± 0.0233 and R^2 = 0.542. Slope is significantly non-zero (p=0.0147). Each point represents mean ± sem.

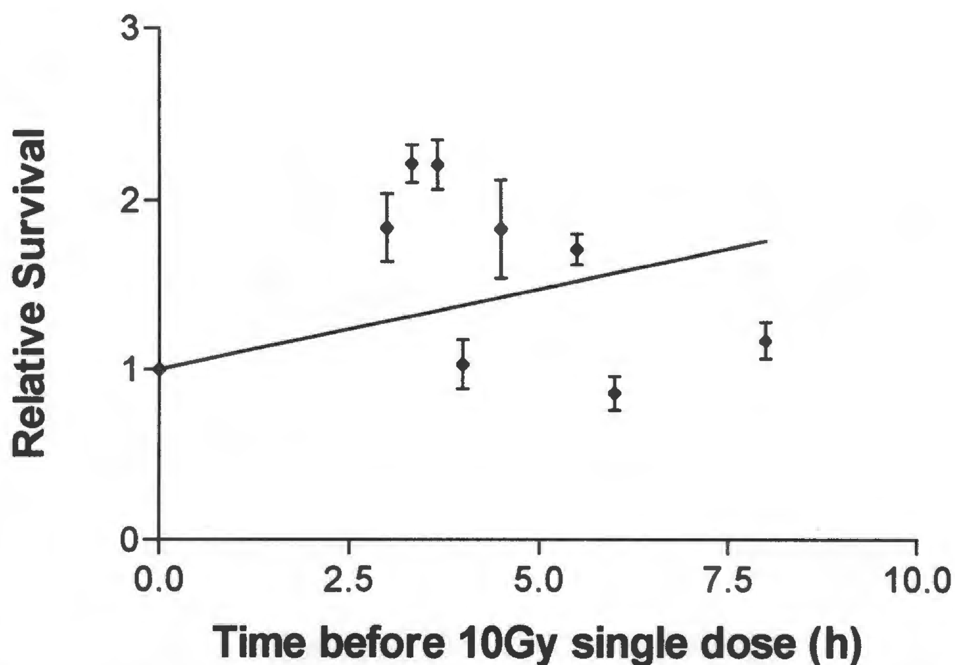


Figure 9.12: Relative survival of CHO cells incubated in McCoy's medium without glucose or glutamine with AOA (Medium G in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = 0.0962 ± 0.0434 and R^2 = 0.377. Slope is not significantly non-zero (p=0.0574). Each point represents mean ± sem.

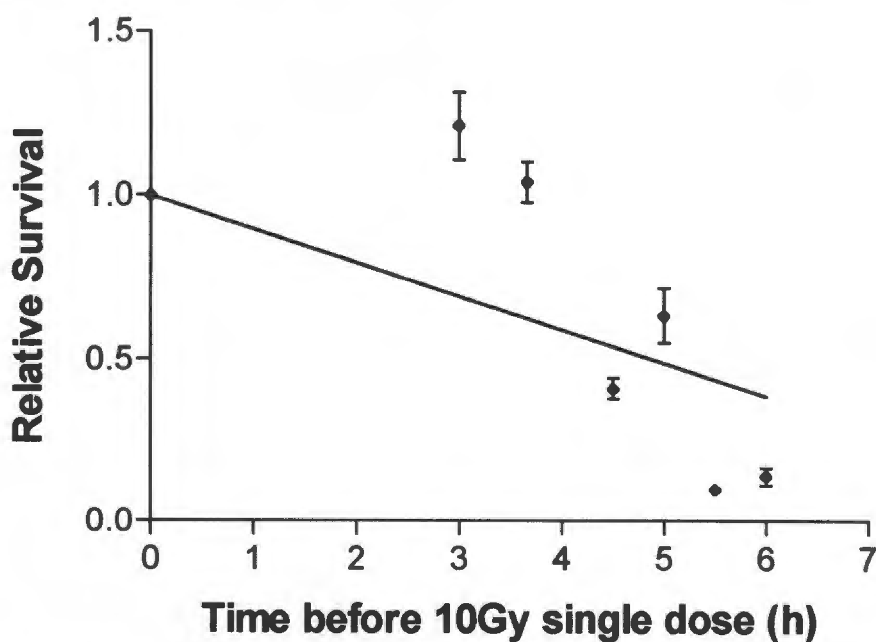


Figure 9.13: Relative survival of CHO cells incubated in McCoy's medium without glucose or glutamine with 2DG and AOA (Medium H in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.103 ±0.0283 and R^2 = 0..686. Slope is significantly non-zero (p=0.0108). Each point represents mean ± sem.

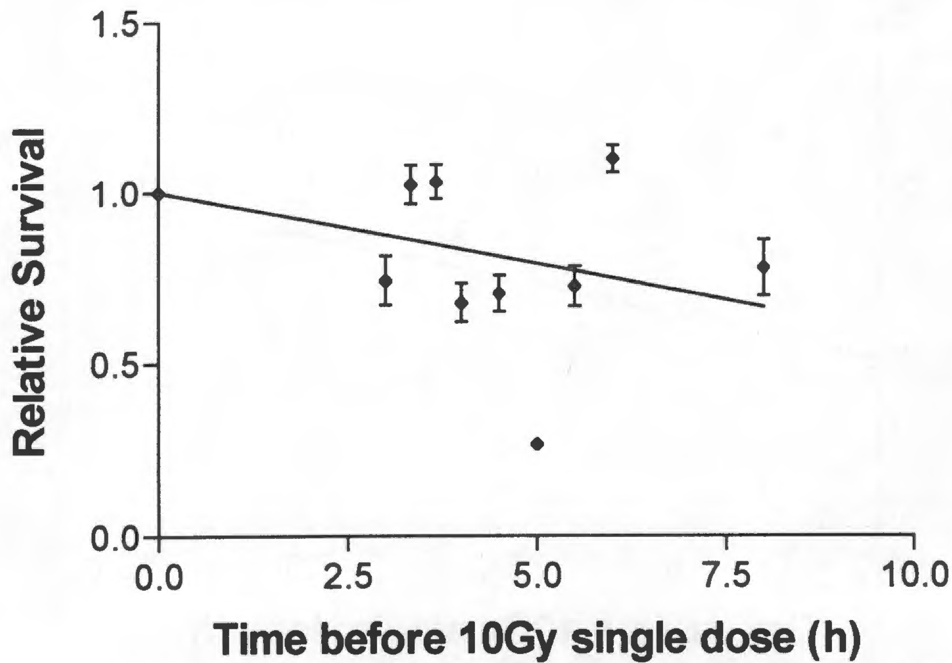


Figure 9.14: Relative survival of CHO cells incubated in McCoy's medium with 2DG and AOA (Medium D in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0423 ± 0.0161 and R^2 = 0.430. Slope is significantly non-zero (p=0.0278). Each point represents mean ± sem.

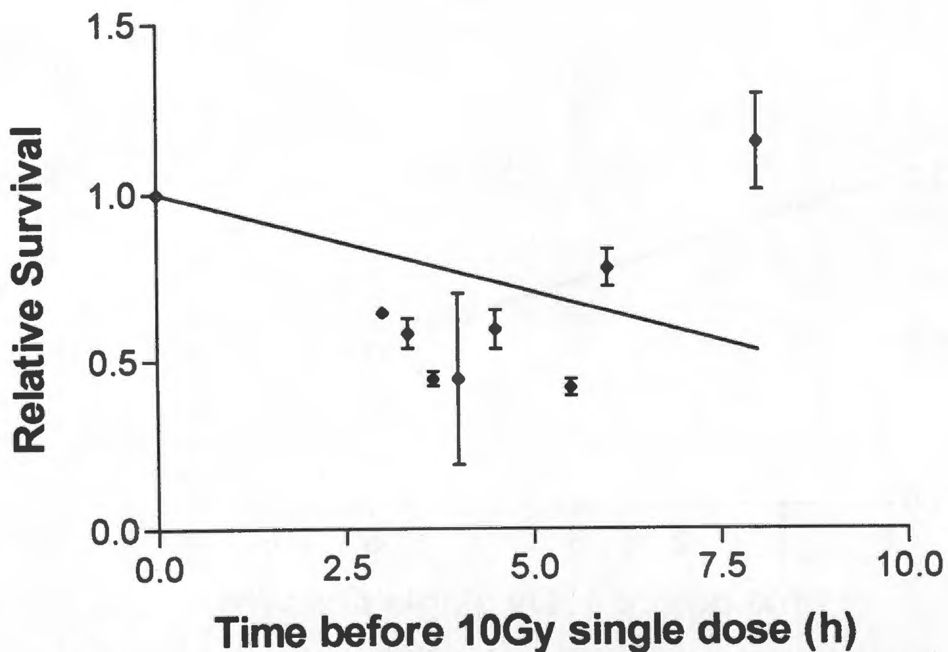


Figure 9.15: Relative survival of CHO cells incubated in McCoy's medium with 2DG (Medium B in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = -0.0591 ± 0.0222 and R^2 = 0.465. Slope is significantly non-zero (p=0.00287). Each point represents mean ± sem.

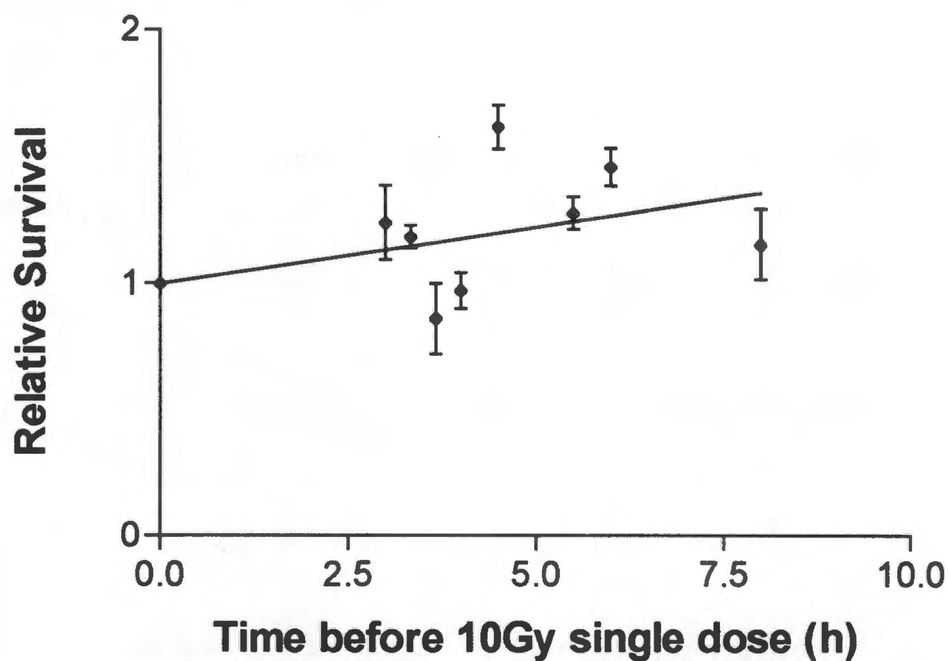


Figure 9.16: Relative survival of CHO cells incubated in McCoy's medium with AOA (Medium C in Table 9.1) for up to 8 hours before 10 Gy. Slope of regression line = 0.0446 ± 0.0157 and R^2 = 0.499. Slope is significantly non-zero (p=0.0220). Each point represents mean ± sem.

Figures 9.17 to 9.32 show the Recovery Ratios of cells kept in the different variations of McCoy's medium between two 5 Gy fractions of radiation. Recovery Ratio was calculated as the ratio of surviving fraction after the two doses of radiation separated by time, and the surviving fraction after two doses given as a single 10 Gy dose (See Chapter 3).

Recovery curves were fitted to the data by non-linear least squares regression according to a mono-exponential equation (Equation 13 in Chapter 3):

$$RR = e^{A(1-e^{-\lambda t})}$$

where RR is Recovery Ratio, t is time between fractions, A is the exponent of e where (e^A - 1) is the maximum possible difference in recovery ratio of a recovery curve and λ represents the rate constant, as described in Chapter 3.

The solid curves represent apparent recovery, dashed curves represent recovery corrected for sensitivity and, for comparison, the curve from Figure 9.17 which shows the recovery kinetics of cells in normal McCoy's is included as a dotted curve. Where curves are not shown fitted to the data points, the fits were found not to converge. No corrected recovery curves are shown for cells in those medium alternatives which did not modify radiosensitivity as seen from certain of the regression lines of Figures 9.1 to 9.17, whose slopes were found to be not significantly non-zero.

Recovery Ratio corrected for sensitivity is determined by modifying the numerators and denominators of the apparent Recovery Ratio, namely the surviving fraction obtained after the two 5 Gy fractions, separated by an interval, and the surviving fraction after two 5 Gy doses with no time between. Each of these values was transformed by an amount which depended upon the change in radiosensitivity to 10 Gy after a time in a test medium, as interpreted by the change in surviving fraction, relative to that found when cells were not exposed to the medium prior to irradiation. Therefore, if there is an increase in radiosensitivity with

time in a certain medium, then the proportion of cell kill after 3 hours (the time corresponding to the initial period of exposure to the test medium before two 5 Gy doses separated by no time are given) will be increased by a certain amount. Similarly, a greater increase in radiosensitivity, which might occur after longer times in the medium, may result in an even greater proportion of cell kill. Thus, the numerator and denominator of the apparent Recovery Ratio must be transformed by increasing the cell survival values by the same proportions as the proportions of cell kill obtained as a result of increased radiosensitivity. In the case of a decrease in radiosensitivity, split dose survival would have to be transformed by reducing cell survival. The confounding influence of radiosensitivity changes can, therefore, be excluded so that results can be interpreted in terms of repair alone.

The corrected recovery curves were fitted to the modified data according to the same model as the apparent recovery curves.

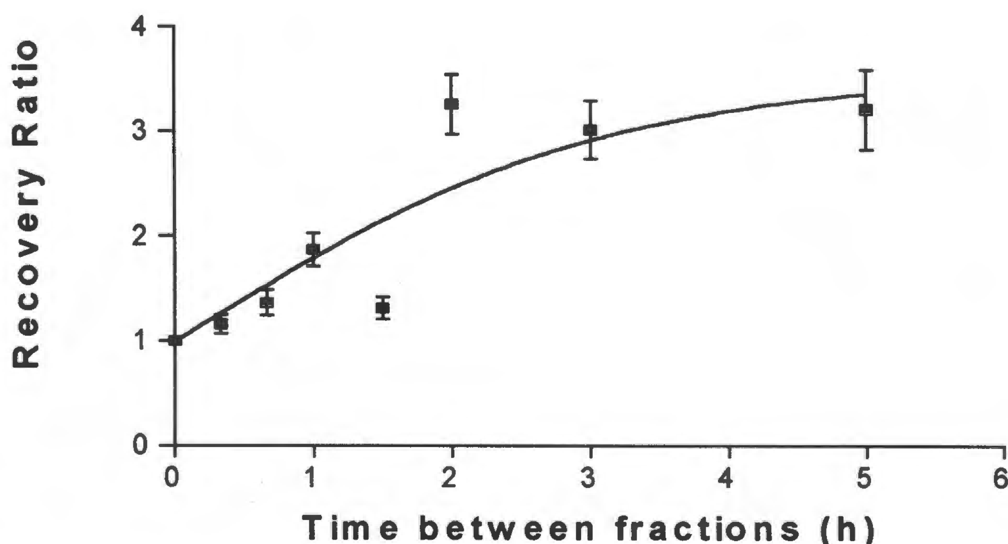


Figure 9.17: Recovery of CHO cells incubated in McCoy's medium (Medium A in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. Each point represents mean ± sem.

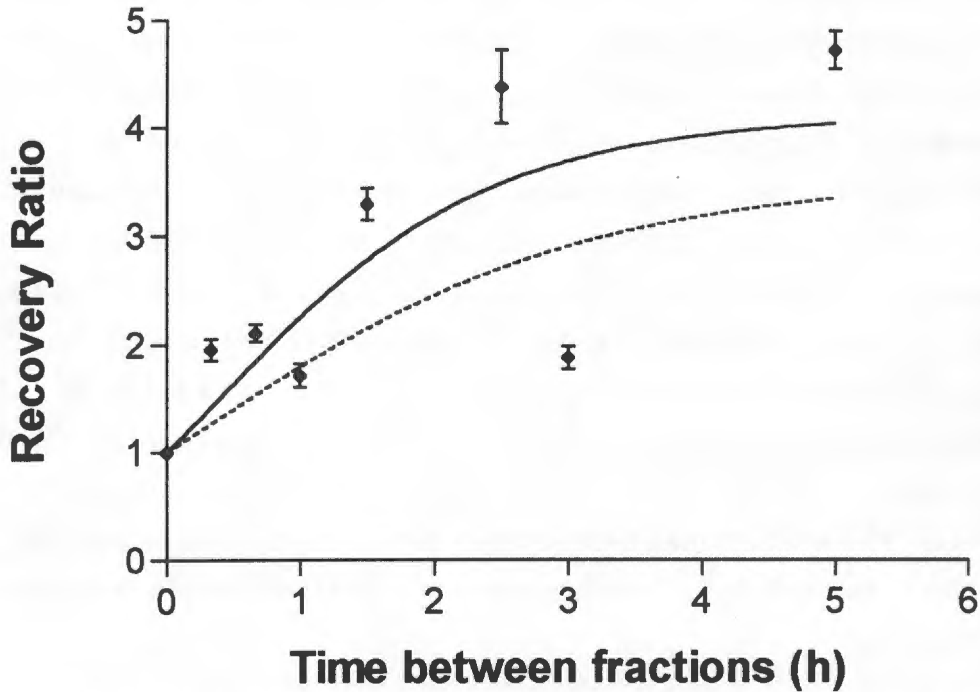


Figure 9.18: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose and glutamine (Medium E in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The dotted curve represents recovery in normal McCoy's from Figure 9.17. Each point represents mean ± sem.

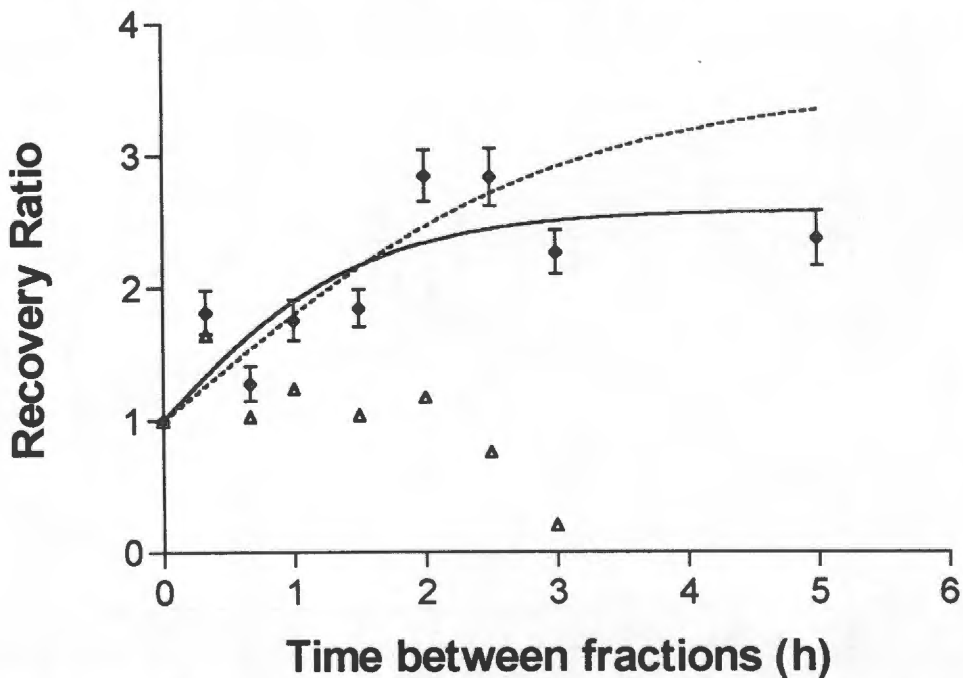


Figure 9.19: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glutamine (Medium M in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

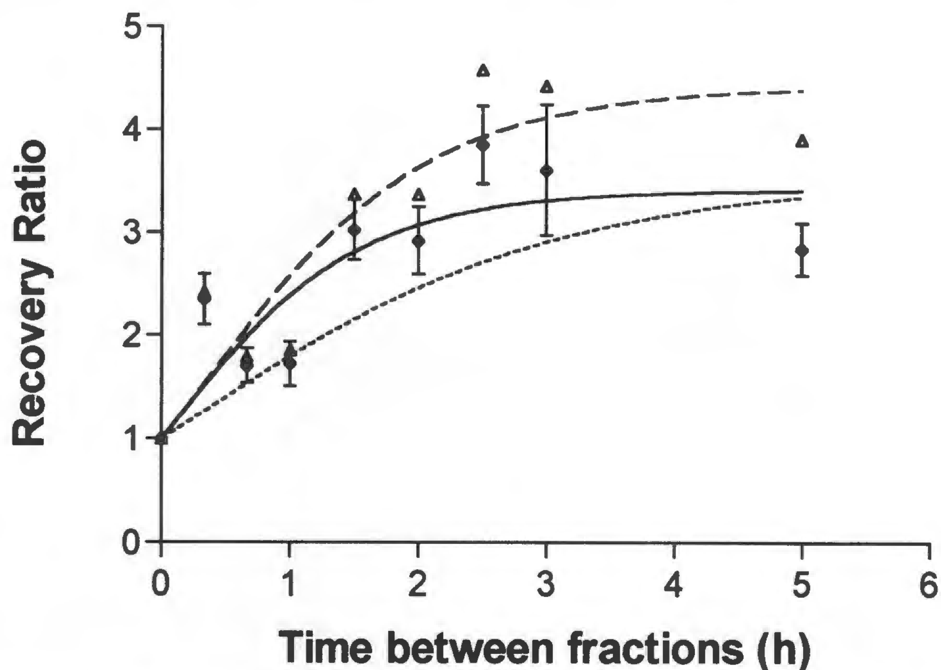


Figure 9.20: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glutamine with 2DG (Medium N in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

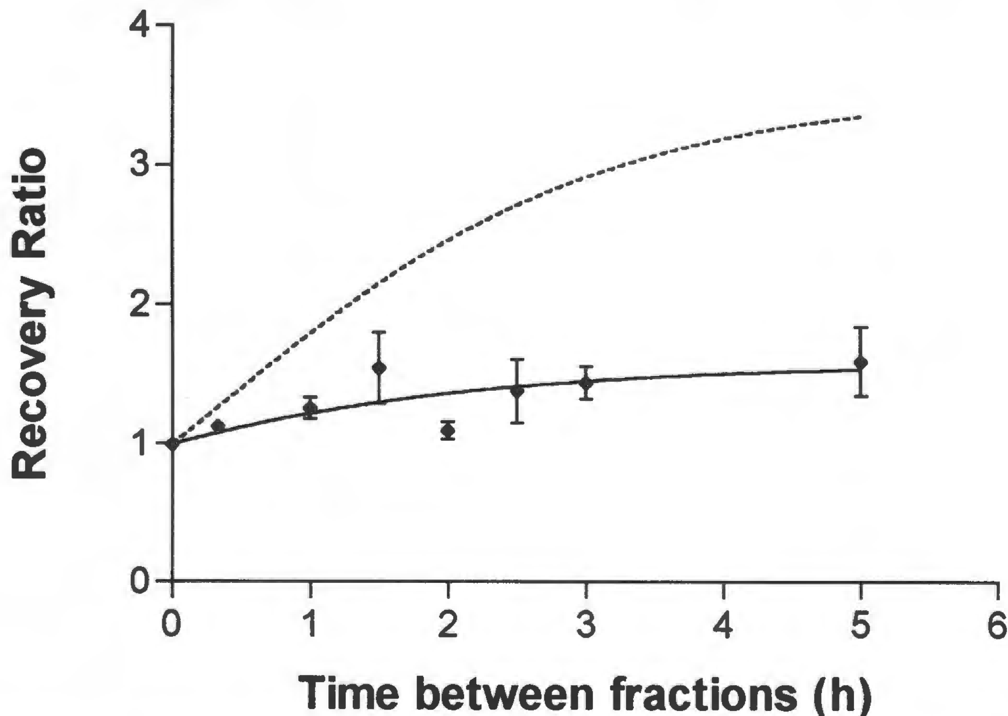


Figure 9.21: The symbols and solid curve represent recovery of CHO cells incubated in McCoy's medium lacking glutamine with 2DG and AOA (Medium P in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

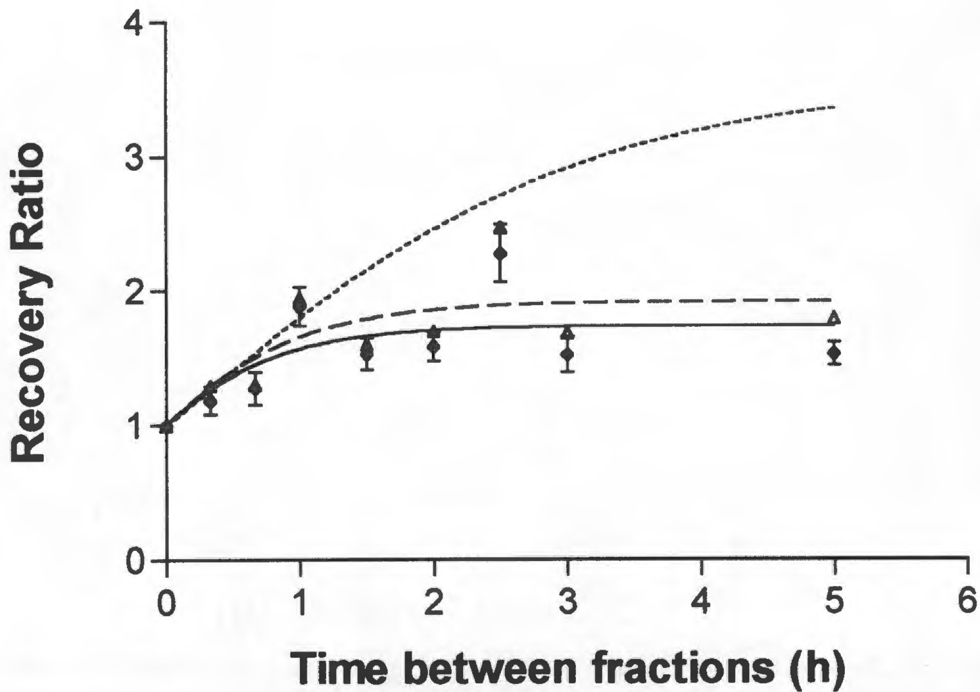


Figure 9.22: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glutamine with AOA (Medium M in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

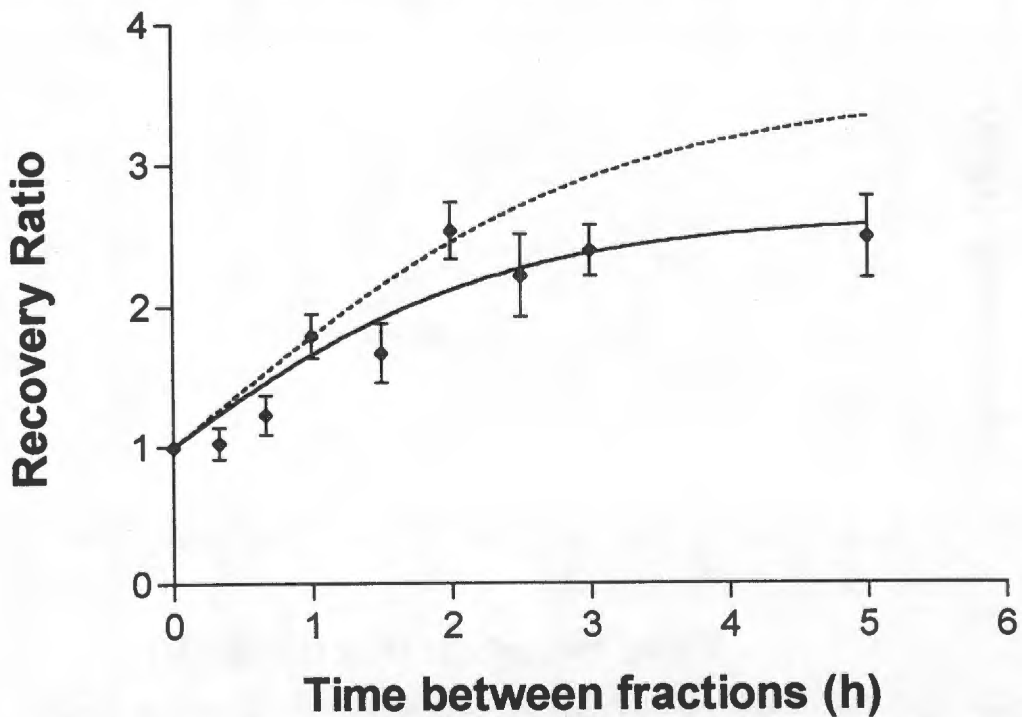


Figure 9.23: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose (Medium I in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The dotted curve represents recovery in normal McCoy's from Figure 9.17. Each point represents mean ± sem.

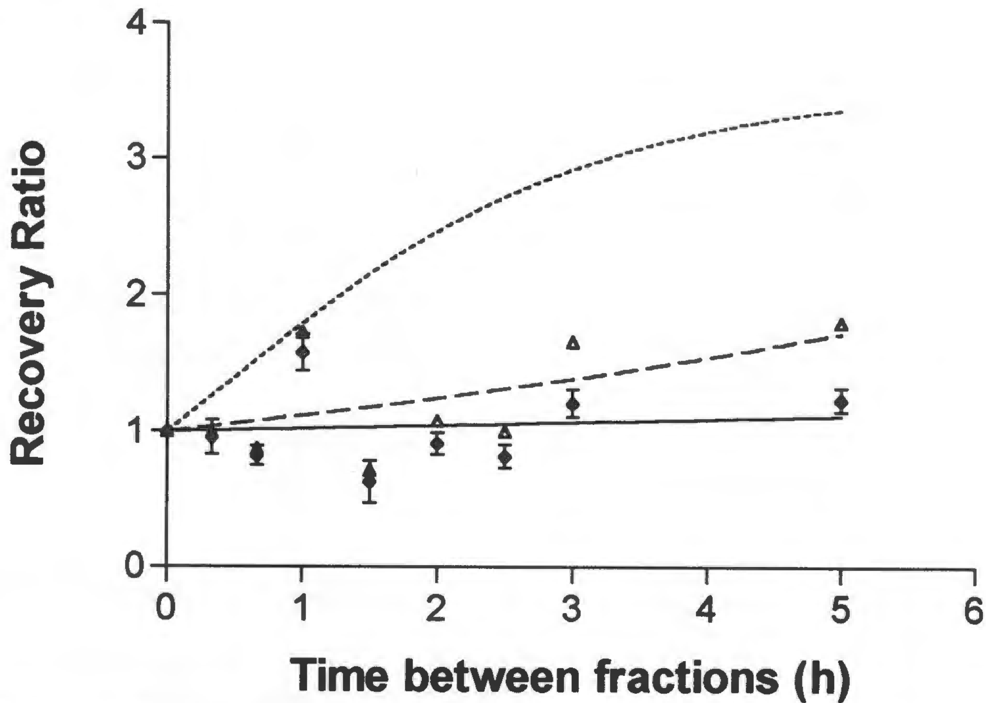


Figure 9.24: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose with 2DG (Medium J in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

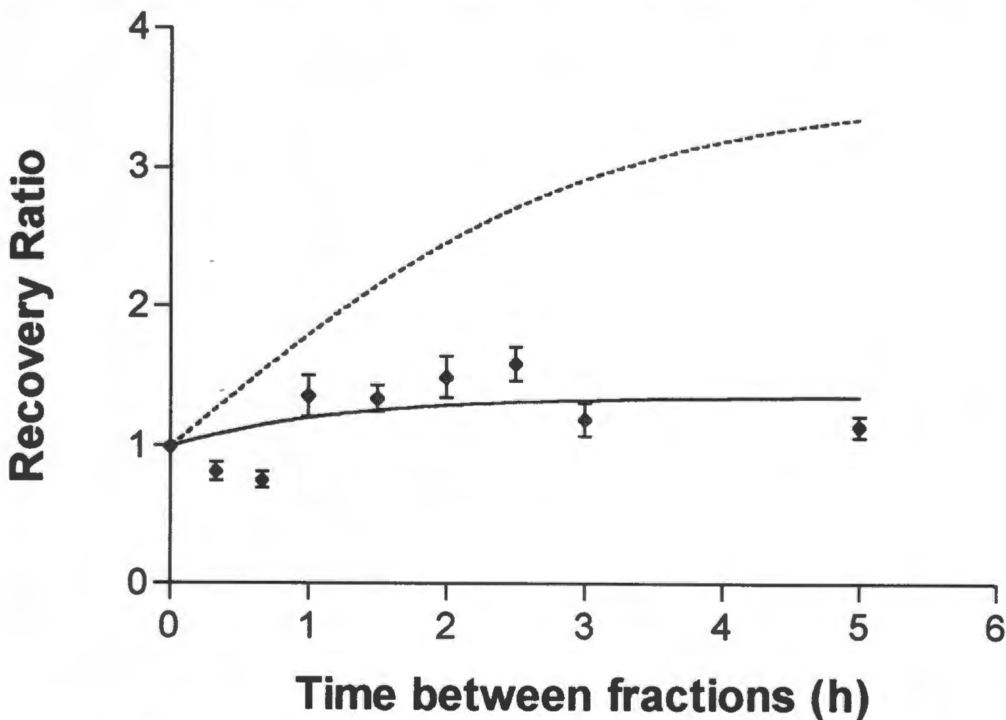


Figure 9.25: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose with AOA (Medium K in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The dotted curve represents recovery in normal McCoy's from Figure 9.17. Each point represents mean ± sem.

