TUMOUR METABOLISM AND RADIOPROTECTION OF NORMAL TISSUE IN BALB/c AND CBA MICE

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TUMOUR METABOLISM AND RADIOPROTECTION OF NORMAL TISSUE IN BALB/c AND CBA MICE

by

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Thesis submitted in fulfilment of the requirements for the Masters Diploma in Technology (Medical Technology) at the School of Life Sciences at the Cape Technikon.

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I declare that this thesis is my own work. It is being submitted for the Masters Diploma in Technology (Medical Technology) at the Cape Technikon, Cape Town.

It has not previously been submitted for any diploma, degree or examination at any other institution. This study was carried out at the Institute for Medical Biophysics, Medical Research Council.

N de Villiers ..... 20/11/92.

Neil Heinrich de Villiers Date

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This work is dedicated to my parents who believed in me and supported me through my studies and to Dr D Szeinfeld who inspired me to do this work through his enthusiasm for and his great knowledge of this subject.

Prediker 1: 13 (ou vertaling)

En ek het my hart daarop gerig om met wysheid te ondersoek en na te speur alles wat onder die Hemel gebeur. Dit is 'n moeilike taak wat God aan die mensekinders gegee het om hulle daarmee te kwel.

Ecclesiastes 1: 13 (N.I.V version)

I devoted myself to study and to explore by wisdom all that is done under heaven. What a heavy burden God has laid on men.

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LIST OF ABBREVIATIONS

ABBREVIATION	FULL NAME
$AcidP_A$	Acid phosphatase activity
AP	Acid phosphatase
aq	aqua
ATP	Adenosine triphosphate
CaNT	Cancer neck tumour
c	concentration
°C	degrees Celsius (unit of temperature)
d	light path length
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
E	Extinction
ϵ	Molar extinction
F-1,6-DPase	Fructose-1,6-diphosphatase
FBM	Final body mass
FC	Food consumption
G-3-PDH	Glycerol-3-phosphate dehydrogenase
G-6-FDH	Glukose-6-fosfaat dehidrogenase

ABBREVIATION	FULL NAME
g	gram (unit of mass)
GTP	Guanosine triphosphate
Gy	Gray (unit for radiation dose 1 Gy = 100 rad)
h	hour (unit of time)
HK	Hexokinase
kg	Kilogram (unit of mass)
LDH	Lactate dehydrogenase
mg/ml	milligram per millilitre (unit of concentration)
μl	micro litre (unit of volume)
min	minute (unit of time)
ml	millilitre (unit of volume)
mm^3	cubic millimetre (unit of volume)
M	Molar (unit of concentration)
MRC	Medical Research Council
NADH	Nicotinamide adenine dinucleotide (reduced form)
NAD	Nicotinamide adenine dinucleotide (oxidized form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NADP	Nicotinamide adenine dinucleotide phosphate (oxidized form)
nm	Nano metre (unit of wavelengths)

ABBREVIATION	FULL NAME
nMol	Nano mol (unit of concentration)
N	Normal (unit of concentration)
n	number of items in a group (statistical symbol)
P	Probability (statistical symbol)
r	Linear regression factor (statistical symbol)
RNA	Ribonucleic acid
SEM	Standard error of the mean (Statistical abbreviation)
SF	Suurfosfatase
SGD	Specific growth delay
SMF	Survival modification factor
SSD	Source to surface distance
s	Standard deviation
TBM	Tumour bearing mice
TM	Tumour mass
TV	Tumour volume
UV	Ultra violet spectrum
VIS	Visible light spectrum
WC	Water consumption
$\bar{X}$	Mean (statistical symbol)

SUMMARY:

The steady state in a tumour rapidly changes with its growth and the subsequent deteriorating blood and nutrient supply. This adaptation in the steady state of the tumour is shown in the increased lactate dehydrogenase and acid phosphatase activity in the tumour during it's growth. These alterations in the tumour metabolism places an increased burden on the body to supply nutrient and to discard the waste products of the tumour. This is demonstrated at the macroscopic level by the decreasing body weight and food intake when the tumour burden increases, and also at the metabolic levels by the responses of certain glycolytic and Cori cycle enzymes. Furthermore three distinct stages were observed in the Cori cycle response to the influence of the tumour namely, a silent or preclinical stage, a hypermetabolic stage and a hypometabolic stage. Although the decreasing body weight cannot be directly linked to the process of gluconeogenesis, the onset of anorexia appeared to coincide with the end of the hypermetabolic stage and the beginning of the hypometabolic stage in gluconeogenesis. This clearly shows that the body's steady state is adversely affected by the presence of the tumour and that the conditions at the metabolic level seem to cause the anorexia. Furthermore, it is well known that the success of cancer therapies depends entirely on the effectiveness of the modality to kill the tumour cell and on the ability of the host to absorb the damage caused by the modality without being destroyed in the process itself. The second part of this study demonstrates the radioprotective effects of ATP at all levels. It is clear from this work that ATP had a bigger influence in protecting the normal tissue than it had on the tumour tissue. This was demonstrated by the response of acid phosphatase (AP) and glucose-6-phosphate dehydrogenase (G-6-PDH) in the tumour and testis. Furthermore, it would seem that ATP has a multifactorial interaction with the cell, two possible mechanisms of protection are indicated by these results. The first of these

interactions is through the receptors of the cell to stimulate enhanced glycolysis, for higher energy production and thus repair. The second possibility is the interaction of ATP with the receptor of the cell to inhibit the production of free radicals and thus damage, as demonstrated by the response of G-6-PDH and AP.

OPSOMMING

Die metaboliese balans van tumore verander vinnig gedurende die groei van die tumor as gevolg van 'n verswakkende bloed en voedingstof voorsiening. Dit word gedemonstreer deur die laktaat dehidrogenase (LDH) en suurfosfatase (SF) aktiwiteit wat verhoog soos die tumor groter word. Hierdie veranderings in die tumor metabolisme plaas 'n verhoogde las op die liggaam om voedingstowwe te voorsien en van afvalstowwe ontslae te raak. Dit word gedemonstreer op makroskopiese vlak deur die verlies in liggaamsmassa en die vermindering van die voedselinname soos die tumor groter word, en op die metaboliese vlak deur die aktiwiteit van sommige glukolitiese en Cori siklus ensieme. Verder word daar drie stadia in die reaksie van die Cori siklus bemerk, naamlik, die stil of prekliniese stadium, die hipermetaboliese stadium en die hipometaboliese stadium. Alhoewel die verlies van liggaamsmassa nie direk verbind kan word aan glukoneogenese nie, wil dit voorkom dat die begin van anoreksie saam val met die end van die hipermetaboliese stadium en die begin van die hipometaboliese stadium. Dit wys duidelik dat die liggaam se metaboliese balans negatief beinvloed word deur die teenwoordigheid van die tumor en dit blyk dat die toestande op die metaboliese vlakke aanleiding gee tot die anoreksie.

Dit is ook verder goed bekend dat die sukses van kankerterapie geheel en al afhanklik is van die effektiwiteit van die modaliteit om tumorselle dood te maak en van die liggaam om die newe-effekte te absorbeer sonder om self vernietig te word. Die tweede gedeelte van hierdie studie demonstreer die radiobeskermende effekte van ATP op alle vlakke. Dit is ook duidelik dat ATP normale weefsel meer beskerm as die tumorweefsel, en word gedemonstreer in die aktiwiteit van SF en glukose-6-fosfaat dehidrogenase (G-6-FDH) in die tumor en testis. Verder blyk dit dat ATP 'n multifaktoriese interaksie met die sel het. Twee

moontlike meganismes waardeur ATP die sel beskerm, word aangedui deur die resultate. Die eerste meganisme geskied deurdat ATP inwerk op die reseptore van die sel en sodoende die glukolise in die sel verhoog wat lei tot meer energie wat geproduseer word en dus 'n groter potensiaal vir herstel. Die tweede moontlike meganisme is die interaksie van ATP met die reseptore van die sel wat die produksie van sommige vry radikale inhibeer en dus ook skade. Die meganisme word gedemonstreer deur G-6-FDH en SF. Dus word die pasient beskerm teen sommige newe effekte van die tipe behandeling.

INTRODUCTION

Chapter 1

INTRODUCTION

The capacity to repair any injury sustained by the body is dependant upon the amount of damage that occurs as well as the state of health that is present in the body at that time. This is a factor that becomes more important when treating certain disease states which require drastic treatments, for example malignant neoplasms.

Some cancer therapies can have as severe effects on the normal tissue as on tumour tissue. Seeing that the body's state of health is already compromised by the presence of the disease, further interference can be very harmful. This is considered one of the limiting factors of aggressive treatment of cancer and could possibly contribute to failure of certain cancer therapies.

To be able to investigate the holistic problem of cancer and its treatment it is necessary to first understand what occurs in a tumour itself, how it influences the body's metabolism, and finally a study of the influence of the treatment on the metabolism of both tumour and normal tissue can be done.

The first part of this thesis is concerned with tumour metabolism and it's influence on the host.

In a tumour we find three types of tissue namely, normoxic, hypoxic and necrotic. This is a unique feature of solid tumours that is due to an abnormal and deficient

vasculature and an abnormal fast growth rate of tumour tissue. The tumour requires vast amounts of nutrients to maintain it's rapid growth. This leads to an altered steady state in the tumour and therefore an altered metabolism. Otto Warburg (1956) was the first to report on these alterations in metabolism when he found that tumour cells could survive for 24 hours without oxygen and tend to have an increased anaerobic metabolism. The fact that tumour cells mostly utilize glucose only up to lactate formation as well as their increased metabolism to cope with the nutrient requirement to facilitate their fast growth rate, places increased stress on the rest of the steady state of the body. The body now has to cope with an increased amount of lactate as well as an increased demand for nutrients. Moreover Kallinowski *et al* (1988) showed that tumours are able to increase it's glucose consumption, depending on it's availability. Thus, the attempts to correct the imbalance in the steady state are futile and costly to the body. This may lead to other abnormalities like anorexia and cachexia, and the body becomes weaker and less capable to repair damage that occurs either through treatment or injury. This study intends to investigate the effect of the tumour on the glucose metabolism in the liver and kidney of the host, as well as its effects on the physiological parameters of the rodent host.

The second part of this thesis intends to evaluate the efficacy of different anticancer modalities. The state of health of the cancer patient is usually the limiting factor in the treatment of this disease. Seeing that all cancer therapies also affect the normal tissue in the vicinity of the tumour, it is essential to reduce the dose of each modality in order not to destroy the healthy tissue surrounding the tumour. This

could have the effect that the dose that reaches the tumour is insufficient to kill the malignant cells, thus rendering the treatment obsolete. Therefore the tumour should either be sensitized to radiation without the normal tissue being affected, or the surrounding tissue should be protected while rendering the tumour tissue unprotected.

Due to the disorganization of the vasculature, drug distribution in a tumour is very poor. This implies that the tumour will receive less of the sensitizing or protecting drug given. For this reason it was decided, for the purpose of this study, to make use of a radioprotectant.

The problem with drugs is that they are artificial and can be toxic to the body. The ideal radioprotection drug would be a chemical substance that normally functions in the body, such as ATP. The radioprotective properties of ATP have been demonstrated by different investigators, in experiments ranging from investigation of DNA damage in spermatogonia (Benova and Baev, 1974;1978; Benova, 1986), to survival studies on monkeys (Nikolov *et al*, 1986). Several researchers have suggested that ATP acts via the purinergic receptors to mediate the radiation response. This is the reason why ATP was considered the radioprotector of choice. The aim of this study was to investigate the possibility of ATP to protect the normal tissue without decreasing the effect of radiation on the tumour to such an extent that it loses its therapeutic value. Furthermore, by protecting the normal tissue, the dose of radiation can be increased without harming the patient.

The aim of this thesis is to further understand the interrelationship between the tumour and the host, and to study the effect of certain treatment modalities on the

holistic system, in order to try and protect the body from the adverse effects of effective cancer therapy modalities.

Study Objective: The objective of this study was to understand the effect of the tumour on the host state of health and to evaluate the radioprotective effect of ATP on the normal tissues.

LITERATURE REVIEW

CHAPTER 2

CHAPTER 2

LITERATURE REVIEW

Since the beginning of time, man has always been searching for ways to improve his quality of life, health and comfort. Thus, mankind has always tried to answer the health questions of his specific era. In this era there are a number of major diseases that concern the human being, for example heart disease and cancer to mention but two. Next to heart disease, cancer is the biggest killer of mankind with approximately 23% of mortalities in the U.S.A. caused by cancer in the last decade. In the westernized countries about 1 000 000 new cases of cancer are identified each year (Robbins *et al*, 1984). Therefore the two major factors motivating cancer research are the intellectual challenge of understanding a problem that is almost unrivalled in complexity, and the need to relieve human suffering (Vincent, 1985).

The transformation of normal cells to malignant cells are induced in various ways, namely by chemical carcinogens, physical carcinogens and viral carcinogens (oncogenic viruses). The ideal modality of cancer therapy will cure the tumour without toxicity, discomfort, suffering or anxiety and will still be affordable to the patient(Vincent, 1985). This ideal is still far from reached. The strategy of cancer treatment depends in part on the tumour characteristics and stage of development. Methods of treatment usually employed are surgery, radiotherapy, chemotherapy, hormone therapy and/or immunotherapy (Louis, 1978).

2.1 SOLID TUMOURS

A tumour can be defined as an abnormal mass of tissue or an abnormal cell population with a capacity for progressive growth (Louis, 1978).

The growth rate of the tumour correlates inversely with the degree of differentiation of the tumour cells. Thus, the more differentiated the cells become, the slower they tend to divide (Robbins *et al*, 1984). The size of a tumour is governed by at least three parameters, namely the cell cycle time, the size of the growth fraction and the rate of cell loss (Louis, 1978). If the cells do not mature, they can live much longer than the normal cells. It is also known that as normal cells become specialized, they tend to lose their ability to proliferate, but in the neoplastic cell its replication capability may be retained along with its specialization (Robbins *et al*, 1984). Due to the rapid growth of solid tumours, most of them have an insufficient neovascularization, which means a decreasing number of nutritive vessels per unit of tissue (Robbins *et al*, 1984; Vaupel *et al*, 1981; Vogel, 1965). Not only are there less nutritive vessels, but also a reduction in vascular space and surface area per unit of tissue volume (Vogel, 1965; Vaupel *et al*, 1981). Furthermore, vascular abnormalities like the broadening in the intercapillary distance, the appearance of lacuna-like, sinusoidal, and cystiform blood vessels and an increase in the number of arteriovenous anastomoses lead to a loss of hierarchy in vessel arrangement. To add to the already abnormal nature of tumour vascularization, the blood vessels become much more fragile, due to the appearance of thin endothelia with wide gaps and a lack of pericytes, which will lead to the blood vessels becoming

distorted and eventually crushed by the tumour cells that proliferate in the limited space within the tumour. Owing to this haphazard manner in which the vasculature pattern develops in malignant tumours, abnormal cellular spatial relationships exist within the tumour. Abnormalities such as a decrease in tumour blood flow per unit of tissue volume, and an increase of vascular flow resistance (Sevick *et al*, 1989; Vaupel *et al*, 1981), caused by the abnormal vasculature pattern discussed above, occur in the microcirculation of the tumour. Due to this higher resistance, vascular prestasis and stasis are found in the tumour, which lead to an arteriovenous shunt in perfusion. Other abnormal features of tumour circulation are that there are no drainage for lacuna-like blood vessels, and the presence of many non-flowing capillaries; micro- and macrothromboses and occlusion of certain vessels also occur, which cause certain regions to become totally ischemic (Vaupel *et al*, 1981; Robbins *et al*, 1984). The growth of the tumour is dependant upon an adequate supply of nutrient and oxygen to the cells which is limited, due to the abnormal vasculature. This results in the malignant tumour having, in addition to oxygenated cells, which may be dividing or non dividing, poorly oxygenated cells(hypoxic cells), which could be located some distance from the blood vessels (Gray *et al*, 1953). There will also be areas within tumours, which have been starved of all nutrients including oxygen, where the cells are necrotic.(Symington,1974; Vogel,1965; Hall,1973;Robbins *et al*, 1984)

2.1.1 Tumour effects on the host

Rapid tumour growth can be associated with the vast detriment to the host(Rapaport,1989). The presence of any tumour can lead to certain local mechanical effects, such as anatomical disfigurement, obstruction and pressure (Louis, 1978; Robbins *et al*, 1984). These effects are determined by the size and location of the tumour. The symptoms the patient experiences are not necessarily produced by the malignant tumour itself, (Robbins *et al*, 1976), but by its pressure on, or obstruction of an essential organ causing it to fail (Louis, 1978; Robbins *et al*, 1984). Hormonal effects can be produced by malignant tumours although benign tumours are known to be more notorious for this phenomenon (Louis, 1978). The symptoms most commonly experienced by cancer patients are those of anaemia, infection, fever, cachexia, carcinomatous neuropathy, pulmonary osteodystrophy and thrombotic complications (Robbins *et al*, 1976; Louis, 1978). The death of a cancer patient is normally due to one or more of the above mentioned complications (Louis, 1978; Robbins *et al*, 1984:254). This study intends to investigate only the phenomenon of cancer cachexia.

2.1.1.1 Cancer cachexia

Cancer cachexia is a state in which the body becomes weak and loss of normal body mass is apparent. (Robbins *et al*, 1976) This is caused by the fact that the body spends more energy than is supplied by the diet. A complicated relationship between food intake and cachexia exists, which involves several physiological

mechanisms such as hunger and satiety control, glucostatic and lipostatic regulation, nitrogen balance and hormonal balance (Guaitani, 1982). Cancer cachexia is a constitutional disease in which the increased resting energy expenditure in a patient with a growing tumour is observed (Zylics *et al*, 1990; Lowry, 1991). The higher energy demand is not only due to tumour growth, but also to higher protein turnover(Vorster, 1990; Zylics *et al*, 1990). This is caused by an acceleration in liver protein synthesis, (Karlberg *et al*, 1983; Warren *et al*, 1985), and also the tumour utilizing the body's supply of glutamine for its own protein production, (Ekman *et al*, 1982; Stein *et al*, 1976; Pratt *et al*, 1958). Thus, there is not only stress on the host's glucose supply, but also on the protein stores of the body, which lead to an ever increasing energy expenditure to provide sufficient building blocks and fuel. With the tumour cell requiring vast amounts of glucose to maintain its energy steady state (Guaitani, 1982), the host is constantly at a negative energy balance(Vorster, 1990). A low blood glucose level and a high blood lactate level is seen in many cancer patients due to the high glucose turnover to lactate in the tumour.(Lowry *et al*, 1980; Ekman *et al*, 1982). This imbalance stimulates the host to produce glucose from other non-carbohydrate sources by the process of gluconeogenesis (Ekman *et al*, 1982). The body can produce glucose by using at least three principle non-carbohydrate sources, namely, lactate, glycerol and the majority of amino acids(Stryer, 1975). Thus, the amino acids can either be used for protein re-synthesis (Zylics *et al*, 1990), or oxidized in gluconeogenesis (Lowry *et al*, 1981), and ureagenesis pathways, to form new glucose for the rest of the body(Stryer, 1975). The process of gluconeogenesis is an energy consuming process which utilize 6 high

energy phosphates whereas anaerobic glycolysis only generate two. This means that there is a net loss of four high energy phosphates, which have to be supplied by the body(Stryer, 1975). Gluconeogenesis is mainly found in the liver and the cortex of the kidney, but very little in the brain and skeletal muscle(Stryer, 1975; Lundholm *et al*, 1976). The main function of this process is to supply enough glucose for the metabolic demands of the brain, muscle and the tumour (Stryer, 1975).

The present study focuses the attention on some aspects of the gluconeogenesis metabolism as a pivotal process in the host's attempt to maintain homeostasis during cancer cachexia. In these studies, the activities of some enzymes of the gluconeogenesis and glycolytic pathways of the liver and kidney of CBA mice bearing the CaNT tumour, were investigated as indicators of the progress of this pathophysiological state in the host.

2.2 RADIATION

After the discovery of X rays and radioactivity in the 1890's, it was also applied in the field of cancer therapy with varying success. The success of this type of therapy was hampered by the discovery of all its inherent dangers, thus doses were limited to that which the patient could safely absorb. In many instances this was less than the dose required to kill the tumour. At present scientists are trying to formulate the radiation, or combinations of treatment, that would have the maximum effect on the tumour without harming the normal tissue.

One can distinguish between two types of radiation, namely ionizing and

non-ionizing radiation. The main difference between ionizing and non ionizing radiation is the size of the energy packets that they carry (Hall,1973). Radiation is considered to be ionizing if the photon size is above 124 eV (Hall,1973).

2.2.1 Types of ionizing radiation

There are two main classes of ionizing radiation namely electromagnetic and particulate radiation.

2.2.1.1 Electromagnetic radiation

The most common examples of electromagnetic radiation in radiotherapy are X- and gamma rays. X-rays are produced extranuclearly by a device that accelerates electrons and then stops them in a target made of either tungsten or gold, part of the kinetic energy of the fast electrons are given off as X-rays. Gamma rays on the other hand are produced intranuclearly by radioactive isotopes, the energy for the rays is obtained from the unstable nucleus of the isotope breaking up to form a more stable element.

2.2.1.2 Particulate radiation

There are two main types of particulate radiation, namely charged and uncharged particles . The only type of uncharged particulate radiation is neutron radiation. However, there are several types of charged particle radiation such as electrons, protons, α particles, negative π mesons, and heavy charged ions.

i) Uncharged particulate radiation

Neutrons: These are particles with no charge, but with the same mass as a protons, thus they cannot be accelerated by an electrical device. This type of radiation is produced by deuteron particles that are accelerated and impinged upon a beryllium target which strips the protons of the deuterium leaving only the fast neutrons. This was considered to be the ionizing radiation of choice for this study.

ii) Charged particulate radiation

Electrons: Electrons are produced in an accelerator called a betatron, the particles are accelerated to close to the speed of light.

Protons: Protons being heavier than electrons (about 2000 times) requires more complex and expensive equipment to accelerate them to a useful speed.

α particles: This type of radiation consists of nuclei of helium atoms (two neutrons plus two protons) which are positively charged, thus they can be accelerated in a similar device as is used for protons. This type of radiation is also produced during decay of radioactive isotopes.

2.2.2 Types of non-ionizing radiation

There are many types of non-ionizing radiation namely radiowaves, radar, visible light, infrared, ultraviolet and microwaves. These types of non-ionizing radiation are all electromagnetic radiation. For the present investigations, the microwave radiation was used in rodent tumours and normal tissue.

2.2.3 The interaction of ionizing radiation and living matter

The interaction of radiation with living matter can take place via two different mechanisms namely, by direct ionization or by indirect ionization.

When a particle has sufficient kinetic energy it can cause direct damage to the macromolecules of the absorbing matter, this is termed direct ionization. This action has three possible routes to follow, cell death can be caused by single- or multi-hits on one target or by multi-targets being hit and damaged. Damage may occur in any part of the cell. Structures most likely to be damaged by radiation are DNA, lysosomes, mitochondria, membranes and in some cases, enzymes (Workman,1985).

When a photon of an electromagnetic radiation interacts with matter, it reacts with the orbital electrons, setting fast electrons in motion. On the other hand if neutrons interact with matter, it reacts with the nucleus, setting fast protons in motion. The difference in particle sizes of electrons and protons is the main reason for the different ionization and excitation tracks through matter, and accounts for the differences in the biological effects between X-rays and neutron radiation (Hall,1973). Both types of radiation produces ion pairs in the ionization process. Although the ion pairs have a very short lifetime of approximately 10^{-10} seconds, they give rise to free radicals. These free radicals do not carry a charge like the ion pairs, but only an excess of energy due to an unpaired electron in the outer shell of the atom. This makes the free radical, which has a longer lifetime, highly reactive and the excess energy can break chemical bonds (Hall,1973). The types of free radicals produced are e-aq, $OH\cdot$ and $H\cdot$. The free radicals are

formed during radiolysis of water in the cell, which can cause damage to the macromolecules such as DNA and RNA. The mechanism of interaction of the radiation with the living matter produced by free radicals, is the indirect effect of radiation.

2.2.4. The clinical application of radiation

The application of radiotherapy for cancer treatment should be considered with three parameters in mind. These are i) the difference in radiosensitivity between the tumour cells to be treated and the normal cells that fall within the treatment area, ii) the difference in the repair capacity of the tumour cells and its surrounding normal cells, and iii) the ability of the organ in question to repair radiation damage. The ideal in radiotherapy is the total eradication of the tumour with no sign of functional or structural damage to the normal tissue. This goal is far from reached, and most regimes are compromises between damage and therapeutic gain (Rubin and Poulter,1978). The problem with radiotherapy in tumours, is that the tumour has a heterogeneous distribution of cells. The tumours basically contain three types of cells, namely, oxic, hypoxic and necrotic cells. These cell types differ in radiosensitivity with the hypoxic cells being much more radioresistant (Gray *et al*, 1953). The aim of increased therapeutic gain is to increase the difference in radiosensitivity between the tumour and the normal cell. This can be achieved by either a radiosensitizer that will only sensitize tumour cells to radiation, or a protector that will only protect normal cells. This study investigated the aspect of potential radioprotection at both

macroscopic and biochemical levels. In many tumours there is an inadequate vascular density and capillary surface area which will lead to poor distribution of any anticancer drug or radiosensitizer, (Corbett and Valeriote,1987; Sevick and Jain, 1989), thus more radiosensitizer may be present in normal tissue than in tumour tissue. Most drugs currently available are not truly cancer-specific, as most tumours are not usually more sensitive to it than the vital stem cells of normal tissues (Corbett and Valeriote,1987). This study focused on the radioprotective properties of ATP in normal and tumour tissue after radiation damage.

2.3. METABOLISM

Homeostatic regulation of the internal environment in animals was stressed by Claude Bernard in the late 19th century. This implies that all the necessary enzyme-catalyzed reactions take place at a rate responsive to changes in the internal and external environments. An example of metabolism displaying changes in the internal environment of the cell, is when damage occurs in the cell and repair mechanisms occur. A cell or organism can be considered diseased if it responds inadequately, or incorrectly, to internal or external stress. These modifications in the steady state of the tumour and normal tissues by internal and external stressors were monitored at the enzymatic levels.

2.3.2 Anaerobic glycolysis

Anaerobic glycolysis consist of two major pathways, namely the Embden-Meyerhof pathway and the Pentose Phosphate shunt.

