METABOLIC RESPONSES TO *IN VITRO* ZINC SUPPLEMENTATION.

By

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ABSTRACT:

The present study was carried out to determine the effects and possible mechanism of action of zinc supplementation on the *in vitro* growth of malignant murine melanoma (B16) and non-malignant monkey kidney (LLCMK) cells.

Cell culture studies showed that zinc supplementation significantly inhibited B16 growth at all the concentrations studied (1, 3, 5 and 10μg/ml). Zinc was also found to inhibit the growth of the LLCMK cells, although to a lesser extent than the B16 cells.

Possible evidence of mobilisation of the essential fatty acids from the membrane phospholipid stores was noted in both cell types. This effect was, however, greater in the B16 cells. Δ^6-desaturase activity was found to be significantly lower in the B16 cells than in the LLCMK cells ($p \leq 0.05$). Zinc supplementation resulted in an increase in the enzymes activity in the LLCMK cells and, at high concentrations, in the B16 cells. An estimation of elongase and Δ^5-desaturase activity with zinc supplementation indicated that zinc had little or no effect on the activity of these enzymes.

B16 cells were found to have higher levels of free radicals than the LLCMK cells. Zinc supplementation resulted in increased free radical formation in the B16 cells, while no effect was observed in the LLCMK cells. Lipid peroxidation increased in both cell types with increased zinc concentrations. The observed effect of zinc supplementation on cell growth may involve these elevated levels of lipid peroxides.

Cyclo-oxygenase activity was found to be greater in the B16 cells than the LLCMK cells. The activity of the enzyme increased with higher concentrations of zinc (10μg/ml) in both cell types. Prostaglandin E_2 levels were found to be lower in the B16 cells compared to the LLCMK cells. The

levels of prostaglandin E_2 in both cell types appeared to be dependent on the levels of the polyunsaturated fatty acid precursors to the prostaglandins.

Zinc was found to inhibit the activity of the enzyme adenylate cyclase in both cell types. The cAMP levels in the LLCMK cells were also found to decrease with zinc supplementation. In the case of the B16 cells, cAMP levels increased at low concentrations of zinc despite a decrease in adenylate cyclase activity, suggesting a possible inhibition of cAMP phosphodiesterase activity at these concentrations of zinc.

It is concluded that although zinc supplementation does have an effect on cell growth, this effect is not mediated through the activation of adenylate cyclase by the prostaglandins resulting in elevated levels of cAMP. A possible mechanism involving lipid peroxidation is proposed.

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LIST OF ABBREVIATIONS:

AA - Arachidonic acid

5´-AMP - Adenosine-5´-monophosphate

ANOVA - Analysis of variance

ATP - Adenosine-5´-triphosphate

B16 - Murine melanoma cell line

BHT - Butylated hydroxytoluene

BSA - Bovine serum albumin

cAMP - Adenosine-3´,5´-cyclic monophosphate

cGMP - Guanosine-3´,5´-cyclic monophosphate

cpm - Counts per minute

DGLA - Dihomo-gamma-linolenic acid

DNA - Deoxyribonucleic acid

EFA - Essential fatty acid

GLA - Gamma-linolenic acid

GPx - Glutathione peroxidase

GR - Glutathione reductase

GSH - Glutathione

HPETE - Hydroperoxy fatty acid

LA - Linoleic acid

LLCMK - Non-malignant monkey kidney cell line

MDA - Malondialdehyde

NADH - Nicotinamide adenine dinucleotide (reduced)

NADPH - Nicotinamide adenine dinucleotide phosphate (reduced)

NBT - Nitroblue tetrazolium

PBS - Phosphate buffered saline

PDE - Phosphodiesterase

PG - Prostaglandin

PUFA - Polyunsaturated fatty acid

RNA - Ribonucleic acid

SEM - Standard error of the mean

SOD - Superoxide dismutase

TBA - Thiobarbituric acid

TCA - Trichloroacetic acid

TLC - Thin layer chromatography

CHAPTER 1: GENERAL INTRODUCTION.