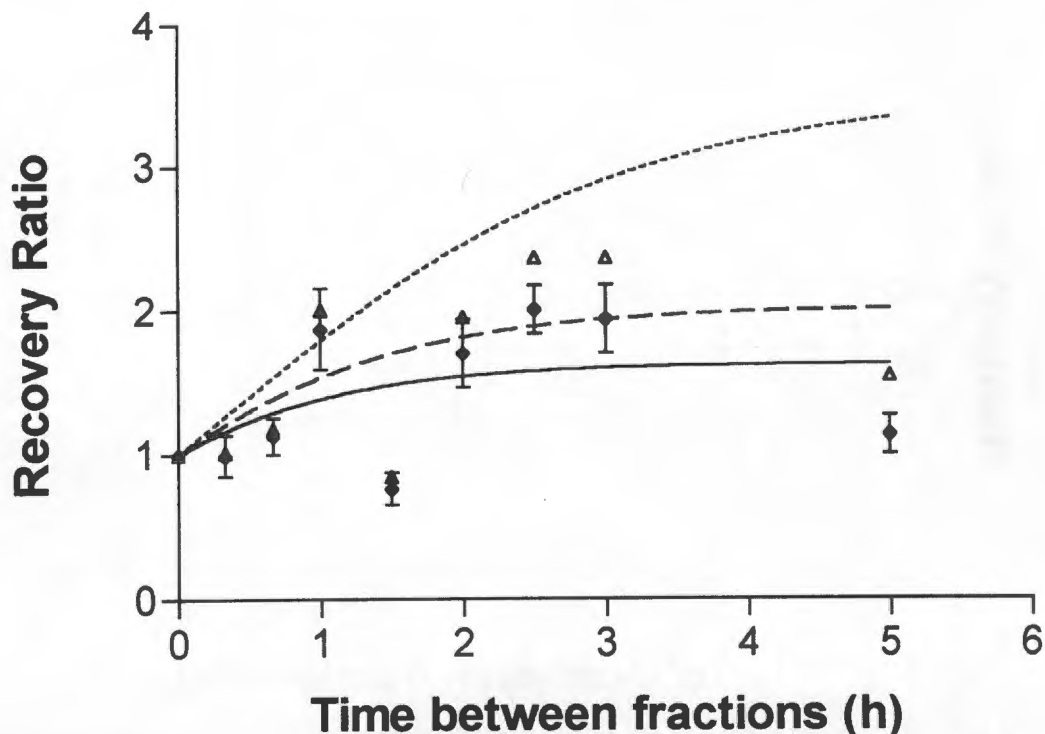


Figure 9.26: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose with 2DG and AOA (Medium L in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

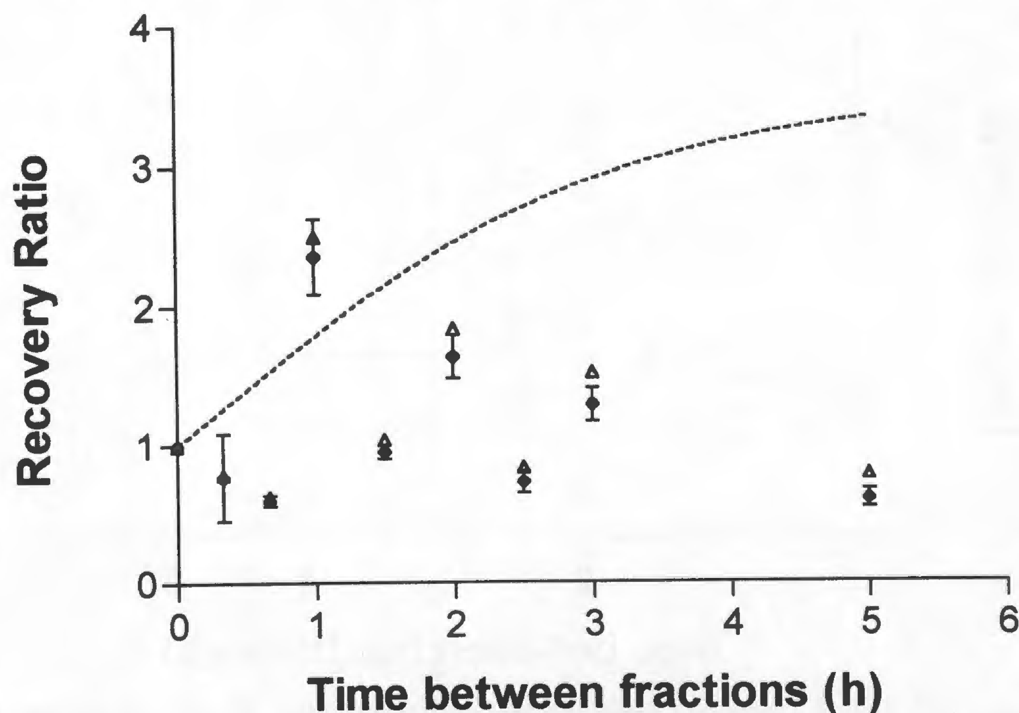


Figure 9.27: The closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose and glutamine with 2DG (Medium F in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

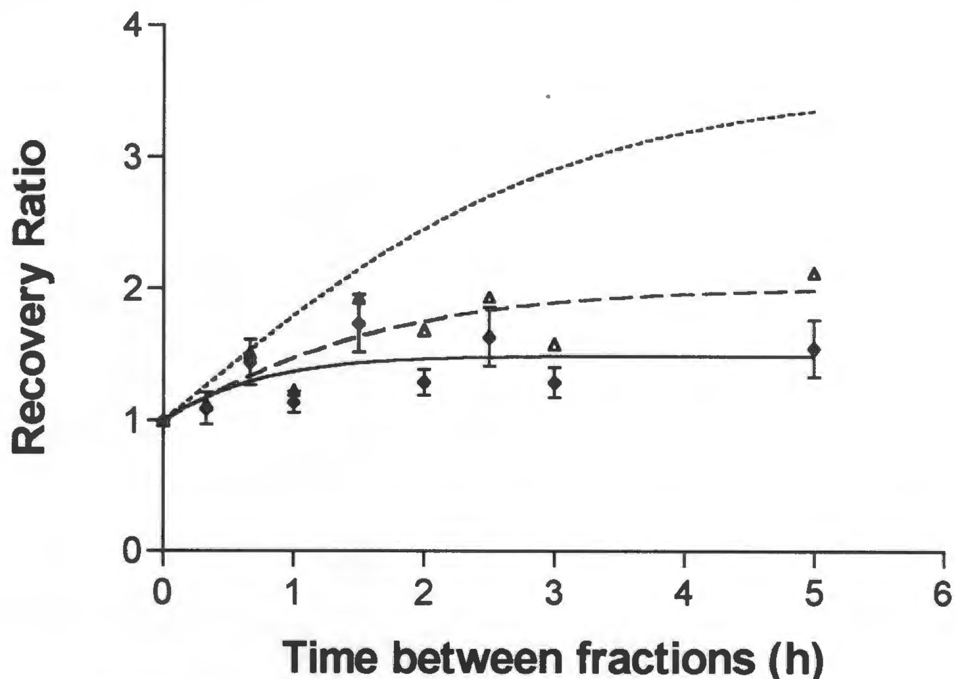


Figure 9.28: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose and glutamine with AOA (Medium G in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

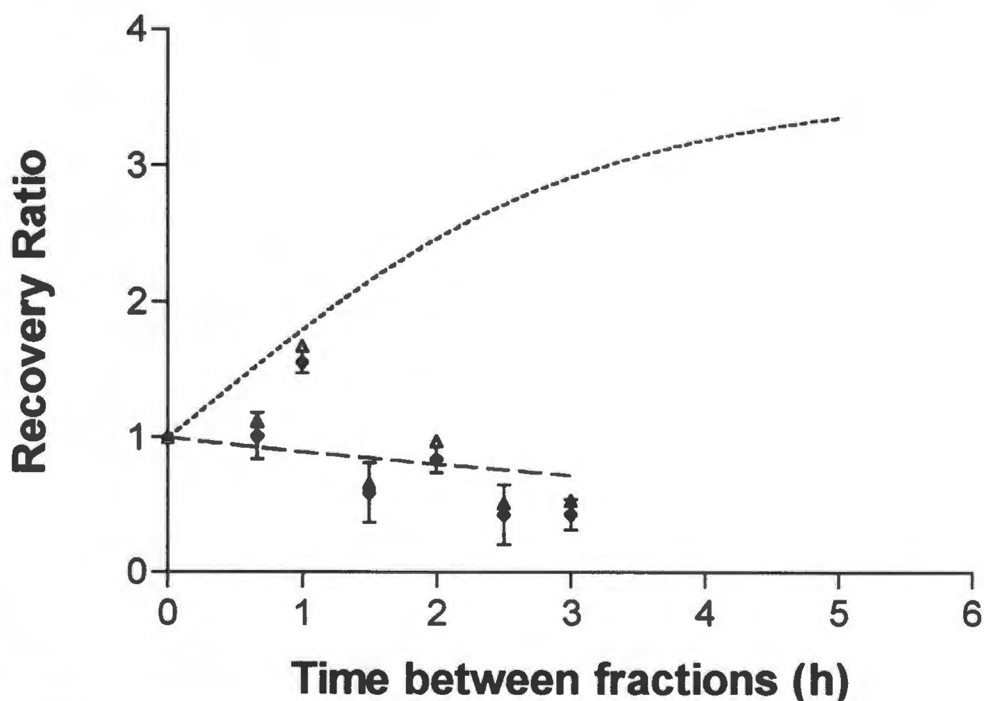


Figure 9.29: The closed symbols represent recovery of CHO cells incubated in McCoy's medium lacking glucose and glutamine with 2DG and AOA (Medium H in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

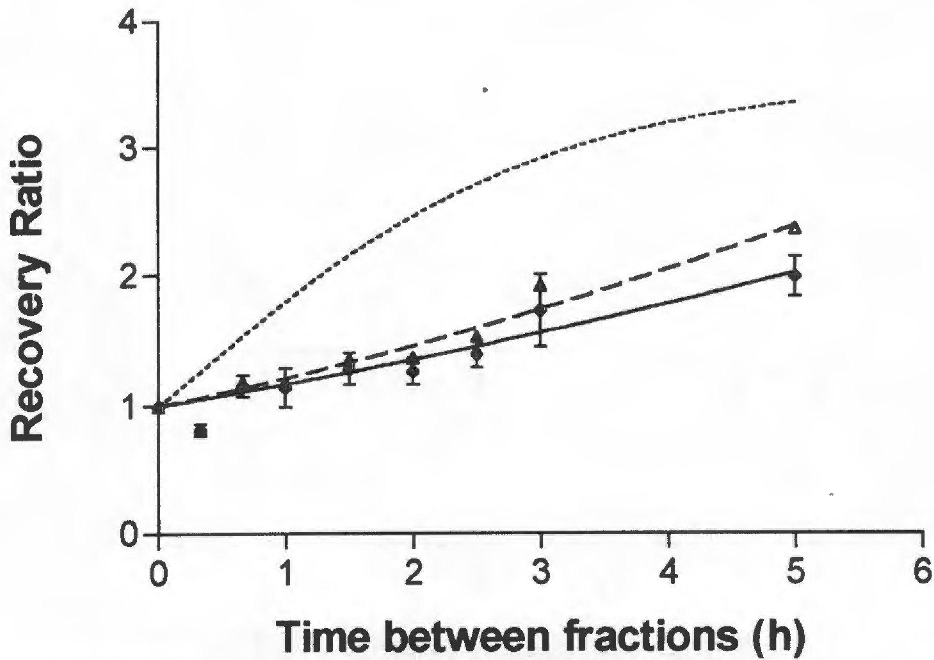


Figure 9.30: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium with 2DG and AOA (Medium D in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

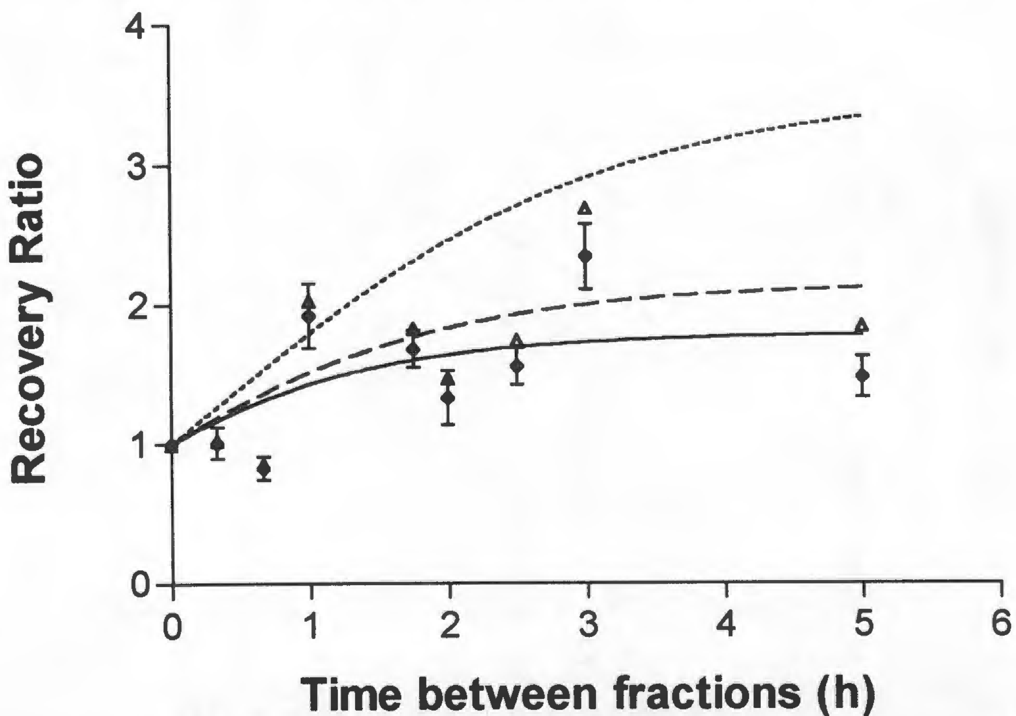


Figure 9.31: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium with 2DG (Medium B in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

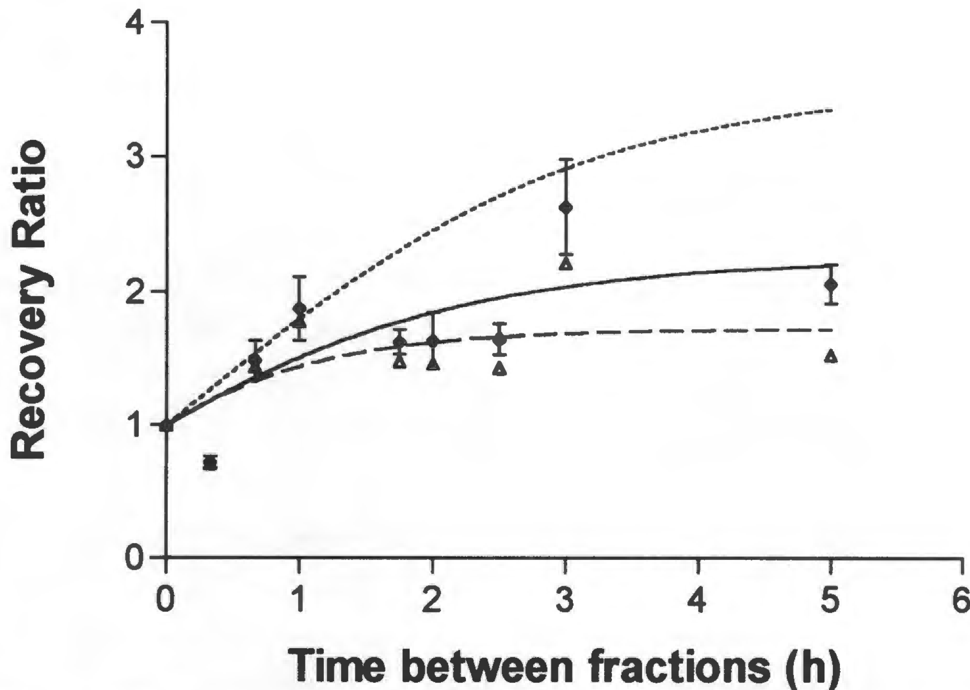


Figure 9.32: The solid curve and closed symbols represent recovery of CHO cells incubated in McCoy's medium with AOA (Medium C in Table 9.1) for 3 hours prior to two 5 Gy fractions separated by up to 5 hours in the same medium. The open triangles and dashed curve represent recovery corrected for sensitivity. The dotted curve represents recovery in normal McCoy's medium from Figure 9.17. Each point represents mean ± sem.

If one assumes that the initial number of repairable lesions induced by the first 5 Gy fraction is the same for each medium type, then the change in recovery ratio with time will represent the relative rate of recovery. The slopes of recovery curves give an indication of the relative rates of repair at any given time between the two fractions. A shift in the curve will represent a change in the slope of the curve and will, therefore, give an indication of an altered repair rate. A left shift indicates an increased rate of repair while a right shift indicates a reduced rate of repair.

Relative rates of repair, for the different variations of McCoy's medium, given by the initial slopes (λA described below) of the recovery curves are shown in Table 9.2. The initial slope is the slope of the tangent to the recovery curve at zero time, that is, the derivative, dRR/dt at t=0.

$$RR=e^{A(1-e^{-\lambda t})} \quad (13)$$

$$dRR/dt = \lambda A e^{A(1-e^{-\lambda t}) - \lambda t} \tag{15}$$

Therefore, at t=0,

$$dRR/dt = \lambda A \tag{16}$$

As described below, if the medium alternatives are divided into groups, certain features, with respect to repair kinetics, become apparent. Only four medium types (media E, M, N and O in Table 9.1) resulted in left shifts in the initial part of the recovery curve relative to that when normal McCoy's medium was used, as shown in Figures 9.18, 9.19 (untransformed curve), 9.20 and 9.22 respectively. Initial slope values (λA) for these curves were greater than that of the curve for normal McCoys medium, as shown in Table 9.2. However, as described below, most medium alternatives resulted in a shift to the right of the initial portion of the recovery curve, that is, an indication of a reduction in repair rate.

Media with AOA but without 2DG: Most media in the group which contained AOA, but not 2DG, produced right shifts in the initial parts of the recovery curves as shown in Figure 9.25 for medium K, Figure 9.28 for medium G and Figure 9.32 for medium C. Initial slopes of recovery curves for these media were shown to be less than that for normal medium. Media K, G and C resulted in reductions in initial slope relative to that for normal medium of 64%, 20-28% and 28-24% respectively (initial and final values of ranges shown represent the initial slope changes relative to those for normal medium for the untransformed and transformed curves respectively). One medium in this group, Medium O, produced left shifts in the recovery curves and produced 20-23% steeper initial slopes than that for normal medium, as shown in Figure 9.22. Medium G, which was depleted of both energy substrates, was not more effective in respect of the alteration of repair rate than medium with both substrates (medium C) as indicated by the similar initial slope values for the two pairs of recovery curves. The steepest initial slope in this group was for medium O, which contained glucose and lacked glutamine, while the medium which produced the shallowest slopes in the group was medium K, which contained glutamine and lacked glucose.

Media with 2DG but without AOA: Most media in the group which contained 2DG, but not AOA, also produced initial right shifts in the recovery curves/data, as shown in Figure 9.24 for medium J, Figure 9.27 for medium F and Figure 9.31 for medium B. Media J and B resulted in reductions in initial slope of recovery curves relative to that for normal McCoys medium of 97-86% and 30-24% respectively. Convergence of the fit of the equation (13) to the data for medium F in Figure 9.27 was not possible so slope values for these data were not determined. Medium N resulted in a left shift of the recovery curves and a 92-91% increased initial slope value relative that for normal medium, as shown in Figure 9.20. It was apparent, when considering the two groups of media above, that a similar reduction in initial slope of recovery curves relative to that for normal medium was produced for medium containing glucose, glutamine and AOA (medium C), and medium containing glucose, glutamine and 2DG (medium B).

Media with neither glucose nor glutamine: For medium types without inhibitors and lacking either glucose (Medium I) or glutamine (Medium M), an initial right shift in the repair curve (or data points, in the case of medium M) was observed, as shown in Figures 9.23 and 9.19 (transformed recovery) respectively. Medium I resulted in an 8% reduction in initial slope relative to that for normal medium. Lack of convergence of the fit of equation (13) to tranformed data in Figure 9.19 resulted in no slope value being determined but it is apparent from the location of data points that a best fit curve would not have appreciable positive slope. As also shown in Figure 9.19, the untransformed recovery curve had an initial slope 36% greater than that for the normal curve. As shown in Figure 9.18, medium which lacked both glucose and glutamine (medium E) resulted in a 54% steeper initial recovery curve slope than that for normal medium.

Media with both 2DG and AOA: Media which contained both inhibitors (Media P, L, H and D), produced right shifts in the recovery curves (or data in the case of medium H), as shown in Figures 9.21, 9.26, 9.29 and 9.30, respectively. Media P, L and D produced 66%, 28-16% and 79-74% reductions in initial slope relative to that for normal

medium, respectively. Lack of convergence of the fit of equation (13) to the data for medium H was not possible so initial slope values were not presented. Nevertheless, no recovery was apparent for cells in this medium, which lacked both energy substrates and contained both inhibitors.

In addition to initial slope values (λA) of recovery curves which were mentioned above, Table 9.2 shows the coefficients of the mono-exponential recovery equations and half times for repair ($t_{1/2}$) of recovery curves shown in Figures 9.17 to 9.32. As discussed in Chapter 3, recovery saturates when recovery ratio is equal to e^A and A value (amplitude) is an indication of the extent of repair. As also discussed previously, λ represents the recovery curve rate constant. A large range of A values and rate constants (λ) were found. The majority of curves show A values less than that of the normal McCoy's curve. In most cases, the fit of the recovery equations to the data was poor as shown by the correlation coefficients being generally much less than 1.

Half times for repair ($t_{1/2}$), shown in Table 9.2, were not used for comparison of repair rates because $t_{1/2}$ values cannot be compared if the saturation values (e^A) of the recovery curves are not equal. $T_{1/2}$ represents the time required for recovery ratio to reach a proportion of the saturation value, e^A, or the time for a proportion of final damage to be repaired. The rate of repair is determined by the amount of initial damage repaired and not by the final amount of damage. Therefore, because the final cellular damage was different for the different media compared, estimation of the rate of repair by the proportion of final damage remaining after irradiation, would be incorrect.

Figures 9.33 to 9.48 show the effect of exposure to different versions of McCoy's medium alone on clonogenic cell survival. No major changes in survival were found with any of the medium types tested except for McCoy's lacking both glucose and glutamine with both 2DG and AOA (medium H, Figure 9.45). Figure 9.49 shows the effect of time in this medium on cell viability assessed by uptake of trypan blue. After 8 hours of exposure to this medium, determination of surviving fraction was not possible because of 100% uptake of trypan blue. In the case of split dose experiments in which cells were exposed to this medium type for 8

hours, that is, an initial 3 hour period followed by 5 hours between fractions, no recovery ratio could be determined at this time point, as shown in Figure 9.29. None of the other media tested showed loss of viability as determined by trypan blue uptake. For medium H, the clonogenic toxicity of which is represented in Figure 9.45, the number of cells plated to determine clonogenic survival was assessed by counting only those cells which excluded trypan blue.

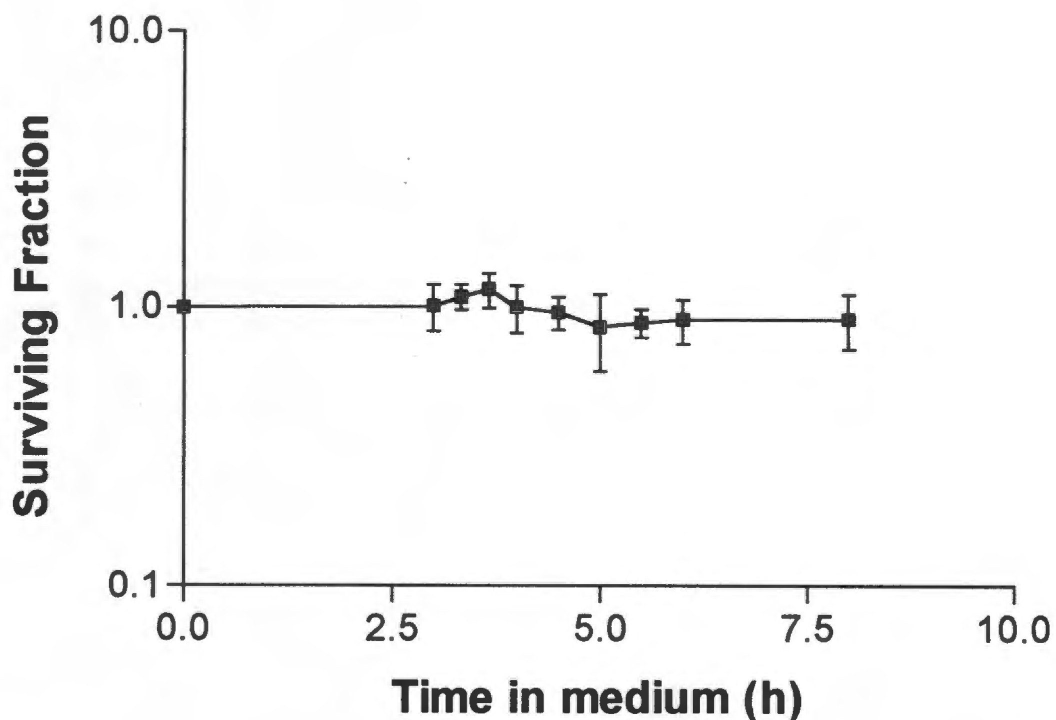


Figure 9.33: Survival of CHO cells incubated in McCoy's medium (Medium A in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

Table 9.2: Coefficients of the fit of equation (13) to recovery curves shown in Figures 9.17-9.32. Coefficients of curves corrected for sensitivity are shown immediately under initial coefficients where applicable. Asterisks indicate where convergence of the fit of the model was not possible. Errors shown represent standard errors.

MEDIUM		A	λ	λA	$t_{1/2}$	R^2
Normal McCoy's	(Fig 9.17)	1.26 ± 0.20	0.62 ± 0.28	0.79 ± 0.37	1.12 ± 0.50	0.79
No glucose and glutamine	(Fig 9.18)	1.41 ± 0.24	0.85 ± 0.51	1.21 ± 0.75	0.81 ± 0.49	0.57
No glutamine	(Fig 9.19)	0.95 ± 0.11	1.12 ± 0.49	1.07 ± 0.49	0.62 ± 0.27	0.70
No glutamine+2DG	(Fig 9.20)	1.23 ± 0.11	1.23 ± 0.49	1.51 ± 0.61	0.56 ± 0.22	0.71
No glutamine+2DG corrected	(Fig 9.20)	1.48 ± 0.11	1.01 ± 0.29	1.51 ± 0.45	0.69 ± 0.20	0.83
No glutamine+2DG+AOA	(Fig 9.21)	0.45 ± 0.14	0.58 ± 0.44	0.26 ± 0.22	1.20 ± 0.90	0.59
No glutamine+AOA	(Fig 9.22)	0.54 ± 0.09	1.71 ± 1.27	0.94 ± 0.71	0.41 ± 0.30	0.51
No glutamine+AOA corrected	(Fig 9.22)	0.65 ± 0.09	1.49 ± 0.90	0.97 ± 0.60	0.47 ± 0.28	0.59
No glucose	(Fig 9.23)	0.97 ± 0.10	0.74 ± 0.22	0.72 ± 0.22	0.93 ± 0.27	0.89
No glucose+2DG	(Fig 9.24)	2.64 ± 847.92	0.009 ± 2.80	0.023 ± 10.54	78.59 ± 24966.43	0.03
No glucose+2DG corrected	(Fig 9.24)	9.11 ± 334.97	0.012 ± 0.48	0.11 ± 5.96	57.76 ± 234.93	0.37
No glucose+AOA	(Fig 9.25)	0.30 ± 0.16	0.94 ± 1.45	0.28 ± 0.46	0.74 ± 1.14	0.35
No glucose+2DG+AOA	(Fig 9.26)	0.48 ± 0.21	1.17 ± 1.57	0.56 ± 0.79	0.63 ± 0.96	0.26
No glucose+2DG+AOA corrected	(Fig 9.26)	0.71 ± 0.22	0.94 ± 0.86	0.66 ± 0.64	0.74 ± 0.68	0.42
No glucose or glutamine+2DG	(Fig 9.27)	*	*	*	*	*
No glucose or glutamine+AOA	(Fig 9.28)	0.40 ± 0.08	1.58 ± 1.12	0.63 ± 0.47	0.44 ± 0.34	0.49
No glucose or glutamine+AOA corrected	(Fig 9.28)	0.70 ± 0.11	0.81 ± 0.36	0.57 ± 0.27	0.86 ± 0.38	0.75
No glucose or glutamine+2DG+AOA	(Fig 9.29)	*	*	*	*	*
Normal medium+2DG+AOA	(Fig 9.30)	2.75 ± 4.84	0.059 ± 0.12	0.16 ± 0.43	11.77 ± 23.38	0.90
Normal medium+2DG+AOA corrected	(Fig 9.30)	3.33 ± 4.33	0.061 ± 0.09	0.20 ± 0.40	11.44 ± 16.93	0.94
Normal medium+2DG	(Fig 9.31)	0.59 ± 0.19	0.93 ± 1.01	0.55 ± 0.62	0.73 ± 0.75	0.42
Normal medium+2DG corrected	(Fig 9.31)	0.77 ± 0.21	0.78 ± 0.63	0.60 ± 0.51	0.89 ± 0.71	0.54
Normal medium+AOA	(Fig 9.32)	0.81 ± 0.19	0.70 ± 0.46	0.57 ± 0.40	0.99 ± 0.64	0.64
Normal medium+AOA corrected	(Fig 9.32)	0.54 ± 0.14	1.11 ± 1.05	0.60 ± 0.59	0.62 ± 0.59	0.48

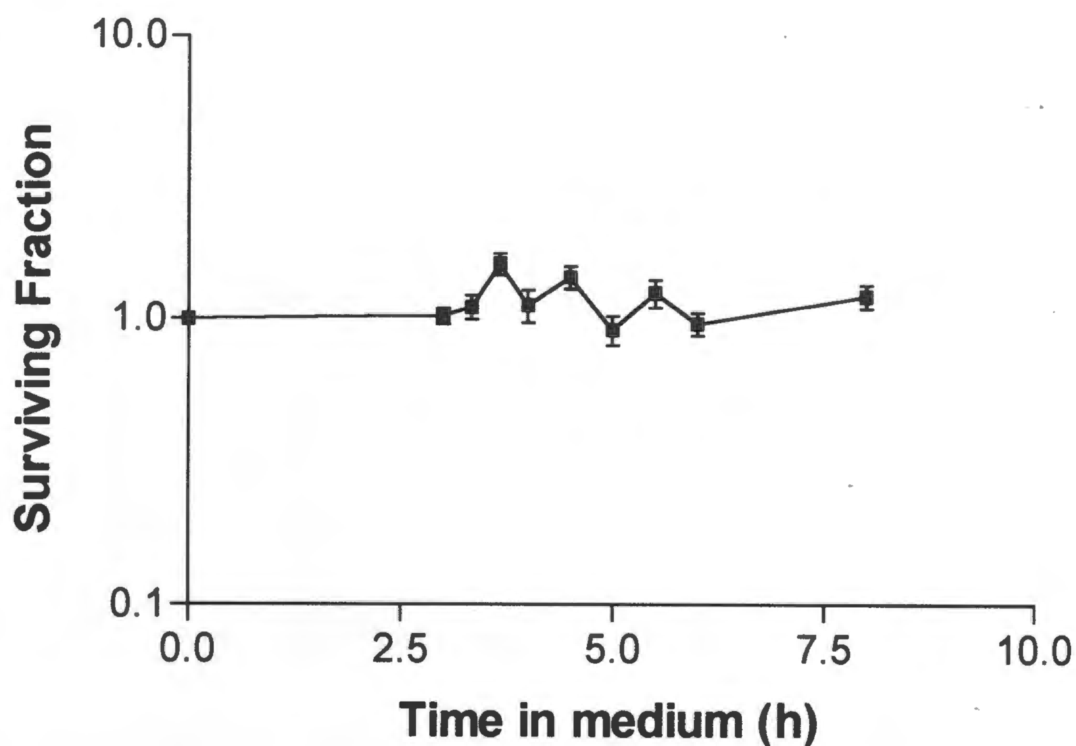


Figure 9.34: Survival of CHO cells incubated in McCoy's medium lacking glucose and glutamine (Medium E in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