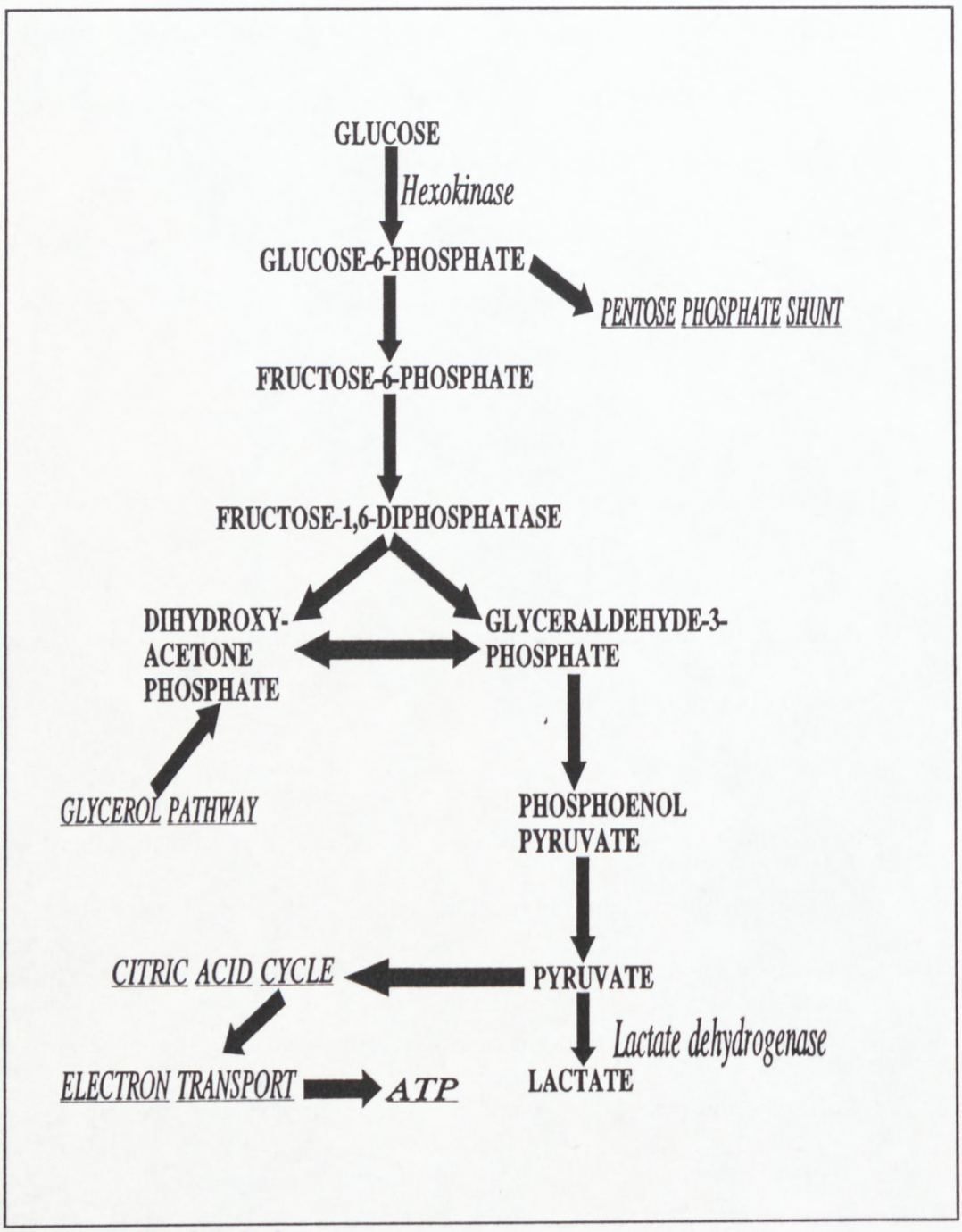

Figure 2.1: *Glycolytic Pathways*

2.3.2.1 Embden-Meyerhof pathway

This mechanism of carbohydrate metabolism is one of the most ancient

mechanisms known to man, and stems from the time that the earth had an atmosphere lacking oxygen. Most of the higher organisms have retained the capability of anaerobic metabolism, and utilize it for the preparation of glucose for aerobic glucose catabolism. The glycolytic pathway consists of 11 separate enzyme steps of which only two were used in this study, namely hexokinase (first step) and lactate dehydrogenase (last step) as shown in Figure 2.1.

i) Hexokinase

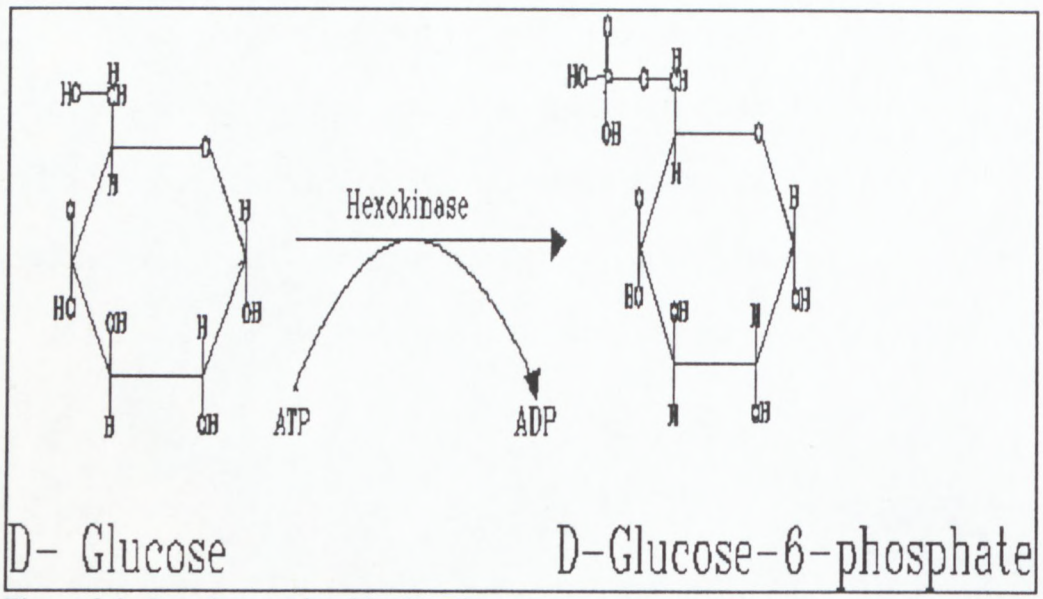

Figure 2.2: ***Reaction catalyzed by Hexokinase.***

The term "kinase" refers to the fact that this enzyme belongs to the group of phosphotransferase enzymes, and the prefix "Hexo" refers to the fact that the enzyme phosphorylates all 6-carbon sugars. This enzyme prepares glucose and other 6-carbon sugars for breakdown (Fig 2.2). The step is not only important for the priming of glucose, but prevents glucose from leaving the cell by phosphorylation, and at the same time creates a gradient so that more glucose can move into the cell.

ii) Lactate dehydrogenase

Lactate dehydrogenase is the last reaction in the Embden-Meyerhof pathway. The reaction of this enzyme is reversible. The direction of this enzyme is dependant on the balance in concentration of the substrate (Pyruvate,NADH), and the product (lactate,NAD^+). Under normal circumstances NADH is produced in the reaction catalyzed by glyceraldehyde dehydrogenase and is oxidised back to

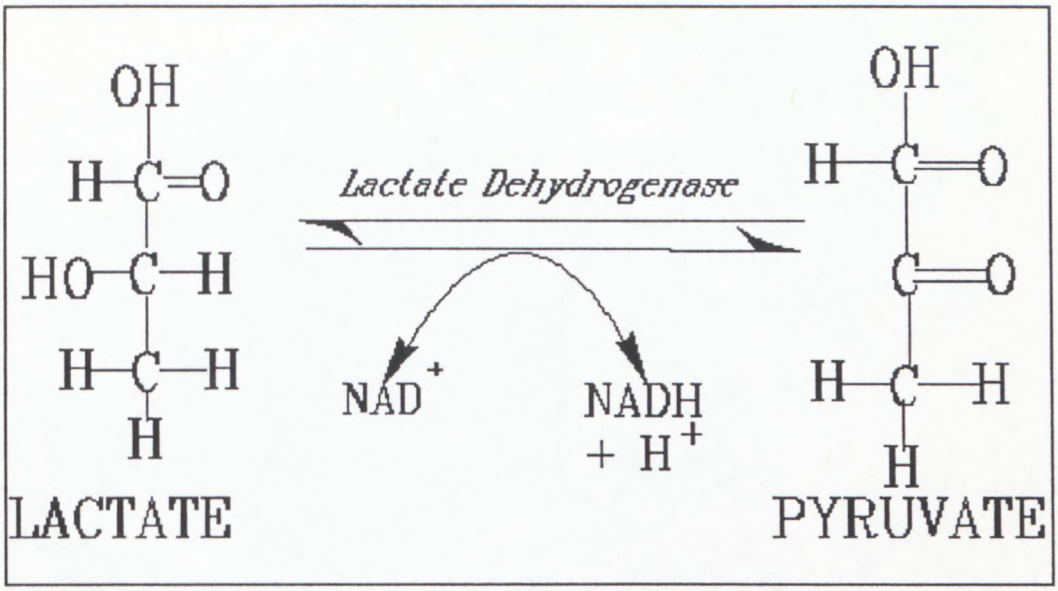

Figure 2.3: ***The reaction catalyzed by Lactate dehydrogenase.***

NAD^+ by the electron transport system in the mitochondria, thus keeping the NAD^+-NADH steady state. If, for any reason, the mitochondria are unable to cope with the supply of pyruvate and NADH, this enzyme (LDH) will oxidize the NADH back to NAD^+ and thus act as a salvage pathway to maintain the NAD^+-NADH steady state. An example of this scenario is found when a muscle is under low tension of oxygen (hypoxia), and requires more energy than the mitochondria can provide, the glycolysis will be increased and the pathway will follow through to lactate as end product. As one can see from the reaction that takes place (Fig 2.3), this enzyme oxidizes NADH to NAD^+ required for glycolysis to continue past the glyceraldehyde dehydrogenase step that needs NAD^+.

2.3.2.2 Glycerol pathway

i) Glycerol-3-phosphate dehydrogenase

This is the second enzyme in the pathway to introduce glycerol into the glycolysis

pathway. This enzyme uses glycerol-3-phosphate and NAD^+ as co-factor, and produces acetone phosphate and NADH. This can either be further metabolized by the glycolysis pathway, or be built back to glucose by gluconeogenesis.

2.3.2.3 Pentose Phosphate shunt

The pentose phosphate shunt is an alternative route for the Embden-Meyerhof pathway in the metabolism of glucose in certain tissues. Organs that contain the necessary enzymes for this pathway are the liver, mammary glands, testes, and adrenal cortex, and 30% to 60% of the normal metabolized glucose of these organs is oxidized by this pathway (Scheve,1984). This pathway has a number of important functions, the first of which is producing NADPH, an extra mitochondrial reducing agent, the second being to convert hexoses into pentoses and so provides a means for 3,4,5,6,7 carbon sugars to enter into the glycolysis pathway. The third function of this pathway is to create ribose-5-phosphate, which acts as a building block of both RNA, DNA and nucleotide three phosphates (ATP,GTP). The fourth function of this pathway is to provide an alternative pathway for glucose oxidation. Two molecules of NADPH are produced per CO_2 molecule; this means that each molecule of glucose produces 12 molecules of NADPH. If this is converted into NADH by a transhydrogenase reaction, 36 molecules of ATP can be formed (3 ATP/NADH). Thus, this pathway is equivalent to aerobic metabolism (Scheve,1984). The enzyme of interest in this pathway is Glucose-6-phosphate dehydrogenase.

i) Glucose-6-phosphate dehydrogenase:

This enzyme is the first in the pentose phosphate shunt. The dehydrogenation of

glucose-6-phosphate to glucono-δ-lactone-6-phosphate, producing NADPH from $NADP^+$, is catalyzed by this enzyme. This enzyme is specific for $NADP^+$ as co-factor.

2.3.3 Gluconeogenesis:

The term gluconeogenesis implies the synthesis of new glucose, or glycogen from sources such as lactate, pyruvate, glycerol, and amino acids. In mammals this process mainly occurs in two tissues, namely the liver and kidney. The conversion of lactate to glucose via the Cori cycle is of physiological importance in the homeostatic process of the body. This is because anaerobic glycolysis does not only produce lactate, but also hydrogen ions, which can cause lactic acidosis if not removed by gluconeogenesis. Glycerol on the other hand is obtained by the hydrolysis of triglycerides. Although the majority of steps in the glycolysis pathway are reversed in the glyconeogenesis pathway, the reactions in glycolysis where ATP is hydrolysed can not be reversed, and alternative enzyme systems are used. There are three enzymes which show an irreversible reaction, namely: (1) Hexokinase, (2) Phosphofructokinase, and (3) Pyruvate kinase. The alternative enzyme for the catalysis of the reverse reaction catalyzed by Phosphofructokinase, is Fructose-1,6-diphosphatase, which we have investigated in this study. This enzyme is a specific indicator of the gluconeogenesis in the cell. Lactate as precursor enters the Cori cycle by the reversal of direction of the enzyme lactate dehydrogenase as discussed previously. Glycerol on the other hand enters the pathway in the same way as for glycolysis, and can either be broken down to energy CO_2 and H_2O, or can be synthesized back to glucose.

2.3.4 Lysosomal degradation:

The lysosome is an organelle that creates an acidic environment, and degrades material from either inside or outside the cell to its basic constituents for the cell to use, or excrete. When a cell is injured, the damaged component is cleaved by hydrolytic enzymes to its component residues. The lysosome contains a specific group of enzymes, namely, the acid hydrolases. This group is made up of five types of enzymes, namely, nucleases, proteases, glycosidases, lipases, and phosphatases. The only one of importance for this study, was the phosphatases. This enzyme is responsible for the lysis of phosphate esters and was first studied as far back as the 1950's (de Duve,1983).

2.3.4.1 Acid phosphatase

Acid phosphatase is one of the acid hydrolases found in the lysosome of the cell. This enzyme catalyses the breakdown of phosphate esters such as phospholipids found in the membrane of the cell; it functions at a pH of $\pm$ 4. The activity of this enzyme has been recognised by many researchers to be an indicator of cell injury (Shah and Bhatavdekar,1983), or cell death (Bowen and Ryder 1974;1976).

2.3.5 Adenosine-5'-triphosphate:

2.3.5.1 Biochemistry

This molecule consists of three different component molecules, namely a ribose molecule, a purine ring and three phosphate molecules. The phosphate tail of ATP is energy rich, and when ATP is hydrolysed to ADP, -36 800 J/mol is

released; this is enough energy to drive non-spontaneous reactions. The pathway that produces the basic building blocks of ATP, starts from the pentose phosphate shunt where ribose-5-phosphate is produced; the AMP basis is produced first, and then phosphorylated to ADP, which is further phosphorylated via other energy producing reactions such as the electron transport system in the mitochondria.

2.3.5.2 Function:

From the time that ATP was discovered, the function of this molecule was thought to be only a high energy yield, and that it only took part in enzyme reactions where energy or phosphates were needed. The function of this molecule in recent years is thought to be somewhat more complex, as this molecule is implicated in a number of different physiological processes other than only energy transference. ATP can act as a neurotransmitter by opening the Ca^{2+} channels (Benham and Tsieu,1987), and is also involved in the physiological regulatory process via the receptor system (Rappaport,1989). It was shown by several researchers that exogenous ATP has a radioprotective effect on tissue (Benova,1978; Szeinfeld,1990). The advantage of ATP for radioprotective studies lies in the fact that it is a compound which naturally occurs in the body.

MATERIALS AND METHODS

CHAPTER 3

CHAPTER 3

MATERIALS AND METHODS:

This chapter deals mainly with the experimental setup of this study and will cover aspects such as the strain of animals, types of tumours, measurement of tumours and treatment by certain drugs and exposure to ionizing and non-ionizing radiation).

3.1 EXPERIMENTAL ANIMALS

Two strains of mice were used in these investigations, namely CBA and BALB/c. The CBA mice were bred and kept, in the animal room of the Radiobiology Group of the Research Institute for Medical Biophysics, MRC, and the BALB/c mice were bred at the MRC Animal Unit of the Research Institute for Nutritional Diseases and kept in the animal room of the Radiobiology Group of the Research Institute for Medical Biophysics, MRC. The mice were fed with mice cubes obtained from the MRC Animal Unit at the Research Institute for Nutritional Diseases. Water and food was allowed ad libitum. The animal room was maintained at 21° ± 1°C.

3.1.1 Experimental mice and tumours

The tumours used was **Ca**rcinoma **N**eck **T**umour (**CaNT**) and

Rhabdomyosarcoma in the CBA and BALB/c mice respectively.

3.1.1.1 CBA mice and the CaNT tumour.

Male CBA mice of approximately six weeks old weighing between 16 to 19 g, were used for experiments. Originally the experimental CaNT tumour was obtained from Professor R. Berry of the Middlesex Hospital Medical School, University of London, England and maintained by serial passage (see 3.1.2).

3.1.1.2 BALB/c mice and the induced transplantable Rhabdomyosarcoma.

A transplantable rhabdomyosarcoma was induced by injection of 0.1 ml of a 1 mg/ml solution of 3-methylcholanthrene in peanut oil subcutaneously into the right thigh region of six to eight week old male BALB/c mice. Rhabdomyosarcoma was found in all mice 3 to 4 months after the injection with 3-methylcholanthrene. In these investigations male BALB/c mice of six to eight weeks old weighing 19 to 25 g were used. The tumour was maintained in the sternal area, by serial passage (see 3.1.2).

3.1.2 Propagation of tumours

The medium used for the propagation was McCoy's 5A medium with L-Glutamine and $NaHCO_3$, at a pH of between 7.2 and 7.4 For the preparation of the tumour cell suspension 5 ml of this medium was used for three tumours. Tumours for propagation were removed by surgical excision after the mice were sacrificed by prolonged exposure to ether. The tumours were diced into small

pieces, in a 65 x 15 mm sterile Plastic petri dish, with sterile blades. After dicing, the total preparation was aspirated through a 12 x 38 mm size needle into a sterile syringe (B-D Plastipak, Promex). The supernatant with the suspended tumour cells was placed in a sterile plastic tube (Falcon), and allowed to settle for a few minutes. From the upperlayer of the preparation, 0.1 ml was injected subcutaneously into the sternum area of both types of mice, using a 0.4 x 13 mm needle. The tumours became visible between 7 to 10 days after injection.

3.1.3 Measurement and volume calculations

Tumour size was measured in three orthogonal directions with a Vernier calliper. The shape of the tumours was assumed to be spherical and the volume was calculated by the following formula:

$$Volume=(\frac{l+b+h}{6})^3\times\frac{4\pi}{3}$$

(3.1) *Equation for tumour volume calculations.*

3.1.4 Types of tissue used

The different tumours and normal tissues for the experiments were selected for cancer cachexia, radioprotection and hyperthermia studies.

3.1.4.1 Cachexia

In this experiment the kidney and liver of CBA mice, bearing different sizes of

the CaNT tumour, were used to measure fructose-1,6-diphosphatase, lactate dehydrogenase and glycerol-3-phosphate dehydrogenase activities in the cancer cachexia studies.

3.1.4.2 Radioprotection

In this section of the work testis and rhabdomyosarcoma tumour tissue from BALB/c mice were used.

3.1.5 Tissue preparation

Mice were killed by cervical dislocation, and the respective tissues surgically removed and placed in ice cold physiological saline containing EDTA. This was homogenised in a Potter type glass homogenizer and centrifuged at 12 000 g in a Superspeed refrigerated centrifuge(Du Pont Sorvall RC-5) at 4°C, and the supernatant used for the enzyme analysis.

3.2 EXPERIMENTAL DESIGN: CANCER CACHEXIA

This part of the work consists of two major sections, namely the physiological parameters and the enzymatic analysis.

3.2.1 Physiological experiment

This experiment was carried out in a metabolic chamber (see 3.2.1.5). Mice of

6-8 weeks old were chosen from an inbred colony of CBA mice. These mice were randomly divided into Tumour Bearing Mice (TBM) and Control groups respectively. The TBM group was inoculated with CaNT tumour (see 3.1.2), and together with the control group, were allocated to the metabolic chambers on the same day. The day of implantation of the tumour cells was considered day zero, and all the following parameters namely food intake, water consumption, urine and faeces production, tumour volume and body mass, were determined from this day onwards until the tumours reached a size of about 2200 mm^3. The measurements were carried out on the 1st, 3rd and 5th day of the week. Tumour size was determined, and volume calculated as described in paragraph 3.1.3..

3.2.1.1 Water consumption

The water consumed by the mice is the difference between the weight of the full closed water bottle and the weight of the residual water bottle on the determination day, plus the weight of the wasted water that was collected in the water waste bottle. The consumption was calculated by the formula 3.2; the units used to express the water consumption was g/24h.

$$WC = \frac{[i(w) - p(w)] - [p(ws) - i(ws)]}{n(d)}$$

(3.2) *Water consumption (WC): n(d)= number of days from each reading; i(w)= initial weight ; p(w)= residual weight of water; p(ws)= present weight of waste bottle; i(ws)= initial weight of waste bottle.*

3.2.1.2 Food consumption

The food container was weighed and filled on every determination day (see 3.2.1). The consumption was deduced from formula 3.3; and was reported as g food consumed per 24 h.

$$FC = \frac{[i(f) - p(f)]}{n(d)}$$

(3.3) *Food consumption(FC): n(d)= number of day from last reading; i(f) = initial weight of food container(full); p(f) = present weight of food container.*

3.2.1.3 Urine and faeces production

The urine and faeces production of the mouse was determined by the difference in the weight of the urine/faeces container when empty, and the weight of the urine/faeces container at the determination day. The urine and faeces production was reported as g/24h (see equation 3.4).

$$PRODUCTION = \frac{[p(c) - i(c)]}{n(d)}$$

(3.4) *Urine and faeces production: n(d) = Number of days from each reading; i(c) initial weight of tube; p(c) final weight of tube.*

3.2.1.4 Body mass of the host

The body mass was measured for both groups, namely the TBM group and the control group, for the duration of the experiment. In the case of the TBM, final mass of the animal was calculated by the following procedure: the masses of the tumours were subtracted from the masses of the mice. There is a 1:1 relationship between the volume and mass of the tumour. Hence, the tumour size was expressed in g of tissue. The final body mass was calculated by formula 3.5.

$$FBM = TM - (TV + 1000)$$

(3.5) *Final body mass (FBM): TM = total body mass; TV = tumour volume*

3.2.1.5 Metabolic chamber

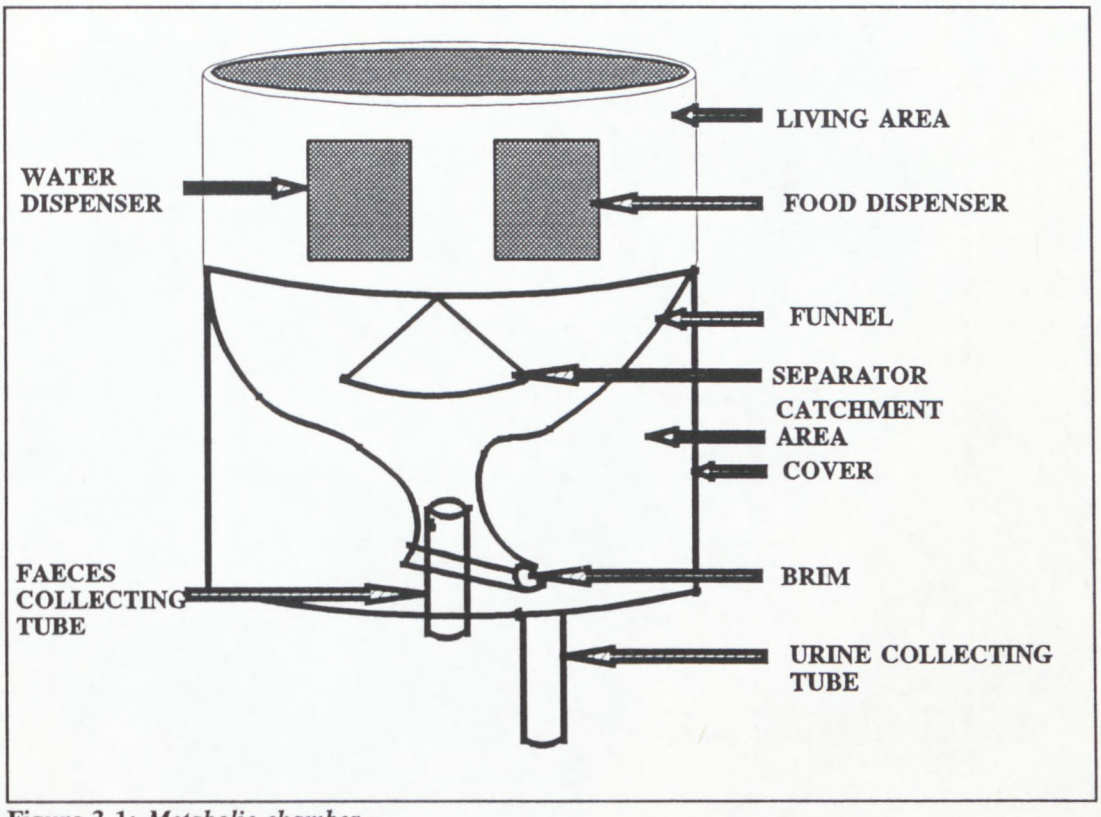

Figure 3.1: *Metabolic chamber*

The metabolic chamber consisted of three major sections, namely food and water dispensing compartments, animal living area and a catchment area.

i) Food and water dispensing units

Food dispenser: The food dispenser consisted of two different parts, namely the tunnel and the food container; the tunnel was situated on top of the food container. The tunnel was just big enough that one mouse could move in it, but could not turn around. The floor of the tunnel had 2 holes, one right above the food compartment and one right above the waste compartment. The waste compartment was the closest to the cage in order to trap all food that was wasted in the process.

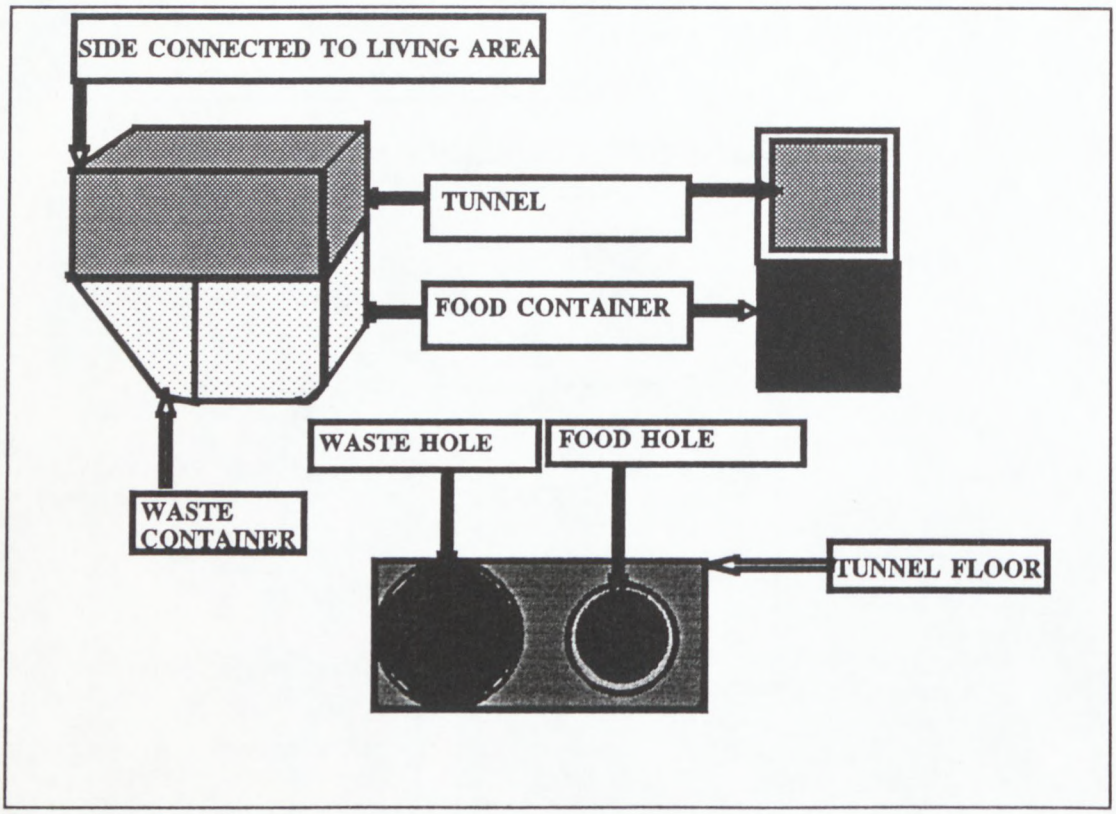

Figure 3.2: *Food dispenser*

Water dispenser:

The water dispenser consisted of two parts, namely the water bottle and the waste bottle. The water bottle was placed at a 45° angle with the cage, the nozzle being above a platform which would trap the water that went to waste. The platform sloped down to the waste bottle and formed a small lip that guided the waste water into the waste bottle.

ii) Living area

The living area was a cylindrically shaped perspex tube with a lid at the top and a stainless steel grid at the bottom. The food and water dispensers slid onto the side of the living area.