Cancer can be defined as a cellular malignancy whose loss of normal controls results in unregulated growth, lack of differentiation, and ability to invade local tissues and metastasise (1).

With improved medical knowledge and treatment of diseases such as scarlet fever, diphtheria, tuberculosis, typhoid fever and dysentery, life expectancy has been increasing since the middle of the 19th century. However, cancer remains a major cause of death, accounting for up to 20% of all deaths, particularly in the middle-aged and older population. Cancer can develop in tissues of any organ at any age (2,3).

Two types of tumours have been identified. Cells growing at an abnormal rate, forming a tumour which cannot invade the surrounding tissues and remains localised in the tissue of origin is called a benign tumour. Tumours with cells which are able to leave the site of origin and reach the blood stream or lymphatic system and so spread to secondary sites are termed malignant tumours. This spread of tumour cells and their ability to establish secondary areas of growth is known as metastasis. It is these malignant tumours which are often lethal to the patient if they are not arrested early in their development (4).

Malignant tumours are divided into three broad groups, namely: the carcinomas which arise in the epithelia; the sarcomas which occur in fibrous tissue and blood vessels and the leukemias and lymphomas which arise in the lymphocytes and in the lymph nodes (2,4).

A unifying factor in cancer is the mutation of DNA. The DNA in normal cells may be transformed, either by an alteration or substitution of one or more of the purine or pyrimidine bases. No single cause for these mutations in the DNA of certain cells can be given as numerous factors have been

identified as promoters of cancer. These include; genetic susceptibility, age, geographic distribution, viruses, environmental factors and immunological disorders (2,5-11).

Cancer cells can readily be distinguished from normal cells. They have a high nucleus-to-cytoplasm ratio, prominent nucleoli, many mitoses, and little specialised structure. The restriction of a normal cell type to a given organ or tissue is maintained by cell-to-cell recognition and by physical barriers. In the case of cancer cells, the cells have the ability to release the enzyme collagenase IV, which degrades collagen IV, a dominant component of basal membranes, thus breaking down these physical barriers (2).

Metastasis is an active process. In order to supply the rapidly dividing mass of cells with oxygen and nutrients, tumours induce a supply of blood vessels to the tumour (a process known as angiogenesis). These newly formed blood vessels tend to be "leaky" and malignant cells can cross the walls with ease (12).

Although lymphatic vessels are also ports of entry for malignant cells, tumours do not induce their own lymphatic network. Because there is no means of draining the excess fluid away from between the cells of the tumour, hydrostatic pressure builds up in the tumour and may aid in the release of individual cells from the cellular mass. The ability of cells to leave a primary tumour has been shown by Fidler and Poste (cited in 13) not to be a random event which can be accomplished by all of the tumour cells. There are certain cells which have a greater capacity to metastasise than others. Once tumour cells have successfully crossed the basement membrane and entered the vascular or lymphatic system, they are carried in the circulation until they reach a secondary site and lodge in a capillary bed or lymph node, either by obstructing a narrow vessel or by adhering to the vascular endothelium.

The majority of the cells entering the capillaries or lymph nodes are destroyed by physical-mechanical and host defense mechanisms, however, a few of the surviving cells begin to invade the vessel wall and eventually leave the circulation. Only those tumour cells which begin to proliferate will initiate a secondary tumour. Proliferation can be stimulated by local growth factors, hormones produced by the host organ and autostimulating growth factors produced by the tumour cells themselves (14). A new metastatic growth will only expand if angiogenesis occurs, ensuring the new cells of nutrients. The secondary tumour will therefore have its own vascular supply and can in this way also become metastatic (15).

The treatment of cancer has taken on many different approaches. The most common of these being surgical excision of the tumour, provided it has been detected early enough and is localised. Localised tumours may also be treated using radiation therapy. In the case of tumours which have already metastasised, chemotherapy is the only successful treatment. This form of treatment aims at the destruction of the tumour and exploits morphological, metabolic and immunological differences between normal and malignant cells to destroy the cancer cells. However, selective toxicity towards cancer cells has not been achieved, resulting in a variety of harmful side effects to the patient. Other problems which exist are that the cancer may initially respond well to drug treatment but then develop some resistance to it; alternatively, the drug may fail to penetrate the tumour either due to an inadequate blood supply or the cell membrane becoming impermeable to the drug (16-19).