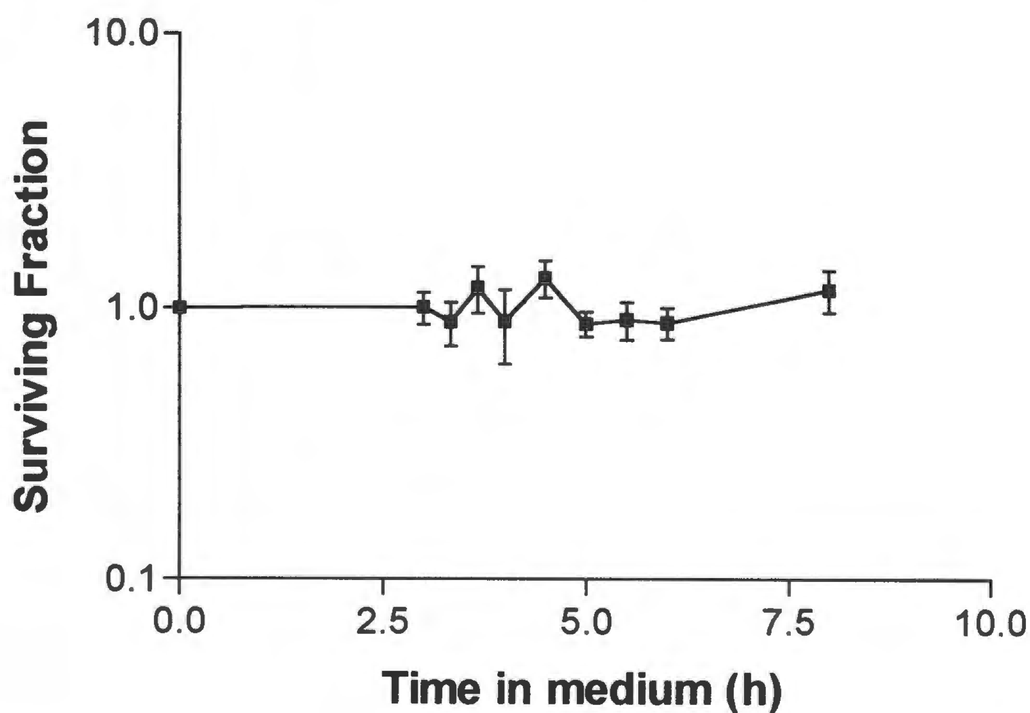


Figure 9.35: Survival of CHO cells incubated in McCoy's medium lacking glutamine (Medium M in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

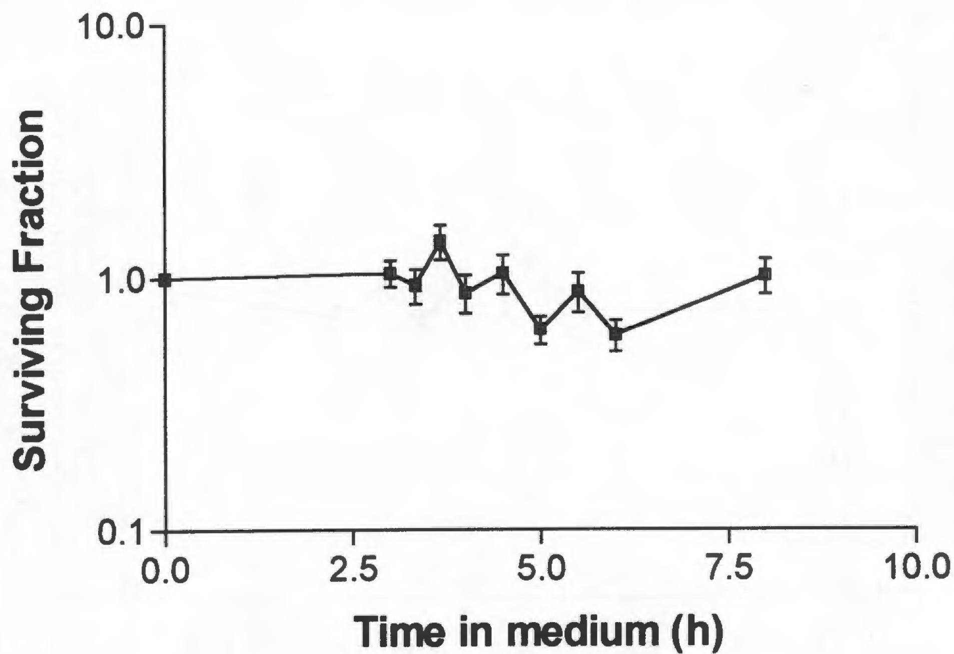


Figure 9.36: Survival of CHO cells incubated in McCoy's medium lacking glutamine with 2DG (Medium N in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

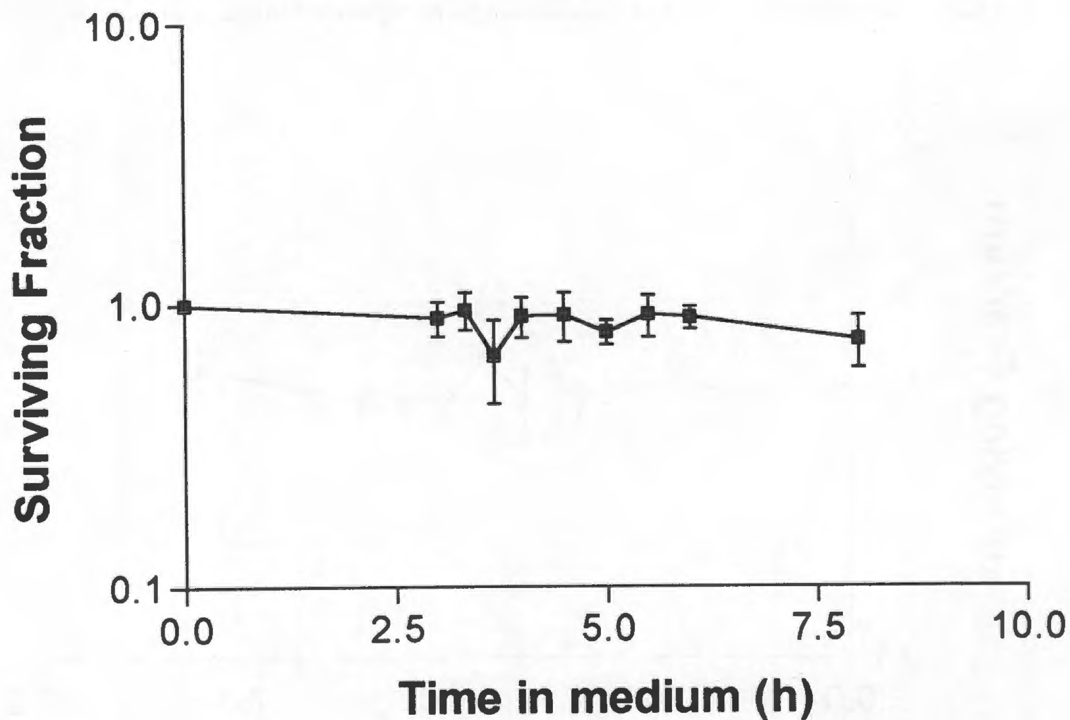


Figure 9.37: Survival of CHO cells incubated in McCoy's medium lacking glutamine with 2DG and AOA (Medium P in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

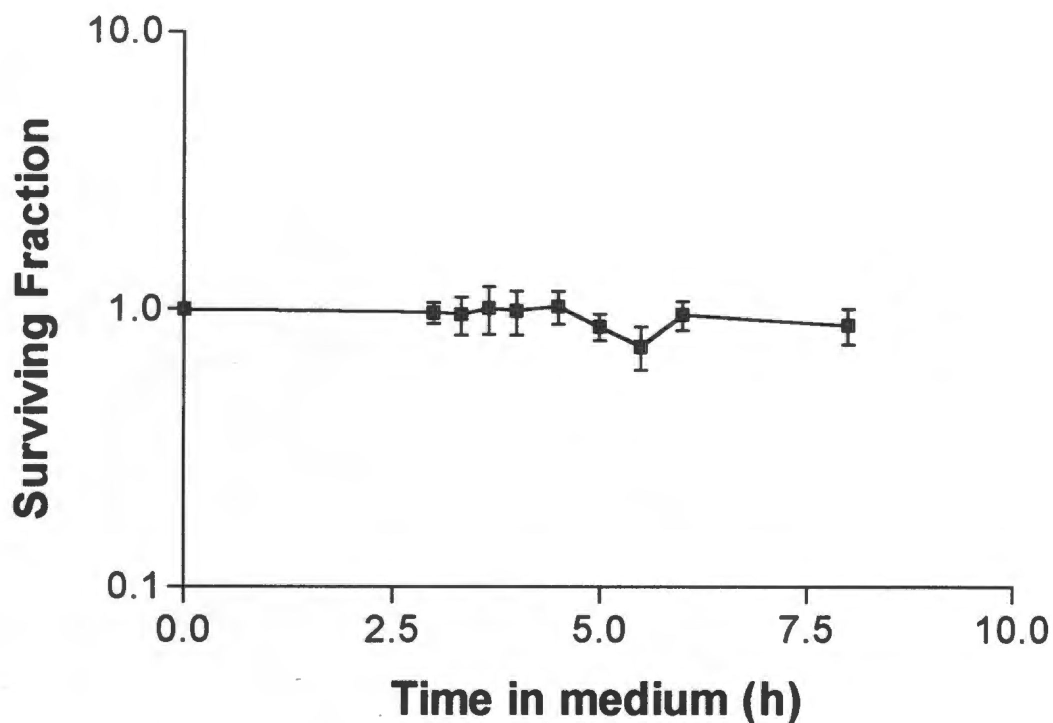


Figure 9.38: Survival of CHO cells incubated in McCoy's medium lacking glutamine with AOA (Medium N in Table 9.1) for up to 8 hours. Points are means ± standard deviations.

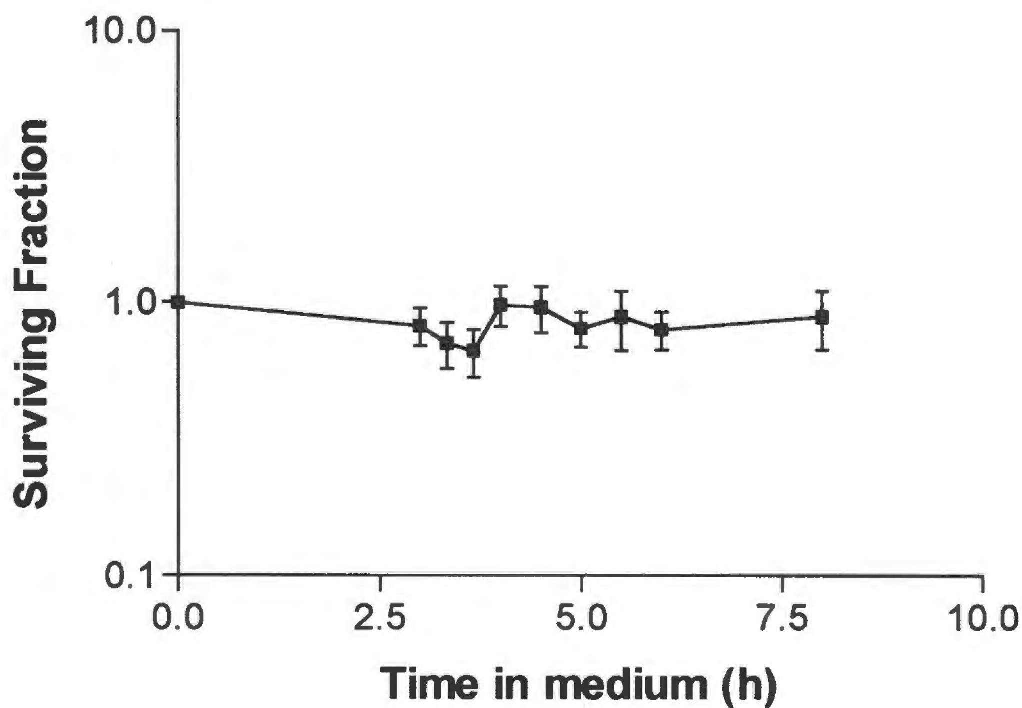


Figure 9.39: Survival of CHO cells incubated in McCoy's medium lacking glucose (Medium E in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

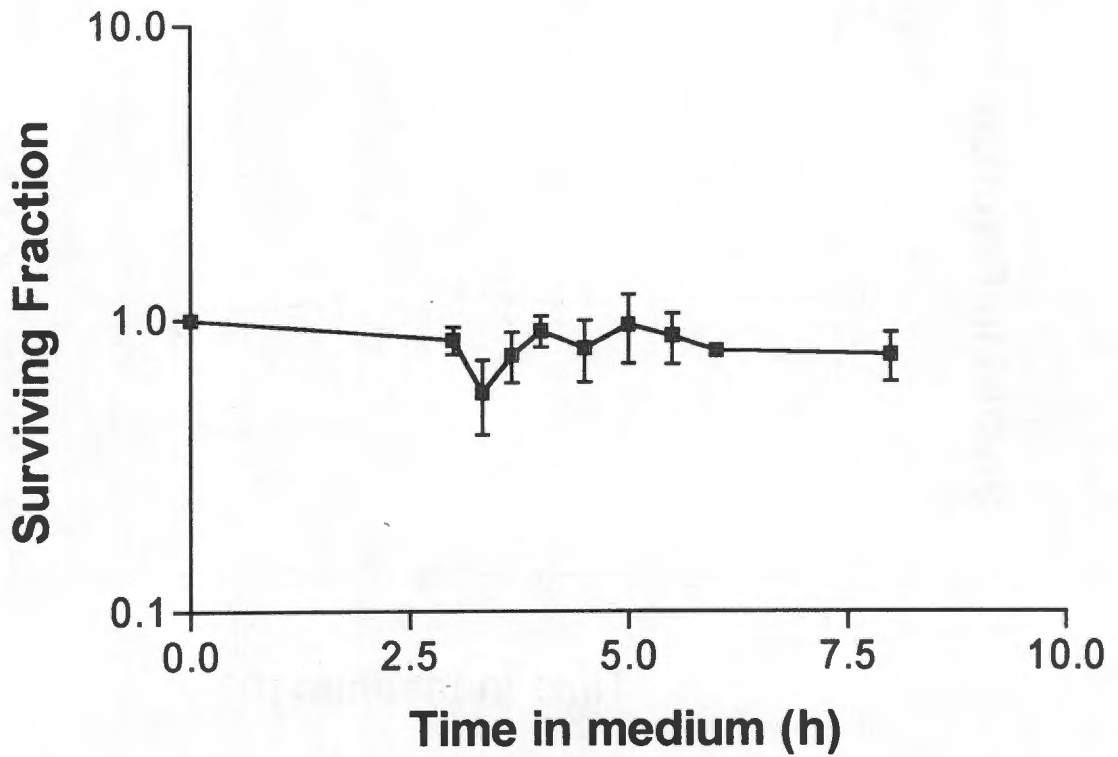


Figure 9.40: Survival of CHO cells incubated in McCoy's medium lacking glucose with 2DG (Medium F in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

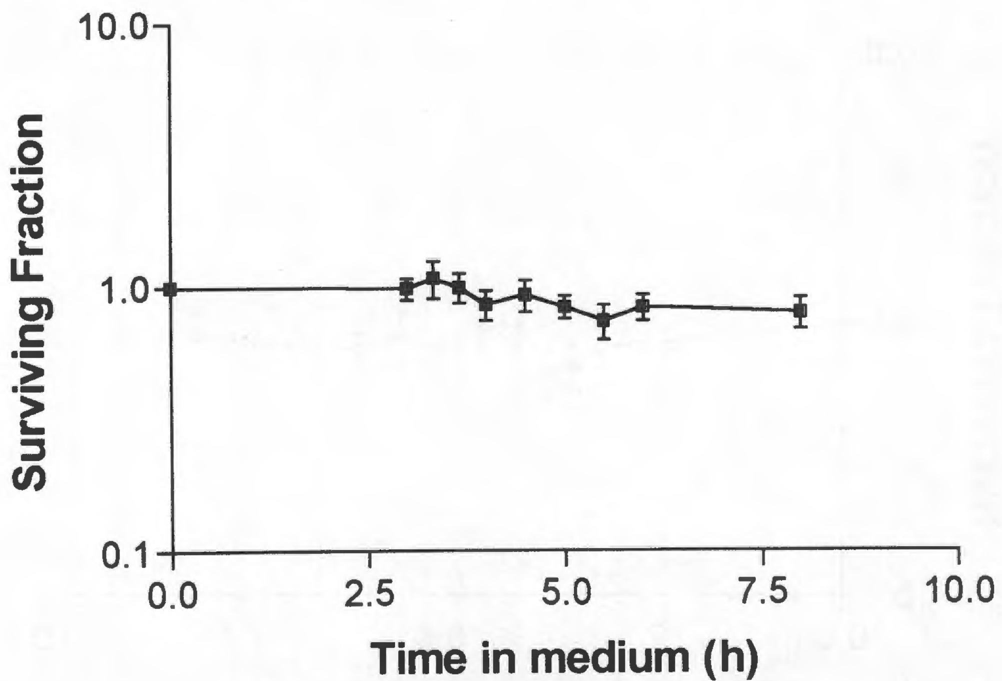


Figure 9.41: Survival of CHO cells incubated in McCoy's medium lacking glucose with AOA (Medium G in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

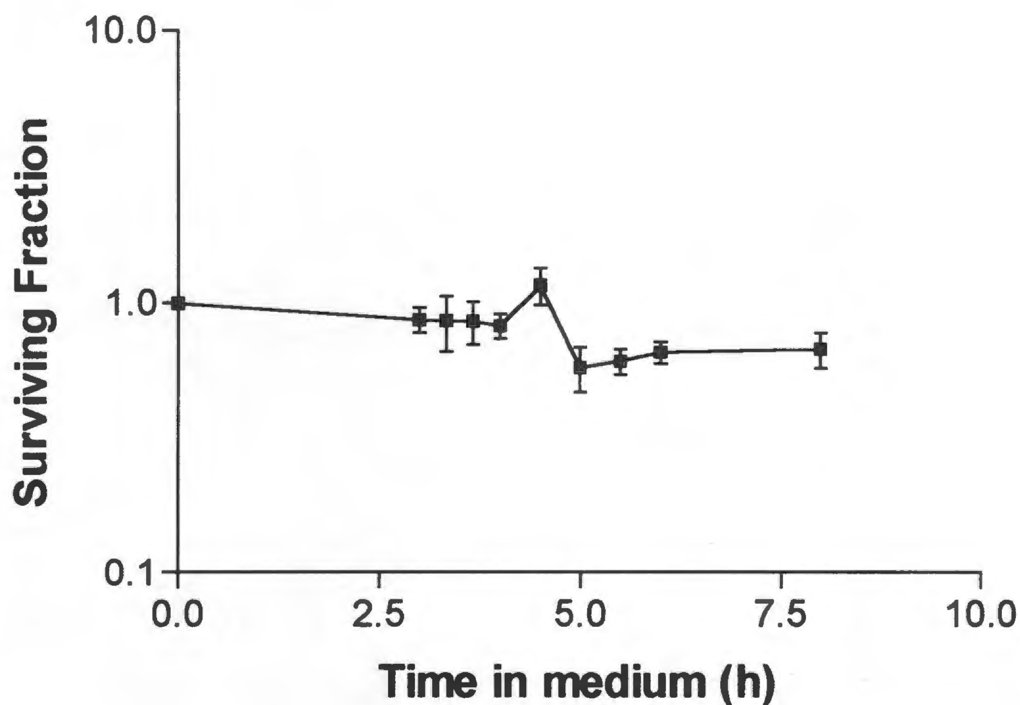


Figure 9.42: Survival of CHO cells incubated in McCoy's medium lacking glucose with 2DG and AOA (Medium L in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

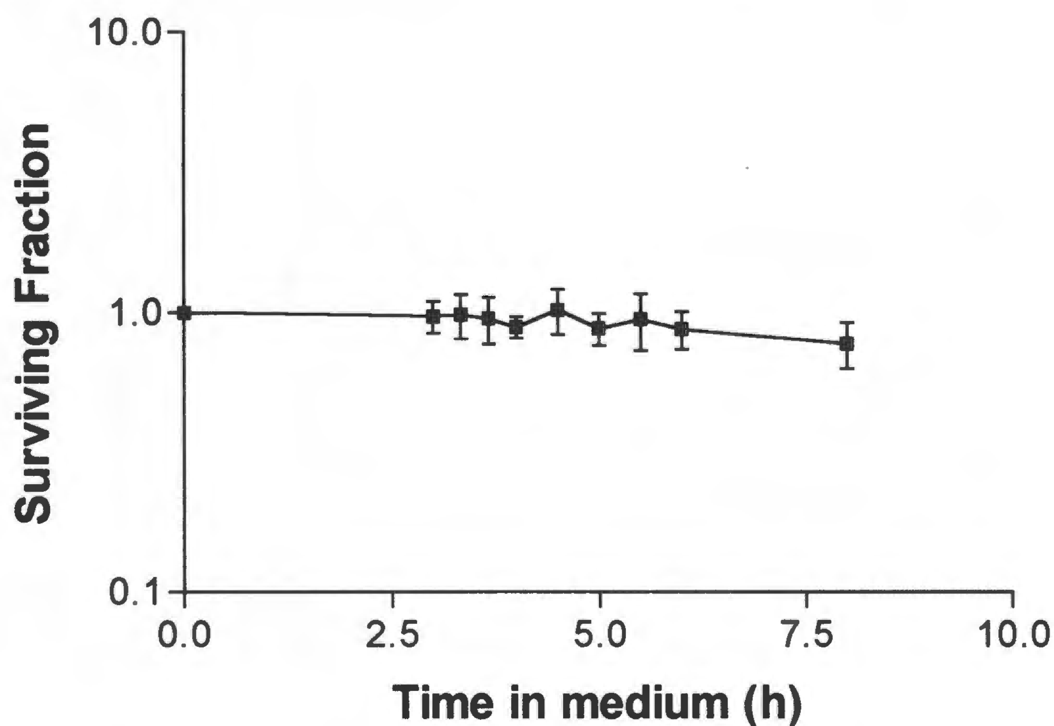


Figure 9.43: Survival of CHO cells incubated in McCoy's medium lacking glucose and glutamine with 2DG (Medium F in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

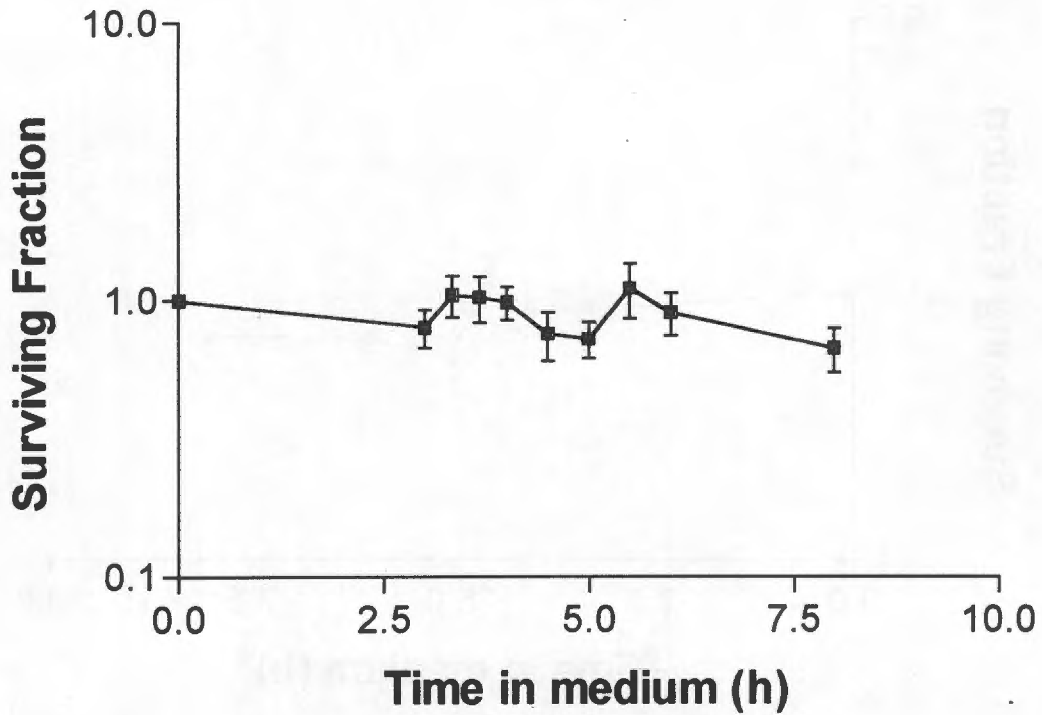


Figure 9.44: Survival of CHO cells incubated in McCoy's medium lacking glucose and glutamine with AOA (Medium G in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

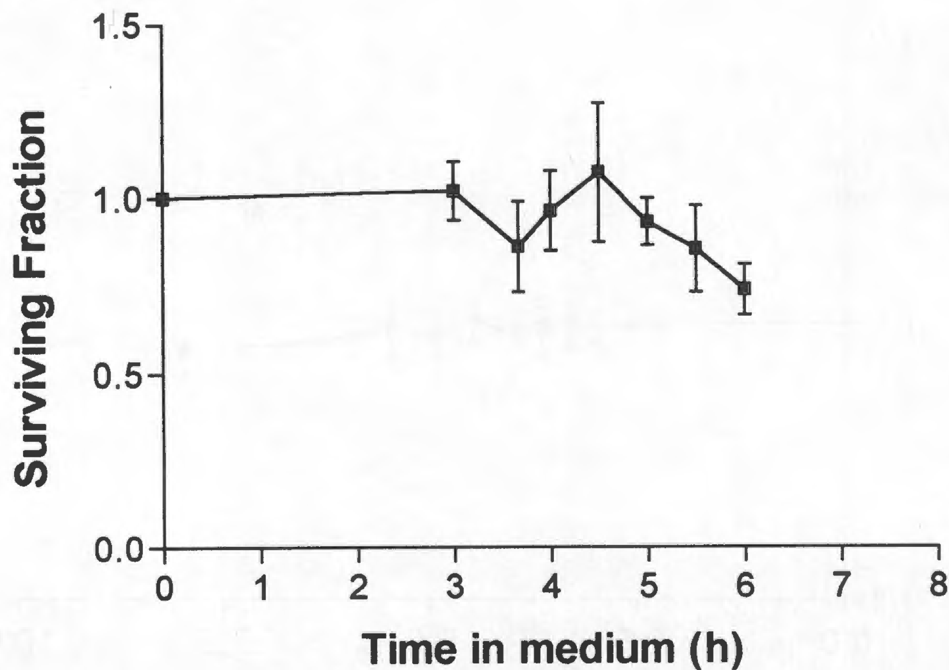


Figure 9.45: Survival of CHO cells incubated in McCoy's medium lacking glucose and glutamine with 2DG and AOA (Medium H in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

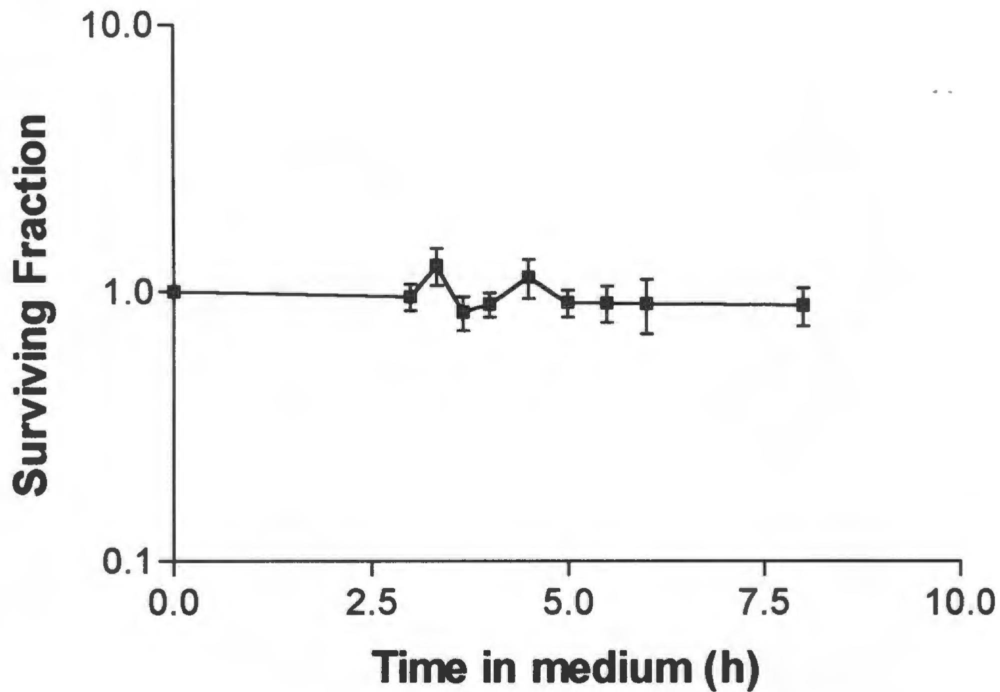


Figure 9.46: Survival of CHO cells incubated in McCoy's medium with 2DG and AOA (Medium D in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

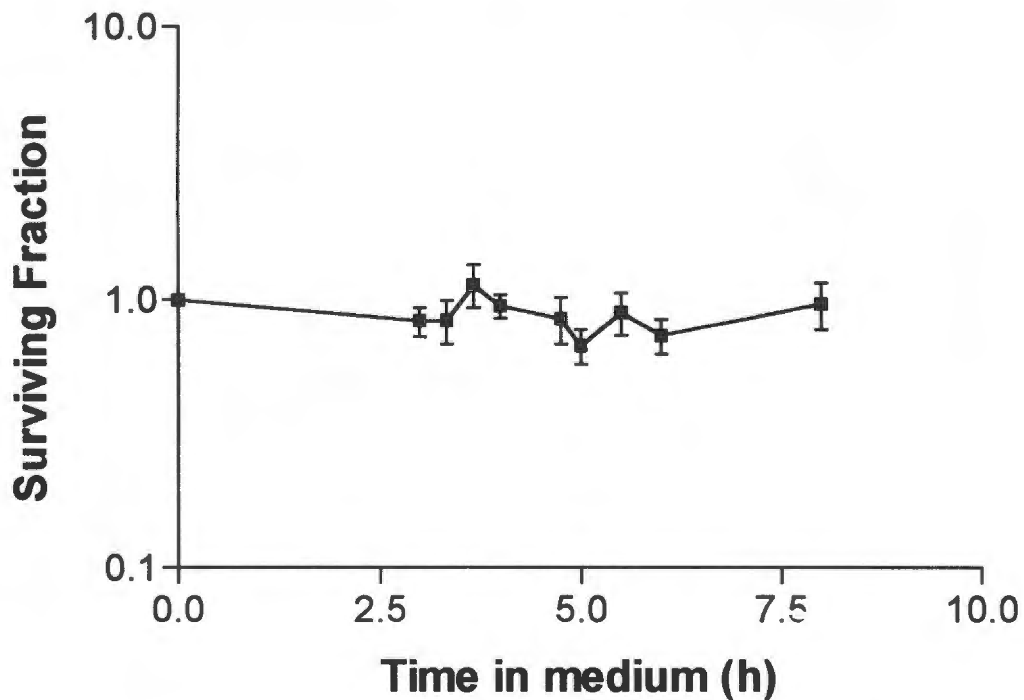


Figure 9.47: Survival of CHO cells incubated in McCoy's medium with 2DG (Medium B in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

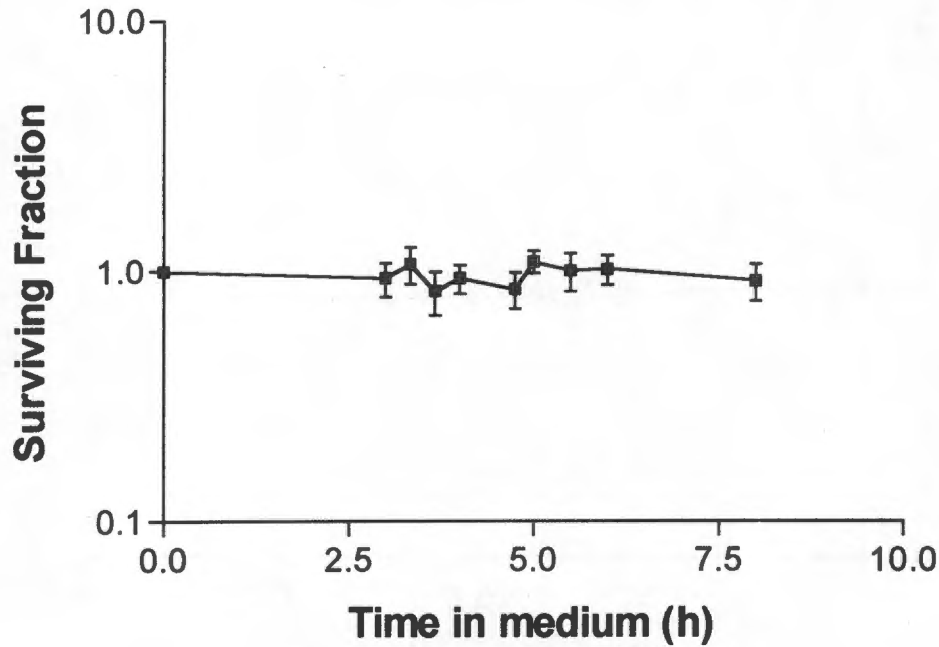


Figure 9.48: Survival of CHO cells incubated in McCoy's medium with AOA (Medium C in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation.