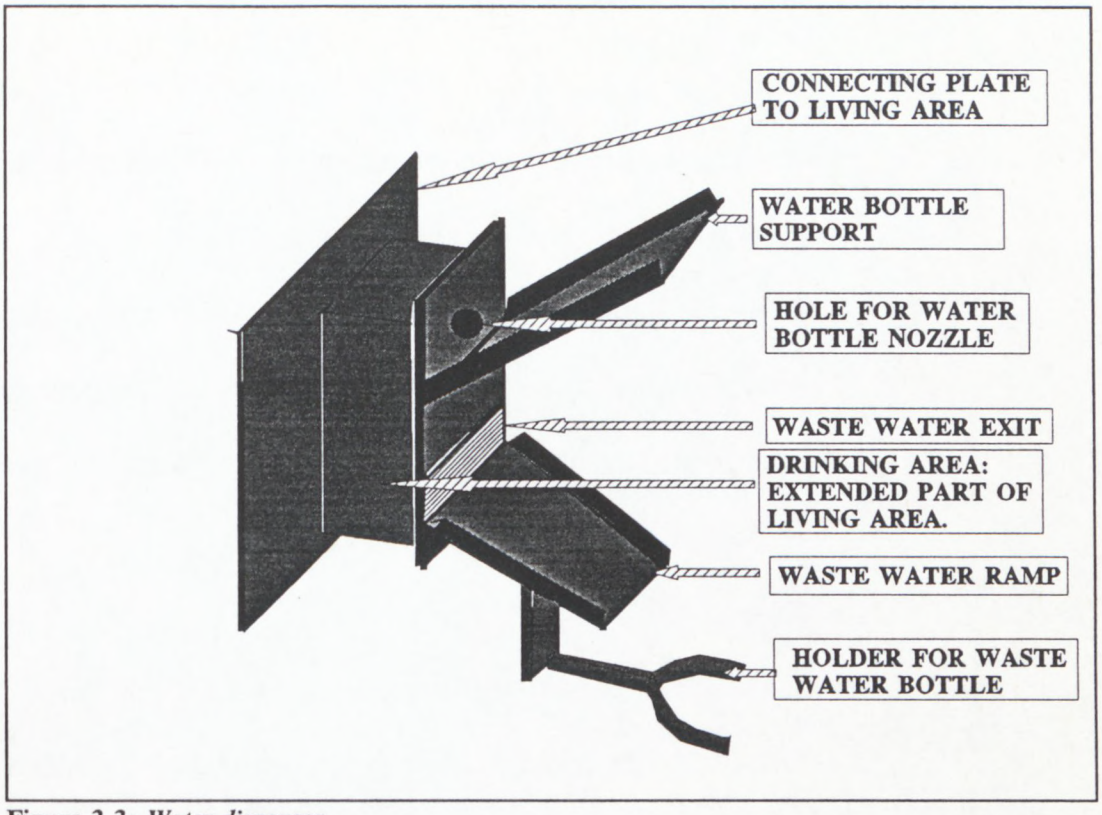

Figure 3.3: *Water dispenser*

iii) Collecting area

The collecting area was situated below the living area and consisted of five separate parts, namely the cover, the separator, the funnel, brim and the collecting tubes.

Cover:

The cover was a round container with two holes in the bottom for the collecting tubes. The funnel hooked onto the top of the container and on the side of the container; there were three hooks that hooked onto the bottom of the living area. The hole for the faeces tube was situated in the centre of the cover, and the hole for the urine tube was situated off centre beneath the lip of the brim.

Separator:

The separator was a cone shaped object of Perspex with four arms that extended from the top of the cone at a right angle direction from each other, running slightly upwards from the cone. The tips of the arms fitted into the holes of the funnel. This ensured that the separator remained in the centre of the funnel. The separator ensured that the urine and faeces were channelled to the sides of the funnel, where further separation occurred.

Funnel:

The funnel used, differed from normal funnels in the sense that the tube of the funnel did not continue to get smaller, but instead slightly increased in diameter after reaching the bottom of the funnel cup. The bottom of the funnel tube had two wings where the brim was attached to the funnel. The bottom of the funnel was also sloped to the one side.

Brim:

The brim was a trough-like part running underneath the edge of the funnel, to channel the urine to the urine collecting tube. The brim clipped onto the wings of the funnel. The brim collected the urine which flowed down the funnel, to guide it to the urine collecting tube.

Collecting tubes:

There were two types of collecting tubes, namely urine tubes and faeces tubes. The only difference between the two were, that the

hooks for attachment to the cover, were at different positions on the tube. On the urine tube, the hooks were smaller and situated at the top of the tube. The faeces tube had the hooks in the middle of the tube. The reason for the hooks in the middle of the tube, was that the tube could reach into the funnel and situate the top of the tube just underneath the neck of the funnel. This tube would then collect all faeces that fell through, and that did not stick to the side of the funnel.

3.2.2 Cachexia enzymes

The enzymes assayed to investigate the phenomenon of cachexia was Lactate dehydrogenase, Glycerol-3-phosphate dehydrogenase and Fructose-1,6-diphosphatase (see ch.2).

3.2.2.1 Lactate dehydrogenase

([R] - Lactate: NAD^+ oxidoreductase; EC 1.1.1.28) This enzyme functions in the anaerobic glycolysis and in the Cori cycle. The method used, was described by Bergmeyer and Bernt (1974).

i) *Method:* The test was carried out on a Philips Pye Unicam PU 8800 UV/VIS spectrophotometer. The buffer used was a phosphate buffer (pH 7.5) containing 6.2 mg pyruvate sodium salt obtained from Sigma Chemical Company, St Louis, USA. NADH di-sodium salt made up in a 1% $NaHCO_3$ solution was used as a colour system. In the performance of the analysis, 1.145 ml of buffer/substrate was

used with 50 μl of the NADH solution and 5 μl of supernatant (sample). This was mixed well and placed in the spectrophotometer and read at a wavelength of 340 nm at 25°C. The change in absorbance was recorded every minute for 3 minutes. The activity of the enzyme was calculated from this change in activity (for more information on the calculation of the activities see 3.4.2.).

PREPARATION OF SOLUTIONS: (30 TESTS)

PHOSPHATE BUFFER AND SUBSTRATE

REAGENT	MASS/VOLUME
K_2HPO_4	700 mg
KH_2PO_4	90 mg
Na-PYRUVATE	6.2 mg
DISTILLED WATER	90 ml

pH: 7.5

NADH SOLUTION:

REAGENT	MASS/VOLUME
NADH-Na_2	14 mg
$NaHCO_3$	15 mg
DISTILLED WATER	1.5 ml

WAVELENGTH: 340 nm

REACTION TEMPERATURE: 25°C

REACTION SOLUTION:

SOLUTION	TEST	BLANK
Phosphate/Pyruvate	1.145 ml	1.195 ml
NADH	0.050 ml	------
Sample	0.005	0.005
MIX WELL AND READ FOR 3 MINUTES AT 340 nm		
Total volume	1.2 ml	1.2 ml

The activity of this enzyme starts to decrease after 3 hours after the sample is taken, thus it is convenient to carry out the test within this time.

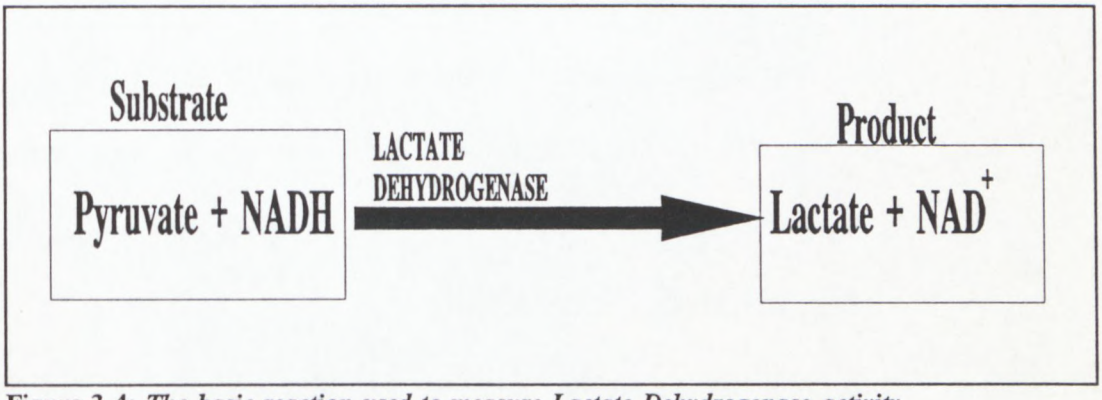

Figure 3.4: ***The basic reaction used to measure Lactate Dehydrogenase activity***

3.2.2.2 Glycerol-3-phosphate dehydrogenase:

(SN - Glycerol-3-phosphate:NAD^+ 2-oxidoreductase; EC 1.1.1.8) Glycerol-3-phosphate dehydrogenase is the enzyme that converts glycerol-3-phosphate to di-hydroxy acetone phosphate for entrance into the glycolytic pathway. The method used to determine the activity was described by Bergmeyer *et al* (1974).

i) *Method:* The analysis was carried out on a Philips Pye Unicam PU 8800 UV/VIS spectrophotometer. The buffer used in this reaction was a triethanolamine buffer with a pH of between 7.5 and 7.6. The substrate for this reaction was di-hydroxy acetone phosphate (DAP) obtained from

Sigma Chemical Company, St Louis, USA. The substrate was prepared by dissolving 3 mg of DAP in 1 ml of distilled water. The colour reagent and co-enzyme for this reaction was NADH. This was prepared by dissolving 10 mg NADH di-sodium salt (Sigma Chemical Company, St Louis, USA) in 1 ml of distilled water. The reaction mixture consisted of 1.140 ml triethanolamine buffer, 20 μl NADH solution, 20μl DAP solution and 20μl of sample. This was mixed well and the change in absorbance monitored every minute for three minutes, at a wavelength of 340 nm, with an incubation temperature of 25°C. The activity of the enzyme was calculated from the change in absorbance, (for more information on the calculation of the activity refer to 3.4.2).

PREPARATION OF SOLUTIONS

SUBSTRATE SOLUTION:

REAGENT	MASS/VOLUME
DI-HYDROXY ACETONE PHOSPHATE (DAP)	3 mg
DISTILLED WATER	1 ml

NADH SOLUTION:

REAGENT	MASS/VOLUME
NADH-Na_2	10 mg
DISTILLED WATER	1 ml

WAVELENGTH: 340 nm

REACTION TEMPERATURE: 25°C

REACTION MIXTURE:

SOLUTION	TEST	BLANK
TRIETHANOLAMINE BUFFER*	1.140 ml	1.180 ml
NADH	0.020 ml	------
DAP	0.020 ml	-------
Sample	0.020 ml	0.020 ml
MIX WELL AND READ AT 340 nm FOR 3 MINUTES		
Total volume	1.2 ml	1.2 ml

* See Hexokinase for buffer (see 3.3.5.1).

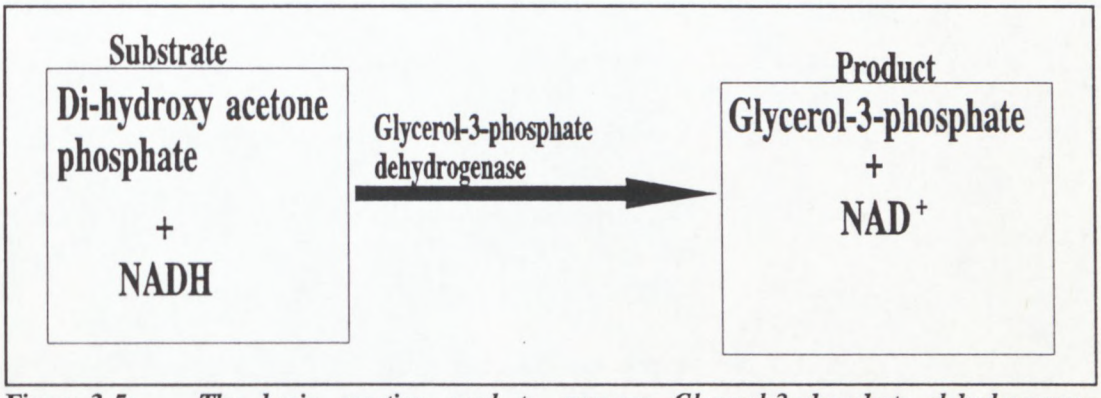

Figure 3.5: *The basic reaction used to measure Glycerol-3-phosphate dehydrogenase activity*

3.2.2.3 Fructose-1,6-diphosphatase

(Hexose diphosphatase; D-Fructose-1,6-bisphosphate 1-phosphohydrolase; EC 3.1.3.11). This enzyme functions in the Cori cycle during anabolic processes and is an accurate indicator of the activity of this cycle in the tissues under present investigation.

i) Method: The measurement was carried out on a Philips Pye unicam PU8800 UV/VIS spectrophotometer. The buffer used for this enzyme analysis was a TRIS buffer with a pH of 7.5. The reaction mixture consisted of 910μl of buffer, 20μl of magnesium chloride solution, 50μl

NADP solution, 120μl mercapto-ethanol solution, 5μl phospho-glucose isomerase enzyme suspension, 5μl glucose-phosphate dehydrogenase enzyme suspension, and 30μl of the sample. This was mixed well before 6μl of the substrate (fructose-1,6-diphosphate) was added. This was then mixed again and the change in absorbance measured, at a wavelength of 340 nm, at a temperature of 25°C. The magnesium chloride solution was prepared by dissolving 1.02 g of magnesium chloride in 10 ml distilled water. The NADP solution was prepared by dissolving 8.5 mg of NADP di-sodium salt in 1ml of distilled water. The mercapto-ethanol solution was prepared by diluting 15.6 mg of mercapto-ethanol in 1ml distilled water. The glucose-6-phosphate dehydrogenase (type VII: from Bakers Yeast) enzyme suspension and the phosphoglucose isomerase (type III: from Bakers Yeast) was obtained from Sigma Chemical Company, St Louis, USA, and their activities was 500 and 5000 units, respectively. The substrate solution was prepared by dissolving 27.9 mg fructose-1,6-diphosphatase trisodium salt (Sigma Grade 98-100%; crystalline), obtained from Sigma Chemical Company, St Louis, USA, in 1ml distilled water. The activity of the enzyme was calculated from the change in absorbance, (for more information on the calculation of the activity refer to 3.4.2).

PREPARATION OF SOLUTIONS

BUFFER SOLUTION:

REAGENT NAME	MASS/VOLUME
TRIS BUFFER (pH 7.5)	12,1 g
DISTILLED WATER	70 ml
HCL 6.3M	(adjust pH to 7.5)

NADP SOLUTION:

REAGENT	MASS/VOLUME
NADP-Na_2	8.5 mg
DISTILLED WATER	1 ml

SUBSTRATE SOLUTION:

REAGENT	MASS/VOLUME
FRUCTOSE-1,6-DIPHOSPHATASE	27.9 mg
DISTILLED WATER	1 ml

MAGNESIUM CHLORIDE SOLUTION:

REAGENT	MASS/VOLUME
$MgCl_2$	1.02 g
DISTILLED WATER	10 ml

MERCAPTO ETHANOL SOLUTION:

REAGENT	MASS/VOLUME
MERCAPTO ETHANOL	15.6mg
DISTILLED WATER	1 ml

ANALYSIS METHOD:

WAVELENGTH: 340 nm

REACTION TEMPERATURE: 25°C

REACTION MIXTURE:

SOLUTION	TEST	BLANK
TRIS BUFFER	0.910ml	1.110 ml
$MgCl_2$	0.020 ml	------
NADP	0.050 ml	-------
MERCAPTO ETHANOL	0.120 ml	-------
PHOSPHO-GLUCOSE ISOMERASE	0.005 ml	-------
GLUCOSE-6-PHOSPHATE DEHYDROGENASE	0.005 ml	-------
Sample	0.030 ml	0.030 ml
MIX WELL, AND THEN ADD THE SUBSTRATE.		
FRUCTOSE-1,6-DIPHOSPHATE	0.006	-------
Total volume	1.2 ml	1.2 ml

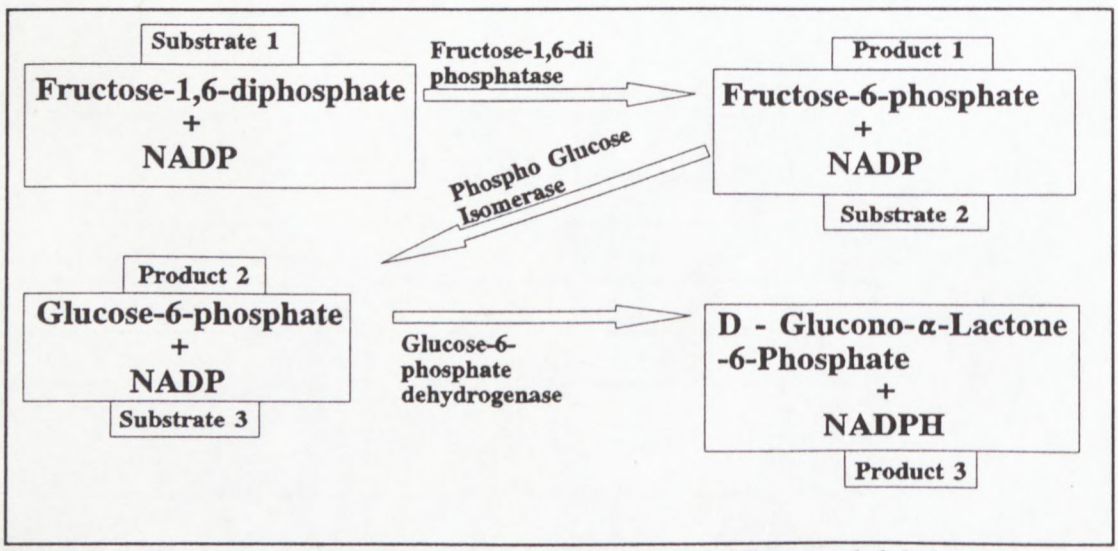

Figure 3.6: ***The basic reaction used to determine the activity of Fructose-1,6-diphosphatase.***

3.3 EXPERIMENTAL DESIGN: RADIOPROTECTION

The radioprotection section of this study consisted of two parts, namely protection against neutron radiation and hyperthermia. This was deemed

necessary as the two modalities are frequently combined for use in clinical trials.

3.3.1. Fast neutron radiation

For the exposure to neutron irradiation, the BALB/c and CBA mice were placed in a nylon phantom for the duration of the exposure in a p(66MeV)/Be neutron therapy beam, (National Accelerator Centre, Faure). A field size of 29 x 29 cm and Source to Surface Distance (SSD) of 150 cm were used for all exposures. The doses used were 2, 3.8 and 6 Gy respectively. Doses were calculated and exposures performed by the physicists at National Accelerator Centre, Faure.

3.3.2 Administration of the drugs

3.3.2.1 Adenosine-5'-Triphosphate

The radioprotective compound used was Adenosine-5'-Triphosphate obtained from Sigma Chemical Company, St Louis, USA. The dose of exogenous ATP was 700 mg/kg body weight. The ATP solution was freshly prepared in double distilled water, prior to injection. The injection of 0.5 ml of the ATP solution was given intraperitonealy 20 minutes before the mice were treated with the radiation source.

3.3.3. Tumour growth delay assay

This section of the study was carried out on BALB/c mice with the

rhabdomyosarcoma. Tumours with a mean size of 176 ± 17 mm^3 were selected for the tumour growth delay assay. This assay was performed at two different doses, 2.0 Gy and 3.8 Gy, with and without prior treatment with ATP. The mice were divided into three groups, controls, 2.0 Gy and 3.8 Gy respectively. The groups receiving 2.0 and 3.8 Gy neutron radiation were further sub-divided into two groups each, with and without prior administration of ATP. The administration of ATP was given as described in paragraph 3.3.2.1, and the exposure was according to that described in paragraph 3.3.1. The tumour volume was measured every weekday, until they reached a volume of approximately 1000 mm^3. The volume measurement and calculation was carried out as described in paragraph 3.1.3. The specific growth delay was calculated by the following equation:

$$SGD = \frac{(tumour\ volume\ tripling\ time\ of\ exposed\ tumours)}{(tumour\ volume\ tripling\ time\ of\ control\ tumours)}$$

(3.6) ***Formula for calculating the Specific Growth Delay (SGD)***

3.3.4. $LD_{50/30}$ mice survival assay

$$SMF = \frac{(\%\ survival\ with\ ATP)}{(\%\ survival\ without\ ATP)}$$

(3.7) ***The Survival Modification Factor (SMF).***

A group of 50 male BALB/c mice was used for this experiment. The mice were divided into two groups, Group 1 (n=30) received only radiation (see paragraph 3.3.1, p 49) at a dose of 6 Gy, and Group 2 (n=20) received radiation at the same dose but with prior treatment with ATP (see paragraph 3.3.2.1, p 49). The

mice were monitored for a period of three months. The number of surviving mice was expressed as a percentage of the total number used in the experiment. The survival modification factor was also calculated by equation 3.7.

3.3.5 Enzyme analysis of radioprotection studies

Mice for this part of the study was exposed to neutron radiation. In the case of neutron radiation, doses (see paragraph 3.3.1, p 49), of 2.0, 3.8 Gy and 6.0 Gy were used. The enzymes measured for the study of radioprotection were hexokinase, glucose-6-phosphate dehydrogenase, lactate dehydrogenase and acid phosphatase. For the method of lactate dehydrogenase see 3.2.2.1.

3.3.5.1 Hexokinase

(ATP: D - Hexose - 6 - phosphotransferase; EC 2.7.1.1). This is the first enzyme in the Embden-Meyerhof pathway of anaerobic glycolytic metabolism. The activity of this enzyme was determined using the method of Beutler (1975).

i) *Method:* The analysis was carried out on a Philips Pye Unicam PU 8800 UV/VIS spectrophotometer. The buffer used in this analysis was a triethanolamine buffer at a pH of 7.5 -7.6. The glucose solution was prepared by dissolving 1g D-glucose in 10 ml of the buffer. The magnesium chloride solution was prepared by dissolving 203mg magnesium chloride in 10 ml distilled water. For NADP solution, 20mg NADP di-sodium salt obtained from Sigma Chemical Company, St Louis, USA, was dissolved in 2ml of distilled water. The ATP solution was

prepared by dissolving 20mg adenosine-5'-triphosphate obtained from Sigma Chemical Company, St Louis, USA, in 2ml of distilled water. The reaction mixture was prepared by adding 500μl buffer, 500μl glucose solution, 100μl magnesium chloride solution, 100μl NADP solution, 50μl ATP solution, 5μl glucose-6-phosphate dehydrogenase enzyme suspension and 10μl of the supernatant (sample) together, mixed well ,incubated for 3 minute at 25°C, and the change read at 340 nm for three minutes. The glucose-6-phosphate dehydrogenase enzyme suspension (type VII: from Bakers Yeast) with an activity of 500 units was obtained from Sigma Chemical Company, St Louis, USA. The activity of the enzyme was calculated from the change in absorbance, (for more information on the calculation of the activity refer to 3.4.2).

BUFFER SOLUTION:

REAGENT	MASS/VOLUME
TRIETHANOLAMINE	3.73 g
DISTILLED WATER	200 ml
HCl	2.225 ml
MIX WELL, AND ALLOW TO COOL.	
DISTILLED WATER	FILL UP TO 500 ml
Adjust the pH to 7.5 - 7.6 with NaOH.	

GLUCOSE SOLUTION

REAGENT	MASS/VOLUME
D-GLUCOSE	1 g
TRIETHANOLAMINE BUFFER	10 ml

MAGNESIUM CHLORIDE

REAGENT	MASS/VOLUME
$MgCl_2$	0.203 g
DISTILLED WATER	10 ml

NADP SOLUTION

REAGENT	MASS/VOLUME
NADP	0.02 g
DISTILLED WATER	2 ml

ATP SOLUTION

REAGENT	MASS/VOLUME
ATP	0.02 g
DISTILLED WATER	2 ml

WAVELENGTH: 340 nm

REACTION TEMPERATURE: 25°C

REACTION MIXTURE:

SOLUTION	TEST	BLANK
TRIETHANOLAMINE BUFFER	500 µl	655 µl
GLUCOSE	500 µl	500 µl
$MgCl_2$	100 µl	100 µl
NADP	100 µl	-----
ATP	50 µl	-----
GLUCOSE - 6 - PHOSPHATE DEHYDROGENASE	5 µl	-----
SAMPLE	10 µl	10 µl
MIX WELL, INCUBATE FOR 3 MINUTES AND THEN READ FOR 3 MINUTES		
TOTAL VOLUME	1.265 ml	1.265 ml

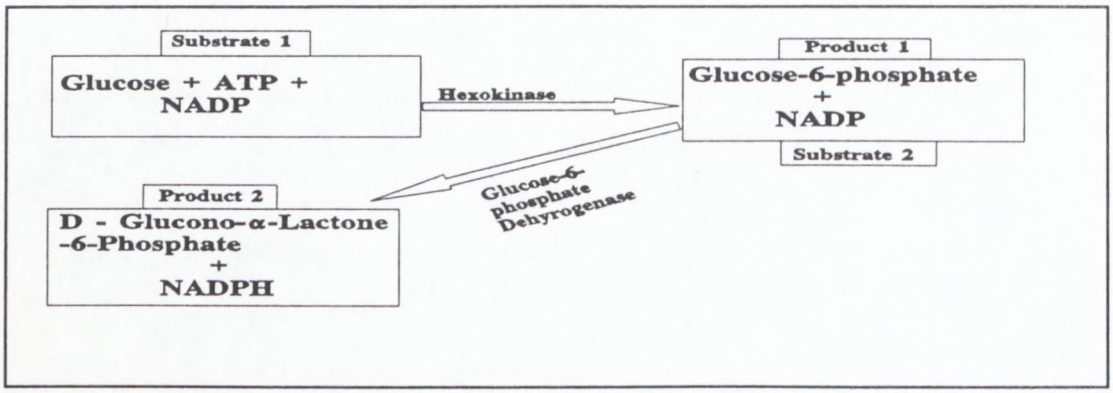

Figure 3.7: *The basic reaction used to measure the activity of Hexokinase*

3.3.5.2 Glucose - 6 - phosphate dehydrogenase

(D-Glucose - 6 - phosphate dehydrogenase: NADP oxidoreductase EC 1.1.1.49). This is the first enzyme in the pentose phosphate shunt. The activity of this enzyme was determined by the method of Löhr and Waller (1974).

i) Method: This enzyme analysis was carried out using a Philips Pye Unicam PU8800 UV/VIS spectrophotometer. The buffer used in this analysis was the same as described for hexokinase (see 3.3.5.1, p. 51). The glucose-6-phosphate solution was prepared by dissolving 13mg D-glucose-6-phosphate di-sodium salt (Hydrate, Sigma Grade: 98-100%), obtained from Sigma Chemical Company, St Louis, USA, in 1 ml distilled water. The magnesium chloride solution was prepared by dissolving 128mg of magnesium chloride in 10 ml distilled water. The NADP solution was prepared by dissolving 25mg of NADP di-sodium salt obtained from Sigma Chemical Company, St Louis, USA, in 1ml of a 1% $NaHCO_3$. The reaction mixture was prepared by adding 1.14ml buffer, 20μl sample, 20μl magnesium chloride, 20μl NADP, mixed well and incubated for 5 minute at 25°C. After five minutes 20μl of glucose-6-phosphate was added, mixed well and the change in absorbance measured

at a wavelength of 340 nm for 3 minutes. The activity of the enzyme was calculated from the change in absorbance, (for more information on the calculation of the activity refer to 3.4.2).