In order to overcome this non-specificity of these drugs, researchers are continuing to search for agents which are selectively toxic to tumour cells. At present, much attention is being focused on immunotoxins. The principle behind immunotoxins is that a toxic agent is linked to a monoclonal antibody which will bind to a particular tumour antigen. The linked toxic agent will destroy the targeted cancer cells but leave normal cells unharmed. In practice, there are still problems with this method of treatment and there is still much research to be done before immunotoxins become standard

therapeutic agents (10,18).

Horrobin (20) proposed that normalizing cancer cells by techniques which are harmless to normal tissues is a more rational aim. The treatment of cancer by means of nutritional compounds has been suggested. These are natural agents, occurring in the average diet, which are not toxic to normal cells even in high doses but which interfere with the proliferation of cells. These compounds do not have the ability to destroy the tumour but may prevent the tumour from metastasising. Nutrients which are capable of preventing metastasis may be of extreme use in the treatment of cancer together with one of the other means of cancer therapy.

It is estimated that over one-third of all cancers result from bad eating habits. There is strong evidence that some food types increase the risks of specific cancers while others lower them. However, the mechanisms involved are still unknown (21). Extensive research has been carried out on a number of selected vitamins and minerals, including vitamins A, C and E and the minerals; calcium, selenium and zinc (22,23). It is the mineral zinc and its effect on malignant cells which forms the basis of this dissertation.

The necessity of zinc for the growth of *Aspergillus niger* was determined by Raulin as early as 1869 (cited in 24). Since then its importance in animal nutrition has been identified.

In mammals and man, sixteen trace metals, of which zinc is one, have been shown to be either essential or beneficial in their nutrition. A trace element is defined as one that makes up less than 0.01% of the body's mass and therefore occurs in living tissues in microgram or less/kg or litre concentrations (25). An element is considered essential when a dietary deficiency results in death or a severe malfunction of the organism and when restoration of physiological levels of that element relieves or prevents such an impairment. The organism can neither grow nor complete its life cycle

without the element in question and its effect cannot be totally replaced by any other element (26).

Large pools of zinc exist in some tissues of the body *viz.* muscle, bone and liver, but these stores are not readily mobilised. Even during dietary zinc deficiency, the concentration of zinc in these tissues show very little change (27).

Rats which are zinc-deficient have shown side effects such as; growth retardation, anorexia (loss of weight), alopecia (loss of hair), hypogonadism (underdeveloped genitals), skin changes (scaly skin and lesions) and mental lethargy (28-33). These effects have been observed in humans suffering from a nutritional deficiency of zinc in countries such as Egypt, Iran and Gambia. The diets of these nations were found to be high in phytate and clay which reduces availability of the mineral for absorbtion by the intestine (29,34). In addition to the above mentioned effects, hepatosplenomegaly (enlarged spleen and liver) and iron-deficiency anaemia were also observed (25,29). These manifestations of zinc deficiency are easily reversed by oral zinc supplementation (35,36).

Dietary zinc deficiency has been shown to have serious implications in prenatal animals as well. Zinc-deficient diets fed to rats throughout the period of pregnancy results in up to half the fertilised embryo's dying during pregnancy. Those which survive to term have lower body weights than normal full-term fetuses. In addition, 90-100% show signs of gross malformations *eg.* brain abnormalities, small or missing eyes, small or missing lower jaw, fused digits and short or absent tails. Heart, lung and urogenital abnormalities have also been observed. These manifestations are irreversible (27,32,37,38).

Many of the pathological signs of zinc-deficiency have been ascribed to changes in the activity of various zinc metalloenzymes. Over 100 enzymes have been identified as requiring zinc for their catalytic activity. Zinc is found to provide the necessary structural integrity to the enzyme.