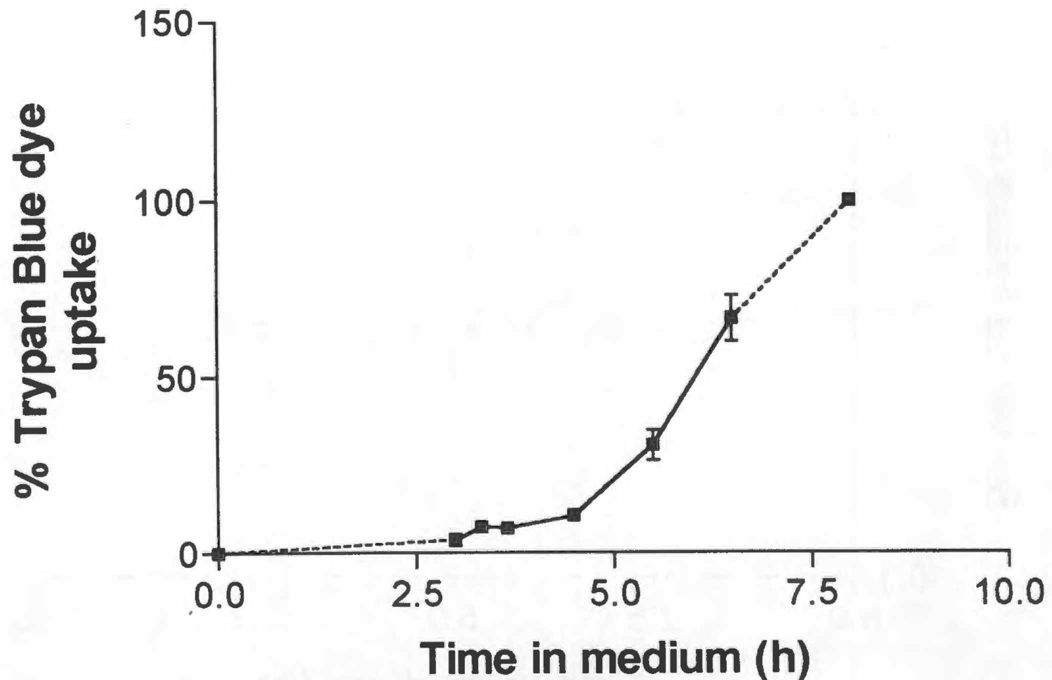


Figure 9.49: Trypan blue uptake of CHO cells incubated in McCoy's medium lacking glucose and glutamine with 2DG and AOA (Medium H in Table 9.1) for up to 8 hours. Each point represents mean ± standard deviation. Where no error bars are shown, errors are hidden due to the size of the symbol.

DISCUSSION

Changes in radiosensitivity were found for cells exposed to most of the medium options listed in Table 9.1, as shown in Figures 9.1 to 9.16. However, no significant radiosensitivity change could be detected in cells exposed to normal McCoy's medium with a full complement of glucose and glutamine, as shown in Figure 9.1. Most variations of McCoy's medium yielded an increased radiosensitivity with time in the medium. The greatest increases in sensitivity were noted for two media lacking glucose and containing 2DG, namely McCoy's medium lacking glucose and glutamine with both AOA and 2DG, as shown in Figure 9.13, and McCoy's lacking glucose with 2DG, as shown in Figure 9.8. The only medium which contained 2DG that failed to show a trend towards increased sensitivity was McCoy's lacking glutamine with both AOA and 2DG, as illustrated in Figure 9.5. However, the 8 hour point does suggest an increase in sensitivity at longer times.

In spite of trying to accumulate as many data points as practicable, the number still appears to be too low for maximal precision as evidenced by poor fits of the regression lines to the data shown in Figures 9.1 to 9.16.

Once radiosensitivity has been taken into account, as described in the Results section, it is assumed that the amount of repairable damage induced by the first 5Gy fraction in a split dose experiment is the same for all media. Therefore, the rate at which recovery ratio increases with time between fractions would reflect the rate at which damage is being repaired, regardless of the level at which recovery rate saturates. A decrease in the initial slope of the recovery curve relative to that for normal medium, therefore, indicates a slower rate of recovery while an increase in the initial slope indicates the reverse.

Figures 9.17 to 9.32 show that, in general, repair rate was reduced in most of the energy supply inhibited media tested as shown by right shifts and reductions in the initial slopes of recovery curves. Four variations of medium, namely, E, M, N and O resulted in an increased rate of

repair, as shown in Figures 9.18, 9.19 (untransformed), 9.20 and 9.22. This could not be adequately explained on the basis of energy supply and repair alone unless the combination of factors in these media resulted in less severe energy depletion. The common factor in these media was a lack of glutamine although other media lacking glutamine did not increase repair rate above that of normal medium. Media lacking glutamine which resulted in a reduced repair rate include medium P (Figure 9.21) and those media also lacking glucose and containing inhibitors (media F, G and H), as shown in Figures 9.27, 9.28 and 9.29, respectively.

The greatest inhibition of repair was found with the medium which lacked both glucose and glutamine and contained both 2DG and AOA (medium H), as shown in Figure 9.29. Although the fit of equation (13) to the data in Figure 9.29 did not converge, it is evident that a best fit recovery ratio curve would not rise above a value of one, which indicates no repair, and that the trend is to fall below this level. This may be radiosensitivity related. Corrected recovery lifts the curve closer to the horizontal but still appears to have a negative slope.

Seymour and Mothersill (1987) showed a similar depression of initial repair rate in split-dose experiments in CHO cells using inhibitors of glycolysis such as sodium fluoride, sodium iodoacetate and sodium arsenate. The extent of repair seemed to have been increased by the presence of sodium iodoacetate or sodium arsenate and decreased by sodium fluoride. All three inhibitors were shown to shift radiation survival curves to the left. Spiro *et al.* (1985) found in split-dose experiments with V79 cells that repair took place as efficiently in Hanks' buffered salt solution without glucose as in normal growth medium containing glucose. However, no repair was apparent with Hanks' buffered salt solution when cells were kept in hypoxia. When Hanks' contained glucose or cells were in culture medium, the presence of hypoxia reduced split dose recovery but did not prevent it completely. These experiments show the importance, in certain cell types, of inhibiting energy supply at the glycolytic level and at the respiratory level in order to achieve effective inhibition of repair.

Repair can be analysed in terms of the rate of repair or in terms of the amount of repair. The saturation exponents (A) of recovery curves, as shown in Table 9.2, indicate the relative maximum repair levels possible according to the best fit of equation (13) to the data. At curve saturation, recovery ratio becomes constant at a value of e^A. The majority of medium types tested prevented cells from recovering to the same extent as in normal McCoy's medium although several media resulted in recovery curve saturation at higher recovery levels. It would seem, therefore, that, in addition to repair rate being altered, the relative extent of repair in different media, as indicated by A values in Table 9.2, was inhibited to different extents and that the extent of repair may not be maximal in normal McCoy's medium, as shown by A values for certain of the media being greater than that of normal medium.

Small alterations in recovery ratio values can have significant effects on the shape of the recovery curve and on the A value. R^2 values considerably less than 1 in many cases show up the erratic nature of the data and its fit by equation (13). If A values were equal for all the medium types tested, rate constants (λ) would give an indication of relative recovery rate. The λ value of normal McCoy's can be used to derive a half life of repair under normal culture medium conditions ($t_{1/2}$= $\ln2/\lambda = 1.12 \pm 0.496$ h). This value is similar to the value of 70 minutes (1.167 h) reported by Stackhouse and Bedford (1993) in CHO cells. They reported a slightly faster value for log-phase cells (50 minutes). Iliakis *et al.* (1992) reported a $t_{1/2}$ of 101 minutes (1.68 h) for PLD repair assessed by immediate and delayed plating. Iliakis *et al.* (1993) quoted a repair $t_{1/2}$ of 66.8 minutes (1.11 h) for PLD repair assessed after response to radiation and hypertonic medium. From split-dose repair curves for CHO cells presented in the publication of Nelson *et al.* (1990), $t_{1/2}$ appeared to be approximately one hour. In the paper of Iliakis *et al.* (1993), a half time for chromosome rejoining was given as 75.8 minutes after 10 Gy.

Radiosensitivity of cells which had been exposed to different variations of McCoy's medium was assessed using a 10 Gy single dose. Any change in sensitivity was then used to modify surviving fractions in the split dose experiments and thus also to modify recovery ratios. If survival is

affected by a certain percentage as a result of being exposed to a medium type for a longer time, then split dose survival must be adjusted by adding or subtracting the same percentage of survival depending on whether sensitivity is increased or decreased respectively at the relevant times. This modification of survival, as a result of two 5 Gy fractions, using the change in sensitivity to 10 Gy given after the same time in the medium as the second 5 Gy fraction, may involve an overestimate of the degree of radiosensitization or protection. This is because, for split doses, at the time of giving the first 5 Gy fraction, cells would have been exposed to medium for a shorter time than the time before they were given the second 5 Gy fraction. The sensitizing effects of the medium would, thus, be less than for the second 5 Gy fraction which was exposed to the medium for longer. The combined effects of the medium on sensitivity, for two 5 Gy doses given after different times in the medium, might, therefore, be less than those for a single 10 Gy dose given after a period which corresponds to the time in medium before the second 5 Gy dose fraction. Thus, the use of 10 Gy single dose to estimate the contribution of radiosensitivity in the split dose experiment may result in an overcompensation of split dose survival. However, once sensitivity has been taken into account using this method, a more representative estimation of recovery from radiation damage can be made.

This manipulation of data may be simplistic and may overestimate the contribution of sensitivity changes but if, even after compensation is made, no recovery can be detected, then it is likely that no recovery occurs. Manipulated data should be considered as relative values and not as definitive values. The region between the initial apparent recovery curve and the manipulated curve can be considered to be an envelope of effect with the true curve falling somewhere within it.

An alternative method of taking sensitivity into account might be to consider what changes in sensitivity to a single 5 Gy dose occur with time in a particular medium. The difference in sensitivity for zero time between fractions and that for greater times between fractions would then be represented by the sensitivity change due to time in medium before a single 5 Gy dose rather than a single 10 Gy dose. The argument

for the 5 Gy method of sensitivity correction makes the assumption that the amount of damage caused by 5 Gy is equivalent to the amount of damage caused by one half of 10 Gy. This would mean that 5 Gy causes the same proportion of damage as when it is given immediately after another 5 Gy dose constituting the second half of a 10 Gy dose. This is not so. The clonogenic survival curve for CHO cells has a typical shoulder region and, thereafter, bends more steeply with dose and so the proportion of cells killed by the first 5 Gy will be less than for the next 5 Gy. Recovery ratio is calculated by dividing the surviving fraction, for a particular time interval between two 5 Gy doses, by the surviving fraction obtained when there is no time between fractions (single 10 Gy dose). Therefore, to use sensitivity data obtained by comparing the effects of the smaller, less effective 5 Gy single doses may not be appropriate.

Final damage after two split doses of radiation may not only be determined by repair of damage but also by the number of lesions produced. By allowing time after one fraction of radiation before giving the second, the damage caused by the first dose of radiation may be allowed to repair itself or may affect the way in which the damage caused by the two doses interacts. Those lesions which are considered to be repairable after a dose of radiation will not be repaired if a subsequent dose converts those repairable lesions to irrepairable lesions. It is conceivable that some lesions induced by radiation are sublethal but irrepairable. If there is interaction between these sublethal lesions and lesions induced by the subsequent doses, then these sublethal lesions may become lethal. It can be speculated that metabolic changes induced in sublethally damaged cells, possibly related to repair mechanisms in the same cells, may affect the efficiency with which damage from subsequent doses interacts with previously incurred irrepairable sublethal damage. Inhibition of repair may, therefore, influence the interaction of two split doses and consequently radiation response, not only by preventing damage from being repaired, but by modifying interaction of lesions which may be dependent on other processes affected by repair.

An environment which inhibits or does not support energy metabolism may influence radiation response in a number of ways. There is probably an interplay of energy dependent processes, namely, repair, damage fixation and misrepair which will determine the cellular radiation response (Jain *et al.*, 1985). If the rate of repair is reduced by energy depletion, reparable damage which would normally be repaired relatively quickly, will persist for a longer period and thus would allow greater interaction with radiation damage resulting from the second dose. This process could occur by an effective reduction in the number of units of repair machinery because some are closed down or are functioning less efficiently. Greater interaction of damage may effectively increase the slope of a recovery curve by increasing the interaction of the doses and reducing the amount of repairable damage after the second dose.

If repair is completely inhibited by extensive energy depletion, all damage produced is essentially irreparable in nature as long as the depleted conditions do not change. If conditions revert to conditions with normal energy supply, repair might occur but at a later time. In the experimental set-up, if cells are exposed to such a severely energy depleted environment that no repair is allowed to occur between fractions, repair would only be permitted after the second dose when cells are replaced in full medium. However, at this point, cells are plated and encouraged to divide and so fixation of damage is likely to occur as a result of cell division. Any division delay induced by prior exposure to depleted medium may hold off fixation of damage while favouring repair. Hence, there may be competition between repair and fixation of damage which may vary depending on the extent of energy depletion.

Potentially lethal damage (PLD) repair may be enhanced by any division delay after the second dose (when cells are plated to assess clonogenicity), possibly as a result of previous exposure to depleted medium. Little (1971) demonstrated that PLD repair could be enhanced by exposure of irradiated cells to conditioned medium. Pohlit and Heyder (1981) showed that more PLD repair took place in conditioned medium than in full nutrient medium. This was explained on the basis that, under normal nutrient conditions, more PLD is converted to irreparable damage while optimal PLD repair is able to take place in

conditioned medium. Kapiszewska and Lange (1988) suggested that experiments which attempt to analyse repair in conditioned media might be explained in terms of competition between PLD repair and PLD fixation. ATP is necessary for misrepair, division (which leads to fixation of damage), and repair itself (Jain *et al.*, 1985). The competition between these processes may lead to different degrees of damage in cells depending on the metabolic changes in the cell.

Although the degree of energy depletion in cells between two fractions of radiation is important with respect to repair and consequently, the response of the cells to radiation, radiation response will also be determined by the amount of damage in cells after the second radiation dose. This is because it is likely that cells exposed to different degrees of energy depletion between fractions may carry residual effects which may affect repair after the second dose fraction. Repair may continue to be inhibited in cells previously exposed to severely depleted medium. A reduction in recovery ratio may, therefore, arise partially from repair after the second fraction being inhibited in addition to the repair inhibition between fractions. To reduce the influence of effects after the second dose in a split dose experiment, when a medium which depletes energy supply is tested, cells might be placed in normal medium for a period before the second dose is given. Radiosensitivity changes may thus be reduced and cells may respond to the second dose in a uniform way for different medium types.

Medium types may differ with respect to their time and duration of action and thus affect the overall radiation response differently. While some media may reduce energy metabolism only slightly, a significant inhibition of energy metabolism may disrupt membrane transport and modify radiation response. Ouabain, which inhibits the action of the ATP dependent sodium-potassium pump in the plasma membrane (Lawrence, 1988), has been shown to modify radiation response in tumour cells.

As mentioned above, the A value represents the relative extent of repair in a split dose repair curve. There are a number of possible reasons for a lower A value. First, less repair might occur because of a reduced

number of repairable lesions. Secondly, less repair may take place after either the first or second dose of radiation as result of the inhibition of repair mechanisms, which may result in a reduced maximum level of recovery.

In summary, the results indicate that McCoy's media containing 2DG can increase radiosensitivity in CHO cells relative to that in normal McCoy's medium. Media containing AOA can alter radiosensitivity causing both an increase and a decrease in sensitivity depending on the presence or absence of glucose and glutamine. Each of 2DG, AOA, glucose depletion, glutamine depletion alone and in most combinations, can significantly reduce split-dose radiation interfraction repair rate but they vary with respect to their ability to affect the extent of repair. Cells in media depleted of glucose tend to be more susceptible to repair inhibition by 2DG but media depleted of glutamine do not seem to more susceptible to repair inhibition by AOA. This might be partly expected as the 2DG to glucose ratio is important with regard to the effectiveness of 2DG. Even though no glutamine may be present in medium, it is possible that cells will make use of intracellular stores of glutamine and thus partially negate the deficiency in the medium. Glycogen within the cells may play a role in energy supply although glycogen levels are normally low in cells other than liver and muscle. Glycogen in cells can contribute to intracellular glucose though it is expected to be depleted fairly soon after glucose starvation. Sevdalian and Zielke (1978) reported that glycogen levels in cultured fibroblasts were rapidly depleted in medium low in glucose (1 mg/100 ml). Levels of glycogen in confluent cells reduced from 120 μg/mg protein to about 60 μg/mg protein in about 4 hours. The absence of either glucose or glutamine from normal medium caused moderate inhibition of repair rate but when both substrates were absent, repair rate seemed to be increased. Slight effects on energy supply may result in less repair inhibition. This may be due to a stimulation of energy metabolism by surface receptors as a result of low or absent extracellular glucose and glutamine which does not occur when only one substrate is absent. Such a stimulation of energy metabolism may stimulate repair resulting in an overcompensation of cellular repair processes in an attempt to survive.

Competition between the opposing processes of repair and fixation of damage may explain certain of these effects as both processes may rely on energy supply (Jain *et al.*, 1985). Different degrees of energy depletion may result in different responses to radiation as a result of differing relative contributions of fixation of damage and repair. It may be speculated that, in those media which resulted in a left shift in the recovery curve, repair inhibition may have been of lesser importance than damage fixation. Consequently, a reduced damage fixation, and therefore, greater cell survival, may have resulted in a steeper initial slope in the recovery curve. For media where the initial slopes of recovery curves were less than that of normal medium, repair inhibition may have been of greater consequence than fixation.

All McCoy's medium variations tested failed to cause appreciable toxicity after exposure to the media alone except that medium which lacked both energy substrates and contained both inhibitors. This medium type resulted in intermitotic death of cells with time in the medium. Such a devastating effect on energy supply may result in not only an inhibition of repair but also in the inhibition of active transport and the disruption of ion homeostasis which could account for the intermitotic death.

CHAPTER 10

THE EFFECT OF 2-DEOXY-D-GLUCOSE ON TUMOUR GROWTH IN VIVO

The tumour growth delay assay is commonly used to assess response of tumours to the effects of drugs or radiation (Kallman, 1987). Cytotoxic agents including ionizing radiation can cause the death of some cells which may result in the shrinkage of tumours. The degree of damage caused by the injurious agent can be assessed by the time taken for the tumour to reach a specified size.

As was discussed in the Introduction to this Chapter, 2DG has been shown to increase the effect of radiation in various systems including certain murine tumours. It was proposed that the effect of 2DG on the growth delay after radiation be examined further in three murine tumours *in vivo* including a rhabdomyosarcoma, a fibrosarcoma and a melanoma. In the case of the fibrosarcoma, AOA was also administered.

METHODS AND MATERIALS

Animals and Tumours

Three strains of mice were used in the experiments, namely, Balb/c, WHT and C57/BL. Mice were bred in the Radiobiology SPF unit and then kept in a clean area for the duration of the experiment. Mice were 6 weeks old at the time of innoculation of tumour cells. Mice were given food and water *ad libitum* (Mouse pellets were obtained from Specialist Animal Feeds, Delft, South Africa).

A rhabdomyosarcoma was grown in the Balb/c group, the Fib/T fibrosarcoma was grown in the WHT group and B16 melanoma was grown in the C57/BL group. The rhabdomyosarcoma was originally induced in our laboratory by the sub-cutaneous administration of 3-methylcholanthrene, and subsequently maintained by serial passage in

the Balb/c strain. B16 melanomas were produced by innoculation into mice of B16 cells from *in vitro* cell culture. Details of origins, induction and inoculation of tumour lines are presented in the Appendix.

Tumours were grown in the right gastrocnemius muscles of mice. The method for passaging of tumours is outlined in the Appendix except that, in this experiment, tumours were inoculated intramuscularly. At the start of treatment, all tumours had grown sufficiently that the diameter of tumour bearing legs was 8.5 mm at their widest girth.

2-Deoxy-D-glucose

0.1 ml of a 20% (20g/100ml 0.9% (w/v) saline) 2DG solution was injected intraperitoneally 20 minutes before 25 Gy irradiation.

Jain *et al.* (1979) conducted experiments to determine the pharmacokinetics of 2DG in mice. It was found that 2DG was rapidly absorbed when administered by the intraperitoneal route. Elimination of an intraperitoneal dose (20% in saline) from the plasma was exponential with a half life of about 90 minutes. A raised blood glucose level was found in the treated group.

Aminooxyacetic acid

0.1 ml of AOA in saline, to give a final concentration in the mouse of 50 mg/kg body weight, was injected intraperitoneally immediately after administration of the 2DG dose.

Control animals were administered 0.1 ml 0.9% (w/v) saline intraperitoneally 20 minutes prior to irradiation. These mice were restrained and sham irradiated as for the test animals.

Irradiation

Tumours were irradiated with an Eldorado 6 Cobalt unit with a fixed lead collimator in a 20 cm x 20 cm field, with full back-scatter, at 80 cm SSD. Mice were restrained in a perspex (lucite) jig with tape while their

right hind tumour bearing legs were irradiated. A 5 mm thick perspex (lucite) plate, supported so that it did not press on the tumours, was placed above the tumours to achieve full build-up. The average dose rate was 0.83 Gy min^{-1}. The bodies of the mice were shielded with 10 cm thick lead blocks. Tumours were irradiated 20 minutes after administration of 2DG and AOA, when applicable.

Tumour Growth Assessment

Tumour mass was determined from tumour-bearing leg diameter measurements according to an empirical relationship established in the laboratory. Figure 10.1 illustrates this relationship. The tumour-bearing leg was assumed to have a circular cross section and its diameter was determined by fitting the tumour-bearing leg into apertures of known diameter, which had been cut into a perspex (lucite) plate. Tumour mass was determined by carefully excising the tumour and weighing it.

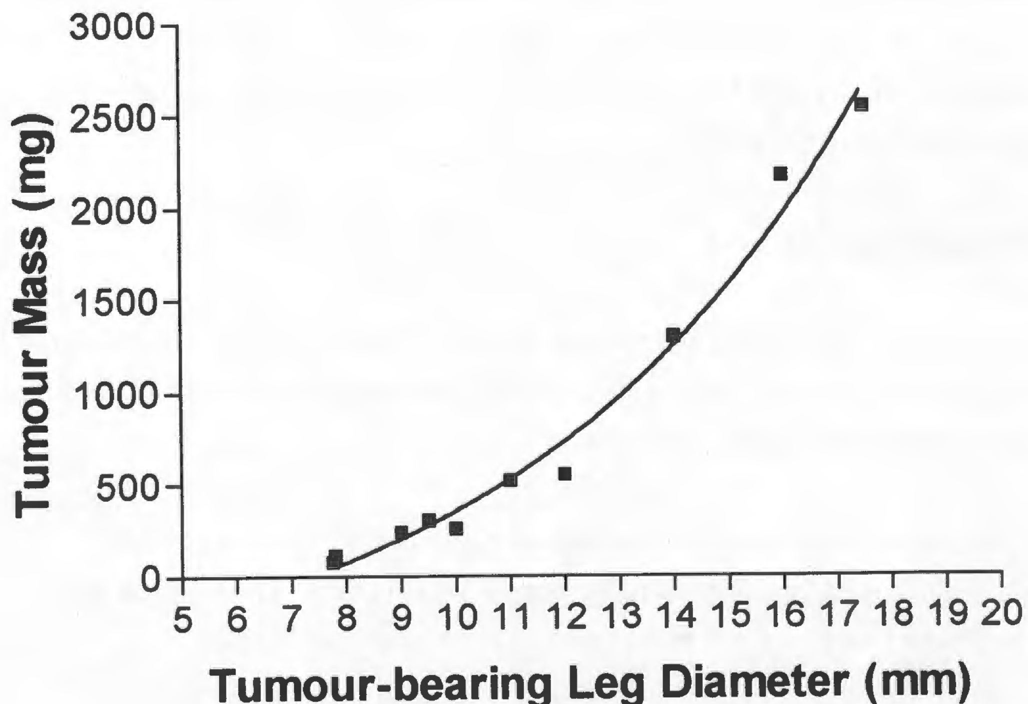


Figure 10.1: Relationship between tumour mass and tumour-bearing leg diameter.

A relationship was also established between tumour mass and tumour volume determined from measurements of displacement of water in a

measuring cylinder. Tumour mass was found to be proportional to tumour volume, as shown by the linear relationship in Figure 10.2.

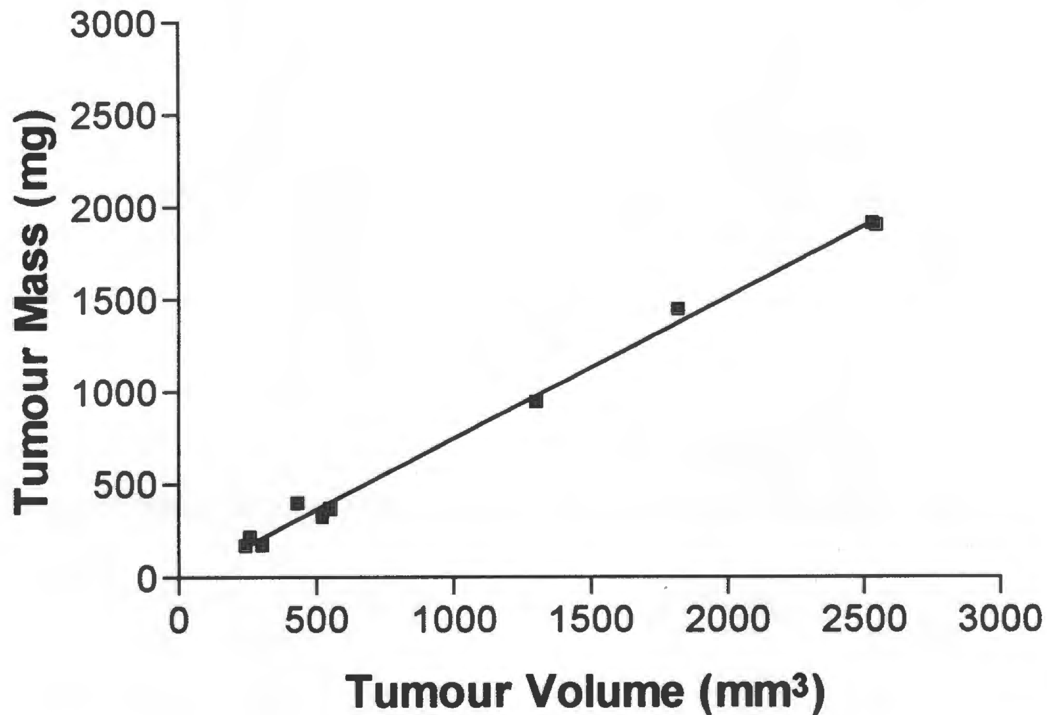


Figure 10.2: Relationship between tumour mass and tumour volume. Data were fitted by linear regression. R^2=0.99.

At the start of treatment, tumour-bearing legs had a diameter of 8.5 mm which indicated an initial tumour mass of 140 mg. After treatment, diameters of individual tumour-bearing legs were recorded over several days until tumours had reached 5 times their original mass (or volume). Response to radiation, inhibitors or a combination of both radiation and inhibitors was assessed from the time taken for tumours to reach 3 times and 5 times their initial masses respectively. To determine whether any significant difference in tumour growth times existed between groups, Student's t-test was performed. Significant difference was considered to be when $p<0.05$.

RESULTS

Table 10.1 shows the times taken for Fib/T tumours to reach volumes 3 and 5 times that of initial volumes after treatment with saline, 2DG alone or 2DG and AOA, both with and without 25 Gy radiation.

No significant difference in the time to reach either of the end points could be found between any two of the unirradiated groups though there was a trend for tumour growth to be delayed in the group treated with 2DG and AOA relative to that treated with saline. Similarly, no statistically significant difference in growth time after irradiation could be found between the group administered only saline and the group given 2DG. A significant shortening of the time to reach both end point volumes was noted in the group given both 2DG and AOA prior to radiation compared to the group which was given only saline prior to irradiation (p=0.02 and p=0.03 for the 3x and 5x end points respectively).

Table 10.1: Fib/T tumour growth time to reach specific volumes in WHT mice after systemic pretreatment with saline, 2DG or 2DG and AOA, followed by radiation. End points, 3x and 5x, refer to 3x and 5x initial tumour volume respectively. Values shown are means ± standard deviations. n=5.

	END POINT	Time after 0Gy (days)	Time after 25Gy (days)
SALINE	3x	3.16 ± 1.04	9.48 ± 1.18
	5x	6.37 ± 1.10	10.82 ± 1.55
2DG	3x	3.25 ± 0.85	10.20 ± 1.78
	5x	6.60 ± 1.22	12.46 ± 4.05
2DG + AOA	3x	4.52 ± 1.80	7.35 ± 0.77
	5x	7.13 ± 0.29	8.33 ± 0.86

The effect of a treatment relative to another can also be interpreted by comparing growth delays, that is, the differences in time for tumours to reach specific end points relative to that for a control treatment. Table 10.2 shows Fib/T tumour growth delays after different treatments relative to saline treatment.

Table 10.2: Growth delay of Fib/T tumours after different treatments relative to saline treatment.

	END POINT	Growth Delay (days)
25 Gy	3x	6.32 ± 1.57
	5x	4.45 ± 1.90
2DG	3x	0.09 ± 1.34
	5x	0.23 ± 1.64
25 Gy + 2DG	3x	7.04 ± 2.06
	5x	6.09 ± 4.20
2DG + AOA	3x	1.36 ± 2.08
	5x	0.76 ± 1.14
25 Gy + 2DG + AOA	3x	4.19 ± 1.29
	5x	1.96 ± 1.40

Table 10.3 shows the times taken for murine rhabdomyosarcomas to reach volumes 3 and 5 times that of initial volumes after treatment with saline or 2DG, both with and without 25 Gy radiation. No significant difference in tumour growth times were found between unirradiated groups administered saline or 2DG. Similarly, no significant difference could be found between irradiated groups. Table10.4 shows growth delays of Balb/c rhabdomyosarcoma after different treatments relative to saline treatment.