GLUCOSE - 6 - PHOSPHATE SOLUTION

REAGENT	MASS/VOLUME
D-GLUCOSE - 6 - PHOSPHATE	0.013 g
DISTILLED WATER	1 ml

MAGNESIUM CHLORIDE

REAGENT	MASS/VOLUME
$MgCl_2$	0.128 g
DISTILLED WATER	10 ml

NADP SOLUTION

REAGENT	MASS/VOLUME
NADP	0.025 g
$NaHCO_3$	0.010 g
DISTILLED WATER	1 ml

ANALYSIS METHOD:

WAVELENGTH: 340 nm

REACTION TEMPERATURE: 25°C

REACTION MIXTURE

SOLUTION	TEST	BLANK
TRIETHANOLAMINE BUFFER	1.140 ml	1.180 ml
SAMPLE	20 µl	20 µl
$MgCl_2$	20 µl	20 µl
NADP	20 µl	-----
MIX WELL AND INCUBATE FOR 5 MINUTES AT 25°C, THEN ADD		
GLUCOSE - 6 - PHOSPHATE	20 µl	-----
MIX WELL AND READ AT 340 nm FOR 3 MINUTES		
TOTAL VOLUME	1.220 ml	1.220 ml

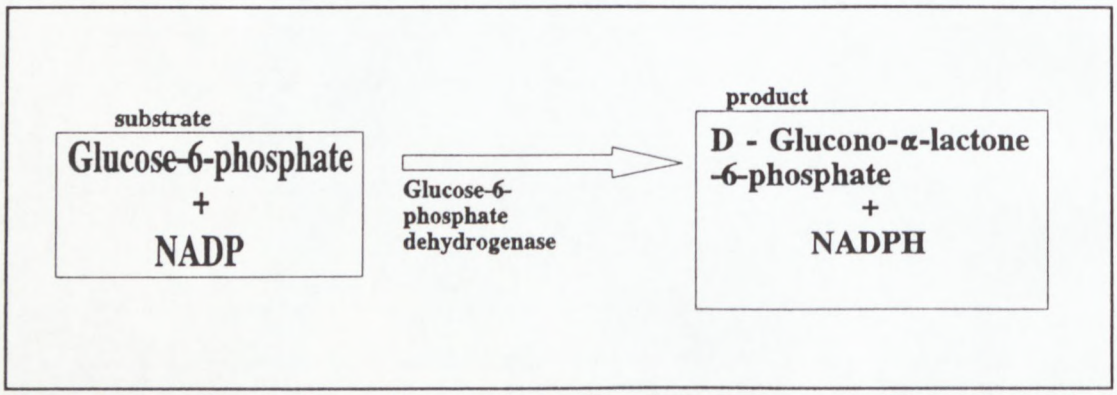

Figure 3.8: *The basic reaction used in the determination of Glucose-6-phosphate dehydrogenase activity.*

3.3.5.3 Acid phosphatase

This lytic enzyme is found in the lysosome and belongs to a group of enzymes called the hydrolases. The specific function of this particular hydrolase is to catalyse the removal of the phosphate group from a phosphate ester. This enzyme is important, because it "cleans" the cell by removing old or damaged macromolecules, breaking it down to its original constituents of which some are re-used by the cell. The activity of this enzyme was determined by the method described by Walter and Schutt (1974).

i) Method: This enzyme analysis was carried out using a Philips Pye Unicam PU8800 UV/VIS spectrophotometer. The buffer used in this

analysis was a citrate buffer with a pH of 4.8, containing 203mg of nitrophenyl phosphate obtained from Boeringher Manheim, Germany. The tartrate solution was prepared by dissolving 9.2g of sodium tartrate in 100ml of distilled water. The reaction mixture was prepared by adding 50μl tartrate solution, 1.18ml buffer/substrate solution and 20μl of sample, mixing it well and incubating it for 30 min at 25°C. After the incubation period the reaction was stopped by adding 2ml of 0.1N NaOH to every tube. A sample blank was prepared in a similar fashion to the test, with the only difference being that the NaOH was added before the incubation period. The difference in absorption at 405 nm between the sample blank and the test was used to calculate the activity of the enzyme, (for more information on the calculation of the activity refer to 3.4.2.).

BUFFER SOLUTION:

REAGENT	MASS/VOLUME
CITRIC ACID	0.41 g
SODIUM CITRATE	1.125 g
4 - NITROPHENYL PHOSPHATE	0.203 g
DISTILLED WATER	100 ml
Adjust the pH to 4.8 with HCl.	

TARTRATE SOLUTION

REAGENT	MASS/VOLUME
SODIUM TARTRATE	9.2 g
DISTILLED WATER	100 ml

WAVELENGTH: 405 nm

REACTION TEMPERATURE: 25°C

REACTION MIXTURE:

SOLUTION	TEST	REAGENT BLANK	SAMPLE BLANK
TARTRATE SOLUTION	50 µl	50 µl	50 µl
BUFFER SUBSTRATE	1.18 ml	1.20 ml	1.18 ml
SAMPLE	20 µl	----	20 µl
NaOH	-----	2 ml	2 ml
MIX WELL AND INCUBATE FOR 30 MINUTES AT 25°C, THEN ADD			
NaOH	2 ml	-----	-----
MIX WELL AND READ AT 405 nm AGAINST SAMPLE BLANK			
TOTAL VOLUME	3.250 ml	3.250 ml	3.250 ml

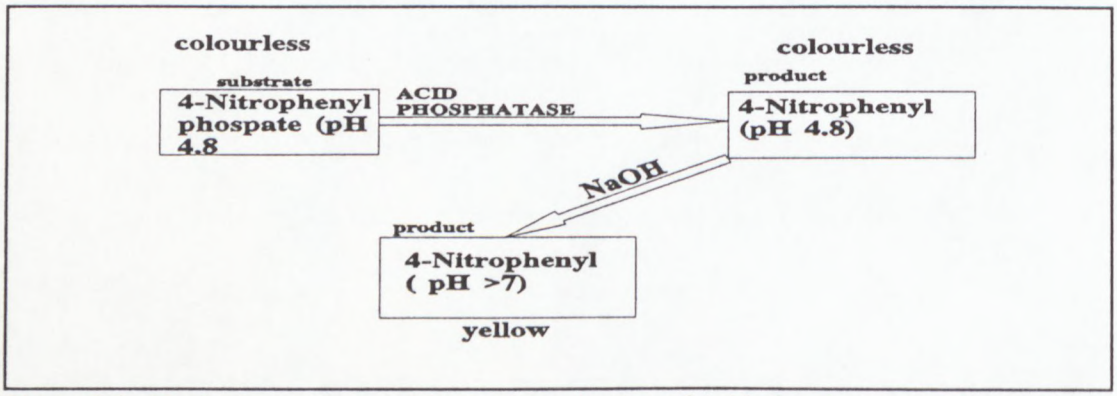

Figure 3.9: ***The basic reaction used in the determination of Acid phosphatase activity.***

3.4 METHODS USED FOR CALCULATIONS AND STATISTICS.

In this section the formulae used for calculating the activity of the enzymes in these studies are given, as well as those used to evaluate statistic data of this work.

3.4.1 Absorption spectrum and extinction coefficient

The Lambert-Beer law shows that, when light passes through matter, some is transmitted and some absorbed.

Where E is extinction; c is concentration; d is the lightpath length, ϵ is the molar

$$E=\varepsilon \times c \times d$$

(3.8) *Lambert-Beer law*

extinction coefficient. ϵ is defined by $\epsilon = \log (I_0/I)$ where I_0 and I are intensities of incident and transmitted radiation respectively.

The Lambert-Beer law further states that the extinction is proportional, both to the concentration c of the substance in question, and the lightpath d. The proportionality constant ϵ is the extinction of the substance in question, at a concentration of unity with a 1 cm lightpath.

The quantity of a substance is expressed in moles which is associated with a volume in cm^3 (i.e. mol/ml), therefore the dimensions of ϵ are (cm^2/mol) as shown by the next formula.

$$\varepsilon = \frac{\log(\frac{I_0}{I})}{c \times d} \qquad cm^2/mol = \frac{1}{(mol/cm^3) \times cm}$$

(3.9) Extinction

However the units for molar extinction more often used is ($M^{-1}.cm^{-1}$), because concentration is usually expressed in mol/litre or M for greater convenience.

3.4.2 Spectrometric methods for enzyme determination

In the determination of all the enzyme activities by spectrophotometric methods, the reaction of the enzyme must be coupled to an indicator system. In this work three colorimetric systems were used namely; NADP-NADPH, NADH-NAD and 4-Nitrophenyl phosphate - 4-Nitrophenyl. The first two systems in this study were used at wavelength of 340 nm and the last one at 405 nm. The indicator can be changed to absorb light at a specific wavelength, or changed to the form

that absorbs less light. The activity of the enzyme is deduced from the amount of change in absorption; these are for kinetic reactions. In an endpoint reaction, the indicator is changed to a form that absorbs light at a specific wavelength. The activity is then deduced from the absorbed light divided by the incubation time. The three different indicator systems can be seen in Figures 3.10 - 3.15.

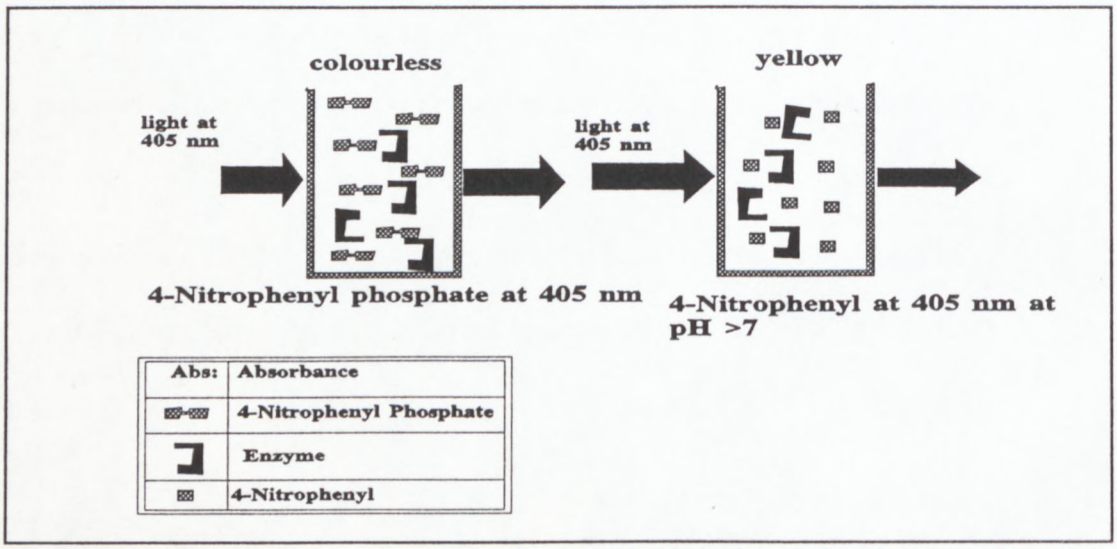

Figure 3.10: *The indicator system used for the determination of Acid phosphatase activity*

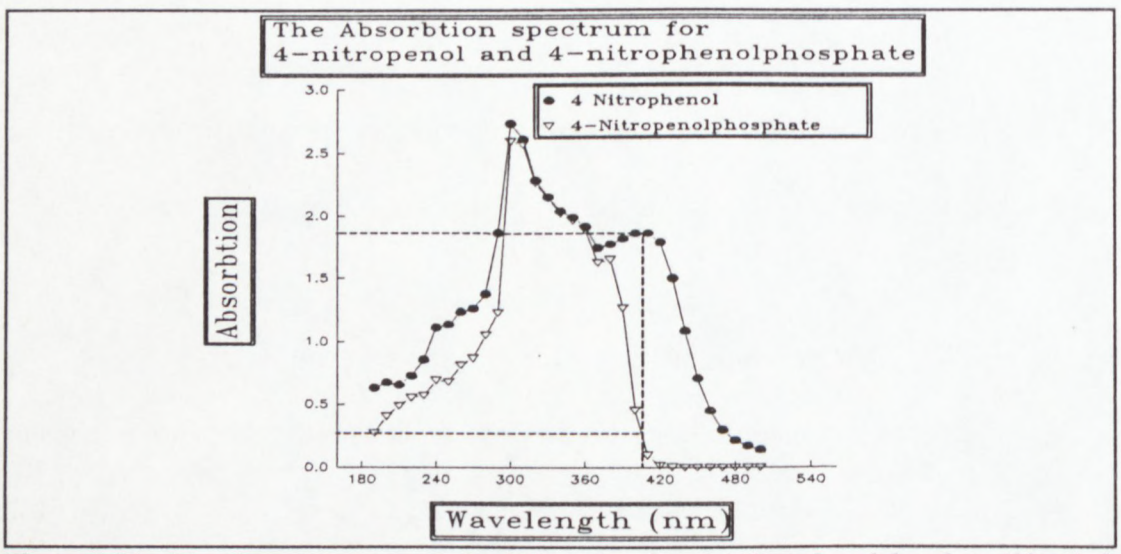

Figure 3.11: *The absorption spectrum for 4-Nitrophenol and 4-Nitrophenolphosphate (10mg/ml)*

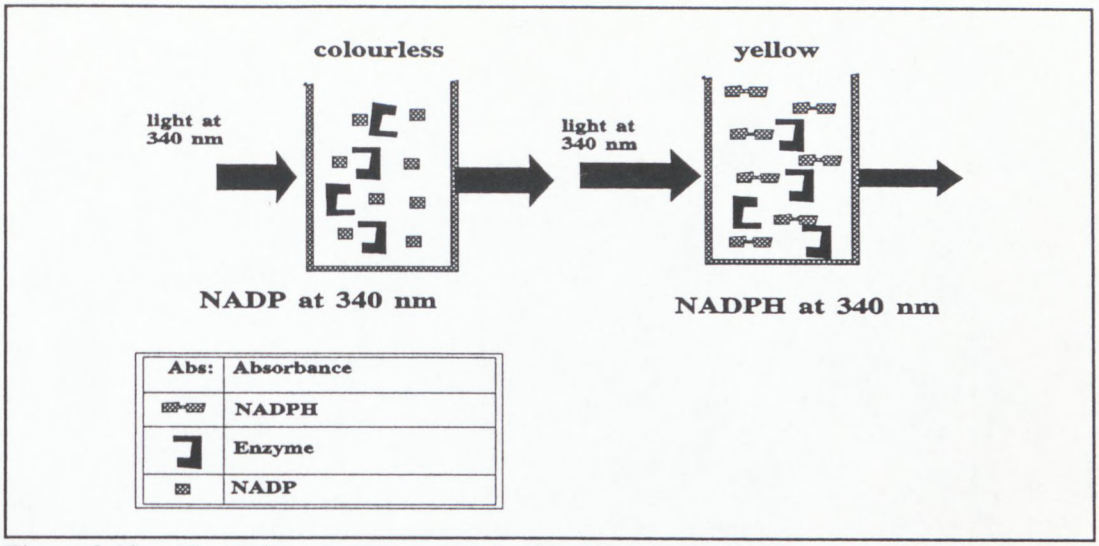

Figure 3.12: *The indicator system used in the determination of Hexokinase and Glucose-6-phosphate dehydrogenase activity.*

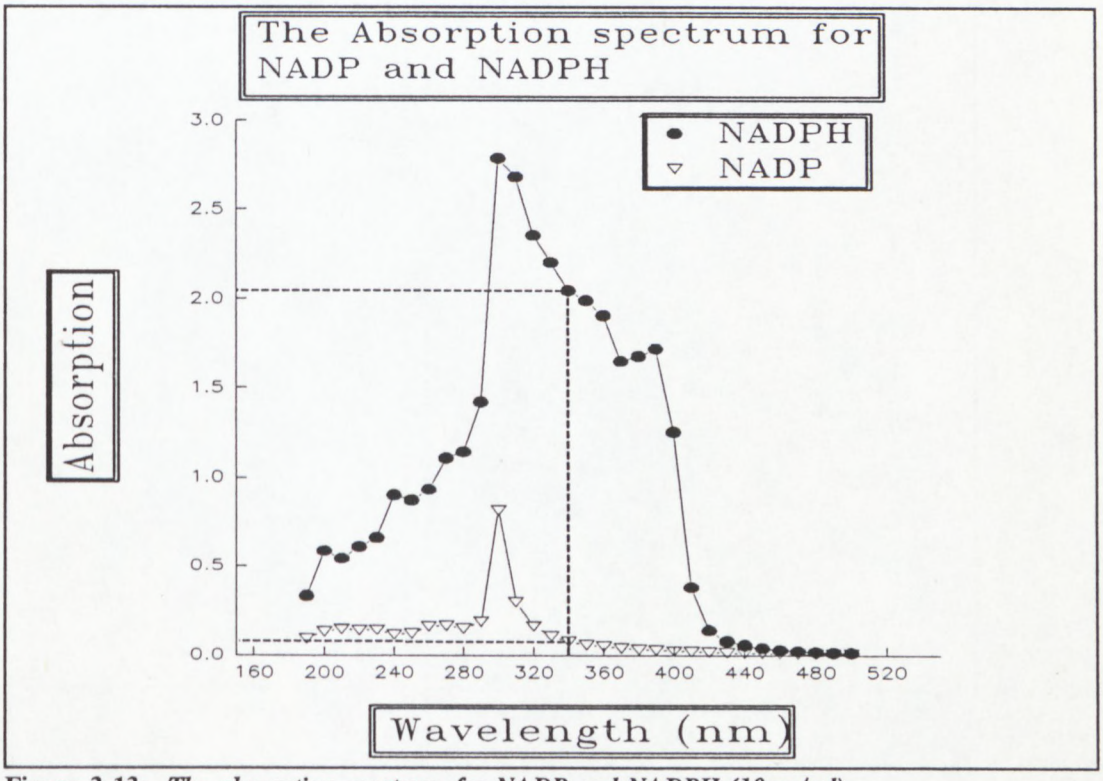

Figure 3.13: *The absorption spectrum for NADP and NADPH (10mg/ml)*

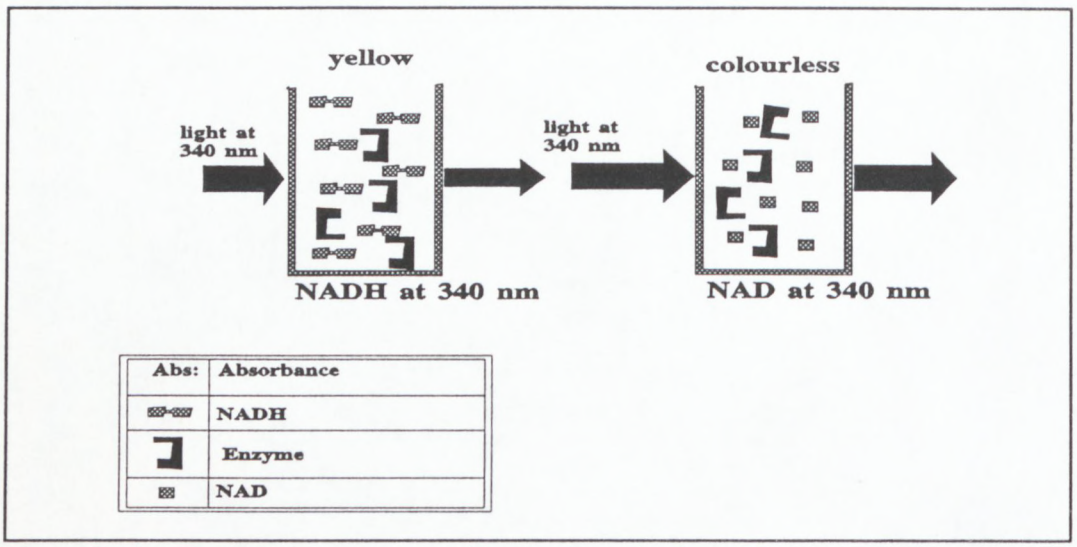

Figure 3.14: *The indicator system used in the determination of Lactate dehydrogenase and Glycerol-3-phosphate dehydrogenase activity.*

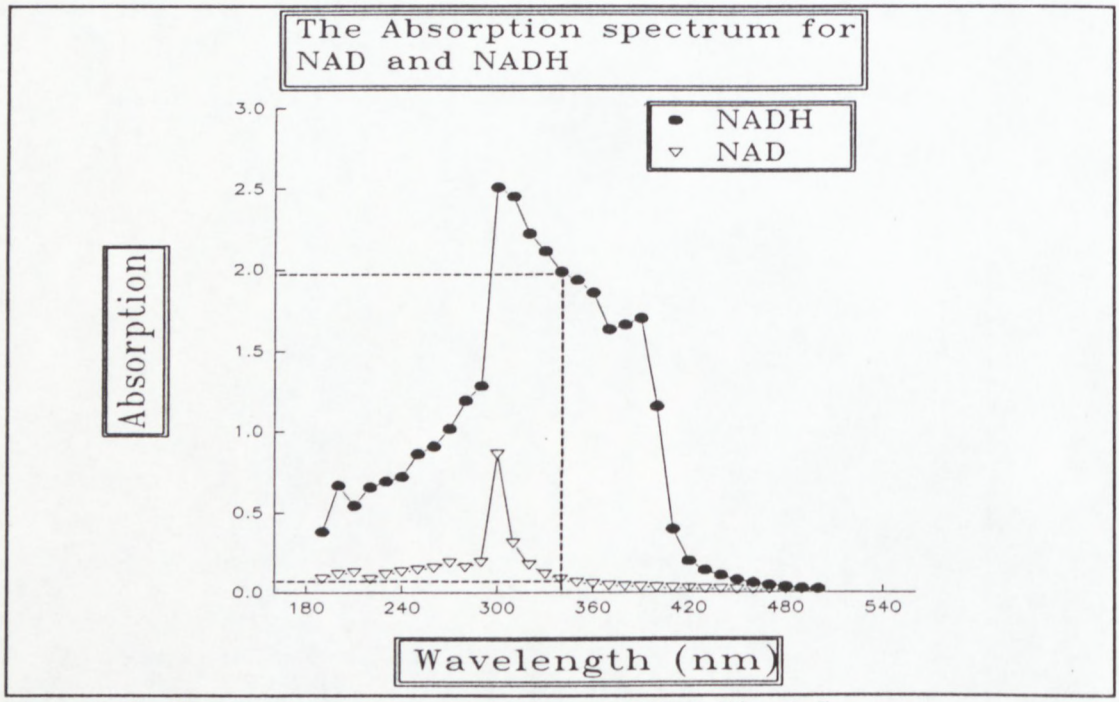

Figure 3.15: The absorption spectrum of NAD and NADH (10mg/ml)

Other symbols used to calculate the activities of enzymes were:

ϵ molar extinction coefficient: NADP or NAD = $6.22 \times 10^{-3} (M^{-1}.cm^{-1})$ or $6.22\ (cm^2/\mu mol)$

4-Nitrophenyl = 18,5 $(M^{-1}.cm^{-1})$

E = extinction change

V = assay volume

v = volume of sample used in assay (ml)

d = lightpath length (cm)

c = concentration (mol/litre) or (nmol/mg)

t = time (min)

t = interval between measurements (min)

The enzymes that made use of the NADP-NADPH in this study were:

1) Hexokinase

2) Glucose-6-phosphate dehydrogenase

3) Fructose-1,6-diphosphatase

The enzymes that used the NADH-NAD colour system in this study were:

1) Lactate dehydrogenase

2) Glycerol-3-phosphate dehydrogenase

The enzyme in this study that used the 4-Nitrophenyl phosphate - 4-Nitrophenyl system was:

1) Acid phosphatase

The rate of substrate conversion or product accumulation was determined by measuring the change in the concentration of the colour substance (NADP,NADH or 4-Nitrophenyl).

$$VA = \frac{V}{\epsilon \times d \times v} \times \frac{\Delta E}{\Delta t}$$

(3.10) *Volume activity (VA) of enzymes.*

The unit used for the activity of acid phosphatase was nmol/10min/ml, and that used for all the other enzymes was μmol/min/ml.

$$SA = \frac{V}{\epsilon \times d \times v \times c_{protein}} \times \frac{\Delta E}{\Delta t}$$

(3.11) *Specific activity (SA) of enzymes.*

The unit used for the activity of acid phosphatase was nmol/10min/mg protein, and that used for all the other enzymes was μmol/min/mg protein.

$$TN = \frac{(substrate converted)}{(c_{enzyme} \times time)}$$

(3.12) *Turnover number of enzymes.*

$$\mu mol\ converted/\text{min}/mg Protein = \frac{(\mu mol/\text{min}/ml)}{(mg protein/ml)}$$

or

$$nmol\ converted/10\text{min}/mg Protein = \frac{(nmol/10\text{min}/ml)}{(mg Protein/ml)}$$

(3.13) *The units for the enzyme assays.*

3.4.3 Statistical methods

The statistical methods used were:

1) mean
2) standard deviation
3) standard error of the mean (SEM)
4) student t test

5) regression coefficient

3.4.3.1 Mean

The mean (x) is given by equation 3.14.

$$(\bar{x})=\frac{\Sigma x}{n}$$

(3.14) *Mean*

Where Σx = the sum of all the observations in the sample population.

n = number of observations.

3.4.3.2 Standard deviation

The standard deviation (s) is given by equation 3.15

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

(3.15) *Standard deviation (s).*

3.4.3.3 Standard error of the mean

The standard error of the mean is given by equation 3.16

$$S.E.M.=\frac{s}{\sqrt{(n)}}$$

(3.16) *Standard error of the mean (S.E.M.)*

3.4.3.4 Student's t test

To ascertain if there was a significant difference between sets of data, a Student's two tailed t test with $(n_1+n_2 - 2)$ degrees of freedom, was used.

$$t = \frac{(\overline{x_1} - \overline{x_2})}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

(3.17) ***Student t test formula.***

Where x_1 and x_2 are the means of the sample populations, s_1 and s_2 are the standard deviations and n_1 and n_2 are the numbers of observations. The value of P read against the t value on the t test tables.

3.4.3.5 Regression coefficients

The equation of the regression line is $y = a + bx$, a and b was calculated by equation 3.18 and 3.19 respectively

$$b = \frac{\sum xy - \frac{(\sum x)(\sum y)}{n}}{\sum x^2 - \frac{(\sum x)^2}{n}}$$

(3.18) ***The equation for the slope (b) of the regression line.***

$$a = \frac{(\sum y)}{n} - \frac{b(\sum x)}{n}$$

(3.19) ***The intercept (a) of the regression line***

The coefficients of regression was given by equation 3.20

$$r^2 = \frac{(\sum xy - \frac{(\sum x)(\sum y)}{n})^2}{(\sum x^2 - \frac{(\sum x)^2}{n})(\sum y^2 - \frac{(\sum y)^2}{n})}$$

(3.20) ***The equation for the regression coefficient (r^2)***

RESULTS

CHAPTER 4

CHAPTER 4

RESULTS

4.1 TUMOUR METABOLISM AND ITS INTERACTION WITH HOST METABOLISM

4.1.1 TUMOUR METABOLISM

4.1.1.1 Lactate dehydrogenase

The activity of LDH in the CaNT tumour showed a marked augmentation between 50 mm^3 and 250 mm^3. Furthermore in tumours with a volume between 250 mm^3 to 550 mm^3 the increase in the activity of this enzyme was gradual. Moreover the activity of the enzyme stayed the same in the tumours with a volume between 550 to 950 mm^3 and then increased slightly in tumours bigger than 950 mm^3 as indicated in Figure 4.1.