Alternatively zinc may participate directly in catalysis (39,40). Examples of zinc metalloenzymes are found in all 6 enzyme classes and include alkaline phosphatase, carbonic anhydrase, nucleotidyl transferases, DNA and RNA polymerase and dehydrogenases (41-43). Evidence also indicates that zinc exerts essential physiological roles which are not related to any of the metalloenzymes *eg.* maintaining the structural integrity of membranes (33).

Zinc deficiency as well as supplementation have both been shown to have an effect on the *in vivo* and the *in vitro* growth of various tumours (44-46).

Specific enzymes related to DNA synthesis have been implicated with the observed effect on cellular growth (37,47). Thymidine kinase is an enzyme which is essential for the synthesis of DNA in rapidly growing tissue. Duncan *et al.* (48) suggest that a reduction in thymidine kinase activity may be responsible for producing the depression of DNA synthesis and the consequent depression in protein synthesis. DNA polymerase, another key enzyme involved in DNA synthesis, has also been shown to have lower activity in both zinc-deficient and zinc supplemented rat embryo's (44,48).

Another mechanism whereby cell proliferation may be affected by levels of zinc is in the metabolism of the ω6-fatty acids (49,50). Deficient levels of zinc have been found to result in reduced Δ^6-desaturase activity, the rate-limiting step in the conversion of linoleic acid to the prostaglandins (50-52). Reduced Δ^5-desaturase activity has also been observed in zinc-deficient rats, while zinc supplementation has resulted in greater activities of both enzymes when compared to controls (52).

A product of the ω6-fatty acid synthesis pathway, the prostaglandins, particularly PGE_1, have been shown to result in the accumulation of cAMP in cells (53,54) which in turn has been suggested to have a regulatory role in cell proliferation (55).

The purpose of the work presented in this dissertation was to attempt to further elucidate the means by which a zinc supplementation may result in the regulation of cell proliferation. The mechanism concentrated on was the regulation of cell growth by cAMP via the ω6-fatty acid metabolic pathway as outlined in Figure 1.1.