Table 10.3: Rhabdomyosarcoma tumour growth time to reach a specific volume in Balb/c mice after systemic pretreatment with saline or 2DG, followed by radiation. End points, 3x and 5x, refer to 3x and 5x initial tumour volume respectively. Values shown are means ± standard deviations. n=5.

	END POINT	Time after 0Gy(days)	Time after 25Gy(days)
SALINE	3x	1.39 ± 0.63	1.82 ± 0.96
	5x	2.82 ± 1.28	6.66 ± 3.19
2DG	3x	1.40 ± 0.50	1.90 ± 0.97
	5x	2.86 ± 0.75	6.72 ± 1.67

Table 10.4: Growth delay of Balb/c rhabdomyosarcomas after different treatments relative to saline treatment.

	END POINT	Growth Delay (days)
25 Gy	3x	0.43 ± 1.15
	5x	3.84 ± 3.44
2DG	3x	0.01 ± 0.80
	5x	0.04 ± 1.48
25 Gy + 2DG	3x	0.51 ± 1.16
	5x	3.90 ± 2.10

Table 10.5 shows the times taken for B16 melanomas to reach volumes 3 and 5 times that of initial volumes after treatment with saline or 2DG, both with and without 25 Gy radiation. No statistically significant difference could be found between unirradiated groups given only saline or 2DG nor could any difference be found between irradiated groups given only saline or 2DG. However, a trend existed for 2DG to delay tumour growth both in unirradiated tumours and in irradiated tumours. Table 10.6 shows growth delays of B16 melanomas after different treatments relative to saline treatment.

Table 10.5: B16 melanoma tumour growth time to reach a specific volume in C57/BL mice after systemic pretreatment with saline or 2DG, followed by radiation. End points, 3x and 5x, refer to 3x and 5x initial tumour volume respectively. Values shown are means ± standard deviations. n=5.

	END POINT	Time after 0Gy(days)	Time after 25Gy(days)
SALINE	3x	2.99 ± 0.43	10.25 ± 4.51
	5x	5.40 ± 1.19	16.56 ± 4.65
2DG	3x	4.01 ± 2.12	10.48 ± 7.19
	5x	6.46 ± 2.24	18.37 ± 2.97

Table 10.6: Growth delay of B16 melanomas after different treatments relative to saline treatment.

	END POINT	Growth delay (days)
25 Gy	3x	7.26 ± 4.53
	5x	11.16 ± 4.80
2DG	3x	1.02 ± 2.16
	5x	1.06 ± 2.54
25 Gy + 2DG	3x	7.49 ± 7.20
	5x	12.97 ± 3.20

DISCUSSION

The effects of 2DG on tumour growth in unirradiated and irradiated tumours were investigated in three murine tumours with differing growth rates. The relative growth rate of each tumour is apparent from the times taken to reach a specific endpoint size after saline treatment. The most rapidly growing tumour studied was the 3-methylcholanthrene-induced rhabdomyosarcoma, followed by the B16, while the least rapidly growing tumour was the Fib/T, as was evident from Tables 10.3, 10.5 and 10.1, respectively. In respect of radiosensitivity, as determined from the growth delays after radiation treatment alone shown in Tables 10.6 and 10.2 respectively, the B16 was the most sensitive, followed by the Fib/T. The rhabdomyosarcoma was the least radiosensitive, by having the shortest growth delay after radiation of the group, as shown in Table 10.4.

As shown in Tables 10.1 and 10.2, growth rates of unirradiated Fib/T tumours were unchanged after exposure to 2DG alone but were slightly reduced after exposure to 2DG and AOA, although the reduction was not statistically significant. Similarly, no effect on growth rate was noted for the rhabdomyosarcoma after treatment with 2DG alone, as shown in Tables 10.3 and 10.4. A slight reduction in B16 tumour growth rate was found after 2DG exposure, as shown in Tables 10.5 and 10.6, but this reduction was also not statistically significant.

2DG combined with radiation resulted in slightly greater reductions in tumour growth rate than did radiation alone in the Fib/T and the B16 tumours (5x endpoint) but not in the rhabdomyosarcoma, as shown in Tables 10.1 and 10.2; 10.3 and 10.4; and 10.5 and 10.6, respectively. However, these reductions were not found to be statistically significant. Tables 10.1 and 10.2 show that Fib/T tumour growth is delayed less after radiation when both 2DG an AOA are administered prior to radiation than when saline is given prior to radiation.

To determine whether the effects of 2DG and radiation produced additive, less than additive or greater than additive effects, growth delays, as a result of test treatments, relative to those for saline treated controls were compared. Additivity is considered to occur when the mean growth delay, after combined 2DG and radiation treatment, equals the sum of mean growth delay, after radiation alone, and that after 2DG alone. Less than additivity will occur when the combined treatment results in a lesser effect than the sum of effects for 2DG and radiation alone. For a greater than additive effect, the reverse will apply.

As shown in Table 10.2, the mean growth delay of the Fib/T tumour after 2DG and radiation appeared to be greater than additive for both tumour volume endpoints. In the case of the B16 melanoma, combined 2DG and radiation appeared to produce less than additive effects for the smaller volume endpoint and approximately additive effects for the larger endpoint, as shown in Table 10.6. For the rhabdomyosarcoma, 2DG had little effect on tumour growth rate and little effect on radiation induced growth delay.

As was discussed in Chapter 3, the extent of cellular energy supply may determine the activity of misrepair and fixation of radiation induced damage. Misrepair and fixation of radiation induced damage may be reduced as a result of reduced cellular proliferation at doses of 2DG which result in only a slight reduction in energy supply, but no inhibition of error free repair (Jain *et al.*, 1985). A reduction in misrepair and fixation with no concommitant reduction in error free repair may result in cells being protected from the effects of radiation. It may be speculated that such a situation may have existed in Fib/T tumours which were exposed to combined 2DG and AOA, but not in tumours exposed to 2DG alone. If there is reciprocal upregulation of metabolic pathways, combined effects of the glycolytic inhibitor, 2DG, and the glutaminolytic inhibitor, AOA, could result in less inhibition of energy supply in tumours exposed to both inhibitors than those exposed to 2DG alone.

2DG has been shown to protect certain normal tissues from the effects of radiation (Kalia *et al.*, 1982). Although tumour cells are the primary target of radiation, there is often a considerable amount of normal tissue present which falls within a radiation field, forming both the normal region in which the tumour grows, and the stroma, which supports tumour cells within the tumour. Growth delay is known to be influenced not only by the extent of damage to tumour cells but also to blood vessels and connective tissue. Any protective effects induced in normal tissue may, therefore, indirectly promote growth in tumour tissue after radiation. This must be considered alongside any increased damage induced in the tumour cells themselves.

In addition to the findings presented in this thesis, it has been shown previously by Jain *et al.* (1977b) that 2DG can enhance the effects of radiation in certain solid tumours *in vivo*. However, no such effects were found for the Balb/c rhabdomyosarcoma investigated in the present work. Several factors may influence tumour response to 2DG which may partially explain why different tumours respond in different ways to such treatment. These factors may include, in addition to those already mentioned above, tumour volume, hypoxic fraction, tumour blood flow, the levels of adenine nucleotides and preference of energy substrate. The

size of the tumour volume end-point used may influence the detection of growth delay effects. The pattern of growth retardation is important as perceived growth delay may be less apparent if too small an endpoint or too large an endpoint is used. The initial volume of tumours at the time of treatment is also important because blood supply may be reduced as tumours increase in size (Stanley *et al.*, 1979) and may affect both supply of drugs and possibly levels of hypoxia within the tumour. The concentrations in tumours of adenine nucleotides, including ATP, which will partly be determined by the extent of energy metabolism, can affect blood flow and so may affect the supply of drugs and the oxygen content of tumours both of which may affect the outcome of treatment. (Daly *et al.*, 1983).

As described previously, 2DG may act more effectively in hypoxic cells. This is because, at low levels of oxygen, oxidative metabolism is inhibited, thus making cells dependent on glycolysis for their energy supply, and so, also susceptible to glycolytic inhibition by 2DG. In well oxygenated cells, the extent of inhibition by 2DG may also depend on how much cells rely on glycolysis for metabolic energy and how efficiently other non glycolytic pathways may be used to process alternative energy substrates. If some tumour types have the capacity to use substrates other than glucose, such as glutamine, then it may be possible, to some extent, for cells to overcome the effects of 2DG. Tumours with larger hypoxic fractions might be expected to respond more effectively to 2DG than tumours which have smaller hypoxic fractions. The hypoxic fractions of two tumours described in the present work namely, the Balb/c rhabdomyosarcoma and the Fib/T were determined by Hendrikse (1989) to be 27% and 45% respectively. This may explain why 2DG, which often has a greater effect in hypoxic cells, seemed to enhance the effects of radiation in the more hypoxic Fib/T and not in the less hypoxic rhabdomyosarcoma. A larger effective dose of 2DG may even have been achieved in the rhabdomyosarcoma because of a greater blood supply, which was reported by Hendrikse (1989), but this may not have been sufficient to cause any noticable effects.

In summary, therefore, 2DG appeared to inhibit the growth of B16 tumours slightly. Similar inhibitory effects were apparent in the Fib/T tumour when AOA was combined with 2DG. In addition, 2DG appeared to enhance the effects of radiation in murine Fib/T and B16 tumours but not in 3-methylcholanthrene induced rhabdomyosarcomas. This may be related to the hypoxic fractions of tumours which may affect the efficacy of the action of 2DG. Although 2DG enhanced radiation effects in Fib/T and B16 tumours, the combined efffect in the former was greater than additive while, in the latter tumour, the combined effect was either additive or less than additive depending on the tumour volume endpoint. These findings suggest that 2DG may influence repair after radiation or may influence radiosensitivity. The combination of AOA, 2DG and radiation which was administered to the Fib/T tumour, was less effective than radiation alone or than 2DG combined with radiation. This may also indicate alterations in repair or radiosensitivity.

CHAPTER 11

METABOLIC CONSEQUENCES OF 2DG AND AOA ADMINISTRATION IN CELLS

The activities of glucose-using pathways, glutamine-using pathways and oxygen consumption were examined in CHO cells which were exposed to variations of McCoy's medium. The results from these metabolic studies are presented in this Chapter and will be discussed with reference to the radiation response of CHO cells in the **Summary and Conclusions** which is presented in Chapter 12. A potential criticism may be levelled at the use of the metabolic studies conducted in unirradiated cells to provide explanations for effects observed in irradiated cells. In addition to medium-induced changes to metabolism, it is possible that there may be radiation induced changes in metabolism as well. However, at the doses used and times involved, it is unlikely that energy metabolism will be greatly affected. Rotin *et al.* (1986) showed that when CHO cells were given a lethal dose of x-rays, they were able to maintain a normal rate of glycolysis for 24 hours and normal energy charge for 6 hours afterwards. Radiation did not affect lactate production.

THE EFFECT OF DEPLETING GLUCOSE AND GLUTAMINE AND THE ADDITION OF 2DG AND AOA ON LACTATE AND CO_2 PRODUCTION IN CULTURED CHINESE HAMSTER OVARY CELLS

In Chapter 9, a number of observations were made concerning the effect of variations of McCoy's culture medium which contained different combinations of 2DG, AOA, glucose depletion and glutamine depletion, on the radiosensitivity and repair capability of CHO cells. It was proposed that the effects noted in certain variations of the tissue culture media may be due to the effects of these media on the activity of the pathways of glycolysis, glutaminolysis or both.

Glycolysis and glutaminolysis have several products which may be analysed to determine the activities of such pathways. Two end products common to both glycolysis and glutaminolysis are lactate and CO_2. The flux of material through each of the pathways to each of these end products can be assessed by using ^{14}C labelled energy substrates. The amount of radiolabel detected in the end products will indicate the amount of energy substrates passing through the pathways.

The first aim of this experiment was to determine the effect of selected variations of McCoy's medium, from those listed in Table 9.1 in Chapter 9, on lactate production and CO_2 production via glycolysis and glutaminolysis. The relative activities of the each of the pathways were determined from the relative quantities of these products which originated from each of the radiolabelled glucose and glutamine energy substrates in the media. From this information, the extent by which glycolysis and glutaminolysis were affected by 2DG and AOA was determined. Another objective was to determine the effect of inhibition of glycolysis or glutaminolysis on the activity of the other.

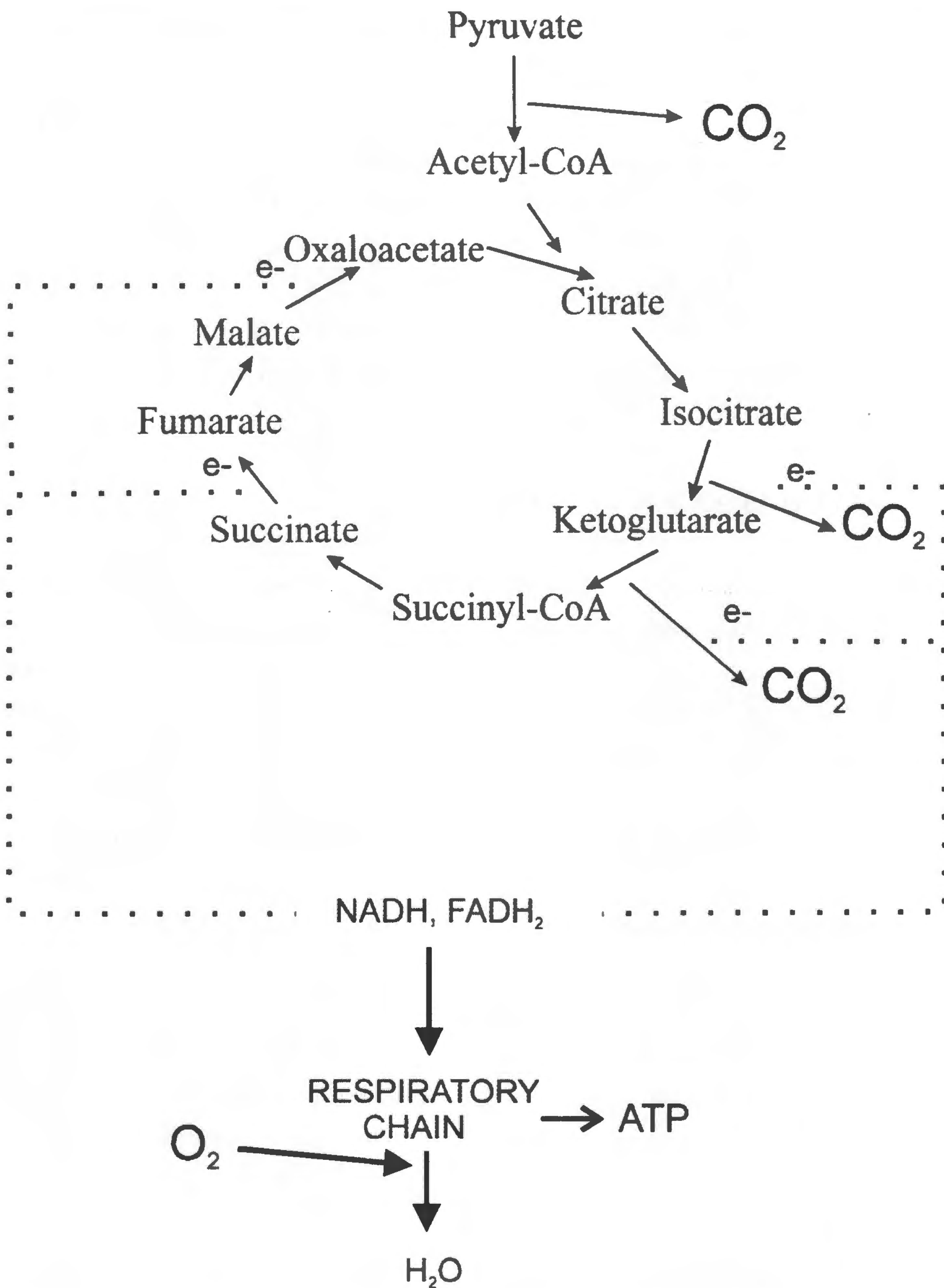


Figure 11.1: Diagram showing stages at which CO_2 is produced from pyruvate oxidation and the citric acid cycle as well as at which points electrons are taken by reducing agents, NAD and FAD .

The production of lactate from glucose and glutamine metabolism is illustrated in Figure 4.2 in Chapter 4. The stages at which CO_2 is produced from pyruvate oxidation and the citric acid cycle are indicated in Figure 11.1 above. If glucose is metabolised via the pentose phosphate pathway, CO_2 may also be produced from carbons at position 1 of glucose. Uniformly ^{14}C-labelled glucose may, therefore, result in $^{14}CO_2$ from labelled glucose metabolised by both glycolysis and pentose phosphate metabolism. However, pentose phosphate is thought to be relatively unimportant in most cells under normal conditions (Krebs, 1974).

METHODS AND MATERIALS

CHO cells were exposed to specific variations of McCoy's culture medium, which contained different combinations of 2DG, AOA, glucose depletion and glutamine depletion, in an isolated, aerobic environment. Either D[U-^{14}C] glucose or L[U-^{14}C] glutamine was added to the media to determine the extent of glucose metabolism and glutamine metabolism respectively. Total lactate, ^{14}C-lactate and $^{14}CO_2$ production by the cells were determined. Lactate from cells was purified by ether extraction, derivatized and separated on an HPLC column. The methods described for determination of lactate were modified and adapted from the methods of Simonides *et al.* (1988) and Persson *et al.* (1991). ^{14}C labelled lactate from ^{14}C-glucose or ^{14}C-glutamine was determined by scintillation counting of eluted lactate. $^{14}CO_2$ from the same media was collected in 5% (w/v) KOH and determined by scintillation counting. More detailed description of these procedures is presented below.

Apparatus

Conway units

Cells were allowed to metabolize in glass Conway dishes. As illustrated in Figure 10.2, Conway dishes have a central well ,which contained 100 μl 5% (w/v) KOH, and a peripheral compartment, which held cells suspended in 1 ml culture medium. Dishes with ground glass flanges were sealed by means of ground glass plates which were lightly smeared

with silicone grease. Gaseous exchange was able to take place freely between the two compartments of the sealed dish.

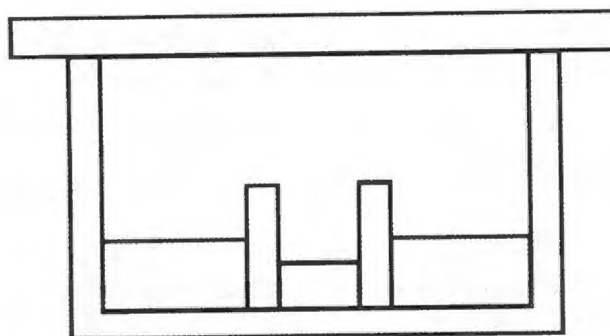


Figure 11.2: Cross section through a Conway dish showing the central well which holds KOH and outer compartment which holds cells in medium.

Scintillation counter

A Beckman LS 1801 liquid scintillation counter was used to detect counts from ^{14}C labelled products.

High pressure liquid chromatography (HPLC)

A LKB Bromma High Pressure Liquid Chromatograph (with variable wavelength detector) was used. The column used and the HPLC running specifications are recorded below.

Reagents

Reagents, including radiolabelled glucose and glutamine, and their sources are listed in the Appendix. All reagents and media were made up freshly immediately prior to assay. Preparation of McCoy's medium variations, including concentrations of 2DG and AOA, are as described for the *in vitro* experiments in Chapter 9. Concentrations of other reagents are recorded in the relevant sections below.

Procedure

CHO cells were grown to confluence in Hams F12 medium containing 10% (v/v) foetal calf serum in plastic tissue culture flasks. Cells were

trypsinized according to standard methods, as outlined in the Appendix, washed 3 times with Hanks' buffered salt solution and resuspended in McCoy's 5a medium with the required complement of glucose, glutamine and inhibitors. After counting, appropriate dilutions were made and $1 x 10^6$ cells in 1 ml medium were transferred to the peripheral compartment of a Conway dish. Uniformly labelled (U-)^{14}C-glucose (10 μCi) (50 μl of 200 μCi/ml solution) was then added to the cells in each dish where applicable. U-^{14}C-glutamine (1.5 μCi) (30 μl of 50 μCi /ml solution) was added to the cells in each dish where applicable. KOH (100 μl 5% (w/v)) was added to the central well of the Conway dish for CO_2 capture. The dishes were placed into the incubator at 37°C immediately after being sealed by placing the silicone-greased glass covers over the tops of the dishes. Cells were harvested 5 hours later. The cells, together with the medium in which they were metabolizing, were analysed for lactate as descibed below. The KOH was transfered from the central well to a 20 ml plastic scintillation vial (Beckman) containing 10 ml scintillation fluid and counted in a scintillation counter.

Preparation of cells and medium for lactate analysis

Dishes were unsealed and the medium was transferred into a polypropylene tube with screw-cap. 0.5 ml trypsin was then added to the peripheral compartment of the dish to detach any adherent cells. The dish was placed in the incubator for 5 minutes to facilitate the action of the trypsin. The dish was agitated to assist cell removal before the contents were also transferred to the tube. One millilitre 6% (v/v) perchloric acid was added to the tube to rupture the cells and to denature any enzymes present. The tubes were vortexed for 30 seconds and allowed to stand for 30 minutes before being frozen in liquid nitrogen and thawed 3 times, to ensure maximum possible cell rupture. The pH of the ruptured cell mixture was adjusted to pH 7.4 with 5 M K_2CO_3. Tubes were centrifuged at 17000 x g for 15 minutes at 4°C to separate cell debris and the $KClO_4$ precipitate from the supernatant.

Extractions

Supernatant (1 ml) was taken and added to a 20 ml glass extraction tube with a Teflon-lined screw cap. 200 µl 1N HCl was added to each sample to acidify it (pH 1.5). 10 ml diethyl ether, from 300 ml previously extracted with 50 ml 50 mM Na_2CO_3, was added to each sample, the tube caps screwed tight and the contents shaken for 10 minutes. Care was taken to release the buildup of pressure in the tubes periodically. This extraction results in the lactic acid from the aqueous phase moving into the ether layer because it is preferentially soluble in organic solvents. In addition, diethyl ether is chemically inert and so will not react with the compound of interest. The tubes were then allowed to settle for 10 minutes to allow the aqueous and ether layers to separate. The ether layer was carefully transferred, without touching the acidified aqueous layer with the pipette-tip, to fresh extraction tubes containing 1ml 2 mM Na_2CO_3 and shaken for 10 minutes. The sodium salt of lactic acid is insoluble in organic solvents and so the lactate moves into the aqueous layer. The ether layer was discarded and the aqueous samples were taken to dryness on a freeze-drier.

Derivatization with bromoacetophenone

Half a millilitre 40 mM ω-bromoacetophenone / 80 mM 18-crown-6 ether in acetonitrile was then added to each dried sample. The tube caps were screwed tight and the tubes were vortexed for 30 seconds and then boiled for 30 minutes at 100°C. Only the tube ends were submerged in the water-bath so that the upper portions of the tubes remained cooler, allowing reflux to occur. The tubes were removed from the water bath, allowed to cool to room temperature and vortexed before samples were injected into the HPLC.

High Performance Liquid Chromatography

The column used for sample separation was a Supelco 25 cm reverse phase C-18 column maintained at 50°C. The mobile phase consisted of 20% (v/v) acetonitrile in water with a flow rate of 1.2 ml/min. Five µl sample was injected on to the column. The run time for each sample was

45 minutes. Lactate was found to be eluted at approximately 11.3 minutes. Absorbance was measured with time and the chromatogram recorded. Detection was at 254 nm.

The total amount of lactate in the sample was determined by calculating the area under the lactate peak of the chromatogram and converting the values obtained to moles using a standard curve, as outlined below. Eluted lactate was collected directly in scintillation vials containing 10 ml scintillation cocktail, for detection of relative amounts of ^{14}C labelled lactate, by liquid scintillation counting.

Lactate Standard Curve

For standard curve determination, $1x10^6$ cells in normal McCoy's medium were allowed to metabolize for 5 hours, as for the experimental controls. As described above, medium from the dishes was transferred to a polypropylene tube, cells were trypsinized and also added to the tube. At this stage, standard amounts of lactate were added to the tubes. Samples were then processed as for test samples above and a standard curve of lactate plotted against area under the peak of the chromatogram corresponding to lactate. The blank values for no added lactate were subtracted. A standard curve is shown in Figure 11.3.

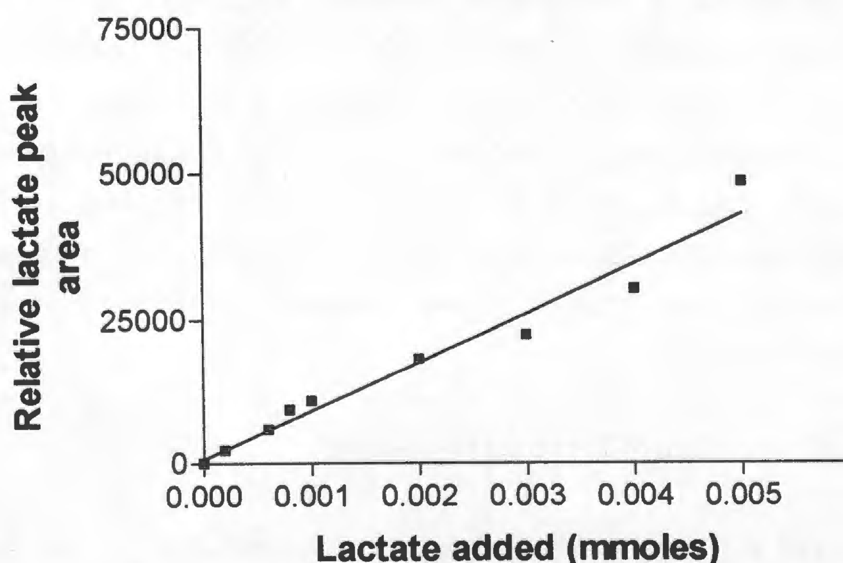
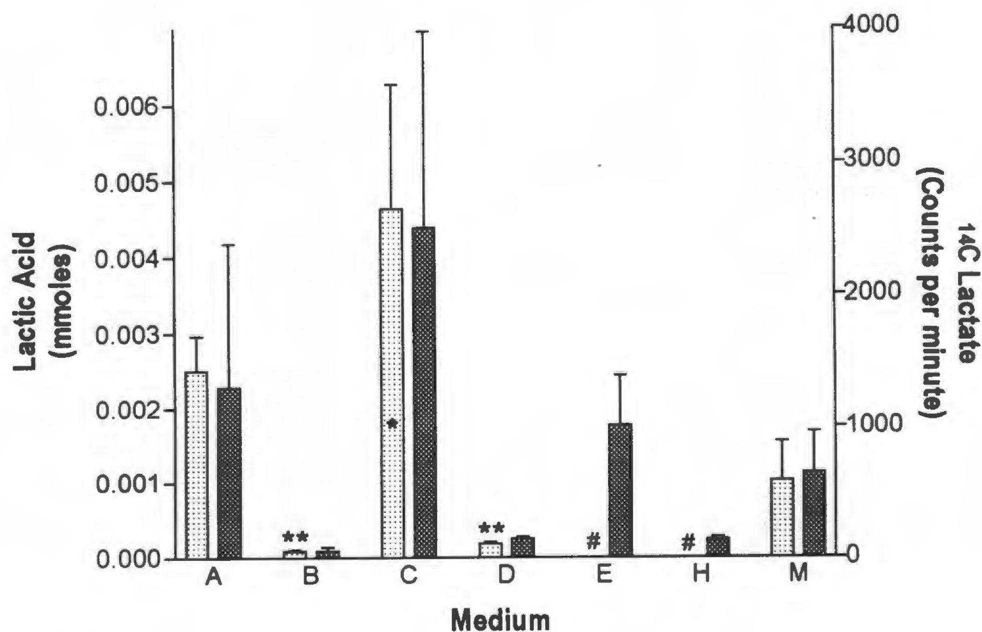


Figure 11.3: Standard curve determined for lactate added to cellular samples relative to the relative lactate peak area. Relative lactate peak area was determined from the difference between relative lactate peak area for cellular samples to which lactate was added, and the relative lactate peak area for control cellular samples. Points were fitted by linear regression. R^2=0.96.

RESULTS

Total lactate and radiolabelled lactate

Figure 11.4 shows the total amounts of lactic acid and relative amounts of ^{14}C-lactate produced by 10^6 CHO cells from ^{14}C-glucose in different variations of McCoy's medium, as listed under the Figure. The amount of labelled lactate follows a similar pattern to the total amount of lactate with respect to medium type.



A: McCoy's medium with glucose, glutamine and ^{14}C-glucose
B: McCoy's medium with glucose, glutamine, 2DG and ^{14}C-glucose
C: McCoy's medium with glucose, glutamine, AOA and ^{14}C-glucose
D: McCoy's medium with glucose, glutamine, AOA, 2DG and ^{14}C-glucose
E: McCoy's medium without glucose or glutamine, with ^{14}C-glucose
H: McCoy's medium without glucose or glutamine, with 2DG, AOA and ^{14}C-glucose
M: McCoy's medium without glutamine, with glucose and ^{14}C-glucose

Figure 11.4: Total lactate produced by $1x10^6$ CHO cells incubated in medium versions A-M shown above and the relative amount of ^{14}C labelled lactate produced by the cells from ^{14}C labelled glucose in the medium. Heights of bars represent means ± standard deviation. Lightly stippled bars relate to the left vertical axis and more densely shaded bars relate to the right vertical axis. # symbols indicate total lactate values too low to be accurately determined. For changes in values relative to those for medium A, significant difference is represented with asterisks. * represents a p-value < 0.05 and ** represents a p-value < 0.01. n=3.

Total lactate was reduced relative to normal McCoy's (medium A) in all medium types tested except C. Student's t-tests showed that these

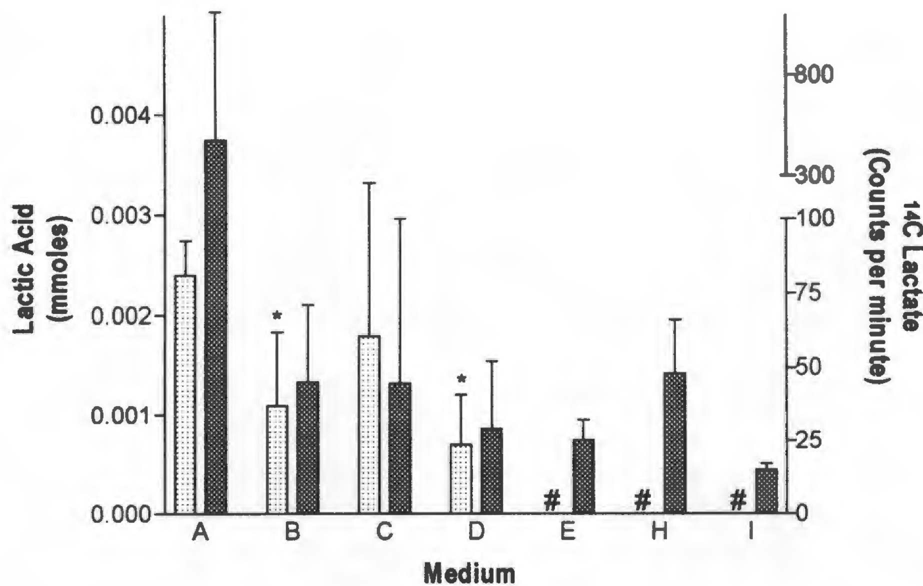
decreases were highly significant. Significant difference was considered to be when $p<0.05$. The reduction in medium M was less dramatic than for the other media tested. Media containing 2DG (media B, D and H) showed especially significant reductions in total lactate relative to that in cells in normal McCoy's medium. Total lactate produced by cells in media E and H was too low to be accurately determined and is represented by # symbols on the graph.

Radiolabelled lactate was also reduced in media containing 2DG (media B, D and H) compared to normal McCoy's medium. An increase was noted in medium C. No significant change was noted for medium E. A 50% decrease in medium M was noted. None of these differences were shown to be statistically significant. Results from media B, D and H were found to be significantly lower than those of medium E ($p=0.0123$; $p=0.0174$; $p=0.0168$ respectively). Values for medium B were significantly lower than for medium M ($p=0.0349$). Although values for media D and H were lower than M, the decreases were found to be not statistically significant ($p=0.0579$; $p=0.0553$ respectively).

Figure 11.5 shows the total amounts of lactic acid and relative amounts of ^{14}C-lactate produced by cells from ^{14}C-glutamine in different variations of McCoy's medium, as listed under the Figure.