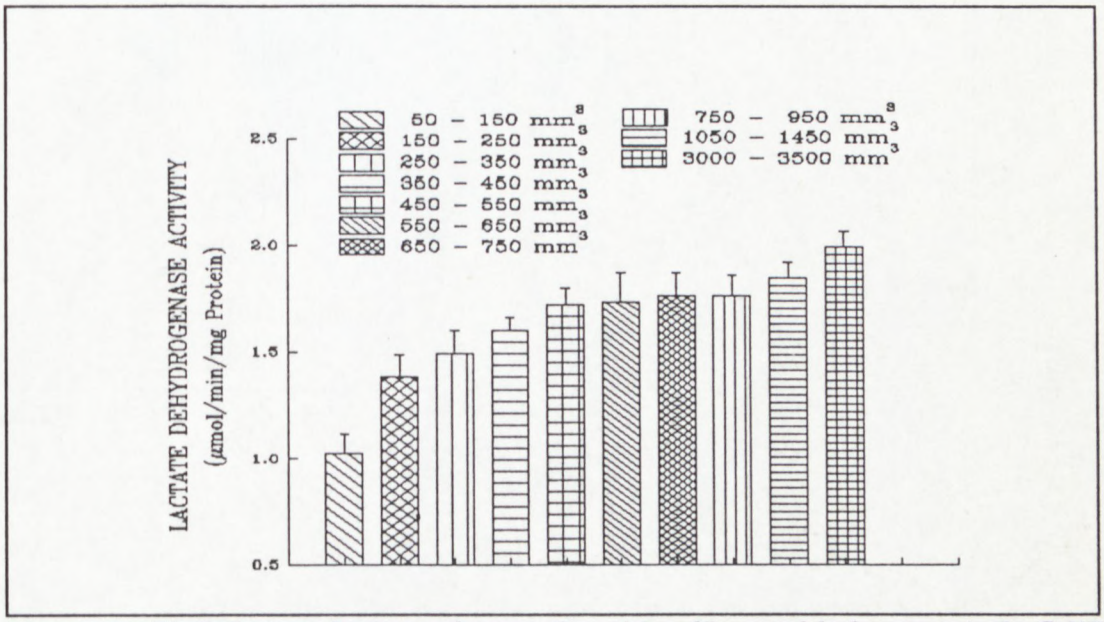

Figure 4.1: ***The influence of tumour volume on the activity of Lactate dehydrogenase in the CaNT tumour. The height of the histogram block represents the mean of approximately 5 samples ± SEM.***

4.1.1.2 Acid phosphatase

The activity of acid phosphatase in the CaNT tumours shows an increase that can be correlated with the increase in tumour volume. In fact there is a linear correlation between the tumour volume and the acid phosphatase activity (Figure 4.2) which satisfies the linear equation 4.1

$$AcidP_A = 0.027TV + 72.25$$

(4.1) *Correlation between acid phosphatase activity and tumour volume.*

The correlation coefficient of this linear line is r = 0.82.

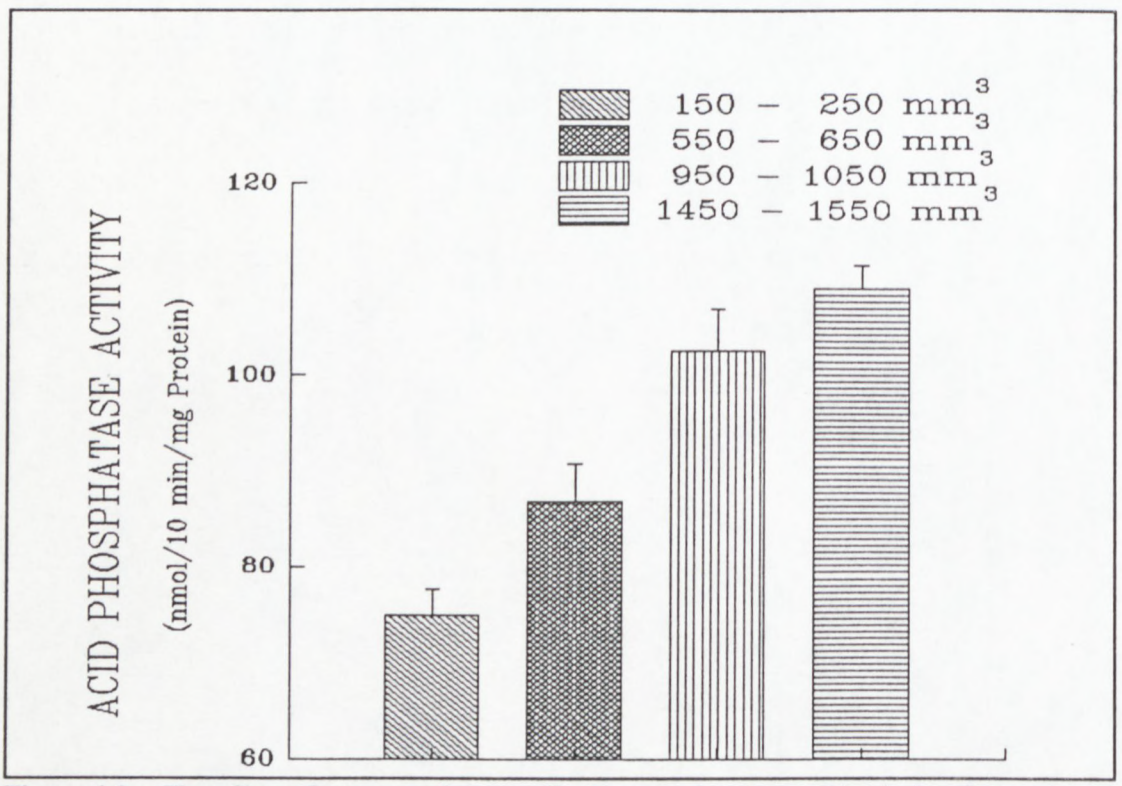

Figure 4.2: *The effect of tumour volume on the lysosomal activity of Acid phosphatase in the CaNT tumour. The height of the histogram block represents the mean of between 5 to 8 samples ± SEM.*

4.1.2 DIETARY PARAMETERS IN THE HOST

4.1.2.1 Tumour growth and body weight

The CaNT tumour, a solid tumour, became visible about 8 days after implantation and was monitored up to a volume of 2213 $\pm$ 327 mm^3 after 26 days after implantation. See Figure 4.3 for the growth curve.

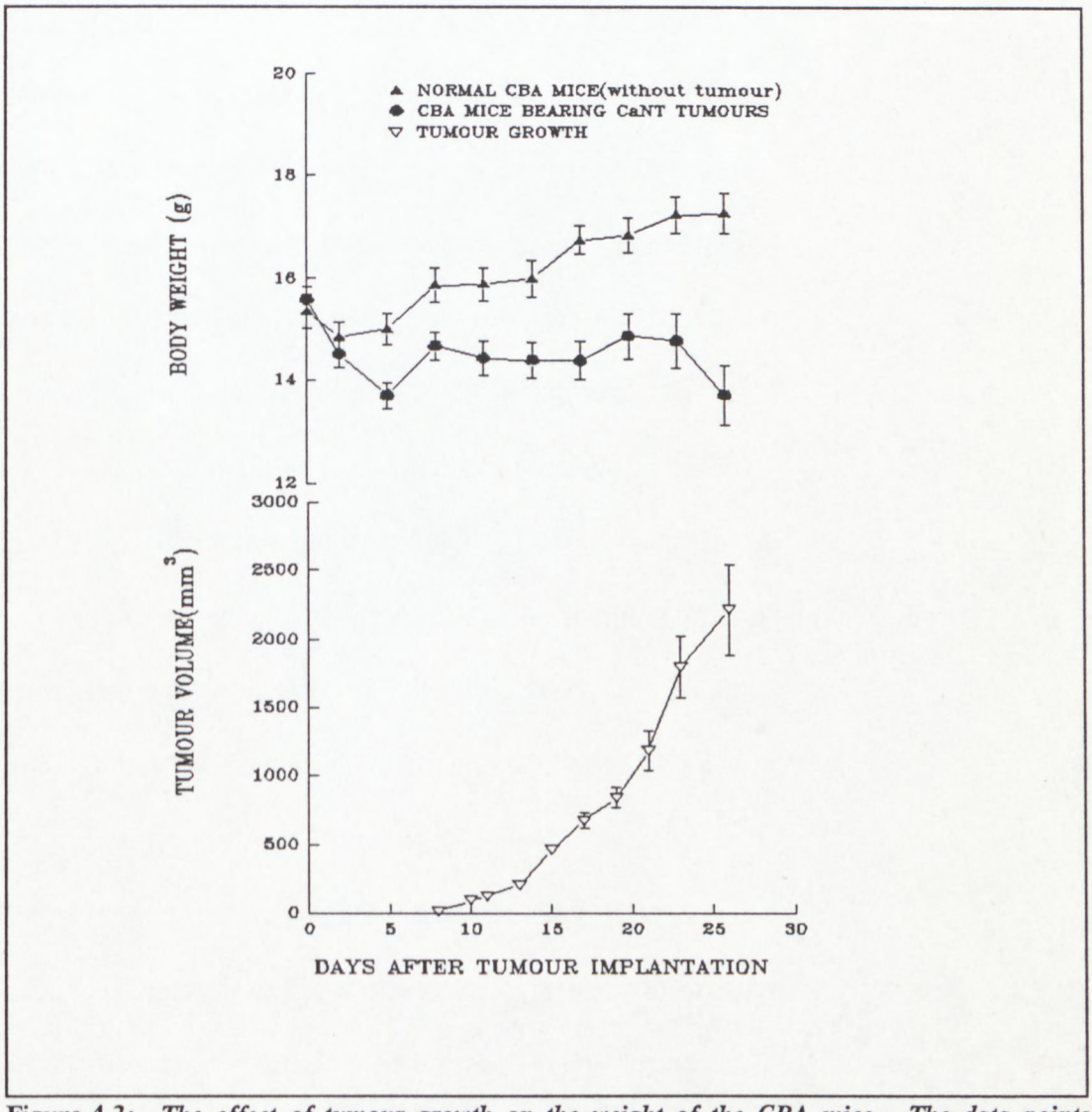

Figure 4.3: ***The effect of tumour growth on the weight of the CBA mice. The data points represent the mean of approximately 13 samples $\pm$ SEM.***

The starting weight of the CBA mice used in the CaNT Tumour Bearing Mice (TBM) group and control group was 15.58 ± 0.26 g and 15.35 ± 0.33 g (±SEM) respectively. There was an initial decline in body mass in all mice (TBM and control group) during the first 5 days after the mice were placed in the metabolic chamber. This decline in body weight may be due to the adaptation of the mice to their new environmental conditions. The decline in body weight of control CBA mice after 5 days was less than the weight loss in the TBM group at the same time ($P < 0.005$). At day 8 there was an improvement in the body weight of both groups of mice. The control group's body weight rose to above their initial body weight, and the TBM group's body weight remained below the initial body weight. The TBM group maintained almost the same weight after 8 days up to 23 days post implantation. The final weight at day 26 was significantly lower (± 1.9g) than the initial weight ($P < 0.01$). At the same time the weight of the control mice continued to increase up to the end of the experiment and reached a final weight of 17.27 ± 0.38 g; this was significantly higher (± 1.9 g) than the initial weight of the control group ($P < 0.01$). The difference in the final weight, at day 26, between the two groups was 3.56 g. This represents a loss of 21 % of the total body weight of the TBM mice. The control and TBM groups weights were significantly different from day 5 onwards ($P > 0.01$). The weight curves are shown in figure 4.3.

4.1.2.2 Food intake

There was a rise in the food consumption at the beginning of the experiment, from 1.84 ± 0.09 g (controls) and 1.71 ± 0.13 g (TBM) to 2.90 ± 0.08 (control) and 2.80 ± 0.12 g (TBM) at 8 days after commencement of the experiment. A steady food consumption in both the control groups and the TBM group was observed between day 8 and day 18 (Figure 4.4).

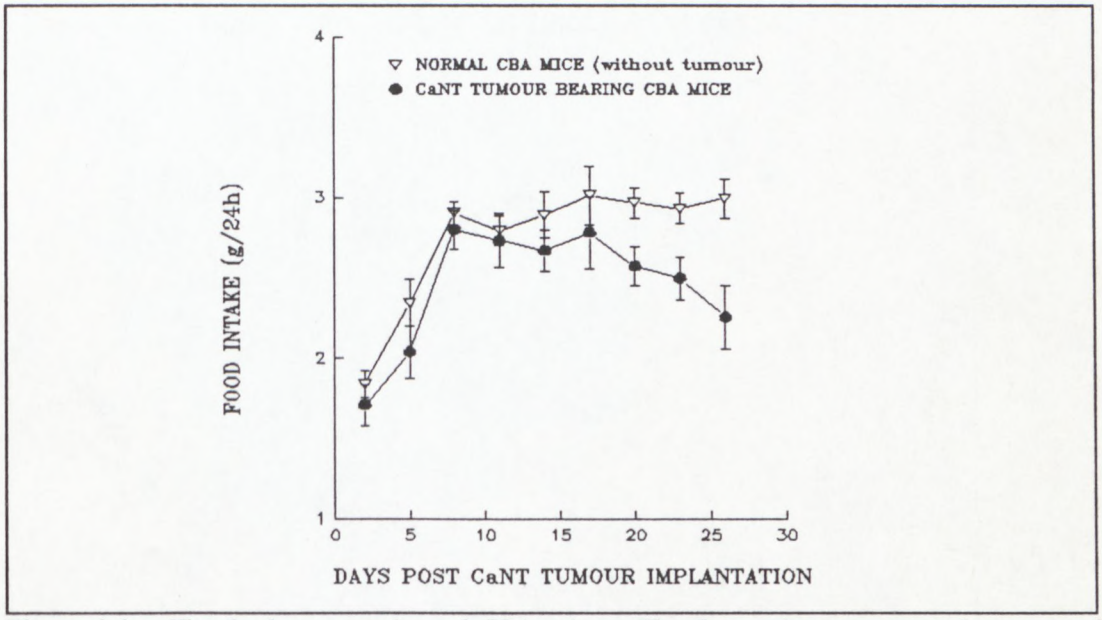

Figure 4.4: ***The food consumption of CBA mice. The data points represent the mean of approximately 13 samples ± SEM.***

Beyond this day the food intake of the TBM group started to decrease and reached its lowest point (2.26 ± 0.19 g) at 26 days after CaNT tumour implantation. Concomitantly the food intake of the control group stayed almost the same, increasing slightly up to (2.99 ± 0.12 g) day 26. The food intake of the controls and TBM groups did not differ before 20 days after implantation. The food intake of the control group was however significantly ($P < 0.05$) higher

compared with the TBM group after day 20. (See figure 4.4)

4.1.2.3 Water intake

The water intake of the CBA control group and that of the TBM group did not differ significantly from one another but as from day 20 onwards the water consumption of the control group showed steady increase, however the water consumption of the TBM group decreased. Results are displayed in figure 4.5.

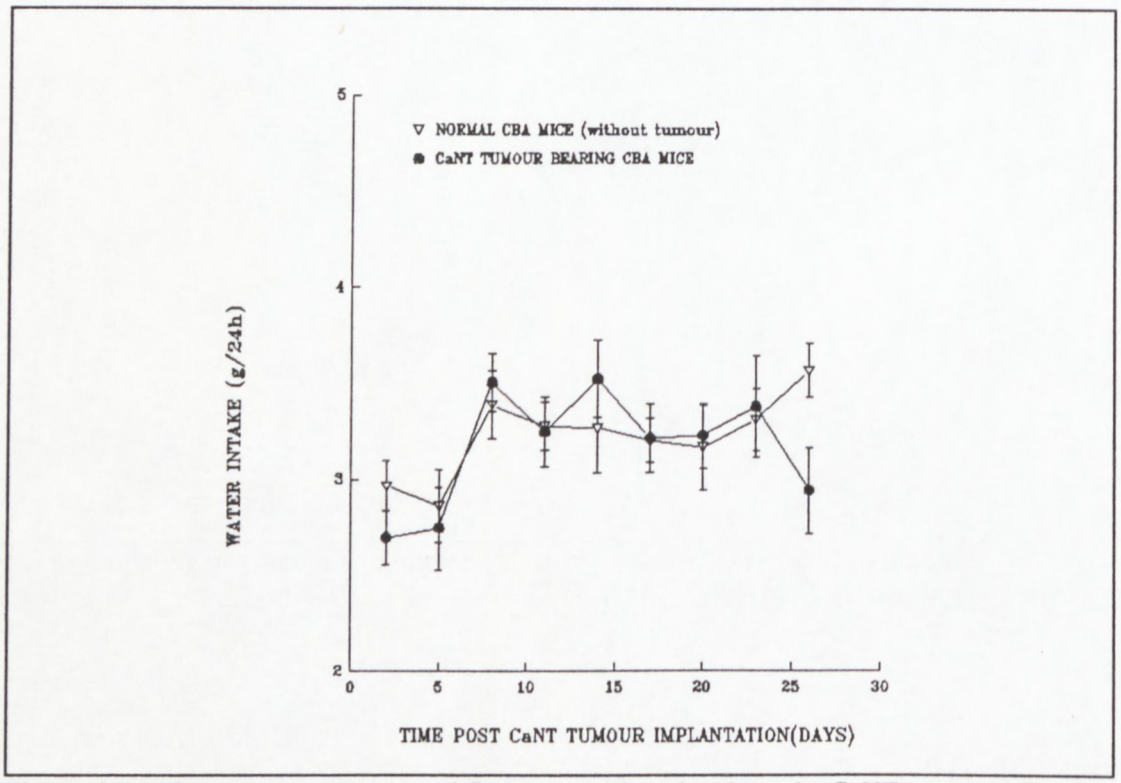

Figure 4.5: ***The water consumption of CBA mice with and without the CaNT tumour. The data points represents the mean of approximately 13 samples ± SEM.***

4.1.2.4 Faeces production

The faeces production in the CBA mice showed an initial period of increase, in both groups (control and TBM) up to about 8 days. Furthermore the faeces production of the control group showed a tendency to increase while the TBM group's faeces production remained almost constant. There was a significant difference ($P < 0.05$) in the production of faeces between the control and TBM groups from day 20 onwards to the 26th day. Results are shown in Figure 4.6.

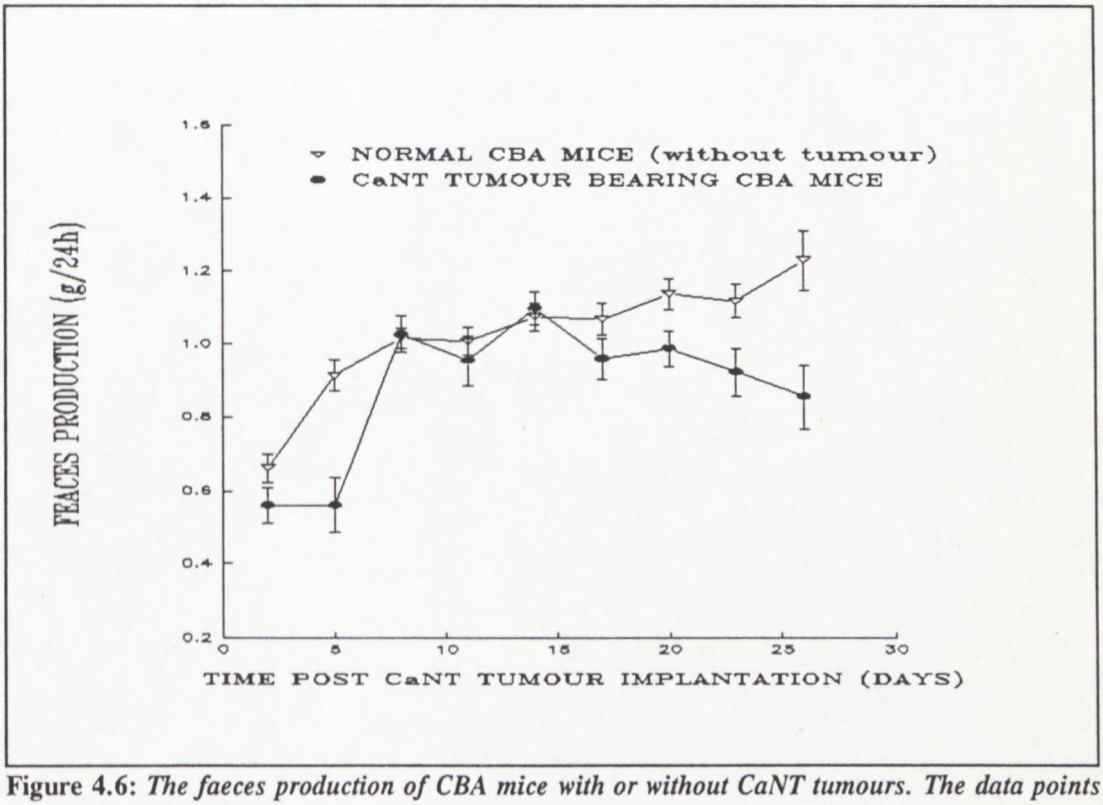

Figure 4.6: ***The faeces production of CBA mice with or without CaNT tumours. The data points represent the mean of approximately 13 samples.***

4.1.2.5. Urine production

The urine production of the CBA control group showed a marked increase up to day 8 after which there was a slight decline in urine output and finally a recovery from day 20 up to day 26. However the TBM group's urinary output appeared to be different from the profile of the control group. It declined slightly at day 5 and then slowly increased to day 13 after which the urine output declined markedly to below its initial urine output to reach a minimum at day 20. The urine production started to increase again after day 20 to reach a maximum at day 26. The TBM urine output differed significantly compared to control urine production ($P < 0.01$) from day 18 up to day 26. The results are shown in Figure 4.7.

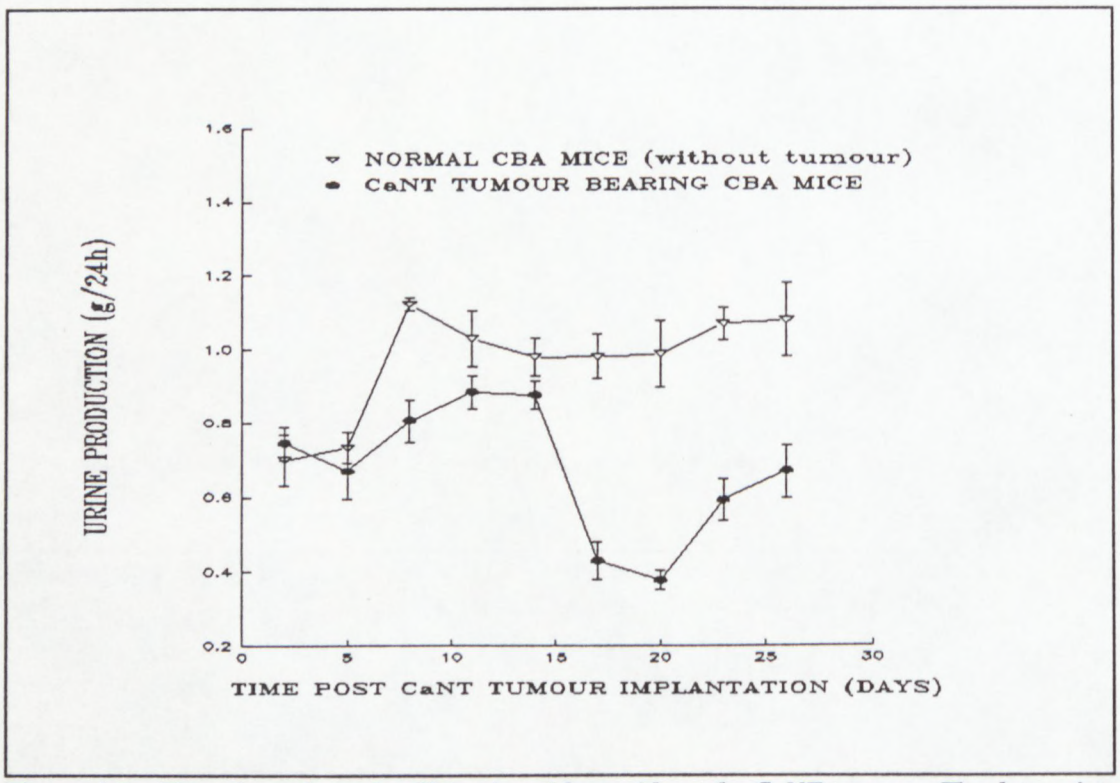

Figure 4.7: ***The urine volume for CBA mice with or without the CaNT tumour. The data points represent the mean of approximately 13 samples ± SEM.***

4.1.3 METABOLISM IN THE HOST

4.1.3.1 Liver

i) Lactate dehydrogenase (LDH)

The activity of LDH in the livers of the CBA mice bearing CaNT tumours indicated an overall increase in the levels of this enzyme versus tumour size. This rise was significant in all sizes when compared to the point of lowest activity of the enzyme except in the volume range 350 -450 mm^3 ($P < 0.05$). The results are displayed in Figure 4.8.

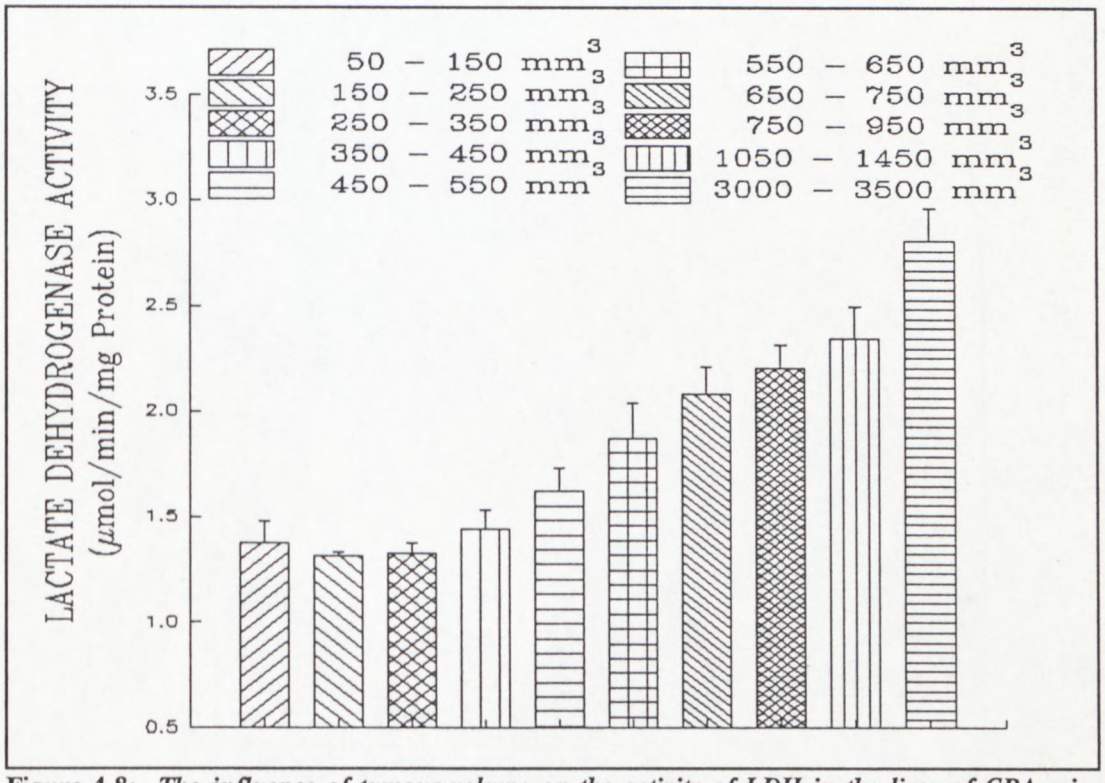

Figure 4.8: ***The influence of tumour volume on the activity of LDH in the liver of CBA mice bearing CaNT tumour. The height of the histogram block represents the mean of approximately 7 samples ± SEM.***

ii) Glycerol - 3 - phosphate dehydrogenase (G-3-PDH)

The specific activity of G-3-PDH in the liver of CBA mice bearing CaNT tumours indicated a steady profile of the tumour growth up to a tumour volume of 450 -550 mm^3. Furthermore there was a significant increase in the activity between tumour volumes 550 - 750 mm^3 reaching maximum activity when the mice tumour volume range reached 650 - 750 mm^3. After this peak of the G-3-PDH activity, a slow decrease was observed up to the biggest tumour volume range (3000 - 3500 mm^3). A significant difference between the activity of G-3-PDH in the liver of mice bearing a tumour larger than 550 mm^3 was observed when compared to the lowest activity measured in the livers of animals with a tumour volume range of 50 - 150 mm^3 ($P < 0.05$).