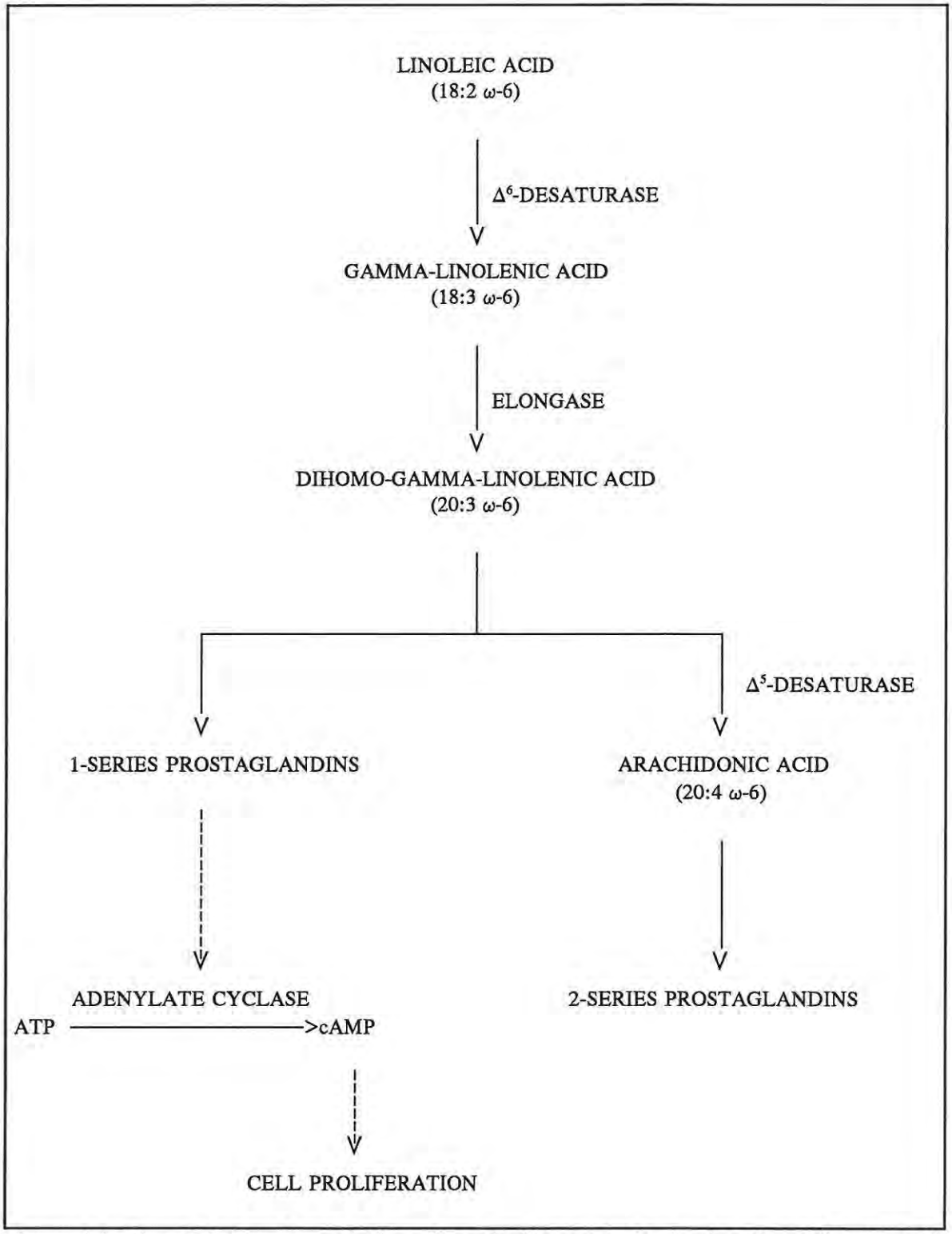

Figure 1.1: The ω6-fatty acid metabolic pathway leading to a reduction in cell proliferation with zinc supplementation.

————> Metabolic pathway

- - - - - -> Suggested pathway

CHAPTER 2: CELL GROWTH.

2.1 INTRODUCTION:

Cells pass through four phases during cell division. The first of these phases is the G_1 phase, during which cells prepare for DNA replication. This is followed by the S phase, in which DNA is replicated. The G_2 phase involves preparing the cells for mitosis and cytokinesis. The final phase is when the cells undergo mitosis. The length of these periods depends on the cell type and conditions of growth. Mitosis is usually the shortest phase (56,57).

In tissue culture systems, normal cell lines stop growing or grow at a greatly reduced rate once the cells have reached confluency. This is a process known as "contact inhibition". Malignant or transformed cells continue growing and dividing even after confluency is reached and so appear to have lost their ability for "contact inhibition". Intracellular levels of cAMP are thought to be involved with this regulation (58).

Most nutrients, including the trace element zinc, are required for the maintenance of cell growth and proliferation. In cells undergoing rapid proliferation, deficiencies of essential micronutrients may limit growth. Inhibition and failure of tumours to grow in zinc-deficient hosts has been shown in various murine models (59-61). In contrast to these reports, tumour growth inhibition in animals receiving exogenous zinc supplements has also been found (46,62-65).

When considering the undesirable pathological consequences of zinc-deficiency (discussed in Chapter 1), zinc supplementation would be a more favourable means of controlling malignant growth as it appears to be non-toxic, even at very high concentrations (44,63).

Data obtained from *in vivo* studies are prone to variations due to the uneven tissue distribution of the zinc and the probability that the zinc is not reaching the necessary cytotoxic levels in the tissue of interest (66,67). To overcome these problems, experiments using *in vitro* cultures can be performed. Although *in vivo* studies are regarded as representing the actual effect of a substance on metabolism, *in vitro* studies may help to form the basis for further investigations *in vivo*, alternatively they may confirm the results already obtained.

In the following study, the effect of zinc supplementation on the *in vitro* growth of normal (LLCMK) and malignant (B16) cells was investigated.