Total lactate was reduced significantly in medium types B, D, I, E and H relative to that in normal McCoy's medium (medium A). # symbols are shown on the graph to represent lactate values which were too low to be accurately determined. No statistically significant difference could be shown between media C, B and D although the trend is a decrease from C to D.

Labelled lactate from ^{14}C-glutamine followed a similar pattern of response to that of total lactate. However, no statistically significant difference in response could be detected between ^{14}C-lactate values for any one of the media tested and normal McCoy's medium. This is largely due to the error on readings which make up the ^{14}C-lactate value for normal McCoy's medium. Nevertheless, the trend shows a larger mean labelled lactate for normal medium relative to other media tested.

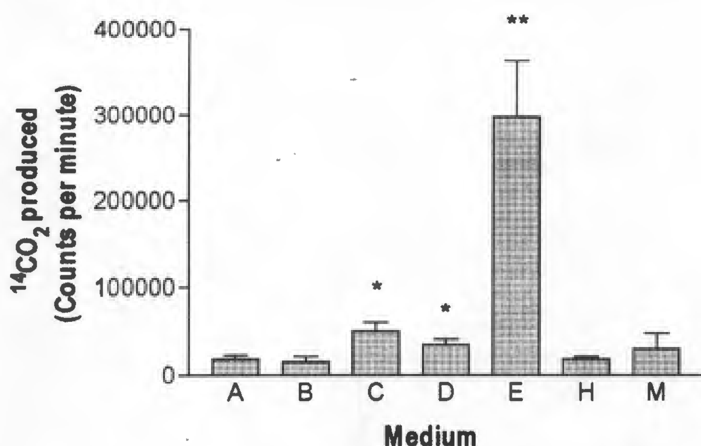


A: McCoy's medium with glucose, glutamine and ^{14}C-glutamine
B: McCoy's medium with glucose, glutamine, 2DG and ^{14}C-glutamine
C: McCoy's medium with glucose, glutamine, AOA and ^{14}C-glutamine
D: McCoy's medium with glucose, glutamine, 2DG, AOA and ^{14}C-glutamine
E: McCoy's medium without glucose or glutamine, with ^{14}C-glutamine
H: McCoy's medium without glucose or glutamine, with 2DG, AOA and ^{14}C-glutamine
I: McCoy's medium without glucose, with glutamine and ^{14}C-glutamine

Figure 11.5: Total lactate produced by $1x10^6$ CHO cells incubated in medium versions A-I shown above and the relative amount of ^{14}C labelled lactate produced by the cells from ^{14}C labelled glutamine in the medium. Heights of bars represent means ± standard deviation. Lightly stippled bars relate to the left vertical axis and more densely shaded bars relate to the right vertical axis. # symbols indicate total lactate values too low to be accurately determined. For changes in values relative to those for medium A, significant difference is represented with asterisks. * represents a p-value < 0.05. n=3.

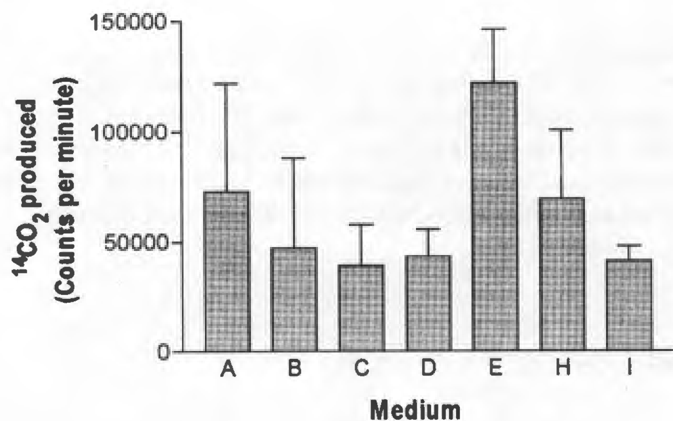
Production of radiolabelled CO_2

Figure 11.6 shows the relative amounts of $^{14}CO_2$ produced by cells in different variations of McCoy's medium containing ^{14}C-glucose, as listed under the Figure. Cells in media C, D and E are shown to produce statistically greater amounts of labelled CO_2 relative to cells in normal McCoy's medium (medium A). Medium E gave a greater than 6 fold increase in mean value over any of the other media tested. The differences were statistically significant, with $p<0.003$ for all cases.



A: McCoy's medium with glucose, glutamine and 14C-glucose
B: McCoy's medium with glucose, glutamine, 2DG and 14C-glucose
C: McCoy's medium with glucose, glutamine, AOA and 14C-glucose
D: McCoy's medium with glucose, glutamine, 2DG, AOA and 14Cglucose
E: McCoy's medium without glucose or glutamine, with 14Cglucose
H: McCoy's medium without glucose or glutamine, with 2DG, AOA and 14Cglucose
M: McCoy's medium without glutamine, with glucose and 14Cglucose

Figure 11.6: Relative amounts of $^{14}CO_2$ produced by 1x10^6 CHO cells in media A-M from ^{14}C labelled glucose. Heights of bars represent means ± standard deviations. n=3. For changes in values relative to those for medium A, significant difference is represented with asterisks. * represents a p-value < 0.05 and ** represents a p-value < 0.01.



A: McCoy's medium with glucose, glutamine and 14C-glutamine
B: McCoy's medium with glucose, glutamine, 2DG, 14C-glutamine
C: McCoy's medium with glucose, glutamine, AOA and 14C-glutamine
D: McCoy's medium with glucose, glutamine, 2DG, AOA and 14C-glutamine
E: McCoy's medium without glucose or no glutamine, with 14C-glutamine
H: McCoy's medium without glucose or no glutamine, with 2DG, AOA and 14C-glutamine
I: McCoy's medium without glucose, with glutamine and 14C-glutamine

Figure 11.7: Relative amounts of $^{14}CO_2$ produced by 1x10^6 CHO cells in media A-I from ^{14}C labelled glutamine. Heights of bars represent means ± standard deviations. n=3.

Figure 11.7 shows the relative amounts of radiolabelled CO_2 produced by cells in different variations of McCoy's medium containing ^{14}C-glutamine, as listed under the Figure.

No statistically significant difference could be detected between the amount of labelled CO_2 formed by the cells in normal McCoy's (medium A) and that in any other medium tested. However, there was a slight non-significant increase in $^{14}CO_2$ production in medium E above that of normal medium. Media C to I yielded non-significant reductions in CO_2 production relative to normal medium. Production of $^{14}CO_2$ by cells in medium E was significantly increased above those for cells in media C, D and I respectively ($p=0.0001$, $p=0.0075$, $p=0.0052$ respectively).

A glucose molecule contains 6 carbon atoms and a glutamine molecule contains 5 carbons. It is these carbons which were labelled in the radiolabelled glucose and glutamine tracers added to the media. Detection of radiolabelled carbons in lactate and CO_2 from those originating in radiolabelled glucose or glutamine indicates that the substrate has been metabolised to these products. The number of labelled carbon atoms in labelled products could be determined from the radioactivity of samples, using the known specific activities of U-^{14}C-glucose and U-^{14}C-glutamine. Therefore, to determine the extent of glycolytic activity, the number of glucose molecules which were completely metabolised to CO_2 and lactate was determined. This was done by determining the radiolabelled carbon content of the products and determining how many glucose carbon skeletons this represented. Lactate contains 3 carbons while CO_2 contains only one. Therefore, every 6 radiolabelled carbons detected in lactate and CO_2 would represent a completely metabolised glucose molecule. Similarly, because glutamine contains 5 carbons, every 5 radiolabelled carbons detected in lactate and CO_2 would represent a completely metabolised glutamine molecule. Thus, the activity of glutaminolysis could be determined from the number of glutamine molecules being completely metabolised to lactate and CO_2.

Presented in Table 11.1 are values which indicate the amounts of ^{14}C-lactate and $^{14}CO_2$ produced from ^{14}C-glucose. Also shown are the

Table 11.1: Amounts of ^{14}C-labelled lactate and CO_2 produced from ^{14}C-glucose by 10^6 cells in media defined under Figures 10.4. Values are means ± standard deviations.

MEDIUM	Lactate (nmol /h/10^6 cells)	CO_2 (nmol /h/10^6 cells)	Lactate/CO_2	Amount of glucose completely metabolised to lactate and CO_2 (nmol/h/10^6 cells)
A	0.20 ± 0.17	0.03 ± 0.007	6.67 ± 5.88	0.11 ± 0.09
B	0.007 ± 0.005	0.06 ± 0.01	0.12 ± 0.08	0.01 ± 0.003
C	0.39 ± 0.23	0.09 ± 0.02	4.33 ± 2.73	0.21 ± 0.12
D	0.02 ± 0.002	0.06 ± 0.03	0.33 ± 0.17	0.02 ± 0.005
E	0.16 ± 0.06	0.55 ± 0.12	0.29 ± 0.13	0.17 ± 0.04
H	0.02 ± 0.003	0.03 ± 0.005	0.67 ± 0.15	0.02 ± 0.015
M	0.10 ± 0.05	0.02 ± 0.008	5.00 ± 3.20	0.05 ± 0.025

lactate/CO_2 ratio and the number of molecules of radiolabelled glucose which were completely metabolised to CO_2 and lactate. The lactate/CO_2 ratio was reduced for cells in media C to H relative to that for cells in normal medium. Moderate decreases of 35% and 25% ocurred for media C and M respectively, while more substantial decreases all of which were greater than 89% occurred for media B, D, E and H. The number of radiolabelled glucose molecules completely metabolised to lactate and CO_2 was reduced in media M, B, D and H and raised in media E and C relative to that in normal medium.

Table 11.2 contains values which indicate the amounts of ^{14}C-lactate and $^{14}CO_2$ produced from ^{14}C-glutamine. Lactate/CO_2 ratio and the number of glutamine molecules completely metabolised to lactate and CO_2 are also shown. The lactate/CO_2 ratio was reduced for media C to H relative to that for normal medium. The number of radiolabelled glutamine molecules completely metabolised to lactate and CO_2 was reduced in media C to H relative to that in normal medium. Media C, B, D and I yielded similar values.

DISCUSSION

Lactate and CO_2 are products of glycolysis and glutaminolysis and, therefore, the metabolism of ^{14}C-glucose or ^{14}C-glutamine via these pathways will result in the production of ^{14}C-lactate and $^{14}CO_2$. If the

Table 11.2: Amounts of ^{14}C-labelled lactate and CO_2 produced from ^{14}C-glutamine 10^6 cells in media A to G defined under Figures 10.5 . Values are means ± standard deviations.

MEDIUM	Lactate (nmol /h/10^6 cells)	CO_2 (nmol /h/10^6 cells)	Lactate/CO_2	Amount of glutamine completely metabolised to lactate and CO_2 (nmol/h/10^6 cells)
A	0.07 ± 0.09	0.12 ± 008	0.58 ± 0.75	0.07 ± 0.06
B	0.006 ± 0.003	0.08 ± 0.07	0.09 ± 0.07	0.02 ± 0.014
C	0.007 ± 0.009	0.07 ± 0.03	0.10 ± 0.04	0.02 ± 0.009
D	0.004 ± 0.003	0.08 ± 0.02	0.05 ± 0.04	0.02 ± 0.005
E	0.003 ± 0.0008	0.20 ± 0.04	0.02 ± 0.007	0.04 ± 0.008
H	0.007 ± 0.003	0.11 ± 0.05	0.06 ± 0.03	0.03 ± 0.01
I	0.002 ± 0.0003	0.07 ± 0.01	0.03 ± 0.004	0.02 ± 0.002

citric acid cycle is inhibited by, for example, hypoxia, or if glycolysis is functioning at a sufficient rate that there is saturation of pyruvate oxidation, then there will be a diversion of glycolytic flux into lactate. Under such conditions, any change in the rate of glycolysis may affect the amount of lactate produced. Hence, lactate production from glucose is sometimes used to determine the extent of glycolysis (Varnes *et al.*, 1984). If glycolysis is functioning at sufficiently low levels that pyruvate oxidation is operating at less than saturation, and the citric acid cycle is not inhibited, then less lactate will tend to accumulate. Under such conditions, more glycolytic flux will be diverted towards the citric acid cycle and ultimately CO_2. Hence, in order to estimate the activity of glycolysis, it may be necessary to detect glycolytic flux to each of the two products, lactate and CO_2. The same argument applies to the estimation of activity of glutaminolysis since both lactate and CO_2 are products of this pathway too.

Glucose may be metabolised by pathways other than glycolysis such as the pentose phosphate pathway (PPP), which can also be used under conditions of energy demand to provide cellular energy. CO_2 is also a product of the pentose phosphate pathway. The carbon atom which is incorporated into this CO_2 is the carbon atom in position 1 of glucose. In the case of radiolabelled tracer experiments presented in this Chapter, uniformly labelled ^{14}C-glucose was used which means that it is possible that some CO_2 detected may have resulted from the activity of this

pathway. However, as was mentioned above, the pentose phosphate pathway is thought to be relatively unimportant in most cells under normal conditions (Krebs, 1974). Glycolysis and the pentose phosphate pathway have some intermediates in common, so under conditions of energy demand, carbons (other than those from position 1) from glucose that is metabolised via the pentose phosphate pathway will enter glycolysis and may eventually be released as CO_2 from the citric acid cycle or may be incorporated into lactate.

The determination of radiolabelled products from radiolabelled energy substrates will reflect both the efficiency of uptake of substrate by the cell and the activity of the metabolic pathways involved. Within the cell, 2DG competes with glucose for binding to the active sites on the hexokinase enzyme. Both 2DG and glucose are phosphorylated by this enzyme to produce 2DG-6-phosphate and glucose-6-phosphate respectively. 2DG-6-phosphate and glucose-6-phosphate then compete for binding to the active sites of glucose phosphate isomerase. 2DG-6-phosphate cannot be metabolised by glucose phosphate isomerase and blocks the metabolism of glucose-6-phosphate thus blocking glycolysis. In addition to enzymatic competition, 2DG competes with glucose for uptake into the cell by competing for the same facilitated diffusion receptor. 2DG thus impedes the uptake of glucose and dilutes the effective concentration of glucose outside the cell. In the experiments presented, the concentration of glucose relative to 2DG in the media used was 0.0158 mmol/ml to 0.01 mmol/ml, that is, a ratio of 1.58:1. This might be expected to result in glucose uptake being reduced to 0.0158/(0.0158+0.01), that is, 61.2% of the uptake expected if glucose was present alone.

Similarly, the amount of ^{14}C-glucose taken up will depend on the concentration of other similar substrate present. When glucose or 2DG is present, less ^{14}C-glucose will be taken up. When both glucose and 2DG are present, even less will be taken up. When the small amount of the radiolabelled tracer is added (to make a concentration of 34.1 nmol/ml) to medium which contains no glucose or 2DG, all the glucose taken up will be radiolabelled. However, the rate of facilitated diffusion depends on the concentration of substrate. Therefore, it is possible that,

for relatively high concentrations of glucose and 2DG, facilitated transport was working at a greater rate and that, at the low concentration of 34.1 nmol/ml, the transport system was working at a lower rate. Consequently, less efficient cellular uptake of radiolabelled glucose may have resulted when the substrate concentration was low.

Total lactate and labelled lactate

As shown in Figures 11.4 and 11.5, CHO cells in normal McCoy's medium containing a full complement of glucose and glutamine were able to produce significant amounts of lactic acid under aerobic conditions. As shown in Figure 11.4, this ability was reduced in medium which lacked glutamine (medium M), in medium which was deficient in glucose and glutamine (medium E) and in those media which contained 2DG (media B, D and H) regardless of the presence or absence of glucose, glutamine or AOA. The most marked reductions in lactate production were achieved in those media which may be proposed as being particularly 'severe', namely, medium H, which lacked both glucose and glutamine and contained both inhibitors, 2DG and AOA, and also medium E, which lacked glucose and glutamine but from which inhibitors were omitted. It is apparent that AOA in the presence of glucose and glutamine (medium C) was unable statistically significantly to affect the cells' capacity to produce lactate. Nevertheless, a non-statistical increase in lactate was shown in Figure 11.4 for this medium while a non-significant decrease was shown in Figure 11.5 for the same medium containing additional trace quantities of labelled glutamine instead of labelled glucose. As shown in Figure 11.5, medium I, which contained glutamine but lacked glucose, resulted in a reduction in total lactate relative to normal medium. Other media which were similar to those represented in Figure 11.4, except that labelled glutamine tracer was substituted for the labelled glucose tracer, resulted in a similar trend in that total lactate was reduced in a similar manner, with the excetion of medium C which was discussed above.

It is apparent from Figure 11.4 and Table 11.1 that the profile of relative production of radiolabelled lactate from radiolabelled glucose for different media is similar to the profile of total lactate production.

However, a statistically significant reduction in labelled lactate from glucose relative to that from cells in normal medium could be detected only in media which contained the glycolytic inhibitor, 2DG (media B, D and H). Medium lacking glutamine but containing glucose (medium M) resulted in a 50% reduction in labelled lactate, relative to normal medium but this reduction was not statistically significant. It was expected that medium C and medium M might have similar effects on lactate levels because, in both these medium types, glucose was present and glutamine usage was prevented. However, medium C resulted in an increase in labelled lactate relative to that for cells in normal medium. Cells in medium E, which contained no glucose and no glutamine, produced labelled lactate levels similar to but slightly less than those for cells in normal medium. Media H and E were similar in that they both lacked both substrates but medium H also contained 2DG and AOA. Both medium types might, therefore, have been expected to result in similar inhibition of both glucose- and glutamine-using pathways. Medium H, however, resulted in much less labelled lactate than did medium E, possibly as a result of more efficient inhibition by the presence of AOA and 2DG. Medium D, which also contained 2DG and AOA but had normal glucose and glutamine levels, resulted in a similar level of labelled lactate as medium H. It would seem, therefore, that the presence of inhibitors is more important than the lack of energy substrates with respect to depression of labelled lactate from glucose.

As shown in Figure 11.5 and Table 11.2, a reduction in the amounts of labelled lactate from labelled glutamine was apparent for cells in all medium types C to H relative to that for cells in normal medium (medium A). Both the presence of AOA or 2DG in normal medium resulted in a reduction in the amount of labelled lactate produced from glutamine, as seen for media C and B in Figure 11.5 and Table 11.2. The greatest reduction in labelled lactate of the group was for medium I, which lacked glucose. The supression of lactate production from glutamine seemed to be less for medium H, which lacked glucose and glutamine and contained both inhibitors, than for medium E, which only lacked glucose and glutamine. Medium D, which contained glucose, glutamine and both inhibitors resulted in a slightly greater suppression of lactate from glutamine than medium H.

Production of labelled CO_2

As shown in Figure 11.6, CO_2 production from glucose by cells in medium C, which contained AOA, was stimulated relative to that for cells in normal medium. CO_2 production by cells in medium D, which contained glucose, glutamine, AOA and 2DG, was approximately double that of normal medium. A large increase in CO_2 production from glucose relative to that for cells in normal medium was also recorded for cells in medium E, which contained no glucose or glutamine (except for trace quantities of ^{14}C-glucose for the assay). This increase in CO_2 production was mirrored by an increase in ^{14}C-lactate production shown for medium E in Figure11.4. Both of these increases may have resulted from an accelerated uptake of radiolabelled glucose possibly due to stimulation of glucose-starved but uninhibited pathways. There was no difference between the extent of CO_2 production from glucose in normal medium and that in medium lacking glucose and glutamine and containing 2DG and AOA (medium H).

It is apparent from Figure 11.7 that cells in medium H, which lacked glucose and glutamine and containing both inhibitors, produced a similar amount of CO_2 from glutamine compared to cells in normal medium. Normal media to which AOA (medium C), 2DG (medium B), or both AOA and 2DG were added resulted in reductions in CO_2 production from glutamine relative to CO_2 produced from glutamine by cells in normal medium. A similar reduction was noted for medium I, which lacked glucose. An increase in CO_2 production from glutamine was found for cells in medium E, which lacked glucose and glutamine (except trace amounts of labelled glutamine used for assay), relative to that for cells in normal medium.

The amount of labelled lactate compared to the amount of labelled CO_2 produced by cells from labelled substrate in the same medium was calculated using the detected activities of the products and specific activities of the labelled substrates. Results of these calculations are shown in Table 11.1 for products from glucose and in Table 11.2 for products from glutamine. Also shown in the Tables are molar lactate/

CO_2 ratios. The lactate/CO_2 ratio gives an indication of the relative number of moles of lactate produced relative to the number of carbon dioxide molecules produced. Any change in this ratio will indicate a change in the molar yield of one product relative to the other and thus indicate a diversion of flux from one product to another. If the lactate/CO_2 ratio increases, this may indicate a relative diversion of flux of material into lactate and away from CO_2. If the ratio decreases, it may indicate a relative diversion of flux away from lactate and towards CO_2. Changes in the lactate/CO_2 molar ratio may give evidence regarding alterations in regulation of pathways and saturation of pyruvate oxidation. Also shown in Table 11.1 and Table 11.2 are the number of molecules of radiolabelled-glucose and -glutamine respectively which were completely metabolised to lactate and CO_2 . The activities of glycolysis/PPP and glutaminolysis were estimated in this way.

As shown in Table 11.1, media C to H resulted in reductions in lactate/CO_2 relative to that for normal medium. This may suggest a diversion of glycolytic/PPP flux away from lactate towards CO_2 in cells exposed to these medium types. The number of labelled glucose molecules completely metabolised to lactate and CO_2 was found to be reduced in media M, B, D and H relative to that for normal medium, as also shown in Table 11.1. These reductions suggested that glycolytic/PPP activity was reduced relative to that in normal cells in cells exposed to media which contained both 2DG and AOA (media D and H), medium containing 2DG (medium B) and in medium lacking glutamine (medium M). From the results, it would seem that glycolysis/PPP was most reduced in medium B. Since the reduction in glycolytic/PPP activity was less when AOA was added to medium B (making it medium D), it would seem that AOA partly counteracted the effect of the medium. Medium C, which contained AOA resulted in an increase in the number of molecules of labelled glucose that were metabolised to lactate and CO_2 relative to that in normal medium suggesting an increase in glycolytic/PPP activity. This is consistent with the concept of reciprocal regulation as medium C (also called medium C in Table 11.2) seemed to result in the inhibition of glutaminolysis as well. The inhibition of glutaminolysis may, therefore, have resulted in an

upregulation of glycolysis/PPP. Medium F also seemed to result in an increased glycolytic/PPP activity relative to that in normal medium. Such an increase in activity may be explained as a compensatory increase in uptake and metabolism of labelled glucose as a result of substrate starvation of the cell.

Table 11.2 shows the amounts of labelled lactate and CO_2 from labelled glutamine. Lactate/CO_2 ratios for cells in media C to H were reduced relative to that for normal medium. This suggests a diversion of flux away from lactate towards CO_2 in each of these medium types. From the number of labelled glutamine molecules completely metabolised to lactate and CO_2, it would seem that glutaminolysis is inhibited in cells in media C to H relative to that in cells in normal medium. Similar glutaminolytic activities resulted from medium containing AOA (medium C), medium containing 2DG (medium B) medium lacking glucose (medium I) and medium containing both inhibitors and glucose and glutamine (medium D). Since the presence of 2DG alone in medium seemed to result in a depression of glutaminolysis and glycolysis relative to that in normal medium, it is possible that reciprocal down regulation of glutaminolysis by an inhibited glycolytic/PPP pathway may have occurred. Therefore, while the inhibition of glutaminolysis may result in the stimulation of glycolysis, the reverse does not seem to hold. A depression of glutaminolysis by the depression of glycolysis may be substrate quantity related in that pathways may rely on inputs from other pathways in order to function. For instance, a sufficient quantity of pyruvate from glycolysis may have to be available for oxidation to maintain the activity of the citric acid cycle and glutaminolysis. It is also possible that circumstances which prevent glycolysis may also prevent glutaminolysis independently.

From the discussion above, it is apparent that cellular lactate/CO_2 ratios for labelled glucose and labelled glutamine were found to be reduced for all media relative to those for normal media, that is a shift in relative flux from lactate to CO_2. Since cells in all media tested also exhibited an inhibition of either glycolysis/PPP or glutaminolysis or both, it seems that interference with glycolysis/PPP or glutaminolysis may result in cells favouring citric acid cycle metabolism. Under conditions which

limit the activity of energy supplying pathways, cells may favour citric acid cycle metabolism and consequent oxidative phosphorylation which is more efficient in providing ATP than glycolysis or glutaminolysis alone.

It has been assumed that, over the 5 hour period during which products of the above pathways accumulated, the activity and regulation of pathways remained constant. It is, however, possible that a number of changes in respect of these factors may have taken place during the 5 hour period and that some of these changes were not resolved owing to the cumulative nature of the results. Different emphasis may be placed on the alternative routes for flux of metabolic material by the cell depending on the demands for energy and the capacity of the pathways involved. The pathways of glycolysis/PPP and glutaminolysis, as mentioned above, have some products in common, namely, lactate and CO_2. It is, therefore, conceivable that the regulation of the activity of one of the pathways will affect the other in some way and that there may be inter-regulation and changing rates of formation of products over an extended period which will not be apparent when considering levels of products after shorter periods.

THE EFFECT OF DEPLETING GLUCOSE AND GLUTAMINE AND THE ADDITION OF INHIBITORS OF GLYCOLYSIS AND GLUTAMINOLYSIS ON OXYGEN CONSUMPTION

The major portion of molecular oxygen consumed by the cell is used for oxidative phosphorylation. The rate of oxygen consumption can be used as a gauge to assess the activity of oxidative phosphorylation. The relative oxidative ATP producing capacity of the cell from all major sources including amino acids, sugars and fatty acids, can, therefore, be indirectly determined.

The aim of the set of experiments described below was to determine the oxygen consumption of CHO cells exposed to different variations of McCoy's medium to evaluate the activity of respiration in these cells.

METHODS AND MATERIALS

Oxygen measurements were made using a chemical microsensor together with a Clark oxygen probe (Diamond Electro-Tech, USA).

The electrode

Polarographic Oxymetry of the bipolar electrode

The polarographic oxygen electrode (Clark electrode), as shown in Figure 11.8, is operated so that the cathode, which consists of a platinum wire, is maintained with a negative potential of 0.75 volts. The external silver/silver chloride anode cylinder is separated from the platinum wire by an epoxy layer.

The tips of the electrodes are in an electrolyte solution (saturated KCl) into which the oxygen to be measured diffuses via a membrane. A current flows when the oxygen is chemically reduced at the cathode surface ($O_2 + 2H_2O + 4e \rightarrow 4OH^-$). Chloride ions from the electrolyte

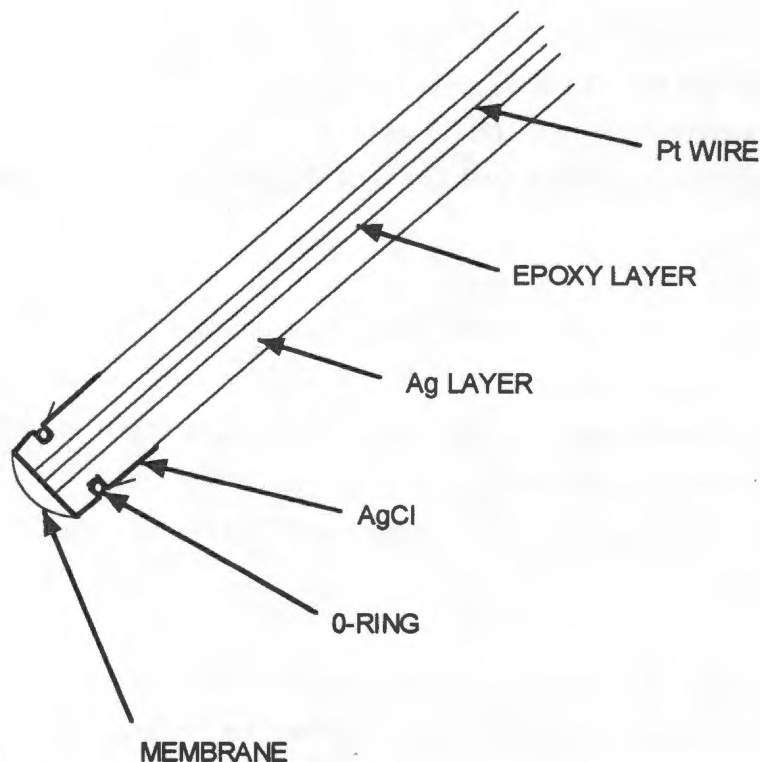


Figure 11.8 : Cross section through the oxygen electrode.

concurrently react with the silver forming more silver chloride ($4Ag^+$ + $4Cl^- \rightarrow$ 4AgCl + 4e-). The probe current is linearly related to pO_2 as the cathode reaction is determined by the membrane being permeable solely to oxygen. The membrane protects the electrodes from chemical and electrical interference from medium, cells or other biological components.

Prior to operation, the silver part (anode) of the probe was 'chloridised' by dipping the end of the probe into 4 M KCl while applying a potential of 1.5 V across the anode and the cathode of the probe so that a coating of chloride ions formed on the anode. The tip of the probe was then dipped into saturated KCl electrolyte solution to collect a drop of electrolyte solution on the tip before a membrane was fitted and held in place over the tip of the probe by a rubber O-ring, which fitted into a groove situated a short distance from the end of the probe. Care was taken not to damage the AgCl layer or to allow air bubbles to get beneath the membrane as this lead to malfunctioning of the probe. Periodically, the tip had to be rejuvenated by cleaning with ammonia water, and rechloridising. The probe had to be stabilised prior to use by

allowing it to stand in saline or other calibrating media for several hours before taking measurements.

Oxygen Chamber

A closed system for measurement of oxygen was used. The electrode was positioned through the centre of a rubber stopper which fitted snugly into the neck of a glass chamber, as illustrated in Figure 10.9. The chamber was filled with medium. To allow the air above the medium to be displaced when the stopper was inserted, a syringe needle connected to a syringe was inserted through the stopper and removed once the stopper was in place, that is, flush with the medium surface. In order that any small bubbles containing O_2 that might be trapped in the system should not influence the oxygen content of the medium during oxygen measurements, the chamber and stopper were assembled whilst under a plastic sheeting tent which was filled with nitrogen gas. Any bubbles would then be nitrogen bubbles.

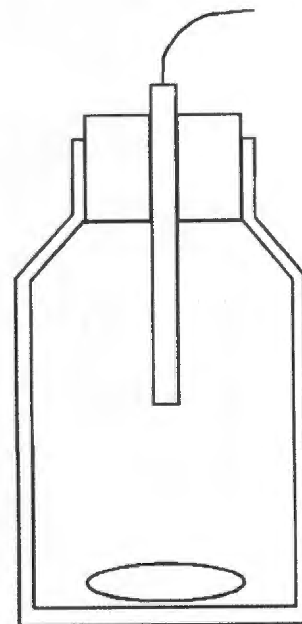


Figure 11.9: Diagram of chamber for measuring oxygen consumption showing position of probe and stirrer magnet (Not to scale). The height of the vial was 5 cm with a base diameter of 2 cm.

The chamber was kept at 37°C in a water bath with a water circulator. Adequate mixing inside the chamber was achieved by means of a small stirrer magnet.

Testing the system

Readings were collected using the chemical microsensor and an additional amplifier (shown in Figure 11.11) constructed in the laboratory to amplify the voltage output of the microsensor so that voltage readings could be recorded via an analogue to digital converter on a personal computer. Voltage readings were initially recorded using the computer program, Data Acquisition Pack (version 2.06), and then imported into Microsoft Excel where regression analysis was conducted.

The apparatus was tested with normal McCoy's medium without cells present and was found to maintain a steady state, that is, minimal voltage drift. A small amount of drift occurred over extended periods of use but the effect of this was minimised by sufficient stabilisation times prior to use and calibration before every individual sample measurement.

As shown in Figure 11.10, a test run with cells in medium containing glucose and glutamine in the chamber yielded relative oxygen values with time in the medium. Initially oxygen readings increased reaching a maximum after 2.5 minutes as the electrode adjusted to the increased temperature (37°C) and stirring of the sample. Thereafter, a steady decrease in relative oxygen content was recorded with time. Rate of oxygen consumption was determined by the slope of the best-fit straight line of the trace after the initial increased reading due to increased temperature and motion. Upon addition of a small volume of an uncoupler of oxidative phosphorylation, sodium azide, to a concentration of 1.66 mM in the chamber, the decrease in relative oxygen was shown to level off as illustrated in Figure 10.10. This indicated that voltage change reflected oxygen consumption.