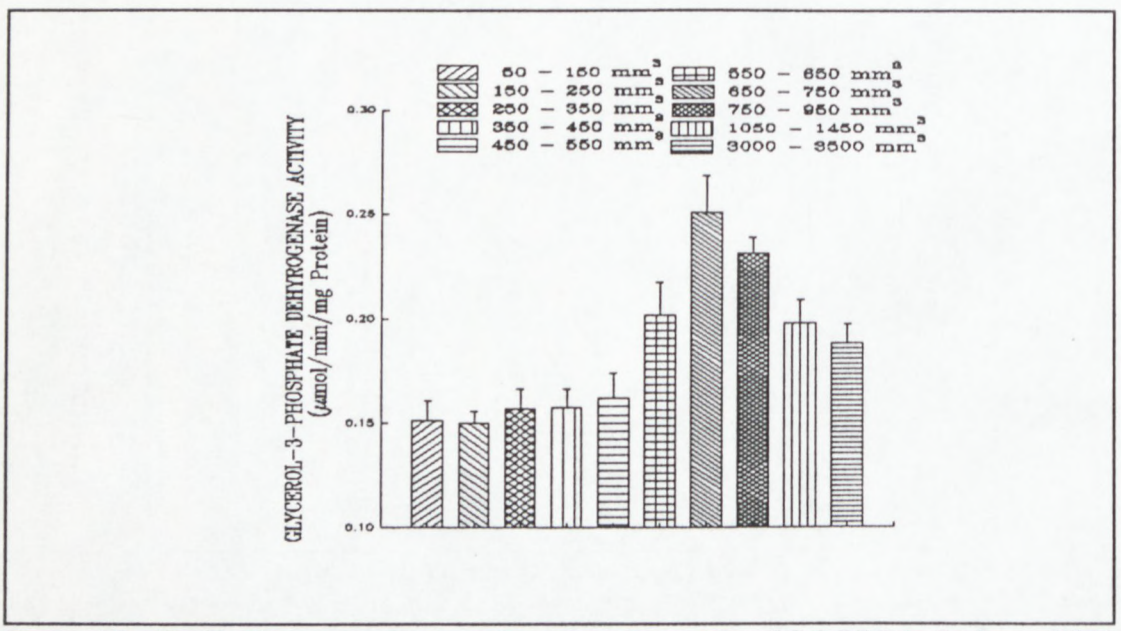

Figure 4.9: ***The CaNT tumour of various sizes versus the activity of G-3-PDH in the liver of CBA mice. The height of the histogram block represents the mean of approximately 8 samples ± SEM.***

There was also a significant decrease in the activity of this enzyme in the liver of CBA mice with tumour size as represented in the histogram blocks from 1050 mm^3 to 3500 mm^3, compared to the highest activity of the enzyme measured, as represented in the histogram block for tumour volume between 650 and 750 mm^3 ($P < 0.05$). The results are illustrated in Figure 4.9.

iii) Fructose - 1,6 - diphosphatase (F-1,6-DPase)

The F-1,6-DPase enzyme response in the liver of the CBA mice with CaNT tumour showed 3 distinct phases, the first was a downward trend when the tumour reached a volume of 50 to 350 mm^3. The second phase, was in the volume range of 350 to 650 mm^3 and showed a significant increase. The point of highest activity of F-1,6-DPase(550 - 650 mm^3) was significantly different from the activity measured in the liver of mice with a tumour in the volume range of 250 - 350 mm^3 (point of lowest activity) ($P < 0.001$).

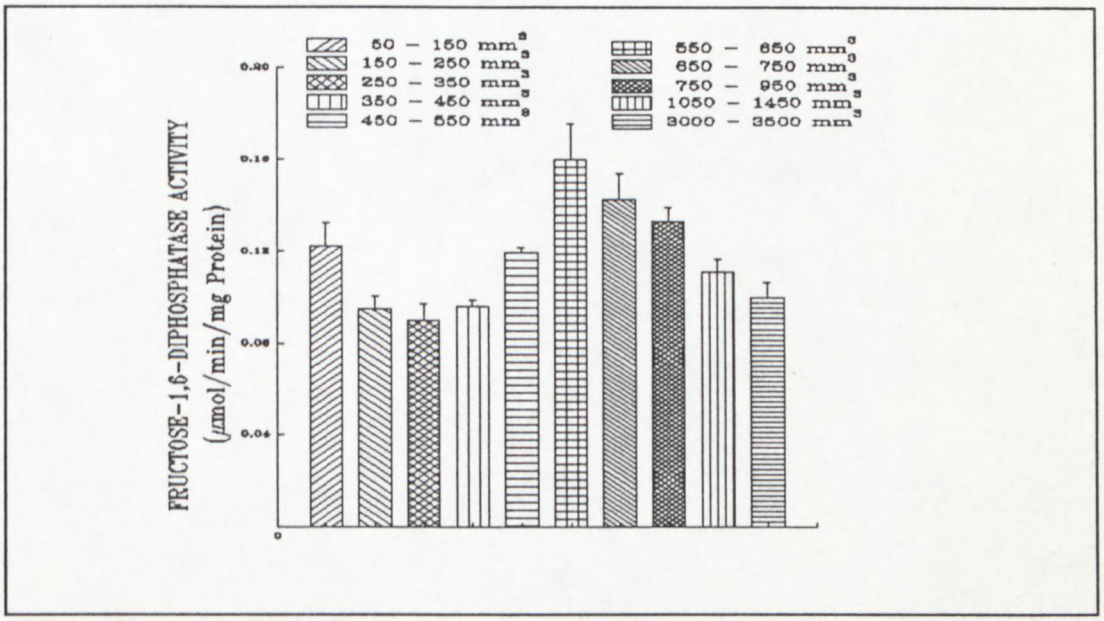

Figure 4.10: ***The CaNT tumour volume of various sizes versus the activity of F-1,6-DPase in the liver of CBA mice. The height of the histogram blocks represents the mean of approximately 7 samples ± SEM.***

The third phase of this enzyme profile demonstrated a decreasing trend in enzyme activity as compared to increased tumour size. The point of lowest activity of F-1,6-DPase(3000 - 3500 mm^3) was significantly lower when compared with the point of highest activity (550 -650 mm^3) ($P < 0.01$). See Figure 4.10 for the results.

4.1.3.2 Kidney

i) Lactate dehydrogenase (LDH)

The activity of LDH in the kidney of the CBA host with CaNT tumour showed a tendency to increase to a maximum level when the tumour reached a volume of 650 - 750 mm^3. There was a significant difference in the hypermetabolic activity of this enzyme in the kidney of mice with a tumour volume interval from 550 mm^3 to 3500 mm^3 compared to the lowest activity of LDH as seen in the kidney of the host mice bearing the smaller tumour size (50 - 250 mm^3) ($P < 0.05$).

The result are displayed in Figure 4.11.

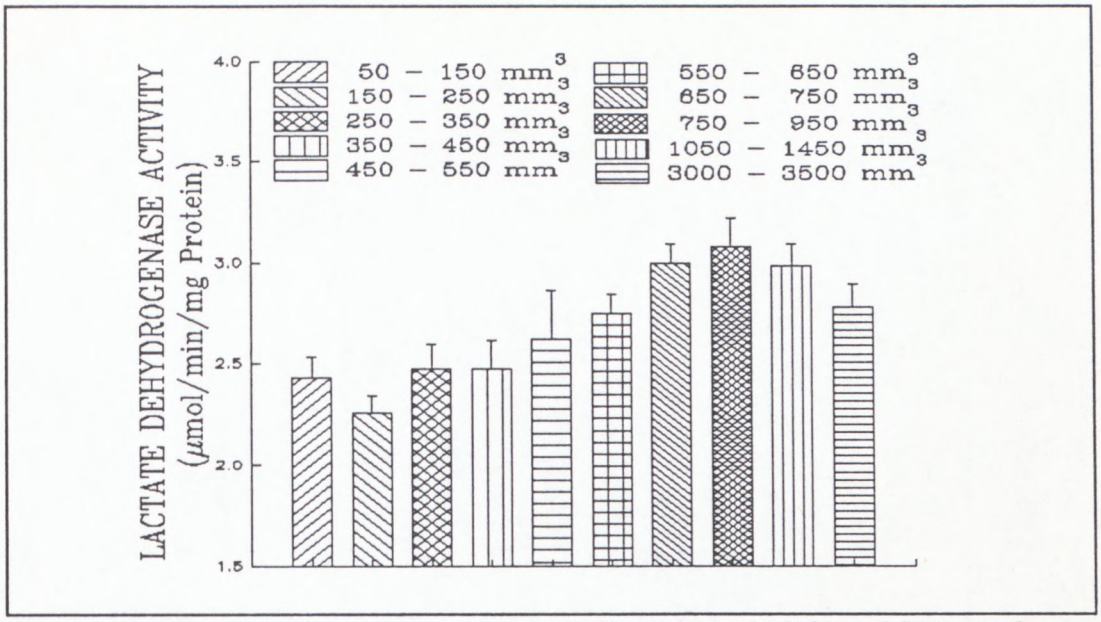

Figure 4.11: ***The effect of tumour growth on the LDH activity in the kidney of CBA mice bearing the CaNT tumour. The height of the histogram block represents the mean of approximately 6 samples ± SEM.***

ii) Glycerol - 3 - phosphate dehydrogenase (G-3-PDH)

The specific activity of G-3-PDH in the kidney of CBA mice with CaNT tumour showed a steady increase in activity up to a tumour size of 550 mm^3, then demonstrated a plateau profile and finally a decrease to almost the initial activity of this enzyme. A significant difference was observed between the highest activity of the enzyme range of 450 -550 mm^3 to the lowest activity in the range of 50 - 150 mm^3 ($P < 0.05$).

The results are shown in Figure 4.12.

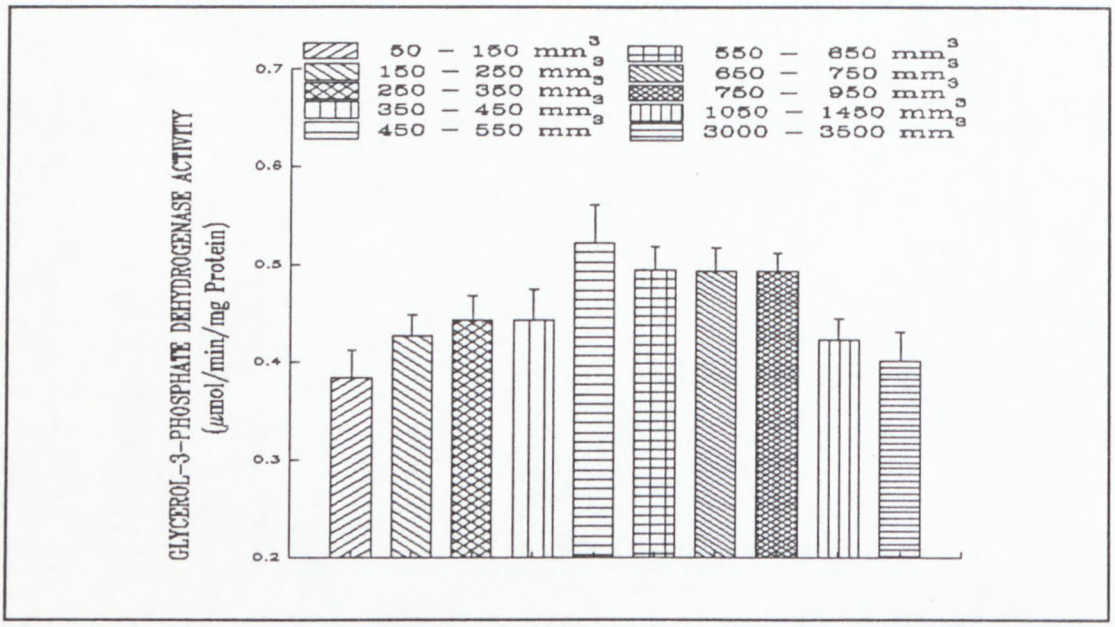

Figure 4.12: ***The response of G-3-PDH in the kidney of CBA mice bearing the CaNT tumour. The height of the histogram blocks represents the mean of approximately 6 samples ± SEM.***

iii) Fructose - 1,6 - diphosphatase (F-1,6-DPase)

The F-1,6-DPase activity in the kidney of CBA mice with CaNT tumour, in the beginning showed a slight decline, the activity of the enzyme then increased to a maximum, when the mice had a tumour of between 650 to 750 mm^3. With tumours larger than 750 mm^3 the activity of the enzyme decreased to almost the initial level. There was a significant difference in activity of this enzyme in the kidney of the host mice with a tumour volume between 450 and 950 mm^3 compared to the activity of the enzyme in the mice with a tumour volume in the range of 50 to 150 mm^3 ($P < 0.05$). The results are presented in Figure 4.13.

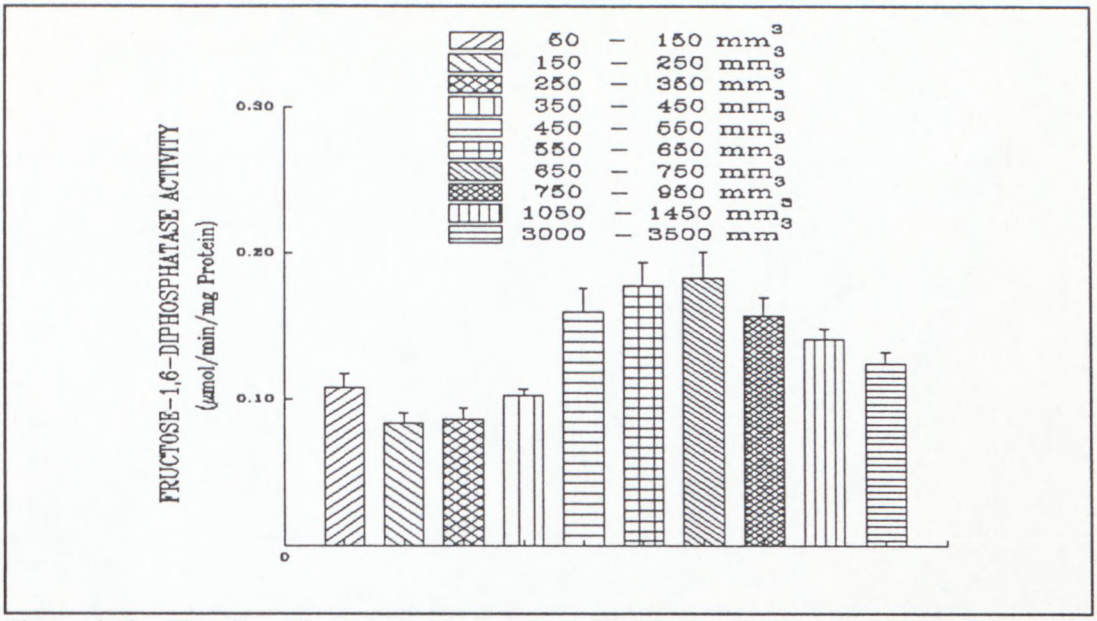

Figure 4.13: *The effect of tumour volume on the F-1,6-DPase activity in the kidney of CBA mice bearing the CaNT tumour. The height of the histogram blocks represents the mean of approximately 6 samples ± SEM.*

4.2 RADIOPROTECTION FROM HIGH LINEAR ENERGY TRANSFER RADIATION

4.2.1 Survival assay

A group of 50 mice were divided into two groups. Group 1 (n=30) received a lethal dose of 6 Gy neutron radiation and Group 2 (n=20) received the same dose of radiation but after prior injection of ATP. In the group that received radiation alone the survival after 30 days were only 40%, but after prior administration of ATP the survival percentage increased significantly to 85%, (see fig 4.14), which means that the survival modification factor (**SMF**) is 2.13 (equation 3.7).

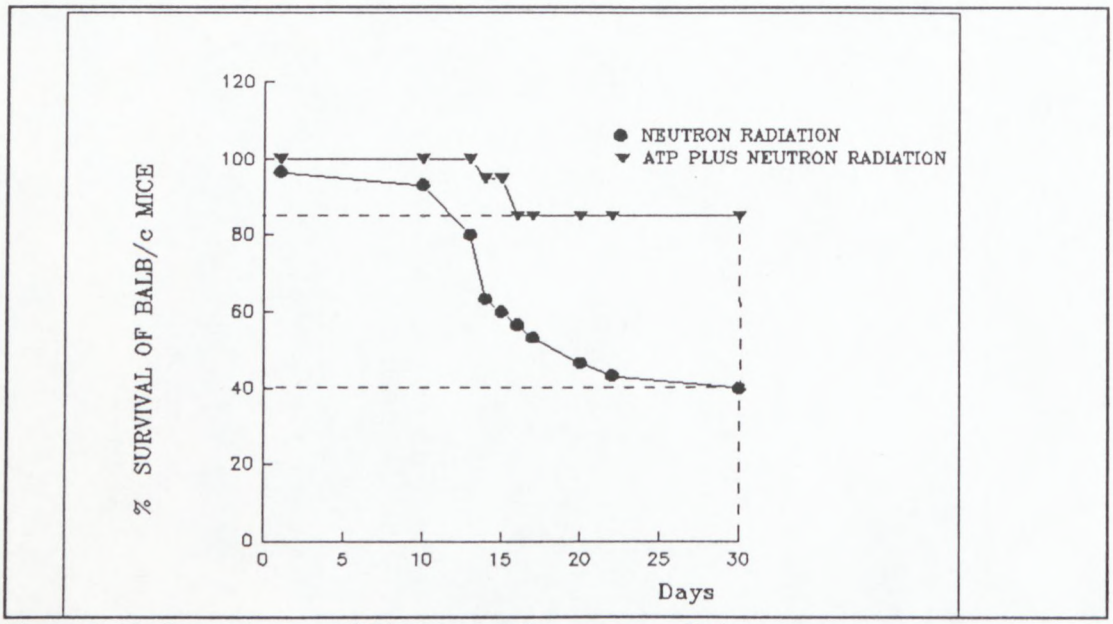

Figure 4.14: *Survival curve of BALB/c at 6 Gy neutron radiation*

4.2.2 Tumour growth delay assay

The mean size of rhabdomyosarcoma tumours, bearing in the BALB/c mice used in the beginning of this experiment, were 176 ± 17 mm^3. A total of 61 animals were used in this experiment. Group 1 (n = 17) was the un-irradiated control group, Group 2 (n = 24) received 2 Gy neutron radiation, and Group 3 (n = 20) received 3.8 Gy neutron radiation. Groups 2 and 3 were subdivided into two groups each, one received radiation alone and the other received ATP prior to radiation treatment.

Treatment	n	Tumour volume tripling time (days)	SGD
Controls	17	3.03 ± 0.19	
2 Gy	12	5.17 ± 0.43	1.71
ATP + 2 Gy	12	3.63 ± 0.38	1.19
3.8 Gy	9	7.35 ± 1.01	2.43
ATP + 3.8 Gy	11	4.80 ± 0.43	1.59

Table 4.1: *The mean tumour tripling times and Specific Growth Delay (SGD).*

The control group had a tripling time of 3.03 ± 0.19 days. The growth of the

tumour that received 2 Gy and 3.8 Gy radiation alone showed a significant delay in growth ($P < 0.01$) when compared to controls. There was also a significant reduction in tumour tripling time after pre-treatment with ATP at 2 Gy and 3.8 Gy ($P < 0.05$) compared to those that received radiation alone. The differential tumour growth delay between the tumour treated with radiation alone and those treated with ATP plus radiation (illustrated in Table 4.1) reflected radioprotection. Finally in this section of the work the specific growth delay of the different exposures were calculated according to equation 3.6(p.56) (see Table 4.1.)

4.2.3 Enzyme analysis

4.2.3.1 Hexokinase (HK)

i) Testis

The animals were exposed either to a lethal dose of neutron radiation (6 Gy) alone or treated with ATP prior to exposure to radiation (6 Gy). The activity of this enzyme was determined after 2,3,5,10 and 14 hours respectively after treatment. After this high dose of radiation the activity of hexokinase sharply decreased below the activity of this enzyme found in unirradiated testis, with maximal decremental activity of the enzyme at 3h after radiation.

The same profile of response was observed after 20 min pre-treatment with ATP, but the initial decrease was much less than with after radiation alone. ATP improved the response of the enzyme when it was added before radiation, this is clearly demonstrated in Figure

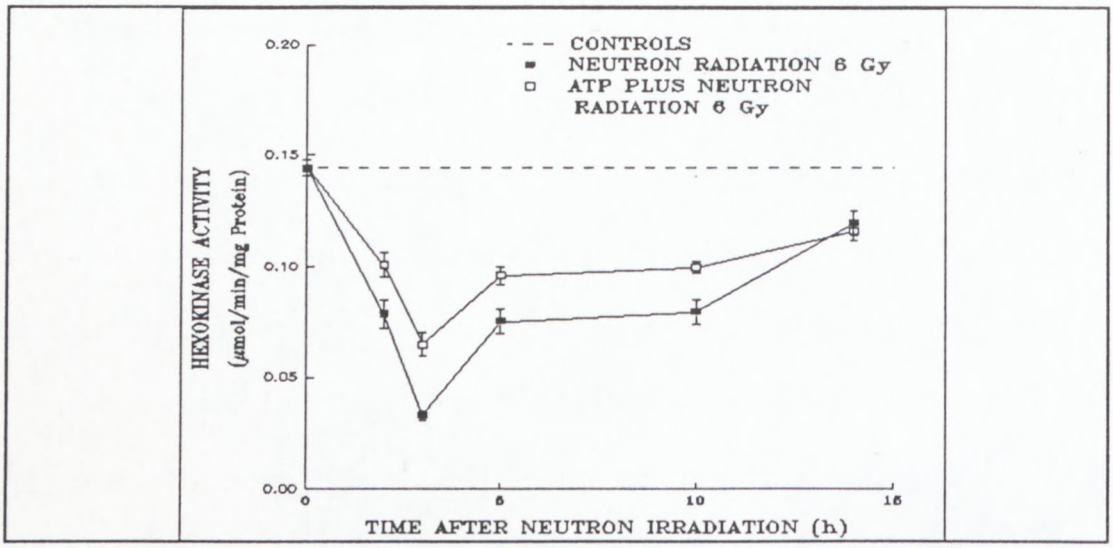

Figure 4.15: ***HK response in the testis of BALB/c mice to 6 Gy of neutron radiation with or without ATP. Each point represents the mean of approximately 5 determinations ± SEM.***

4.15 and the difference between the two treatments was most pronounced 3 hours after the mice were irradiated. The activity at all times were significantly higher in the animals that received ATP before radiation ($P < 0.05$) compared to those that were treated with radiation alone, with the only exception at 14 h.

ii) Tumour

When a lethal dose of 6 Gy was administered alone there was a great decrease in the activity at 3 hours after exposure ($P < 0.02$) when compared to un-irradiated controls. However when used in combination with exogenous ATP administered 20 minutes before radiation there was a marked recovery of the enzyme ($P < 0.001$) when compared to those that received radiation alone (see Figure 4.16).

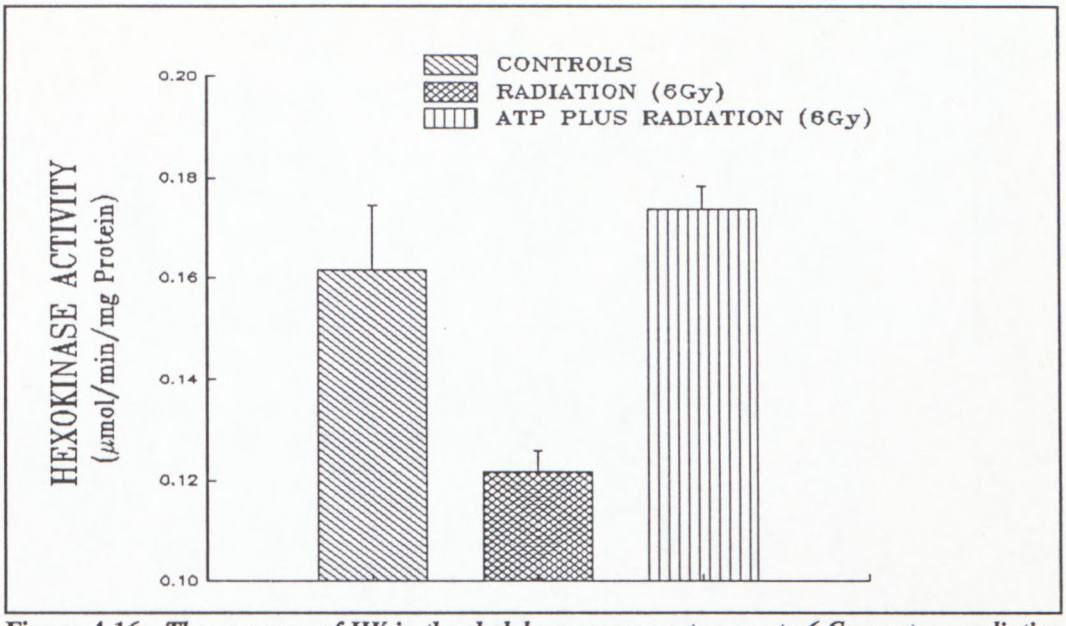

Figure 4.16: ***The response of HK in the rhabdomyosarcoma tumour to 6 Gy neutron radiation with and without ATP. The height of each histogram represents the mean of approximately 6 samples ± SEM.***

4.2.3.2 Glucose - 6 - phosphate dehydrogenase (G-6-PDH)

i) Testis

The enzyme activity increased significantly in the testis of BALB/c mice after neutron radiation alone ($P < 0.05$) but when the mice were pre-treated with ATP, the specific activity of G-6-PDH recovered to about the activity found in testis of unirradiated mice. There was a significant difference after ATP was combined with the radiation ($P < 0.05$) when compared with radiation alone (Figure 4.17).

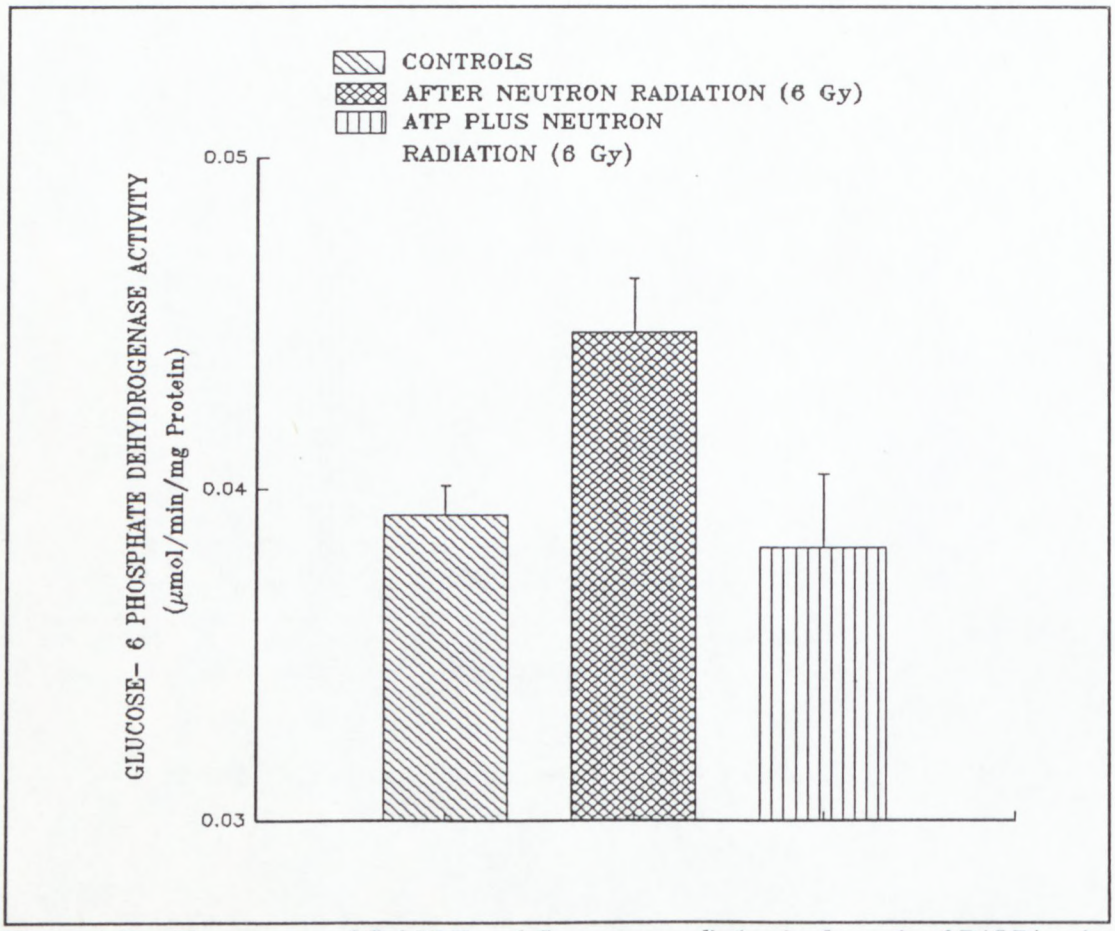

Figure 4.17: ***The response of G-6-PDH to 6 Gy neutron radiation in the testis of BALB/c mice with or without ATP. The height of the histogram represents the mean of approximately 7 determinations ± SEM.***

ii) Tumour

The enzyme activity after 6 Gy neutron radiation alone increased significantly compared to the activity found in untreated tumours($P < 0.0002$). However in the tumours that received ATP 20 min prior to radiation exposure the activity of the enzyme increased slightly above the activity of the untreated tumours($P < 0.03$). Furthermore the increase found in tumours that were pre-treated with ATP prior to radiation was significantly lower than that observed in tumours that received only radiation($P < 0.0001$). This is illustrated in Figure 4.18.

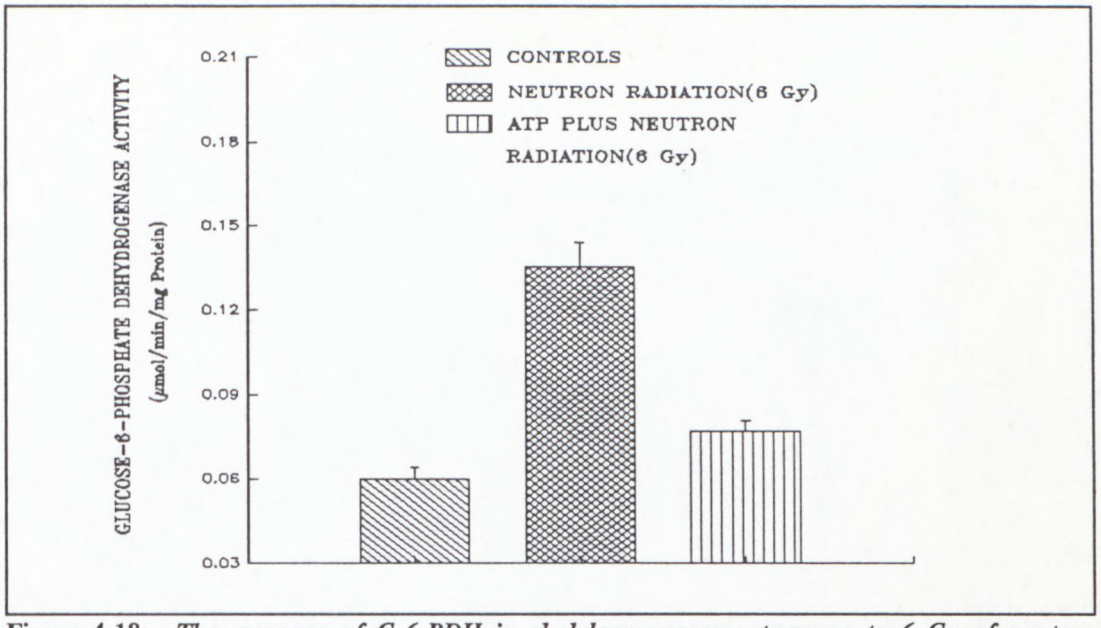

Figure 4.18: *The response of G-6-PDH in rhabdomyosarcoma tumours to 6 Gy of neutron radiation with or without ATP. The height of the histogram block represents the mean of approximately 6 determinations ± SEM.*

4.2.3.3 Lactate dehydrogenase (LDH)

i) Testis

The activity of LDH after a lethal dose of neutron radiation (6 Gy) significantly decreased ($P < 0.01$). When the mice were treated with ATP 20 minutes prior to exposure to radiation, the activity markedly increased above the activity found in the testis of mice that were untreated($P < 0.02$) as demonstrated in Figure 4.19. This implies that there is also a significant difference between the response of BALB/c mice receiving only radiation and those that were injected with ATP before radiation ($P < 0.02$).

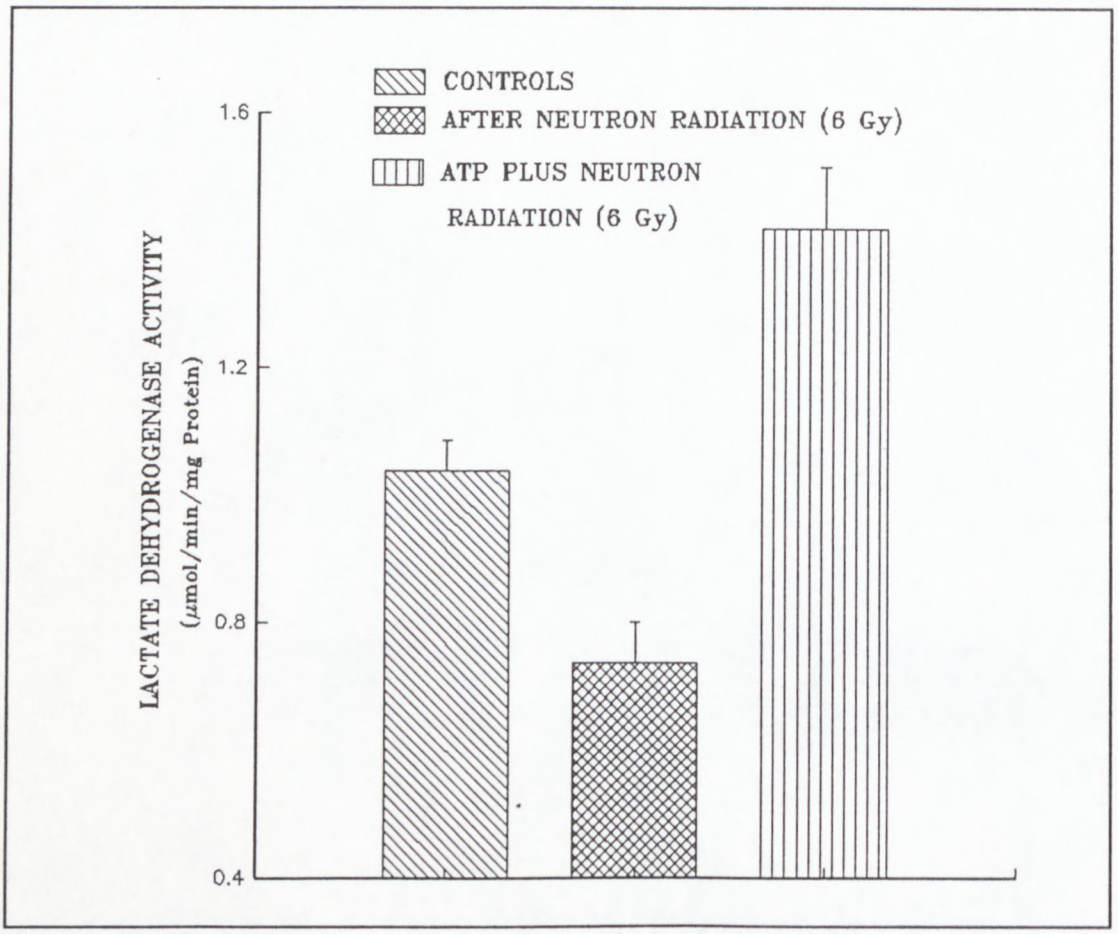

Figure 4.19: ***The LDH response to 6 Gy neutron radiation in the BALB/c testis with or without ATP. The height of each histogram block represents the mean of at least 6 determinations ± the SEM.***

ii) Tumour

When a whole body lethal dose of 6 Gy neutron radiation were given to the BALB/c mice bearing rhabdomyosarcoma tumours, the activity of LDH in the tumour was markedly decreased in relation to the control activity ($P < 0.0001$). However when the mice were treated with ATP 20 minutes before radiation the activity of this enzyme increased significantly above the controls ($P < 0.03$).

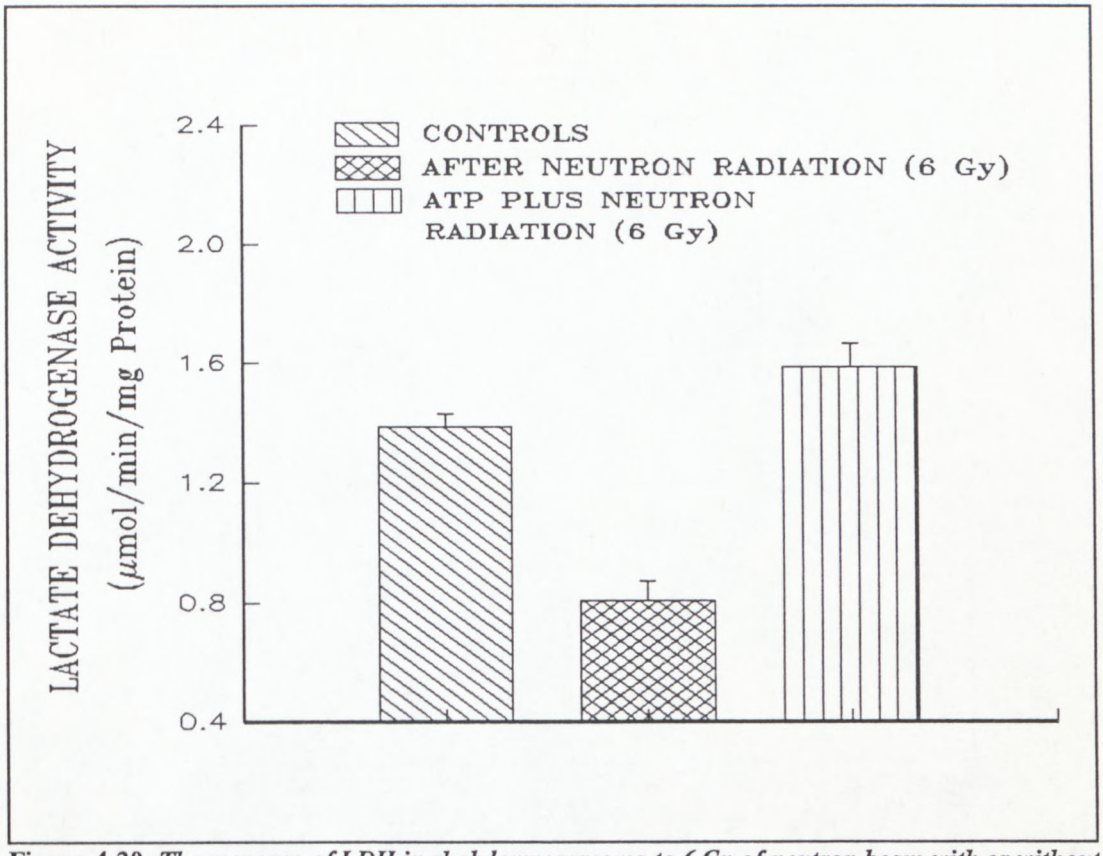

Figure 4.20: ***The response of LDH in rhabdomyosarcoma to 6 Gy of neutron beam with or without ATP. The height of the histogram block represents the mean of approximately 6 determinations ± SEM.***

4.2.3.4 Acid phosphatase (AP)

i) Testis

There was a significant augmentation in the activity of this lytic enzyme($P < 0.01$) after radiation alone at a dose of 3.8 Gy. The enzyme activity found in the testis of animals that received ATP before exposure to 3.8 Gy radiation was almost the same as that observed in the testis of animals that received no treatment. There was a significant difference between the response of this enzyme in the testis of animals that received radiation alone and those that received ATP before radiation ($P < 0.02$). This is shown in Figure 4.21.

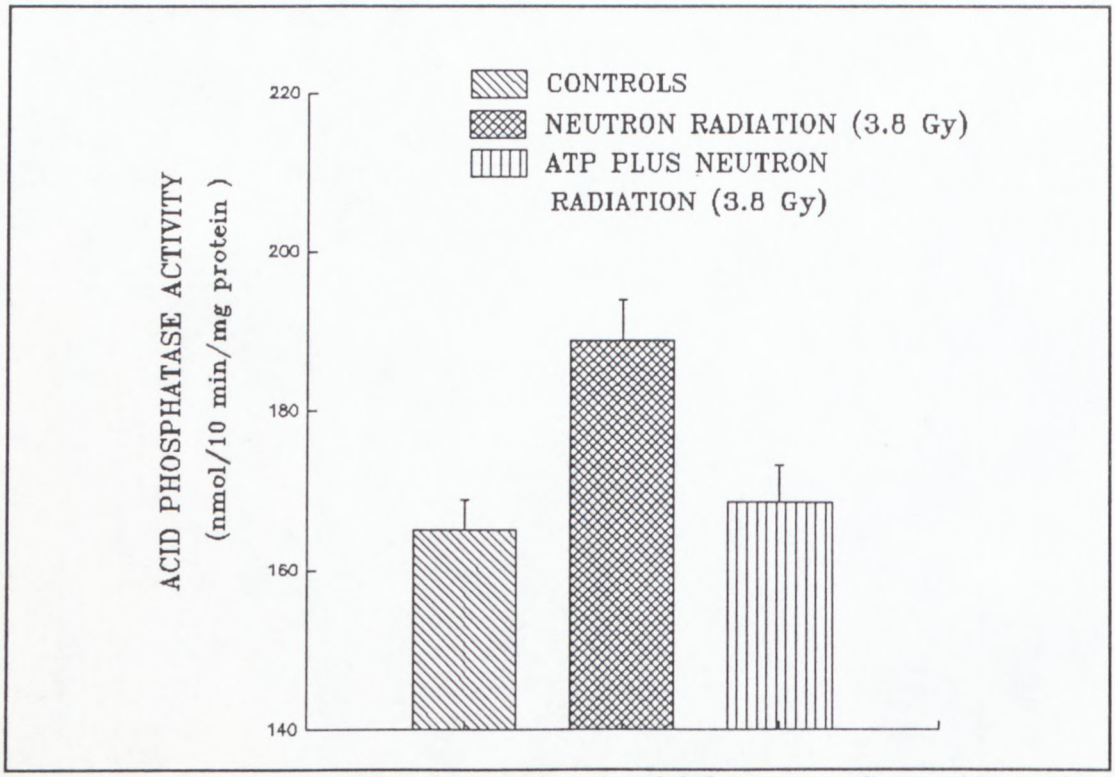

Figure 4.21: ***The response of AP in the BALB/c testis to 3.8 Gy neutron radiation with or without ATP. The height of the histogram block represents the mean of approximately 6 determinations ± SEM.***

With a lethal dose of 6 Gy neutron radiation given to the mice, the activity of AP was monitored at different times up to 72 hours. The enzyme activity in the testis of animals that received only a dose of 6 Gy neutron radiation rose strongly in the first hour and continued to do so up to 24 hours after radiation. At 48 and 72 hours respectively the activity of the enzyme was lower than at 24 hours, but still significantly higher than in the testis of control mice.

However, when the mice were treated with ATP before whole body exposure to neutron radiation, the response of testicular AP was slower at all times. All points were significantly different ($P < 0.03$) from the controls except

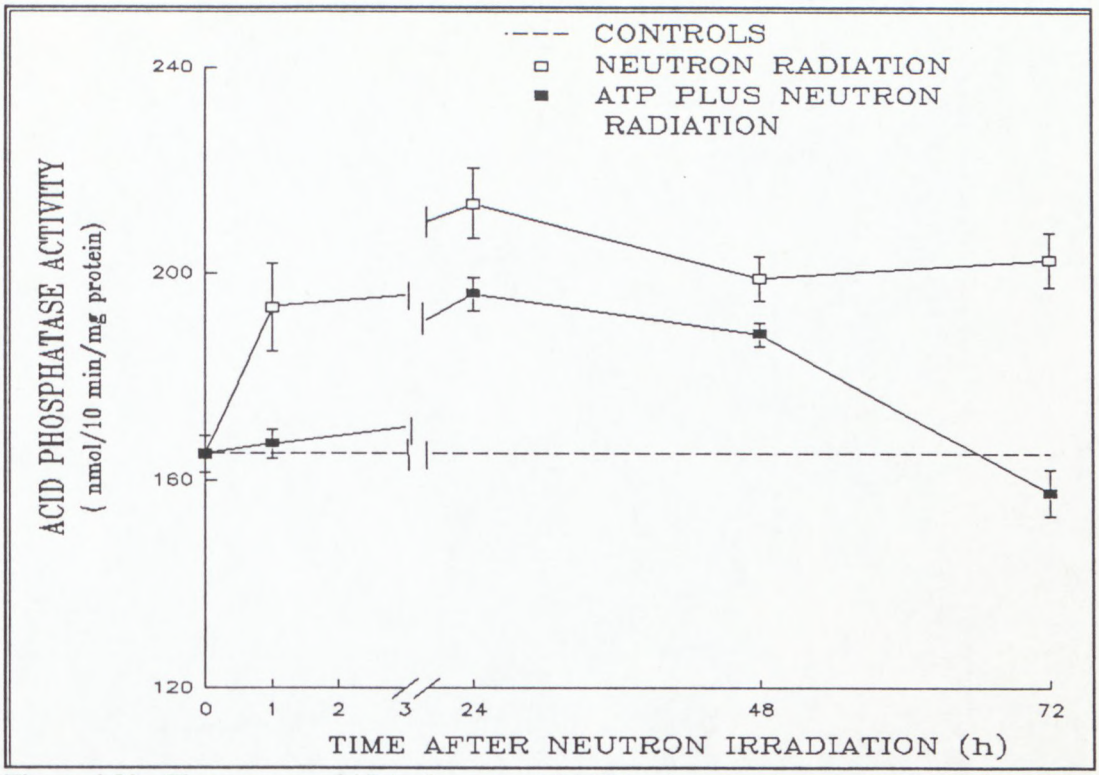

Figure 4.22: ***The response of AP in the testis of BALB/c mice at various times after 6 Gy neutron beam with or without ATP. Each data point represents the mean of approximately 6 determinations ± SEM.***

those at 1 and 72 hours after radiation in the animals that received ATP before exposure. The activity of the enzyme in the group that received ATP prior to radiation, was significantly lower ($P < 0.05$) than the activity of the enzyme in the mice that received only radiation at all times after radiation. This is illustrated in Figure 4.22.

ii) Tumour

The activity of AP in the rhabdomyosarcoma tumour was determined after exposure to three different doses (2.0, 3.8, 6.0 Gy respectively) of neutrons at different times up to 144 hours after radiation as shown in Figure 4.23.

Furthermore, the activity of AP was measured one hour after radiation at a dose of 3.8 and 6 Gy after pre-treatment with ATP 20 minutes prior to

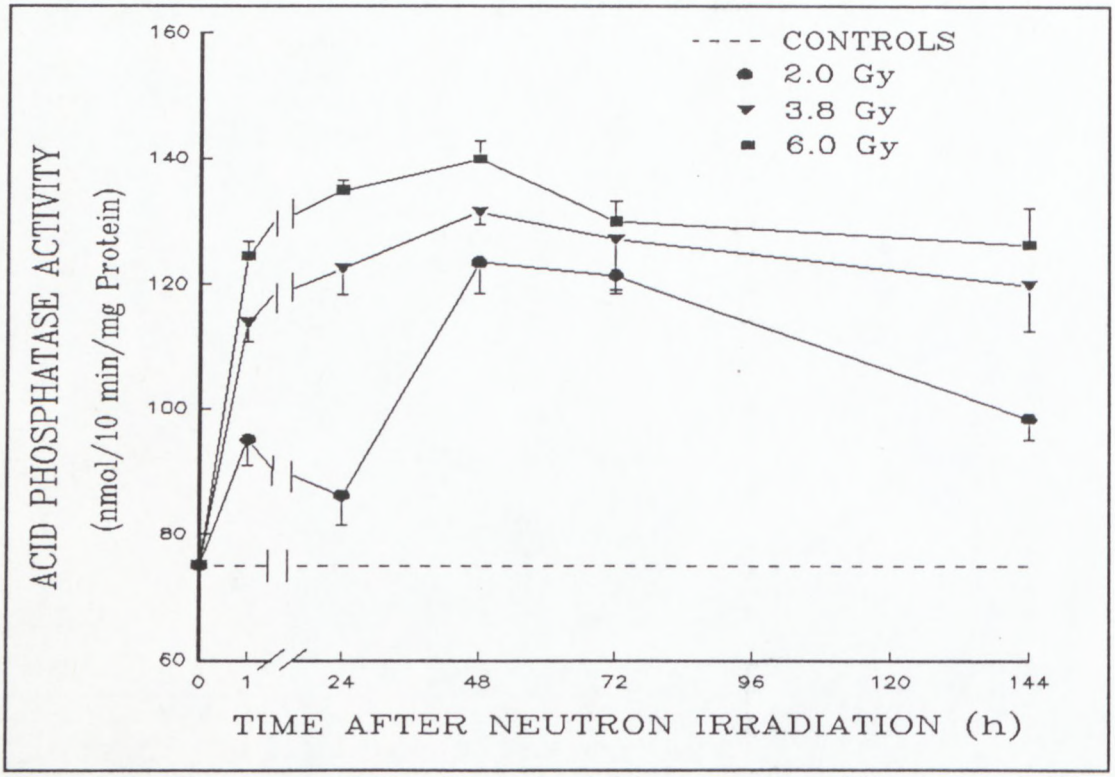

Figure 4.23: ***The response of AP activity in the rhabdomyosarcoma to different neutron doses. Data points represent the mean of between 3 to 8 determinations ± SEM.***

radiation as shown in Figures 4.24 and 4.25 respectively. The activity of this enzyme in the tumours of mice exposed to radiation increased significantly above the activity observed in tumours of animals not exposed to radiation, at all doses and after all times ($P < 0.05$) except after 2 Gy at 24 hours where there was no significant difference .

The activity of AP in tumours which received prior treatment with exogenous ATP indicated an augmentation, but this was markedly less than the tumours that received only radiation ($P < 0.05$).

There was also a significant difference between the AP activity in the tumours of animals that received ATP pre-treatment and the unirradiated control tumours of animals that received only radiation with the $P < 0.05$. This is demonstrated in Figure 4.24 and 4.25.

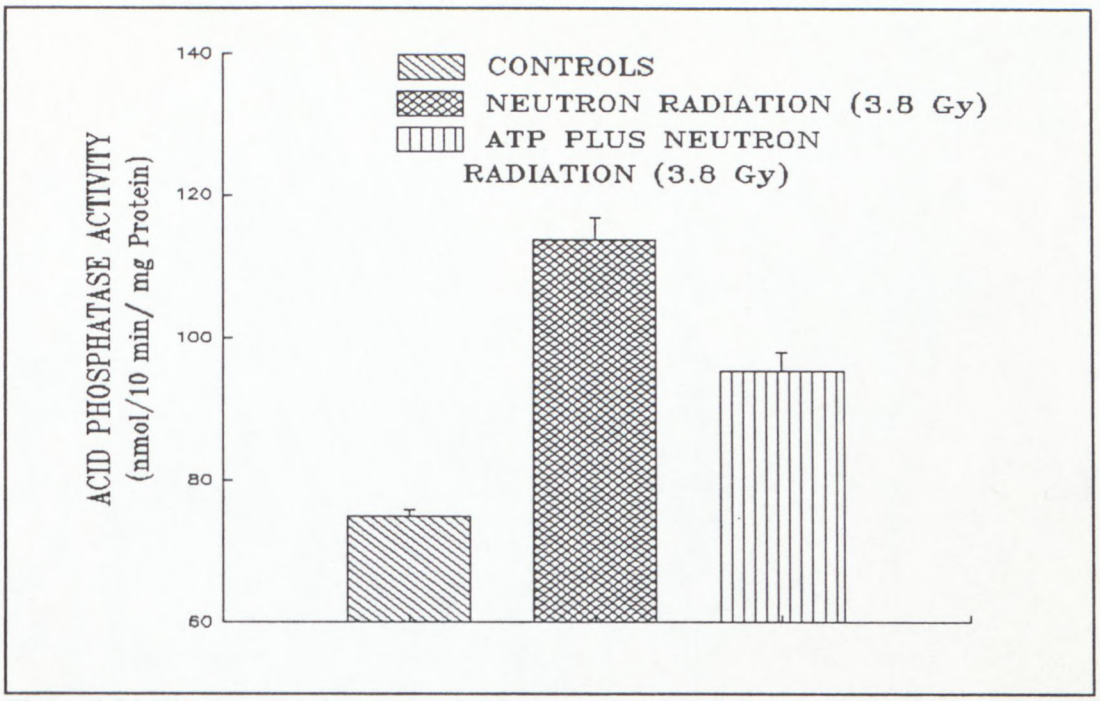

Figure 4.24: *The response of AP in the rhabdomyosarcoma to 3.8 Gy neutron radiation, within and without ATP. The height of the histogram block represents the mean of approximately 6 determinations ± SEM.*

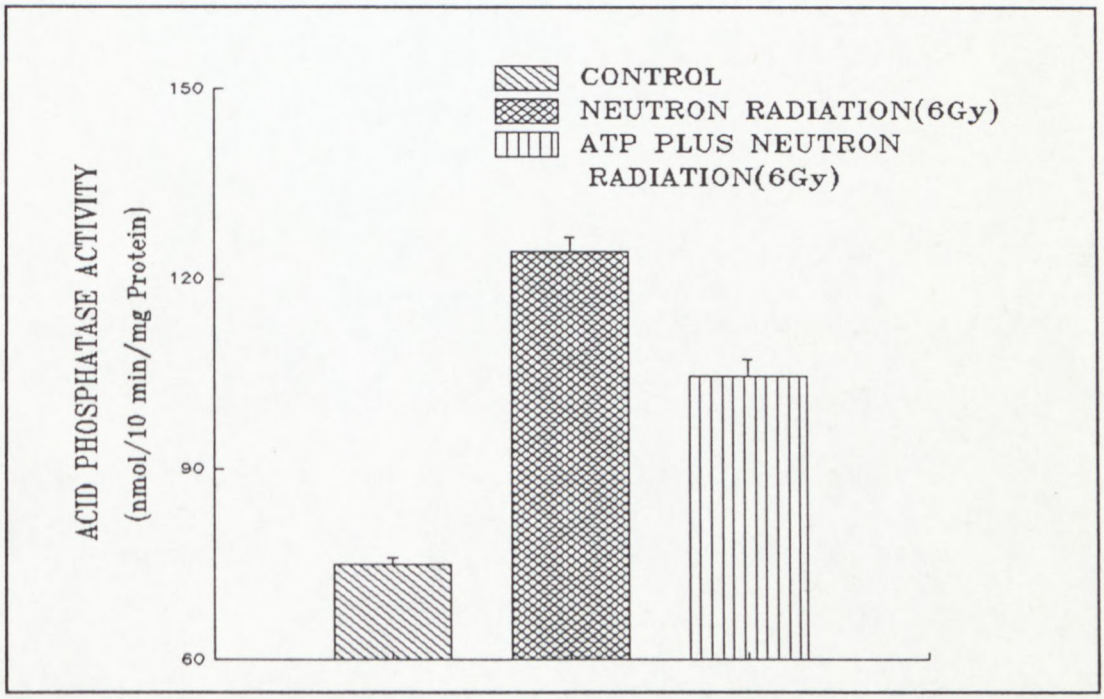

Figure 4.25: *The response of AP in the rhabdomyosarcoma after 6 Gy neutron radiation with and without ATP. The height of the histogram block represents the mean of approximately 6 determinations ± SEM.*

CONCLUSIONS

CHAPTER 5

CONCLUSIONS

The most effective treatment in cancer therapy is achieved when an in depth understanding of the basic metabolism, and vasculature pattern of the tumour and its holistic interrelationship, with the detrimental physiological changes in the host, is gained. The steady state in the tumour rapidly changes with tumour growth, and subsequent deterioration of the blood and nutrient supply. This adaptation in the steady state of the tumour is shown in the increased lactate dehydrogenase and acid phosphatase activity in the tumour, during it's growth. These alterations in the tumour metabolism places an increased burden on the body to supply nutrient, and to discard the waste products of the tumour. These demands for more nutrients and the ability of the tumour to trap great amounts of these nutrients, have a detrimental influence on the steady state of the host. This was demonstrated by this study at the macroscopic level, by the decreasing body weight and food intake when the tumour burden increased, and also at the metabolic levels by the responses of certain glycolytic and Cori cycle enzymes. Furthermore, three distinct stages were observed in the Cori cycle response by the influence of the tumour, namely a silent or preclinical stage, a hypermetabolic stage and a hypometabolic stage. Although the decreasing body mass cannot be directly linked to the process of gluconeogenesis, the onset of anorexia appeared to coincide with the end of the hypermetabolic stage and the beginning the hypometabolic stage during gluconeogenesis. This clearly shows that the body's steady state is adversely affected by the presence of a tumour, and that the conditions at the metabolic level seem to cause the anorexia that was observed in these experiments. This indicates that the body's capacity to resist any adverse condition might be changed with the growth of the tumour, therefore this phenomena must be taken into account when planning the therapy.