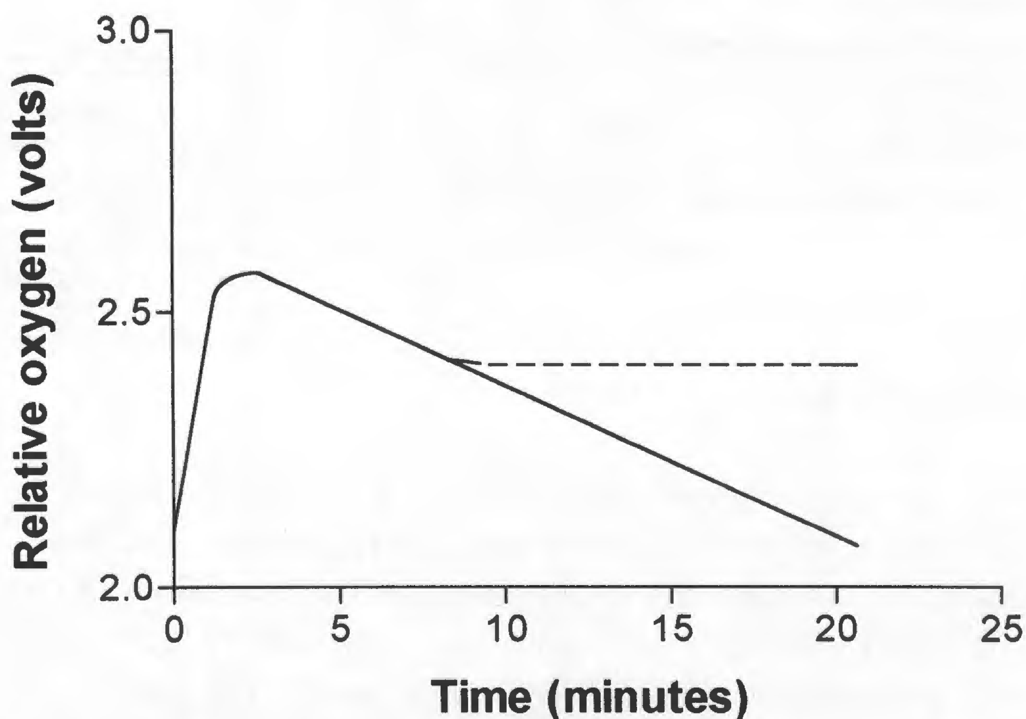


Figure 11.10 : Oxygen consumption curve for CHO cells in normal McCoy's medium. The dashed line shows how the consumption curve levels after the addition of azide to the system. The initial rise in the curve represents the period of adjustment of the probe to the temperature and motion of the medium in the chamber.

The probe electrode was calibrated immediately prior to reading each oxygen consumption curve by bubbling air, to achieve 21% O_2, or N_2, to achieve 0% O_2, through a cell-free mixture for several minutes. The range from 0 to 21% O_2 was represented by a 2.56 V difference in output.

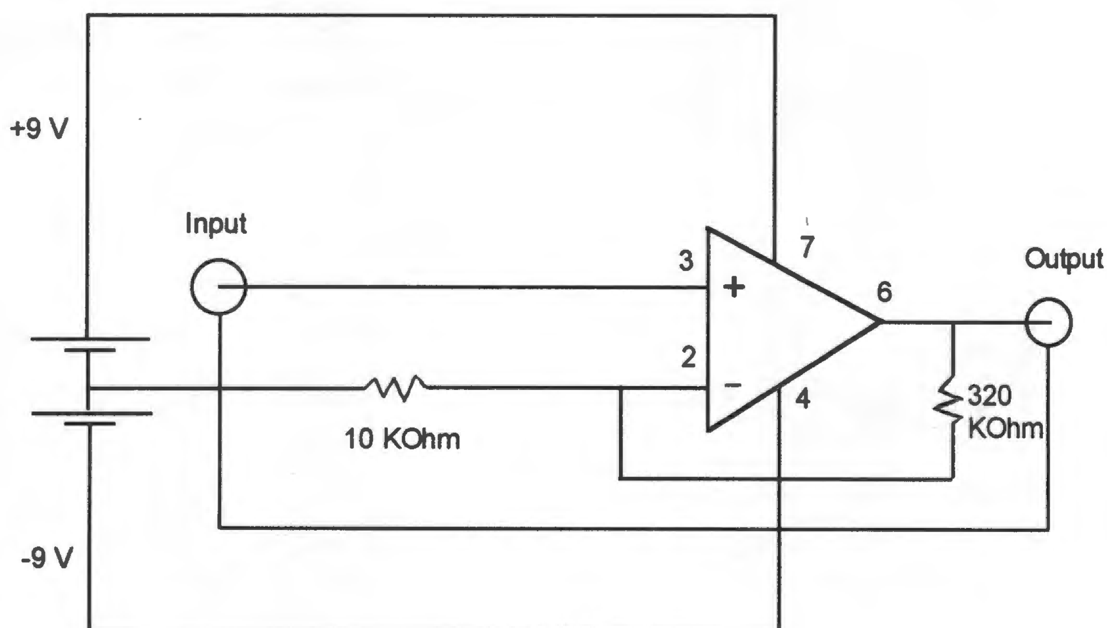


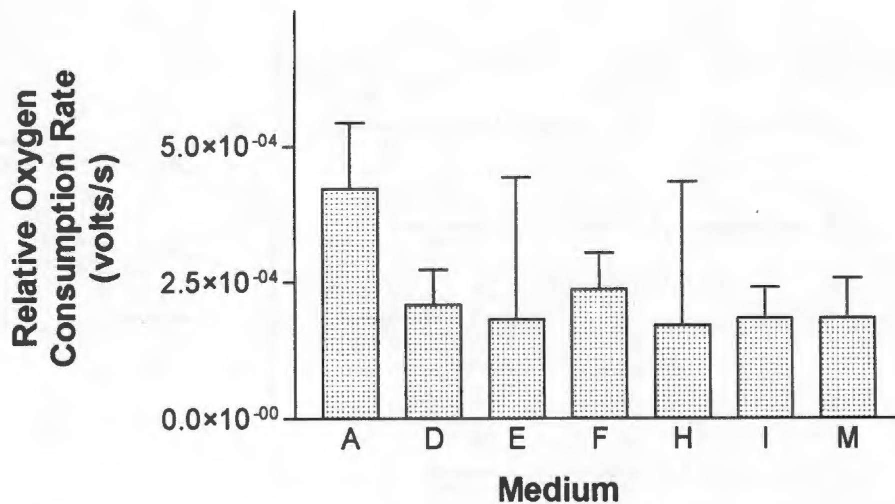
Figure 11.11: Amplifier constructed to amplify signal from microsensor so that data could be recorded on a personal computer.

Experimental Procedure

CHO cells were grown to confluence in plastic tissue culture flasks in Hams F12 medium containing 10% (v/v) foetal calf serum and antibiotics (50 mg/l penicillin, 50 mg/l neomycin, 50 mg/l streptomycin). Cells were trypsinised, centrifuged and resuspended in McCoy's medium as listed under Figure 11.11. A sample of cells was counted using a haemocytometer. Appropriate dilutions were made and the cells placed into the incubator at 37°C for 5 hours. Cells ($3x10^6$) in 6ml of medium were added to the glass chamber for oxygen consumption measurements. Inversion of the sample in a capped test tube several times prior to addition of sample to the chamber ensured that the medium containing the cells was fully aerated.

RESULTS

Figure 11.9 illustrates the oxygen consumption rate of CHO cells in full McCoy's medium and in nutritionally compromised versions of the medium. Student's t-test was applied to determine whether there was any significant difference in oxygen consumption by cells between any of the groups. The rate of oxygen consumption in medium A was significantly greater than in any of the other media tested. The high degree of



A: McCoy's medium with glucose and glutamine
D: McCoy's medium with glucose, glutamine, 2DG and AOA
E: McCoy's medium without glucose or glutamine
F: McCoy's medium without glucose or glutamine, with 2DG
H: McCoy's medium without glucose or glutamine, with 2DG and AOA
I: McCoy's medium without glucose, with glutamine
M: McCoy's medium with glucose, without glutamine

Figure 11.12: Relative oxygen consumption rate of $3x10^6$ CHO cells incubated in media A-M. Heights of bars represent means ± standard deviations. n=12 for medium A. n=5 for media D-M.

significance of these differences was apparent by no p-values being greater than 0.02. However, no statistically significant difference in oxygen consumption could be found between any of the groups M to H.

DISCUSSION AND CONCLUSIONS

Sets of experiments presented prior to this one have focused on glucose-using and glutamine-using pathways. Although glucose and glutamine

are thought to be important energy substrates, other substrates may also be used. The rate of oxygen consumption of cells exposed to different variations of McCoy's medium discussed above may be used to assess overall respiratory activity regardless of the energy substrates involved.

The results shown in Figure 11.12 suggest that cells which are nutritionally compromised with respect to glucose and glutamine were less able to consume oxygen per unit time. Secondly, the absence of either glucose or glutamine resulted in a similar degree of depression of oxygen consumption rate. This degree of depression was common to media containing the inhibitors 2DG or AOA or both.

These data may imply that inhibition of the glycolytic pathway or glutaminolytic pathway can negatively affect the activity of oxidative phosphorylation. It would seem that the degree of effect on respiration is not compounded by both pathways being inhibited concurrently. Inhibition of either glycolysis or glutaminolysis will limit the amount of substrate available to the citric acid cycle from these pathways and, therefore, may regulate the production of reduced electron carriers (NADH, $FADH_2$) by the citric acid cycle. A limited supply of electron carriers will limit electron transport, oxidative phosphorylation and consequently oxygen consumption. It is apparent that oxygen consumption is not completely inhibited but just reduced.

CHAPTER 12

SUMMARY AND CONCLUSIONS

Metabolic energy plays a pivotal role in determining the functional integrity of cells. It is the multi-faceted nature of energy metabolism which makes it an attractive candidate for manipulations which will induce cellular changes. The complexity of energy metabolism and that cells rely on different metabolic pathways to different extents can be seen as problematic from the point of view of researchers who want to manipulate such pathways. However, it is this complexity which makes the cell potentially more controllable because it offers a variety of foci for metabolic intervention which may allow modification of cellular function.

The manner in which cells respond to the effects of radiation is also dependent upon the efficacy of cellular energy supply. In Chapters 8 to 11, a number of experiments were presented in which the relationship between energy metabolism and radiation response was examined. It has been suggested that cellular ATP levels may be altered in response to radiation. In Chapter 8, a set of experiments were described which were undertaken to determine the effects of radiation on the levels of ATP in certain biological systems. Results suggested that cellular ATP levels of B16 cells *in vitro*, murine livers or CaNT tumours *in vivo* did not change appreciably after irradiation. Hence, the hypothesis that *there is a change in ATP levels after irradiation*, proposed in Chapter 7, is considered to be false. Results also failed to confirm that the intracellular concentration of ATP after radiation was dependent upon the energy of the radiation beam. The hypothesis, *any change in cellular ATP content after irradiation of cells is beam energy dependent*, is, therefore, also false.

In Chapters 9, 10 and 11, two sets of experiments were presented. In the one set, presented in Chapter 10, the effect of the glycolytic inhibitor,

2DG, was investigated in murine tumours *in vivo*. The administration of 2DG appeared to cause a slight delay in growth of B16 melanomas but not in the other two tumour types tested. In addition, 2DG also appeared to enhance the growth inhibition effects of radiation in B16 and Fib/T tumours but not in a chemically induced rhabdomyosarcoma. The combination of AOA, an inhibitor of glutaminolysis, and 2DG with radiation was less effective in reducing Fib/T tumour growth than was radiation alone.

The set of experiments presented in Chapter 9 attempted to determine the effect of variations of McCoy's medium, containing different combinations of 2DG, AOA, glucose omission, and glutamine omission, on repair and radiosensitivity of CHO cells *in vitro*. In Chapter 11, results from changes in the activities of metabolic pathways associated with energy metabolism were presented. Glycolytic/PPP activities, glutaminolytic activities and the relative rates of oxygen consumption by cells were determined in CHO cells kept in certain selected medium types as used in the repair and sensitivity experiments. Results suggest that exposure of cells to medium containing 2DG can increase the radiosensitivity of CHO cells relative to that of cells in normal medium. Hence, the hypothesis, *2-deoxyglucose increases the radiosensitivity of cells* , is not false. Medium containing 2DG with glucose and glutamine present was found to result in reduced activities of glycolysis/PPP and glutaminolysis. Exposure of cells to medium containing AOA may also result in a modification of radiosensitivity but the nature of the change depends on the presence of glucose and glutamine. The hypothesis that *aminooxyacetic acid increases the radiosensitivity of cells*, is, therefore, not false under certain conditions. Medium containing AOA with glucose and glutamine present resulted in a reduction in glutaminolysis but a stimulation of glycolysis/PPP which may be explained on the basis of reciprocal upregulation of glycolysis/PPP by depressed glutaminolytic activity. From the effects of medium containing 2DG noted above, it would seem that a depressed glycolysis/PPP does not stimulate glutaminolysis in this cell-line. From the above discussion, the hypothesis that *2-deoxyglucose and aminooxyacetic acid alter the activities of glycolysis and glutaminolysis respectively*, is not false

provided that the pentose phosphate pathway is relatively less active than glycolysis.

Most combinations of 2DG, AOA, omission of glucose, and omission of glutamine from medium resulted in a reduced rate of repair relative to that in normal medium but different combinations varied with respect to the extent of repair. Both hypotheses, *2-deoxyglucose inhibits repair of radiation damage*, and, *aminooxyacetic acid inhibits repair of radiation damage*, were found not to be false. Cells depleted of glucose were found to be more susceptible to repair inhibition by 2DG but cells in medium depleted of glutamine did not seem to be more susceptible to AOA with respect to rate of repair. The absence of glucose or glutamine from medium resulted in moderate reductions in repair rate. However, when both glucose and glutamine were omitted from medium and no inhibitors were present, repair rate was increased. In this medium, cells were found to have an increased rate of glycolysis/PPP relative to that for cells in normal medium. It might be suggested, therefore, that this stimulation of glycolytic/PPP activity may have been able to support repair efficiently from any glycogen or glucose still present within the cell. This increased glycolytic/PPP activity may have been a response to the stimulus of substrate starvation.

Medium lacking both glucose and glutamine and containing both AOA and 2DG resulted in the greatest reduction in cellular repair rate of the media tested. This medium also resulted in an increase in radiosensitivity and was able to cause both mitotic and intermitotic death of cells. In this medium, the activities of glycolysis/PPP, glutaminolysis and oxidative metabolism were reduced relative to those in normal cells. All other medium variations tested failed to cause appreciable toxicity alone in respect of either mitotic or intermitotic cell death but most were able to modify cellular radiation response relative to that of cells in normal medium. In most cases, those media containing both 2DG and AOA reduced cellular repair rate more, relative to that of normal medium, than media with the same glucose and glutamine content and containing either 2DG or AOA alone. In the case of medium containing glutamine but no glucose, the combination of 2DG and AOA was less effective than either of the inhibitors alone with regard to inhibition of repair. In

addition, the most extreme sensitivity effects were noted for a medium containing both inhibitors. Therefore, from the preceding discussion, the hypothesis, *the effects of 2-deoxyglucose and aminooxyacetic acid interact with one another*, is not false. Oxygen consumption by cells in medium containing both 2DG and AOA was reduced relative to that of cells in normal medium. The hypothesis that *2-deoxyglucose and aminooxyacetic acid alter the activity of oxidative metabolism*, was, therefore, also not false.

The evidence presented above, strongly suggests an association between energy supply from glycolysis/PPP and glutaminolysis, and radiation response. Furthermore, it is suggested that the activity of these energy supplying pathways can be modified in such a way that radiation response can be manipulated. The hypothesis that *the alteration of radiation response of cells exposed to 2-deoxyglucose and aminooxyacetic acid is due to the effects of these substances on energy metabolism*, would seem not to be false. However, the interaction between pathways clearly is complex. Therefore, the response of different cell-types to different media, doses of inhibitors and doses of radiation should be investigated further in order to be able to fine-tune and manipulate radiation response in a predictable way.

The *in vitro* experiments conducted using CHO cells indicate certain responses of this cell-line to radiation in the presence of culture media which were modified with respect to their content of glucose and glutamine and the presence of 2DG and AOA. Although some effects of 2DG on radiation response have been investigated previously and the results produced in this thesis verify in part the role of 2DG as a repair inhibitor, it is the role of AOA as a modifier of radiation response and its combined use with 2DG that is novel. Also of relevance is that such radiomodifying effects can occur using doses of 2DG and AOA which are not toxic. Although the use of inhibitors of two important metabolic pathways to alter radiation sensitivity and repair suggested that radiation response could be modified through interference with two separate routes for cellular energy production, it is the conceptual approach of interfering with multiple energy generating pathways to achieve different degrees of effect that is of greatest importance. This

work indicates that the approach used, that is, the use of inhibitors of energy metabolism to modify radiation response was a valid one. The same approach can, therefore, be applied to other cells which may respond differently but the same rationale will apply.

It is beneficial with regard to radiation therapy of cancer to discover ways of modifying the response of cells to radiation without producing toxicity. Substances which do not produce cellular toxicity of their own but which modify the response of cells to radiation can potentially be used to improve radiotherapy. In order to achieve a therapeutic benefit, a greater radiation response would be required in tumour tissue than in normal tissue. It is postulated that a protocol for therapeutic benefit could be designed if certain criteria are fulfilled. First, the pattern of energy metabolism and use of energy substrates by tumours would have to be different to that of normal tissue. Secondly, details regarding the reliance of normal and tumour energy metabolism on particular metabolic pathways and their ability to upregulate other compensatory pathways must be known. Differences in the reliance of tumour and normal tissue on specific energy supplying pathways could then be exploited through administration of suitable inhibitors. If energy supply, which determines the degree of repair, could be inhibited more in irradiated tumour cells than irradiated normal cells, a greater tumour response and a therapeutic gain could be achieved.

Investigations described in this thesis have shown an approach in which energy inhibitors can modify cellular radiation response. Cogniscent that more work is required, it is clear that, with appropriate knowledge of individual tumour and normal energy metabolisms and a variety of inhibitors of energy metabolism, the efficacy of radiotherapy can be improved.

APPENDIX

STANDARD METHODS, REAGENTS AND MEDIA

TISSUE CULTURE REAGENTS AND MEDIA

McCoy's 5a Medium

INORGANIC SALTS	mg/l
$CaCl_2$ (anhydrous)	100.00
KCl	400.00
$MgSO_4$ (anhydrous)	97.67
NaCl	6460.00
$NaH_2PO_4.H_2O$	580.00
$NaHCO_3$	2200.00
OTHER COMPONENTS	
Bacto-peptone	600.00
D-glucose	3000.00 (If added)
Glutathione (reduced)	0.50
Phenol red	10.00
AMINO ACIDS	
L-Alanine	13.90
L-Arginine HCl	42.10
L-Asparagine	45.00
L-Aspartic acid	19.97
L-Cysteine	31.50
L-Glutamic acid	22.10
L-Glutamine	219.20 (If added)
Glycine	7.50
L-Histidine HCl.H2O	20.96
L-Hydroxyproline	19.70
L-Isoleucine	39.36
L-Leucine	39.36
L-Lysine HCl	36.50
L-Methionine	14.90
L-Phenylalanine	16.50
L-Proline	17.30
L-Serine	26.30
L-Threonine	17.90

L-Tryptophan	3.10
L-Tyrosine (Disodium salt)	26.10
L-Valine	17.60
VITAMINS	
Ascorbic acid	0.50
Biotin	0.20
Choline chloride	5.00
D-Ca pantothenate	0.20
Folic acid	10.00
i-inositol	36.00
Nicotinamide	0.50
Nicotinic acid	0.50
Para-aminobenzoic acid	1.00
Pyridoxal HCl	0.50
Pyridoxine HCl	0.50
Riboflavin	0.20
Thiamine HCl	0.20
Vitamin B12	2.00

Trypsin 0.05%

	g/l
NaCl	11.24
KCl	0.40
$NaHCO_3$	0.58
Trypsin powder 1:250 (Difco, Detroit, USA)	0.50
Di sodium EDTA (BDH, Poole, UK)	0.20

Trypsin was stored frozen at -10°C and thawed just prior to use.

Ethylenediaminetetra-acetic acid (EDTA)

Phosphate buffered saline	1L
Di sodium EDTA EDTA (BDH, Poole, UK)	0.2g/l

EDTA was stored at 4°C.

Hanks' Balanced Salt Solution (HBSS) (without glucose)

	g/l
$CaCl_2$	0.14
KCl	0.40
KH_2PO_4	0.06
$MgCl_2.6H_2O$	0.10
$MgSO_4.7H_20$	0.10
NaCl	8.32
$NaHCO_3$	0.35
$Na_2HPO_4.7H_20$	0.09

TISSUE CULTURE TECHNIQUES

Cells were maintained by serial passage in Sterilin plastic tissue culture flasks in media containing L-glutamine, sodium bicarbonate buffer, 10% (v/v) foetal calf serum (Highveld Biological, South Africa) and antibiotics (50 mg/l penicillin, 50 mg/l neomycin, 50 mg/l streptomycin (Highveld, South Africa)). Cells were incubated in a humidified atmosphere containing 95% air and 5% CO_2 at 37°C in a water-jacketed incubator.

Trypsinization of cells *in vitro*

Medium above cells was discarded and replaced with 1 ml EDTA (in the case of 50 ml plastic tissue culture flasks), rinsed and the EDTA discarded. A further 1 ml of EDTA was placed on the cells and left for 2 minutes before being discarded. Cells were quickly washed with 1 ml trypsin which was then discarded and replaced with another 1 ml trypsin. Flasks were then left in the incubator at 37°C until the cells became detached from the plastic. The trypsin and cells were then transferred to a sterile 12 ml polypropylene test tube with cap, 5 ml medium added and centrifuged at 190 x g for 10 minutes. The supernatant was removed and

the cell pellet was disaggregated by gentle tapping of the tube. Cells were resuspended in 5 ml of medium.

Cell concentration was then assessed using a haemocytometer, suitable dilutions made and cells plated into new flasks for serial passaging, clonogenic assays or taken for other purposes.

Staining and assessment of clonogenic survival

Medium was discarded from flasks containing colonies. Colonies were rinsed with phosphate buffered saline and then stained with 5 ml 0.1% (w/v) crystal violet solution. Stain was left in contact with the cells for 15 minutes before being discarded and the cells rinsed with water several times to get rid of excess stain. Flasks were then allowed to dry and colonies counted to assess surviving fraction. A colony was considered to be a group of at least 50 cells.

$$\text{Surviving fraction} = \frac{\text{colonies counted}}{\text{cells plated x plating efficiency}}$$

where

$$\text{plating efficiency} = \frac{\text{number of cells plated after no treatment}}{\text{number of cells plated}}$$

EXPERIMENTAL ANIMALS

Experimental mice were bred and housed in the Specific Pathogen Free mouse unit of the Radiobiology Laboratories, Department of Radiation Oncology, University of Cape Town/ Groote Schuur Hospital. Mice were given food and water *ad libitum*. Laboratory mouse cubes were supplied by Specialist Animal Feeds, Delft, South Africa.

Passaging of tumours

Tumour-bearing mice were killed by extended exposure to ether. Tumours were then excised using clean instruments and disaggregated in a plastic Petri dish containing cold (4°C) sterile McCoy's 5a medium without foetal calf serum or antibiotics by chopping with two scalpels used with a scissor-like action to create a cell suspension. The mixture was allowed to settle for about one minute to allow any large tumour particles to settle. Medium containing cells was aspirated through a 1.2 x 38 mm syringe needle and transferred to a sterile polypropylene test tube. Suitable dilutions were made and 0.1 ml of the cell suspension containing 200 000 cells was inoculated into mice. After about 2 weeks, tumours became visible. Tumours were used for experimental purposes when they reached their desired size or could be excised and either passaged as described above or stored in liquid nitrogen.

3-Methylcholanthrene-induced Rhabdomyosarcoma

Rhabdomyosarcomas were originally induced in Balb/c mice by injecting 0.1 ml of a 1mg/ml solution of 3-methylcholanthrene in arachis oil into the flanks of mice. Tumours developed in 3 to 4 months and were then passaged, as described above, in the sternal regions of mice.

Fib/T Tumour

The Fib/T tumour was obtained originally from Dr N. McNally, Mount Vernon Hospital, Northwood, UK. Tumours were passaged in the gastronemius muscle.

CaNT Tumour

The CaNT tumour was obtained originally from Professor R. Berry, Middlesex Hospital Medical School, University of London, UK. Tumours were passaged subcutaneously in the sternal region.

B16 Tumour

This tumour was inoculated from cells which had been grown in culture, trypsinized and diluted accordingly so that 0.1 ml of a 200 000 cells/ml suspension could be injected into the gastrocnemius muscles of mice.

LIST OF REAGENTS

CHAPTER 8

1) Perchloric acid, Saarchem Pty Ltd, South Africa
2) K_2CO_3, LASEC, South Africa
3) Luciferase-luciferin reagent, Sigma Chemical Company, USA.
4) Tris(Hydroxymethyl)aminomethane (Tris), Saarchem Pty Ltd, South Africa.
5) Adenosine triphosphate (ATP), Sigma Chemical Company, USA.

CHAPTER 9

1) Hams F12 medium, Highveld Biological, South Africa.
2) McCoy's 5a medium, Highveld Biological, South Africa (Formulation is listed above).
3) Foetal Calf Serum (FCS), Highveld Biological, South Africa.
4) Antibiotics (Streptomycin, Neomycin, Penicillin) (Formulation is listed above)
5) Hanks' Buffered Salt Solution (HBSS), made up (Formulation is listed above).
6) Trypsin (without glucose) and EDTA (Formulation is listed above).
7) 2-deoxy-D-glucose, Sigma Chemical Company, USA.
8) Aminooxyacetic acid, Sigma Chemical Company, USA.
9) Trypan blue, Gibco, UK.
10) NaCl (For saline), Merck, Germany.

CHAPTER 11

1) Hams F12 medium, Highveld Biological, South Africa.
2) McCoy's 5a medium, Highveld Biological, South Africa.
3) Foetal Calf Serum, Highveld Biological, South Africa.
4) 2-Deoxy-D-glucose, Sigma Chemical Company, USA.
5) Aminooxyacetic acid, Sigma Chemical Company, USA.
6) D-[U-^{14}C]Glucose aqueous solution, Amersham, UK. Specific activity: 10.8gbq/mmol, 293mCi/mmol.
7) L-[U-^{14}C]Glutamine aqueous solution, Amersham, UK. Specific activity: 10.1gbq/mmol, 274mCi/mmol.
8) Diethyl Ether, Associated Chemical Enterprises, South Africa.
9) NaCl, Merck, Germany.
10) HCl, BDH Ltd, UK.
11) L(+)Lactic acid, Sigma Chemical Company, USA.
12) ω-Bromoacetophenone, Sigma Chemical Company, USA.
13) 18-crown-6 ether, Sigma Chemical Company, USA.
14) Acetonitrile, Baxter Corporation, Burdick and Jackson, USA.
15) Ready Gel liquid scintillation cocktail, Beckman, USA.
16) K_2CO_3, LASEC, South Africa.
17) Na_2CO_3, BDH Ltd, UK.
18) KCl, Saarchem, South Africa.
19) Sodium azide, BDH, UK.

REFERENCES

Alper T. (1979). Cellular Radiobiology. Cambridge University Press, Cambridge.

Altman K.J., Gerber G.B. and Okada S. (Eds) (1970). Radiation Biochemistry. Academic Press, New York.

Barban S. and Schulze H.O. (1961). The effects of 2-deoxyglucose on the growth and metabolism of cultured human cells. J.Biol. Chem. 236: 1887-1890.

Betel I. (1967). Decreased nucleotide content as the cause of decreased ATP synthesis in thymus nuclei after radiation. Int. J. Radiat. Biol. 12: 459-466.

Billen D., Strehler B.L., Stapleton G.E. and Brigham E. (1953). Postirradiation release of adenosine triphosphate from Escerichia coli B/r[1]. Arch. Biochem. 43: 1-10.

Bodansky O. (1975). Biochemistry of human cancer. Academic Press, New York.

Cay O., Radnell M., Jeppsson B., Ahren B. and Bengmark S. (1992). Inhibitory effect of 2-deoxy-D-glucose on liver tumor growth in rats. Cancer Res. 52: 5794-5796.

Chadwick K.H. and Leenhouts H.P. (1981). The molecular theory of radiation biology. Springer-Verlag, Berlin.

Chapman J.D., Webb R.G. and Borsa J. (1971). ATP pool levels in synchronously growing Chinese hamster cells. J. Cell Biol. 49: 229-233.

Chien K.R., Abrahams J., Serroni A., Martin J.T. and Farber J.L. (1978). Accelerated phospholipid degradation and associated membrane dysfunction in irreversible, ischaemic injury. J. Biol. Chem. 253: 4809-4817.

Clement J.J., Song C.W. and Sand T.T. (1978). Tumor cell respiration following irradiation. Radiol. 126: 507-510.

Coe E.L., Garcia E.N., Ibsen K.H. and McKee R.W. (1963). Respiration and glycolysis in X-irradiated Ehrlich Mouse Ascites Carcinoma cells. Radiat. Res. 20: 586-592.

Cole A., Meyn R.E., Chen R. Corry P.M. and Hittelman W. (1980). Mechanisms of cell injury. In: Radiation Biology in Cancer Research. Meyn R.E. and Whithers H.R. (Eds), Raven, New York.

Crabtree H.G. (1935). The differential effect of radium radiation on the carbohydrate metabolism of normal and tumour tissues irradiated at low temperature. Biochem. J. 29: 2334-2343.

Curtis S.B. (1986). Lethal and potentially lethal lesions induced by radiation - a unified repair model. Radiat. Res. 106: 252-270.

Daly J.W., Kuroda Y., Phillips J.W., Shinizu H. and Ui M. (Eds) (1983). Physiology and Pharmacology of Adenosine Derivatives. Raven, New York.

Dalrymple G.V., Sanders J.L., Baker M.L. and Wilkinson K.P. (1969). The effect of 2,4-dinitrophenol on the repair of radiation injury by L cells. Radiat. Res. 37: 90-102.

Dikomey E. and Franzke J. (1986). Three classes of DNA strand breaks induced by X-irradiation and internal beta-rays. Int. J. Radiat. Biol. 50: 893-908.

Dwarkanath B.S. and Jain V.K. (1989). Energy linked modifications of the radiation response in a human cerebral glioma cell line. Int. J. Radiat. Oncol. Biol. Phys.17: 1033-1040.

Dwarakanath B.S., Adhikari J.S., Kapoor N. and Jain V. (1995). Hematoporphyrin derivatives potentiate the radiosensitizing effects of 2-DG in cancer cells. Poster presentation, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Edwards J.C., Chapman D., Cramp W.A. and Yatvin M.B. (1984). The effects of ionizing radiation on biomembrane structure and function. Pro. Biophys. Molec. Biol. 43: 71-93.

Eigenbrodt E., Fister P. and Reinacher M. (1985) - Chapter 6, Regulation of carbohydrate metabolism vol.II, Beitner R. (Ed.), CRC, Boca Raton.

Elkind M.M. and Sutton H. (1959). X-ray damage and recovery in mammalian cells in culture. Nature 184: 1293-1295.

Elkind M.M., Swain R.W., Alescio T., Sutton H. and Moses W.B. (1965). Oxygen nitrogen recovery and radiation therapy. In Cellular Radiation Biology, p442-461, Williams and Wilkins, Baltimore.

Eskey C.J., Koretsky A.P., Dolmach M.M. and Jain R.K. (1993). Role of oxygen vs. Glucose in energy metabolism in a mammary carcinoma perfused ex vivo: Direct measurement by 31P NMR. Proc. Natl. Acad. Sci. USA, 90:2646-2650.

Evans C.W. (Ed) (1991). The metastatic cell - Behaviour and Biochemistry. Chapman Hall, London. 117-118.

Farber J.L., Chien K.R. and Mittnacht S. (1981). The pathogenesis of irreversible cell injury in ischaemia. Am. J. Pathol. 102: 271-281.

Fellenz M.P. and Gerweck L.E. (1988). Influence of extracellular pH on intracellular pH and cell energy status: Relationship to hyperthermic sensitivity. Radiat. Res. 116: 305-312.

Fidler I.J. and Hart I.R. (1982). Biological diversity in metastatic neoplasms: origin and implications. Science 217: 998-1003.

Freeman M.L. and Sierra E. (1984). An acidic environment reduces the fixation of radiation damage. Radiat. Res. 97: 154-161.

Freeman M.L., Holahan E.V., Highfield M.S., Raaphorst G.P., Spiro I.J. and Dewey W.C. (1981). The effect of pH on hyperthermic and X-ray induced cell killing. Int. J. Radiat. Oncol. Biol. Phys. 7: 211-216.