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Furthermore, it is well known that the success of cancer therapies depends entirely on the effectiveness of the modality to kill the tumour cell, and on the ability of the host to absorb the damage caused by the modality without being detrimentally affected in the process. This was the motivation for the second part of this study, i.e. to protect the host from the adverse conditions created by certain cancer therapies like neutron radiation. This part of the work demonstrated the radioprotective effects of ATP at all levels. It is clear from this work that ATP had a bigger influence in protecting the normal tissue, than it had on the tumour tissue. The difference in protection can clearly be seen in the response of acid phosphatase (AP) and glucose-6-phosphate dehydrogenase (G-6-PDH) in the tumour and testis. From these results, it would seem that ATP has a multifactorial interaction with the cell, and two possible mechanisms of protection are indicated by these results. The first of these interactions is through the purinergic receptors of the cell to stimulate enhanced glycolysis, for higher energy production and thus repair. This interaction is well established and has been described by many researchers previously, (Lee and Filkins, 1987), and is also demonstrated in this study by the activity of HK and LDH. The second mechanism is the interaction of ATP with the A_2 receptor of the cell to inhibit the production of free radicals and thus damage. This mechanism has only been described in neutrophils (Cronstein *et al,* 1983;1985;1988; Foreman, 1989), but the activities of G-6-PDH and AP would seem to support this theory as

ATP's second radioprotective interaction. Further investigation into this mechanism of interaction of ATP with the cell after radiation should be undertaken in future studies. The differential protection afforded by ATP can be seen as a function of the distribution of the drug in the tumour and normal tissue, and therefore further work is still warranted to determine the concentration that will afford the maximum differential protection to the host body.

The protection of normal tissue by either ATP is essential if the neoplastic tissue is to be eradicated without any harmful effect to the host body, which is already in a weakened state. The progressive weight loss described in this study can be related to increased morbidity and mortality, and represents a powerful predictor of the host response that could be used in cancer therapy. This knowledge could be used in conjunction with the selective radioprotective properties of ATP on the normal tissue, to maximise tumour cell death. This could improve the effectiveness of radiotherapy and lessen the side effects the cancer patient has to suffer whilst undergoing radiotherapy.

DISCUSSION

CHAPTER 6

CHAPTER 6

DISCUSSION

6.1 TUMOUR METABOLISM AND SOME OF ITS INFLUENCES ON THE CBA MURINE HOST

The tumour metabolism investigation performed in this study attempts to give a holistic view of the interdependent relationship of the host, during impaired metabolism and tumour growth.

In the constitutional disease of cancer cachexia, the blood flow of the neoplastic tissue is impaired and deteriorate with the growth of the tumour(Sevick and Jain, 1989; Vaupel, 1975; 1977; Endrich *et al*, 1979; Vaupel *et al*, 1981). If the body does not respond by supplying more oxygen and nutrients to the tumour, the amount of oxygen and nutrients will become less per cell, and thus an abnormal steady state will arise in the tumour. It is well known that the oxygen tension in tissue can only be increased in three ways, namely 1) higher degree of oxygen dissociation from haemoglobin,(Robbins and Angell, 1976; Walter and Israel, 1979); 2) increased blood flow and, 3) increase in the number of the circulating red blood cells (Rapaport, 1971). The last two options are neutralized by the poor blood supply to the tumour, and the first possibility is very limited in scope. Thus, there will be a higher demand for nutrients to fuel the energy needs of the tumour cells under reduced oxygen tension (Kallinowski *et al*, 1985; Vaupel *et al*, 1987). The concentration of oxygen and nutrients in the tumour decreases with the increase in the distance the cell is situated from the vasculature (Szeinfeld, 1988). Hence, the cells lying more than about 150 nm away from the capillary, can exist under hypoxic

and anoxic states(Vaupel *et al*, 1987). Despite the fact that most tumour cells are under physiological stress, the tumour still continues to proliferate. There are two possible mechanisms to explain how the tumour copes with the necessary amounts of nutrients required for its proliferation. The first is that the tumour cells under stress, send chemical messages (Vincent,1985; Shapot, 1972; Theologides, 1979) to the body to increase the nutrient supply, and/or the second mechanism is that the nutrients are removed from the bloodstream so quickly, that the host tissue becomes nutrient-deprived (Vincent,1985:23; Theologides,1972; Shapot, 1972). The host's tissues then sends out signals to the body store to request an increased amount of nutrients, which alter the physiological homeostatic mechanisms(Herzfeld and Greengard, 1972; Lundholm *et al*, 1978; 1982) This results in increased energy expenditure (Fenninger and Mider, 1954), with the consequent release of host metabolites which are trapped by the growing tumour (Theologides, 1979). Thus, this contributes to promote weight loss, anaemia and marked asthenia in the host (Theologides, 1974).

6.1.1 The influence of tumour growth on the metabolism of rodent solid tumours

The metabolic disturbance during tumour growth, is reflected in part in the increased lactate dehydrogenase activity that is observed in this study (Figure 4.1. see p 70), which may reflect the degree of hypoxia in the tumour. The tumour cells under drastically decreased oxygen tension mostly metabolize glucose to lactate as end product, and not to CO_2 and H_2O as found in normal cells (Vincent,1985; Segura *et al*, 1989). This indicates an

impairment of some tumour cells to metabolize glucose fully (Medina *et al*, 1988; Greenhouse and Lehninger, 1977). This impairment will force the tumour cell to take in and catabolize more glucose to maintain the energy expenditure (Vincent,1985; Inculet *et al.*, 1987 ; Vorster, 1990). In experiments performed by Kallinowski *et al* (1988), it was found that the increase in glucose uptake by tumour was directly linked to the amount of glucose available in the bloodstream of the host. This supported the idea that the tumour removes the nutrients from the blood as soon as they become available. Furthermore, it was found that most tumours released lactate and that the concentration was directly related to the glucose uptake (Kallinowski *et al*,1988).

A further sign of the metabolic deterioration when the tumour increased in size was the augmentation of the activity of the lysosomal enzyme, acid phosphatase (Figure 4.2 see p 71), which suggests that there was a higher amount of injury (Shah and Bhatavdekar, 1983; Szeinfeld and de Villiers, 1991), or cell death (Bowen and Ryder 1974,1976) in the larger tumour.

6.1.2 Tumour incidence on the dietary parameters of the host

The cancer cachexia model used in this study for the determination of the effect of tumour burden on the host dietary parameters, showed steady but reduced body mass up to 23 days after tumour implantation, whereafter there was a further reduction in body mass. These experimental results were coincident with Theologides (1972), who stated that the carcass can either remain the same mass, or even increase for a short period after which it will

decline. The body mass of the control mice on the contrary showed an overall increase, which may be explained by the fact that the mice were juvenile and still growing. Thus, the almost constant mass of the TBM group can be seen as a constitutional disorder of growth of the animals, due to the tumour burden.

Although the solid tumours demanded a vast amount of nutrients due to their fast growth (tumour doubling time = $\pm$ 2.3 days), it was found in this study that the host's body was not able to assimilate more nutrients. These results concord with the work of Vorster (1990), in which she reported that in the disorder of cancer cachexia, a degree of anorexia is present in almost all cases. This is clearly illustrated in Figure 4.4 (see p 74), where a decline is observed in the food consumption starting after day 18, after tumour implantation when the mean volume was $\pm$ 700 mm^3. These results are similar to those obtained by Inculet (1987) in F344 rats. Furthermore the faeces production indicated a similar response, as the food consumption to tumour volume and the ratio between the food intake and faeces production were similar for both the tumour-bearing mice (TBM) and the controls. This indicated that the decreased faeces production in mice bearing the tumour burden, can be associated with the degree of anorexia reflected by the food consumption of the TBM group. Therefore the shortage of nutrient that develops in the host during cancer cachexia, is not due to malabsorption of the food but induced by the anorexia.

Although the water intake and food intake usually correlates with each other under normal conditions, in the case of this model, the water consumption

of the TBM's and controls showed no significant difference up to day 23 after tumour implantation, in spite of the fact that the food consumption of the experimental animals decreased from day 18 after tumour implantation, (Figure 4.5 see p. 75). Despite the fact that there was no difference in the water consumption of the TBM group and controls, the urine production of the TBM group showed a substantial decrease from day 13 after tumour implantation, together with partial recovery in urine output after day 20 after tumour implantation (see Figure 4.7, p. 77). This reduction of urine output was clearly the result of water retention due to either fluid retention by the tumour, oedema of the whole body, or kidney failure (Vincent,1985). Several researchers have stated that patients with advanced cancer often have increased intra-cellular and extra-cellular water content, (Waterhouse *et al*, 1951; Craig, 1957; Theologides, 1979; Lundholm *et al*, 1980; Karlberg *et al*, 1983). Furthermore, Theologides (1972;1979) implicated the water balance of the patient as one of the factors that can alter the mass of the patient. Thus, this could be responsible for the slight increase that developed in body mass of TBM's at day 20 after tumour implantation, as well as the decrease in body mass after the recovery in urine output which took place 23 days after tumour implantation.

6.1.3 The effect of the CaNT tumour burden on the host metabolism

The results obtained in this, as well as other reports on the dietary parameters of the host, indicate a decrease in the intake of nutrients, which implies that a state of anorexia develops in the cancer patient

(Theologides,1972; 1979; Guaitani, 1982; Inculet *et al*,1987; Zylicz *et al*, 1990; Vorster, 1990) .

Waterhouse (1974) and Kallinowski *et al* (1988) states that there is an increase lactate production in the tumour, as is demonstrated in this study by the activity of LDH in the tumour (Figure 4.1. see p 70). The body has to take the lactate from the blood in order to maintain pH and prevent an lactic acidosis (Waterhouse, 1974). The removal of lactate from the blood is performed mainly by the liver and kidney where it can be metabolized back to glucose by gluconeogenesis (Waterhouse, 1974; Holroyde *et al*,1975).

Lactate dehydrogenase activity in the liver and kidney of CBA mice rose constantly, when tumour volumes exceeded 350 mm^3 and 450 mm^3 respectively (see Figures 4.8, p 78 and 4.11, p 82 respectively), when compared to the activity found in the liver when the tumour burden was between 50 -150 mm^3. This implies that there is an increase in lactate utilization during cancer cachexia. The liver and kidney are not considered hypoxic tissues, and the rise in lactate metabolism can therefore only be ascribed to one of the following reasons: a) the first possibility is that the tissues mentioned above utilise lactate to produce energy, or b) the second reason could be that glucose is produced from lactate by the gluconeogenesis pathway (Waterhouse, 1974; Holroyde *et al*,1975). According to Inculet *et al* (1987), the main substrate for the gluconeogenesis in the F344 rat liver is thought to be lactate.

According to several researchers, there is a profound alteration in lipid metabolism with a marked increase in the mobilization of free fatty acids from the adipose tissues (Theologides ,1979; Eden *et al*,1985; Vorster, 1990). Due to the higher lipid metabolism, glycerol is released in large quantities. This phenomena has been used by many investigators to assess the rate of lipid breakdown (Jeevanandam, 1986; Groundwater *et al*, 1990; Klein and Wolfe, 1990). In the constitutional disorder of cancer cachexia, the alteration in lipolysis of the host is directly linked to the tumour burden (Hollander, 1986). Ekman *et al* (1982) suggests that increased gluconeogenesis in the sarcoma-bearing rats utilize glycerol from the increased lipolysis as main substrate, rather than lactate and alanine.

Two distinct profiles in the activity of glycerol-3-phosphate dehydrogenase were found in the liver and kidney, in this study. This could indicate a difference in the availability of this substrate to the two tissues. In the liver, the enzyme activity only increased when the tumour burden exceeded 550 mm^3, and reached its maximum activity when the tumour volume was between 650 to 750 mm^3, while in the kidney the enzyme activity increased from the beginning and reached its peak activity when the size of the tumour was between 450 to 550 mm^3. Several investigators found that increased lipolysis can occur in the early stages of tumour development (Kralovic, 1977; Theologides, 1979; Ekman *et al*, 1982; Beck and Tisdale,1987), this is consistent with the results found in the glycerol-3-phosphate dehydrogenase activity in the kidney. A possible explanation for the late response of G-3-

PDH in the liver, could be that the tissue is not surrounded by adipose tissue and thus do not receive the high levels of glycerol, due to great utilization elsewhere in the body, the kidney on the other hand is surrounded by adipose tissue and will take up the glycerol from altered lipid metabolism more quickly. Furthermore, the two tissues seem to complement each other on the metabolism of this substrate, with the liver starting to use more glycerol after the kidney reached its peak activity. The decrease in the activity of this enzyme after the tumour burden became larger than 1050 mm^3, could be due to a diminished amount of body and dietary fatty acids.

From the response in G-3-PDH and LDH activities, it is clear that the precursors for the gluconeogenesis, namely lactate and glycerol, were increased. Several investigators have demonstrated that the higher concentration of lactate in the blood contributes to an increase in the activity of the Cori cycle (Reichard *et al*, 1963; Holroyde *et al*,1979). The question remained whether the gluconeogenesis is increased in the tumour-bearing host, and whether the actual mass loss in cancer cachexia can be linked to this process. Zylicz *et al* (1990) proposed that the phenomenon of cancer cachexia has three distinct phases, namely the preclinical or silent phase, the hypermetabolic phase and the hypometabolic phase. In our study, the response of the gluconeogenesis enzyme fructose-1,6-diphosphatase in the liver and kidney, demonstrated three similar phases. There was a decrease in this enzyme activity when the tumour burden reached between 50 to 350 mm^3 in size in both tissues. This was probably due to the increased demand for glucose to both the tumour and other body tissues, which could still be

supplied by the glycogen that was broken down to glucose (Waterhouse *et al*, 1979). Therefore the initial phase, namely the silent or preclinical phase, reflected that there was no need to produce glucose by gluconeogenesis. In the second phase, (that is between 350 to 650 mm^3 for the livers and up to 750 mm^3 in the kidney), the need for gluconeogenesis was increased, probably because the supply of glycogen was depleted due to the tumour in its exponential phase of growth, and therefore the body required alternative sources of glucose (Zylicz, 1990). In the third phase of cancer cachexia, the response of fructose-1,6-diphosphatase showed a decrease in activity that took place in the liver and kidney with the tumour burden larger than 650 and 750 mm^3, respectively. This could be due to the body's energy steady-state becoming disrupted with the high energy expenditure(Warnold *et al*, 1978) of gluconeogenesis, and the lower energy intake present in the host body at this stage of cancer cachexia (Shapot, 1972; Theoglides, 1979; Zylicz, 1990).

Furthermore, the decrease in food intake only occurred after the gluconeogenesis pathway reached its peak activity during the hypermetabolic phase, reflected in the activity of F-1,6-DPase. This can indicate that the anorexia is not the cause of the abnormal steady-state in the body, but rather a symptom of it (Lundholm, 1980; Hollander, 1986). It seems that the body surrenders to the adverse conditions present in the constitutional disease of cancer cachexia. This can be clearly observed in the hypometabolic phase, and was demonstrated in this work at the dietary and metabolic levels. This

progressive mass loss of the mice can be related to increased morbidity and mortality and represents a powerful predictor of the host response which can be used in cancer therapy (Roh *et al*, 1984).

6.2 ATP AS RADIOPROTECTOR

An intricate problem with cancer therapy is the possibility of damaging vital organs in the body beyond the capacity of repair during the process of treatment. In radiotherapy there are some of the criteria which should be taken into account in an effort to eradicate a neoplasm. The first of these to consider, is the differential radiosensitivity which exists between the tumour cells and the surrounding normal cells. The second factor is the relative ability of the surrounding normal and tumour cells to repair themselves, and the third factor is the ability of the host organ to repair itself (Rubin and Poulter,1978).

The radiation dose to be used in the radiotherapy to obtain the best results, is another problem. If the dose to be used is too low, in order to prevent damage to the surrounding normal tissue, the chances of the recurrence of the tumour is highly probable(Field *et al*,1968). On the other hand, if the dose used is a tumour curative dose, unacceptable damage to normal tissue can occur (Szeinfeld and de Villiers, 1991b). Thus, a compromise between the dose which inflict minimal damage to normal tissue, and the dose causing maximum destruction to tumour tissue, will be the optimal therapeutic dose. This condition limits the success of radiotherapy in the case of highly radioresistant tumours.

Therefore, the ultimate goal in radiotherapy is either to protect only the normal tissue and not the tumour tissue, or to sensitize only the tumour tissue in order to eradicate the cancer without harming the normal tissue(Tubiana,1971).

Several researchers have shown ATP and other adenyl nucleotides to have radioprotective properties (Grant *et al*, 1976; Tikhomirova and Yashkin, 1983) in normal tissue (Kaufman *et al*, 1982; Vorozhtsova *et al*, 1987; Pospisil *et al*, 1988; Szeinfeld and de Villiers, 1992), and therefore this study concentrated on the radioprotection action of ATP in normal and tumour tissue.

The protective characteristics of ATP against radiation damage was demonstrated in this study by the mouse survival assay, where ATP afforded an increase in the survival percentage of 45 % of lethally exposed BALB/c mice as shown in Figure 4.14 (see p.85). This correlates well with the results of Tikhomirova *et al* (1984), who showed that the survival of CBA and CS7B1 hybrid F1 mice increased from 63% to 80 % after ATP was administered prior to 9 MeV proton radiation. Nikolov *et al* (1986) also demonstrated that the survival of monkeys improved from 5% to 50 % when exogenous ATP was administered prior to exposure to a dose of 8.3 Gy gamma radiation. Langendorf and Langendorf (1971) also showed that cAMP enhanced the protective effect of ATP from 44% to 70%, although cAMP had no protective properties when administered on its own.

Furthermore, the protective effect of ATP on the tumour tissue was demonstrated in our study by changes in the SGD of the rhabdomyosarcoma tumour for the two doses, 2 Gy and 3.8 Gy, used respectively. This demonstrates some form of differential protection, when macroscopic parameters are taken into consideration.

Thus, to understand the mechanism involved in the radioprotection action of ATP, as seen at the macroscopic level, it is necessary that one takes into consideration the basic physiology of a normal cell. A normal cell is regulated by its internal and external environment as well as the nerve and hormonal activity in the close proximity of the cell(Mc Nally *et al*, 1979). In the case of any abnormality in the cell or in its environment, the cell will respond to repair the steady state, and will also send distress signals to the rest of the body to supply nutrients, or to increase bloodflow. The body usually responds to any insult by producing adrenaline and noradrenaline, to enhance the retaliatory response. Therefore, when looking at the cell's retaliatory response, one has to look at the cell's repair function and hence the metabolic processes which facilitate the repair and energy production.

ATP and other adenosine nucleotides have been suggested by several researchers to have the functional characteristics of a neurotransmitter (Burnstock, 1972; 1976; 1981; Brown *et al*, 1979; Kolb and Wakelam, 1983; Burnstock and Kennedy, 1986; Szeinfeld, 1990), acting on the receptors (Akasu *et al*, 1981; Benham and Tsieu, 1987; Foreman, 1989), causing a

metabolic cascade to enhance glucose metabolism (Szeinfeld *et al*, 1991).

Our studies investigated the hexokinase (HK) and lactate dehydrogenase (LDH) activities in the Emden-Meyerhof pathway as a reflecting measurement of enhanced or decreased glucose metabolism(Tozer *et al*, 1987). It is known that HK is the first enzyme in the glucose metabolism pathway. Thus, its activity reflect the total amount of glucose metabolized by the cell, be it via the Emden Meyerhof pathway, or Pentose phosphate pathway.

Evidence of exogenous ATP's regulatory effects on cells are seen in the enhancement of glucose metabolism, as demonstrated in the response of the HK activity in the BALB/c testis after a lethal dose of neutron radiation (Szeinfeld and de Villiers, 1991b). As can be seen from Figure 4.15 (see p. 87), the exogenous ATP inhibited the drop of the enzyme activity up to 90% at three hours after radiation with a lethal dose of neutron radiation. In the rhabdomyosarcoma tumour the activity of HK was also enhanced after ATP was administered, and demonstrated radioprotection of only 48% in the tumour. The effect of enhanced anaerobic glycolysis, by administration of exogenous ATP was also seen in the LDH activity of both the BALB/c testis, as well as in the rhabdomyosarcoma tumour. In both tissues there was an enhancement of the activity of LDH after administration of exogenous ATP, prior to whole body exposure to a lethal dose of 6 Gy neutron radiation. When ATP was given to the mice before exposure to radiation, it seemed

that most of the glucose metabolism was channelled down the Embden - Meyerhof pathway, as an increase in the activity of HK became evident and a reduction in the activity of G-6-PDH was found, when compared to those mice that were only exposed to radiation. This lead to more pyruvate being formed, and the fact that the LDH activity was higher than in the controls, indicated that the mitochondria of the tissues could not cope entirely with the amount of pyruvate being produced. This imbalance between anaerobic glycolysis and aerobic glycolysis can be explained in one of two ways, namely 1) the anaerobic glycolysis is producing pyruvate at a rate higher than the aerobic metabolism could utilise, 2) or there was still some mitochondrial damage which impaired their function and resulted in an accumulation of pyruvate. This pyruvate was then converted to lactate to maintain the NAD^+/NADH steady-state. In the case of the testis, the explanation for the increase in LDH activity above the controls, is most probably due to the first reason given which may be similar to the lactate production seen in the muscle, when subjected to strenuous exercise. However, in the tumour the second reason is the most probable one to explain the increase of activity of LDH above the control level. This theory is supported by Setälä (1984) who found that not only had the tumour a reduced number of mitochondria, but that their function was also impaired. Finally, the improvement in the response of HK and LDH activities after pretreatment with ATP before exposure to a lethal dose of neutron radiation, indicates an increase in the glucose utilization for energy production and thus a greater potential for repair.

One of the functions of the pentose phosphate shunt is, to produce ribose for the production of DNA and RNA. The activity of glucose-6-phosphate dehydrogenase (G-6-PDH), as the first enzyme of the pentose phosphate shunt, was measured and reflected the process that facilitates DNA repair. The results indicated an increase in the activity of G-6-PDH after exposure to radiation alone, which can be correlated with a possible higher DNA production due to radiation DNA damage. This is in agreement with the work of Savitski *et al* (1985), who reported that the activity of G-6-PDH was enhanced, while the activity of fructose diphosphate aldolase was decreased after an absolute lethal dose of gamma radiation. Furthermore, Szeinfeld (1989) also reported an augmentation in the activity of this enzyme after a high dose of X-irradiation. However, after the mice were pre-treated with ATP before radiation, the enzyme activity in normal tissue returned to almost the same activity found in the testis of mice that received no treatment, and the enzyme activity in the tumour only rose by 29%, indicating less production of ribose for DNA and RNA synthesis than after radiation alone. This can be seen as an indication of less DNA damage.

Furthermore, Benova and Baev (1974;1978) reported that ATP used in combination with AET and serotonin, reduced the genetic damage to spermatogonia (Benova, 1986). Further conformation of ATP's action to prevent damage is seen in the work of Cronstein *et al* (1985), where they found that adenosine interacted with the receptors on neutrophils and decreased the amount of superoxide radicals produced by them after

stimulation, by approximately 40 % (Cronstein *et al*, 1983; 1988). If this applies to all cells after radiation, it will imply that ATP not only enhances the glucoregulatory response, but also suppresses the production of some free radicals and thus lessens the oxygen effect of radiation. In view of the fact that most cell damage after radiation is caused by free radicals (De *et al*, 1983; Dizdaroglu and Bergtold, 1986; Surendra and Rastogi, 1990), the fact that ATP inhibit the production of free radicals will imply less damage to all parts of the cell including the DNA (Workman, 1985; Bump and Brown,1990; Powell and McMillan,1990). This protection against damage to the DNA could be the reason for the decrease in the activity of G-6-PDH after pretreatment with ATP, prior to radiation exposure.

The pathway for production of ATP, DNA and RNA consists of two separate parts namely, the pentose phosphate shunt and the *de novo* biosynthesis pathway for adenine nucleotides. The first mechanism discussed for the G-6-PDH activity correlates well with the response seen in acid phosphatase (AP) activity, which is an indicator of damage, after doses of 3.8 Gy and 6 Gy neutron radiation. It is seen in Figures 4.21 (p 93), 4.22 (p 94), 4.24 (p 96) and 4.25 (p 96), that the activity of this enzyme was less after prior administration of ATP before radiation treatment in all tissues, and at all doses tested. In the testis, the activity of AP one hour after irradiation was almost the same as that found in the testis of untreated mice, showing that the initial damage in both 3.8 Gy and 6 Gy were either prevented or repaired. In Figure 4.22 (see p 94) it is shown that not only is the initial

damage to the testis reduced, but the long term damage as well. In the tumour however, there was still much initial damage even though it was considerably reduced at all doses. This demonstrates that ATP reduces the initial damage caused by radiation, and also modifies the late damage in the cell (Figure 4.22, see p 94). The radiation dose effect (Figure 4.23, see p 95), demonstrates the greatest difference between the different doses during the first 24 hours after radiation. Thus, by reducing the acute injury to the cell, the survival of the cells, tissues, organs, and animals were improved. Finally, the difference in the response of AP after pre-treatment with ATP prior to radiation between tumours and testis, can be seen as a differential radioprotection afforded by ATP.

In the light of the evidence that exists in the literature as well as results obtained in this laboratory, the possibility of at least 2 major actions for the intervention of ATP in the process of radiation damage are demonstrated. The first is the action of ATP on the purinergic receptors enhancing the glucoregulatory processes shown by other researchers, as well as by our laboratory (Lee and Filkins, 1987; Szeinfeld *et al*, 1990) , secondly the adenyl nucleotides possibly acts on the A_2 receptors reducing the formation of some free radicals (Cronstein *et al,* 1983;1985;1988), and thus damage. Finally, the results of this study show that ATP afford higher protection to the testis than to the tumour, and thus can be used to enhance the differential effect of radiation in therapy. Further work is warranted to ascertain the dose of exogenous ATP which will enhance the differential protective effect between the tumour and normal tissue.

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