Freeman M.L., Raaphorst G.P., Hopwood L.E. and Dewey W.C. (1980). The effect of pH on cell lethality induced by hyperthermic treatment. Cancer 45: 2291-2300.

Freshney R.I.(1987). Culture of Animal Cells. A Manual of Basic Technique. Second Edition. Alan R. Liss, New York.

Friedberg E.C., Walker G.C and Siede W. (1995). DNA Repair and Mutagenesis. ASM, Washington.

Ganong W.F. (1987). Review of medical physiology. Thirteenth Edition. Appleton and Lange, East Norwark

Gerweck L.E., Urano M., Koutcher J., Fellenz M.P. and Kahn J. *et al.* (1989). Relationship between energy status, hypoxic cell fraction and hyperthermic sensitivity in murine fibrosarcoma. Radiat. Res. 117: 448-458.

Gerweck L.E., Koutcher J.A., Zaidi S.T.H. and Seneviratne T. (1992). Energy status in the murine FSaII and MCaIV tumours under aerobic and hypoxic conditions: An in -vitro analysis. Int. J. Radiat. Oncol. Biol. Phys. 23: 557-561.

Gerweck L.E., Rhee J.G., Koutcher J.A., Song C.W. and Urano M. (1991). Regulation of pH in murine tumor and muscle. Radiat. Res. 126: 206-209.

Gerweck L.E., Seneviratne T. and Gerweck K.K. (1993). Energy status and radiobiological hypoxia at specified oxygen concentrations. Radiat. Res. 135: 69-74.

Glacken M.W. (1988). Catabolic control of mammalian cell culture. Biotechnol. 6: 1041-1050.

Gonzalez-Mateos F., Gomez M.E., Garcia-Salguero L., Sanchez V. and Aragon J.J. (1993). Inhibition of glycolysis by amino acids in ascites tumour cells. Specificity and mechanism. J.Biol. Chem. 268: 7809-7817.

Goodhead D.T. (1995a). Physics of radiation action: Microscopic features that determine biological consequences. Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Goodhead D.T. (1995b). Personal communication.

Gores G.J., Nieminen A., Fleishman K.E., Dawson T.L., Herman B. and Lemasters J.L. (1988). Extracellular acidosis delays onset of cell death in ATP-depleted hepatocytes. Am. J. Physiol. 255 (Cell Physiol. 24): C315-C322.

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

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

Gridley D.S., Nutter R.L., Mantik D.W. and Slater J.M. (1985). Hyperthermia and radiation in vivo: Effect of 2-deoxy-D-glucose. Int. J. Radiat. Oncol. Biol. Phys. 11: 567-574.

Gupta V., Rangala N.S. and Belli J.A. (1986). Enhancement of radiation sensitivity by postirradiation hypoxia. Radiat. Res. 106: 132-136.

Guyton A.C.(1986). Textbook of medical physiology. Seventh edition. W.B. Saunders Company, Philadelphia.

Hall E.J. (1972). The effect of hypoxia on repair of sublethal radiation damage in cultured mammalian cells. Radiat. Res. 49: 405-415.

Hall E.J. (1994). Radiobiology for the Radiologist. Fourth Edition. J.B. Lippincott, Philadelphia.

Halperin M.L., Connors H.P., Relman A.S. and Karnovsky M.L. (1969). Factors that control the effect of pH on glycolysis in leukocytes. J. Biol. Chem. 244: 384-390.

Hamilton E., Fenell M. and Stafford D.M. (1995). Modification of tumor glucose metabolism for therapeutic benefit. Acta Oncolgica 34: 429-433.

Hanka L.J. (1979). Introduction: Possibilities for biochemically rational chemotherapy for some malignancies with depleting enzymes and antimetabolites of specific amino acids. Cancer Treat. Rep. 63: 1009-1011.

Hasegawa K., Anraku Y., Kasahara M., Akamatsu Y. and Nishijima M. (1990). Isolation and characterization of Chinese hamster ovary cell mutants defective in glucose transport. Biochim. Biophys. Acta 1051: 221-229.

Hatanaka M. (1974). Transport of sugars in tumor cell membranes. Biochim. Biophys. Acta 355: 77-104.

Haspel H.C., Wilk E.W., Birnbaum M.J., Cushman S.W. and Rosen O.M. (1986). Glucose deprivation and hexose transporter polypeptides of murine fibroblasts. J. Biol. Chem. 261: 6778-6789.

Hendrikse A.S. (1989). Effect of Breathing Hypoxic Gas Mixtures Followed by Irradiation on Tumour Cell Survival in Experimental Mouse Tumours. PhD Thesis, University of Cape Town, South Africa.

Hirst D.G. (1986). Oxygen delivery to tumours. Int. J. Radiat. Oncol. Biol. Phys. 12: 1271-1277.

Hlatky L., Sachs R.K. and Ring C.S. (1989). Reducing the hypoxic fraction of a tumour model by growth in low glucose. Br. J. Cancer 59:375-380.

Hlatky L., Alpen E.L. and Yee M.K. (1986). Differences in the X-ray sensitivity of cells in different regions of the sandwich, a diffusion limited system for cell growth. Radiat. Res. 108: 62-73.

Holahan E.V., Stuart P.K. and Dewey W.C. (1982). Enhancement of survival of CHO cells by acidic pH after X-irradiation. Radiat. Res. 89: 433-435.

Holahan P.K., Knizner S.A., Gabriel C.M. and Swenberg C.E. (1988). Alterations in phosphate metabolism during cellular recovery of radiation damage in yeast. Int. J. Radiat. Biol. 54: 545-562.

Hopper S. and Segal H.L. (1962). Kinetic studies of rat liver glutamic-alanine transaminase. J. Biol. Chem. 237: 3189-3195.

Hornsby P.J. (1982). The role of vitamin E in cellular energy metabolism in cultured adrenocortical cells. J. Cell. Physiol. 112: 207-216.

Hornsby P.J. and Gill G.N. (1981). Regulation of glutamine and pyruvate oxidation in cultured adrenocortical cells by cortisol, antioxidants and oxygen: Effects on cell proliferation. J. Cell. Physiol. 109: 111-120.

Huber K.R. (1988). Uptake of glutamine antimetabolites 6-diazo-5-oxo-L-norleucine (DON) and acivicin and resistant tumor cell lines. Int. J. Cancer 41: 752-755.

Ibsen K.H., Coe E.L. and McKee R.W. (1960). Some factors influencing respiration and glycolysis in Ehrlich ascites tumor cells. Cancer Res. 20: 1399-1407.

Iizuka M., Ando K. and Aruga T. (1995). Effects of reoxygenation and cell density on repair of potentially lethal damage., Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Iliakis G. (1988). Radiation induced potentially lethal damage: DNA lesions susceptible to fixation. Int. J. Radiat. Biol. 53:541-584.

Iliakis G., Okayasu R, Varlotto J., Shernoff C. and Wang Y. (1993). Hypertonic treatment during premature chromosome condensation allows visualization of interphase chromosome breaks repaired with fast kinetics in irradiated CHO cells. Radiat. Res. 135: 160-170.

Iliakis G., Wang Y., Pantelias G.E. and Metzger L. (1992). Mechanism of radiosensitization by halogenated pyrimidines: Effect of BrdU on repair of DNA breaks, interphase chromatin breaks, and potentially lethal damage in plateau-phase CHO cells. Radiat. Res. 129: 202-211.

Jacobs A.E.M., Oosterhof A. and Veerkamp J.H. (1990). 2-Deoy-D-glucose uptake in cultured human muscle cells. Biochim. Biophys. Acta. 1051:230-236.

Jain V.K., Holtz G.W., Pohlit W. and Purohit S.C. (1977a). Inhibition of unscheduled DNA synthesis and repair of potentially lethal X-ray damage by 2-deoxy-D-glucose in yeast. Int. J. Radiat. Biol. 32: 175-180.

Jain V.K., Porschen W. and Feinendegen L.E. (1977b). Optimization of cancer therapy: Part II - Effects of combining 2-deoxy-D-glucose treatment with gamma-irradiation on Sarcoma-180. Indian J. Exp. Biol. 15: 714-718.

Jain V.K., Purohit S.C. and Pohlit W. (1977c). Optimization of cancer therapy: Part I - X-ray induced potentially lethal damage by 2-deoxy-D-glucose in Ehrlich ascites tumour cells. Indian J. Exp. Biol. 15: 711-713.

Jain V.K., Kalia V.K., Gopinath P.M., Naqvi S. and Kucheria K. (1979). Optimization of cancer therapy: Part III - Effects of combining 2-deoxy-D-glucose treatment with gamma-irradiation on normal mice. Indian J. Exp. Biol. 17: 1320-1325.

Jain V.K., Gupta I. and Lata K. (1982). Energetics of cellular repair processes in a respiratory-deficient mutant of yeast. Radiat. Res. 92: 463-473.

Jain V.K., Kalia V.K., Sharma R., Maharajan V. and Menon M. (1985). Effects of 2-deoxy-D-glucose on glycolysis, proliferation kinetics and radiation response of human cancer cells. Int. J. Radiat. Oncol. Biol. Phys. 11: 943-950.

Jennings R.B. and Reimer K.A. (1981). Lethal myocardial ischaemic injury. Am. J. Pathol. 102: 241-255.

Joiner M.C. and Marples B. (1992). Does induced repair determine the lethal effects of very low radiation doses? Br. J. Radiol. Supplement 24: 74-78.

Kale R.K. and Samuel D. (1987). Effect of radiation on cellular function: Study on calcium uptake. Indian Journal of Experimental Biology 25: 816-821.

Kalia V.K., Jain V.K. and Otto F.J. (1982). Optimization of cancer therapy: Part IV - Effects of 2-deoxy-D-glucose on radiation induced chromosomal damage in PHA-stimulated peripheral human leukocytes. Indian J. Exp. Biol. 20:884-888.

Kallman R.F. (Ed.) (1987). In Rodent tumor models in experimental cancer therapy. Pergamon Press, New York.

Kapiszewska M. and Lange C. (1988). The effects of reduced temperature and/or starvation conditions on the radiosensitivity and repair of potentially lethal damage and sublethal damage in L5178Y-R and L5178Y-S cells. Radiat. Res. 113: 458-472.

Kappos A. and Pohlit W. (1972). A cybernetic model for radiation reactions in living cells. I. Sparsely ionizing radiations; stationary cells. Int. J. Radiat. Biol. 22:51-65.

Kaufmann W.K., Kaufmann D.G., Stenstrom M. and Grisham J.W. (1982). Requirements for adenosine triphosphate in DNA repair in isolated hepatic nuclei. Biochem. Biophys. Res. Comm. 108: 1040-1047.

Keifer J. (1971). The importance of cellular energy metabolism for the sparing effect of dose fractionation with electrons and ultra-violet light. Int J. Radiat. Biol. 20: 325-336.

Kelland L.R. and Steel G.G. (1988). Inhibition of recovery from damage induced by ionizing radiation in mammalian cells. Radiother. Oncol. 13: 285-299.

Kellerer A.M. and Rossi H.H. (1972). The theory of dual radiation action. Curr. Top. Radiat. Res. Q. 8: 85-158.

Kellerer A.M. and Rossi H.H. (1978). A generalized formulation of dual radiation action. Radiat. Res. 75: 471-488.

Kim J.H., Kim S.H. and Alfieri A.A. (1988). Selective killing of glucose-deprived hypoxic cells by hyperthermia. Radiat. Res. 116: 337-342.

Kimura H., Ikebuchi M., Komatsu K. and Aoyama T. (1995). Potentially lethal damage repair in cells from severe combined immunodeficient mouse. Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany

Klouwen H.M. and Appelman A.W.M. (1967). Biochemical radiosensitivity of lymphoid tumors. Cancer Res. 27: 255-260.

Koch C.J., Meneses J.J. and Harris J.W. (1980). The effect of extreme hypoxia and glucose on the repair of potentially lethal and sublethal radiation damage by mammalian cells. Radiat. Res. 70: 542-551.

Kovacevic Z. and Morris H.P. (1972). The role of glutamine in the oxidative metabolism of malignant cells. Cancer Res. 32: 326-333.

Krebs H.A. (1972). The Pasteur effect and relations between respiration and fermentation. Essays Biochem. 8: 1-34.

Krebs (1974) in Weber (Ed). Advances in Enzyme Regulation. 5: 409-434.

Kvamme E. and Svenneby G. (1961). The effect of glucose on glutamine utilization by Ehrlich ascites tumor cells. Cancer Res. 21: 92-98.

Lange C.S., Reddy N.M.S., Mayer P.J. and Nori D. (1995). PLD is delayed for hours after irradiation. Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Laszlo J., Harlan W.R., Klein R.F., Kirshner N., Estes E.H. and Bogdonoff M.D. (1961). The effect of 2-deoxy-D-glucose infusions on lipid and carbohydrate metabolism in man. J.Clin. Invest. 40: 171-176.

Lawrence T.S. (1988), Ouabain sensitizes tumor cells but not normal cells to radiation. Int. J. Radiat. Oncol. Biol. Phys. 15: 953-958.

Lehninger A.L. (1970). Biochemistry The Molecular Basis of Cell Structure and Function. Worth, New York.

Ling C.L., Robinson E. And Shrieve D.C. (1988). Repair of radiation induced damage - dependence on oxygen and energy status. Int. J. Radiat. Oncol. Biol. Phys. 15: 1179-1186.

Little J.B. (1971). Repair of potentially -lethal radiation damage in mammalian cells: enhancement by conditioned medium from stationary cultures. Int. J. Radiat. Biol. 20: 87-92.

Mangiardi J.R. and Yodice P. (1990). Metabolism of the malignant astrocytoma. Neurosurgery, 26: 1-19.

Marchese M.J., Zaider M. and Hall E.J. (1987). Potentially lethal damage repair in human cells. Radiother. Oncol. 9: 57-65.

Marples B. and Joiner M.C. (1993). The response of Chinese Hamster V79 cells to low radiation doses: evidence for enhanced sensitivity of the whole cell population. Radiat. Res. 133: 41-51.

Mathews C.K. and van Holde K.E. (1990) Biochemistry. Benjamin/Cummings, Redwood City.

Matsudaira H., Furuno I. and Otsuka H. (1970). Possible requirement of adenosine triphosphate for the rejoining of x-ray induced damage breaks in the DNA of Ehrlich ascites tumour cells. Int. J. Radiat. Biol. 17: 339-347.

McComb R.B. and Yushok W.D. (1964). Metabolism of ascites tumor cells IV. Enzymatic reactions involved in adenosinetriphosphate degradation induced by 2-deoxyglucose. Cancer Res. 24:198-205.

McGilvery R.W. and Goldstein G.W. (1983) (Eds). Biochemistry a functional approach. Third edition. W.B. Saunders, Philadelphia.

McKeehan W.L. (1982). Glycolysis, glutaminolysis and cell proliferation. Cell Biol. Int. Rep. 6: 1041-1050.

Medina M.A., Quesada A.R., Marquez F.J., Sanchez-Jimenez F. and Nunez de Castro I. (1988). Inorganic phosphate and energy charge compartmentation in Ehrlich ascites tumour cells in the presence of glucose and/or glutamine. Biochem. Int. 16: 713-718.

Medina M.A., Sanchez-Jimenez F., Marquez F.J., Perez-Rodrigues J. Quesada A.R., and Nunez de Castro I. (1988). Glutamine and glucose as energy substrates for Ehrlich ascites tumour cells. Biochem. Int. 16: 339-347.

Merchant T.E., Alfieri A.A., Glonek T. and Koutcher J.A. (1995). Comparison of relative changes in phosphatic metabolites and phospholipids after irradiation. Radiat. Res. 142: 29-38.

Meuller-Klieser W., Walenta S., Paschen W., Kallinowski F and Vaupel P. (1988). Metabolic imaging in microregions of tumors and normal tissues with bioluminescence and photon counting. J. Natl. Cancer Inst. 80: 842-848.

Meuller-Klieser W. (1995). A response modifier in multicell spheroids. Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Mohanti B.K., Rath G.K., Anantha N., Kannan V., Das B.S., Chandramouli B.A.R., Banerjee A.K., Das S., Jena A., Ravichandran R., Sahi U.P., Kumar R., Kapoor N., Kalia V.K., Dwarakanath B.S. and Jain V. (1996). Improving cancer radiotherapy with 2-deoxy-D-glucose: Phase I/II clinical trials on human cerebral gliomas. Int. J. Radiat. Oncol. Biol. Phys. 35: 103-111.

Mothersill C. and Seymour C.B. (1986). Effect of lactate on the recovery of CHO-K1 cells from gamma radiation damage. Acta. Radiologica Oncol. 25: 71-76.

Murray R.K. (1987) Biochemical properties of cancer cells. In Tannock I.F. and Hill R.P. (Eds) The basic science of oncology. Pergamon, New York.

Nakada H.I. and Wick A.N. (1956). The effect of 2-deoxyglucose on the metabolism of glucose, fructose, and galactose by rat diaphragm. J. Biol. Chem. 222, 671-676.

Nagle W.A., Moss A.J., Roberts H.G. and Baker M.L. (1980). Effects of 5-thio-D-glucose on cellular adenosine triphosphate levels and deoxyribonucleic acid rejoining in hypoxic and aerobic Chinese hamster cells. Radiol. 137: 203-211.

Nayler W.G. (1981). The role of calcium in the ischaemic myocardium. Am. J. Pathol. 102: 262-270.

Nelson J.M., Braby L.A., Metting N.F. and Roesch W.C. (1990). Multiple components of split-dose repair in plateau-phase mammalian cells: A new challenge for phenomenological modelers. Radiat. Res. 121: 154-160.

Newsholme E.A. and Leach A.R. (1983) Biochemistry for the Medical Sciences. Wiley and Sons, Chichester.

Nias A.H.W., Swallow A.J., Keene J.P. and Hodgson B.W. (1973). Absence of a fraction effect in irradiated HeLa cells. Int. J. Radiat. Biol. 23: 559-569.

Noda L., Kuby S.A. and Lardy H.A. (1954). Adenosine triphosphate-creatine transphosphorylase, IV equilibrium studies. J. Cell Biol. 210: 83-95.

Okunieff P.G., Koutcher J.A., Gerweck L., McFarland E., Hitzig B., Urano M., Brady T., Neuringer L. and Suit H.D. (1986). Tumor size dependent changes in a murine fibrosarcoma: use of in vivo ^{31}P NMR for non-invasive evaluation of tumor metabolic status. Int. J. Radiat. Oncol. Biol. Phys. 12: 793-799.

Patrick M.H. and Haynes R.H. (1964). Dark recovery phenomena in yeast. II. Conditions that modify the recovery process. Radiat. Res. 23: 564-579.

Paul J. (1970) Cell and Tissue Culture. Fourth Edition. E. and S. Livingstone, Edinburgh.

Pedersen P.L. (1978). Tumor mitochondria and the bioenergetics of cancer cells. Prog. exp. Tumor Res. 22: 190-274.

Persson M., Bleiberg B., Kiss D. And Miles J. (1991). Measurement of plasma acetate kinetics using high-performance liquid chromatography. Analyt. Biochem. 198: 149-153.

Phillips R.A. and Tolmach L.J. (1966). Repair of potentially lethal damage in X-irradiated HeLa cells. Radiat. Res. 29:413-432.

Pohlit W. and Heyder I.R. (1981). The shape of dose-survival curves for mammalian cells and repair of potentially lethal damage analyzed by hypertonic treatment. Radiat. Res. 87:613-634.

Powers W.E. and Tolmach L.J. (1963). A multicomponent X-ray survival curve for mouse lymphosarcoma cells irradiated in vivo. Nature 197:710-711.

Purohit S.C. and Pohlit W. (1982). Experimental evaluation of the glucose antimetabolite, 2-deoxy-D-glucose (2-DG) as a possible adjuvant to radiotherapy of tumors: I. Kinetics of growth and survival of Ehrlich ascites tumor cells (EATC) in vitro and of growth of solid tumors after 2-DG and X-irradiation. Int. J. Radiat. Oncol. Biol. Phys. 8: 495-499.

Purohit S.C. and Pohlit W. (1983). Proceedings of the 7th International Congress of Radiation Research, Amsterdam.

Raaphorst G.P., Azzam E.I., Sargent M. and Feeley M.M. (1988). Reduced pH increases recovery from radiation damage potentially leading to cell death and to in vitro transformation. Int. J. Radiat. Biol. 54: 1031-1040.

Raaphorst G.P. and Dewey W.C. (1979). A study of the repair of potentially lethal and sublethal radiation damage in Chinese hamster cells exposed to extremely hypo- or hypertonic NaCl solutions. Radiat. Res. 77: 325-340.

Racker E. (1965) in Mechanisms in Bioenergetics, Lecture 18, Academic Press, New York.

Racker E. (1976) A new look at mechanisms in bioenergetics, Academic Press, New York.

Racker E., Johnson J.H. and Blackwell M.T. (1983). The role of ATPase in glycolysis of Ehrlich ascites tumor cells. J. Biol. Chem. 258: 3702-3705.

Reddy N.M.S., Stevenson A.F.G. and Lange C.S. (1989). Trypsinization and the radiosensitivity of mitotic and log phase Chinese hamster V79 cells exposed to 250 kVp X-rays. Int. J.Radiat. Biol. 55: 105-117.

Reddy N.M.S., Mayer P.J. and Lange C.S. (1990). The saturated repair kinetics of Chinese hamster V79 cells suggests a damage accumulation-interaction model of cell killing. Radiat. Res. 121: 304-311.

Reinhard R.D. and Pohlit W.E. (1977). Aspects of the regulatory function of adenosine triphosphate on the radiation sensitivity of saccharomyces cerevisiae. Proceedings of an international symposium on the radiobiological research needed for the improvement of radiotherapy. International Atomic Energy Agency, Vienna. IAEA-SM-212/17139-149.

Reitzer L.J., Wice B.M. and Kennel D. (1979). Evidence that glutamine, not sugar, is the major energy source for cultured HeLa cells. J. Biol. Chem. 8: 2669-2676.

Retelewska W. and Leyko W. (1978). Effect of ionizing radiation on the energy metabolism of hog lymphocytes. Radiat. Res. 75: 336-347.

Robin E.D., Murphy B.J. and Theodore J. (1984). Coordinate regulation of glycolysis by hypoxia in mammalian cells. J.Cell. Physiol. 118: 287-290.

Robbins S.L. (1974) Pathologic Basis of Disease. W.B. Saunders, Philadelphia.

Rofstadt E.K., DeMuth P., Fenton B.M. and Sutherland R.M. (1988). ^{31}P Nuclear magnetic resonance spectroscopy studies of tumor energy metabolism and its relationship to intra capillary oxyhemaglobin saturation status and tumor hypoxia. Cancer Res. 48: 5440-5446.

Rojas A. and Joiner M.C. (1989). The influence of dose per fraction on repair kinetics. Radiother. Oncol. 14: 329-336.

Rotin D., Robinson B. and Tannock F. (1986). 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.

Sauer L.A., Stayman J.W. and Dauchy R.T. (1982). Amino acid, glucose and lactic acid utilization in vivo by rat tumors. Cancer Res. 42: 4090-4097.

Scherer E., Steffer C. and Trott K. (1991)(Eds). Radiopathology of Organs and Tissues. Springer-Verlag, Berlin.

Sevdalian D.A. and Zielke H.R. (1978). Depletion of cellular glycogen during the early logarithmic growth phase of human fibroblasts. Experentia 34: 843-844.

Seymour C.B and Mothersill C. (1981). The radiobiological effect of lactate on cells in culture. Int. J. Radiat. Biol. 40: 283-291.

Seymour C.B. and Mothersill C. (1987). The effect of glycolysis inhibitors on the recovery of CHO-K1 cells from split-dose irradiation. Acta Oncologica 26: 367-371.

Simonides W.S., Zaremba R., van Hardeveld C. And van der Laarse W.J. (1988). A nonenzymatic method for the determination of picomole amounts of lactate using HPLC : its application to single muscle fibres. Analyt. Biochem. 169: 268-273.

Skog S., Tribukait B. and Sundius G. (1982). Energy metabolism and ATP turnover time during the cell cycle of Ehrlich ascites tumour cells. Exp. Cell Res. 141: 23-29.

Skog S., Tribukait B. and Sundius G. (1983). Energy metabolism and ATP turnover time during the cell cycle in roentgen irradiated Ehrlich ascites tumour cells. Acta Radiologica Oncology 22: 369-379.

Skog S., and Tribukait B. (1986). Cell size following irradiation in relation to cell cycle. Acta Radiologica Oncology 25: 269-273.

Skog S., Nordell B., Ericsson A.,Tribukait B. and Nishida T. (1986). Changes in energy metabolism following roentgen irradiation of in vivo growing Ehrlich ascites tumour cells studied by ^{31}P magnetic resonance spectroscopy. Acta Radiologica Oncology 25: 63-69.

Snowdowne K.W., Freudenrich C.C. and Borle A.B. (1985). The effects of anoxia on cytosolic free cacium, calcium fluxes, and cellular ATP levels in cultured kidney cells. J. Biol. Chem. 260: 11619-11626.

Spence A.M., Graham M.M., Abbot G.L., Muzi M. And Llewellen T.K. (1988). Blood flow changes following 137Cs irradiation in a rat glioma model. Radiat. Res. 115: 586-594.

Spiro I.J., Kennedy K.A., Stickler R. and Ling C.C. (1985). Cellular and molecular repair of X-ray-induced damage: Dependence on oxygen tension and nutritional status. Radiat. Res. 101: 144-155.

Sridhar R., Koch C.J., Stroude E.C. and Inch W.R (1978). Cell survival in V79 multicell spheroids treated with dehydroascorbate, 5-thio-D-glucose and 2-deoxy-D-glucose. Br. J. Cancer 37: Suppl. III. 141-144.

Stackhouse M.A. and Bedford J.S. (1993). An ioizing radiation-sensitive mutant of CHO cells: irs-20. II. Dose-rate effects and cellular recovery processes. Radiat. Res. 136: 250-254.

Stanley J.A., Shipley W.U. and Steele G.G. (1979). The influence of tumour size on hypoxic fraction and therapeutic sensitivity of Lewis lung tumour. Br. J. Cancer 36: 107-108.

Stanley P.E. and Williams S.G. (1969). Use of the liquid scintillation spectrometer for determining adenosine triphosphate by the luciferase enzyme. Analyt. Biochem. 29: 381-392.

Sumbilla C.M., Zielke C.L., Reed W.D. Ozand P. T. and Zielke H.R. (1981). Comparison of the oxidation of glutamine, glucose, ketone bodies and fatty acids by human fibroblasts. Biochim. Biophys. Acta. 675: 301-304.

Sutherland R., Freyer J., Mueller-Kleiser W., Wilson R., Heacock C., Sciandra J. and Sordat B. (1986). Cellular growth and metabolic adaptations to nutrient stress environments in tumor microregions. Int. J. Radiat. Oncol. Biol. Phys. 12: 611-615.

Sutherland R.M. (1986). Importance of critical metabolites and cellular interactions in the biology of microregions of tumors. Cancer 58: 1668-1680.

Sutherland R.M., Ausserer W.A., Laderoute K.R. and Murphy B.J. (1995). Hypoxia-induced modulation of signal transduction and gene expression: Relevance to malignant progression in human tumors. Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Swanson C.P. and Webster P.L (1985). The Cell.5th Edition. Prentice-Hall, Englewood Cliffs.

Szeinfeld D. (1987) Investigation of some biochemical parameters relating to energy metabolism in experimental rodent tumours after exposure to ionizing radiation and magnetic fields. PhD Thesis, University of Cape Town, South Africa.

Tamulevicius P. and Streffer C. (1995). Metabolic imaging in tumours by means of bioluminescence. Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Tannock I.F., Steele D and Roberts J. (1986). Influence of reduced concentration of L-glutamine on growth and viability of cells in monolayer, in spheroids, and in experimental tumours. Br. J. Cancer 54: 733-741.

Thames H.D. (1985). An 'incomplete-repair' model for survival after fractionated and continuous irradiations. Int. J. Radiat. Biol. 47: 319-339.

Thames H.D. (1989). Repair kinetics in tissues: alternative models. Radiother. Oncol. 14: 321-327.

Tobias (1985). The repair misrepair model in radiobiology: comparison to other models. Radiat. Res. Suppl. 8: s77-95.

Tozer G., Suit H.D., Barlai-Kovach M., Brunengraber H. and Biaglow J. (1987). Energy metabolism and blood perfusion in a mouse mammary adenocarcinoma during growth and following X-irradiation. Radiat. Res. 109: 275-293.

Tozer G.M., Myers R. And Cunningham V.J. (1991). Radiation induced modification of blood flow distribution in a rat fibrosarcoma. Int. J. Radiat. Biol. 60: 327-334.

van Putten L.M. and Kallman R.F. (1968). Oxygenation status of a transplantable tumor during fractionated radiotherapy. J. Natl. Cancer Inst. 40: 441-451.

Van Steveninck J. (1968). Transport and transport-associated phosphorylation of 2-deoxy-D-glucose in yeast. Biochim. Biophys. Acta. 163: 368-394.

Varnes M.E., Tuttle S.W. and Biaglow J.E. (1984). Nitroheterocycle metabolism in mammalian cells. Stimulation of the hexose monophophosphate shunt. Biochem. Pharmacol, 33: 1671-1677.

Vaupel P.W., Frinak S. and Bicher H.I. (1981). Heterogeneous oxygen partial pressure and pH distribution in C3H mouse mammary adenocarcinoma. Cancer Res. 41: 2008-2013.

Vaupel P. (1995). Blood supply, O_2-status and metabolic microenvironment in human tumors. Congress proceedings, Tenth International Congress of Radiation Research, Wurzburg, Germany.

Verma A., Sharma R. and Jain V.K. (1982). Energetics of DNA repair in UV-irradiated peripheral blood leukocytes from chronic myeloid leukaemia patients. Photochem. Photobiol. 36: 627-632.

Villa-Trevino S., Shull K.H. and Farber E. (1966). The inhibition of liver ribonucleic acid synthesis by ethionine. J. Biol. Chem. 241(20): 4670-4674.

Wenner C.E. and Tomei L.D. (1981). In The transformed cell. Cameron I.L. and Pool T.B. (Eds). Academic Press, New York.

Wheeler K.T. (1987). A concept relating DNA repair, metabolic states and cell survival after irradiation. In Radiation Research, Proceedings of the 8th Congress of Radiation Research. Fielden E.M., Fowler J.F., Hendry J.H., Scott D. (Eds). Taylor and Francis, Edinburgh.

Wheeler K.T. and Nelson G.B. (1987). Saturation of a DNA repair process in dividing and non-dividing mammalian cells. Radiat. Res. 109: 109-117.

Whittaker P.A. and Danks S.M. (1978). Mitochondria: Structure, function and assembly. Longman, London.

Wice B.M., Reitzer L.J. and Kennel D. (1981). The continuous growth of vertebrate cells in the absence of sugar. J. Biol. Chem. 256: 7812-7819.

Wick A.N., Drury D.R., Nakada H.I. and Wolfe J.B. (1957). Localization of the primary metabolic block produced by 2-deoxyglucose. J. Biol. Chem. 224: 963-969.

Woodward G.E. and Hudson M.T. (1954). The effect of 2-deoxy-D-glucose on glycolysis and respiration of tumor and normal tissues. Cancer Res. 14: 599-605.

Yushok W.D. (1964). Metabolism of ascites tumor cells II. Inhibition of respiration by nonglycolyzable sugars phosphorylated by hexokinase. Cancer Res. 24: 187-192.

Zanelli G.D. and Lucas P.B. (1976). Effect of stress on blood perfusion and vascular space in transplanted mouse tumours. Br. J. Radiol. 49:382-383.

Zielke H.R., Ozand P.T., Tildon J.T., Sevdalian D.A. and Cornblath M. (1978). Reciprocal regulation of glucose and glutamine utilization by cultured human diploid fibroblasts. J. Cell. Physiol. 95: 41-48.

Zielke H.R., Ozand P.T., Tildon J.T., Sevdalian D.A. and Cornblath M. (1976). Growth of human diploid fibroblasts in the absence of glucose utilization. Proc. Natl. Acad. Sci. USA 73: 4110-4114.

Zielke H.R., Zielke C.I. and Ozand P.T. (1984). Glutamine: a major energy source for cultured mammalian cells. Federation Proc. 43: 121-125.

Zimmer K.G. (1961). Studies on quantitative radiation biology. Oliver and Boyd, Edinburgh.

Zwartouw H.T. and Westwood J.C.N. (1958). Factors affecting growth and glycolysis in tissue culture. Br. J. Exp. Path. 39: 529-539.