2.2 MATERIALS AND METHOD:

2.2.1 Culture media:

Basal medium was prepared by dissolving 12g of Eagles Basal medium (with Hanks salts) (Highveld Biological (Pty) Ltd., RSA) in 10*l* of Milli-Q water. Ascorbic acid (0.05g), serine (0.10g), glycine (0.06g) and $NaHCO_3$ (7.50g) were added. Fifty millilitres of a sodium benzylpenicillin and streptomycin sulphate (1×10^7 IU/*l* and 1×10^7 μg/*l* respectively) antibiotic solution was added (Nova Industries (Pharm.) (Pty) Ltd., RSA). The final solution was sterilised by passing it through a sterile filtration unit (Millipore corp., (USA). This was then incubated at 37°C, to test for sterility, until further use.

2.2.2 Trypsin solution:

NaCl (8.0g), KCl (0.4g), D-Glucose (1.0g), $NaHCO_3$ (0.58g), EDTA (0.2g), Phenol red indicator

(0.02g) and Trypsin (0.033g) were dissolved in 1*l* of Milli-Q water. A 10ml aliquot of sodium benzylpenicillin and streptomycin sulphate (1×10^7 IU/*l* and 1×10^7 μg/*l* respectively) antibiotic solution was added. This solution was stored at -20°C until required.

2.2.3 Cell lines:

B16F10BL6 murine melanoma cells (hereafter referred to as B16 cells) were used as a representative cell line of malignant cell growth. Untransformed cell growth was demonstrated using monkey kidney cells (LLCMK). Both cell lines were obtained from Highveld Biological (Pty) Ltd., RSA.

2.2.4 Cell Culture Procedure:

Sterile techniques employed include: working on a lamina flow bench, swabbing the hands and surface of the bench regularly with 95% alcohol, autoclaving all equipment before use and sterile-filtering all solutions used.

Experimental growth medium was prepared by adding 10% foetal calf serum to 100ml bottles containing basal medium. Zinc-supplemented media was prepared by adding zinc, at final concentrations of 1, 3, 5 and 10 μg/ml, to each of the prepared growth medium bottles. The zinc used was an atomic absorption spectroscopy standard solution (1000 μg/ml) (SMM Chemicals (Pty) Ltd., RSA).

The zinc in the standard was solubilised in perchloric acid. For this reason a control was used where the growth medium contained 1ml concentrated perchloric acid (the equivalent amount of acid in the highest zinc treatment), in order to ensure that any effect on the cell growth was due to the zinc and

not the acid. Another control, containing only foetal calf serum, was also prepared.

The pH of all the experimental media was adjusted to between 7.2 and 7.4 (physiological pH) using 1M NaOH.

Flasks of cells which were 75% or more confluent were used in the experiments. The medium was decanted from the flasks and 10ml of trypsin solution was added to the flasks to free the cells. These flasks were incubated for approximately 10 minutes at 37°C, after which the cells lift off the surface. These were transferred to sterile tubes with caps, and centrifuged at 1000g, at 4°C, for 8 minutes using a refrigerated bench-top centrifuge (Hettich Universal K2S). The trypsin was then decanted and the cell pellets resuspended in 1ml growth medium.

The number of cells present in each culture was determined by enumeration using a haemocytometer (Neubauer, W. Germany). A drop of the cell suspension was transferred into the well of the haemocytometer and a cover slip was placed in position. Each culture was counted in duplicate, and the total number of cells present in the culture was calculated.

For each experiment, thirty flasks ($25cm^2$) (each treatment was replicated five times) were then seeded with an aliquot of the cell suspension, equivalent of 3×10^5 cells and 15ml of appropriately supplemented growth media was added to each flask. These flasks were then incubated at 37°C, for a period of 5 days, after which fresh growth media was prepared and the medium in each flask was replaced.

Once the first flask had reached confluency (about 7 days), the cells were harvested (as above) and the cell pellets were resuspended in 1ml of phosphate buffer. The approximate number of cells in each flask was then determined by enumeration, using a haemocytometer as previously described.

2.2.5 Statistical analysis:

The results obtained were analyzed using a one-way ANOVA followed by the Student-Newman Keuls Multiple Range Test.

2.3 RESULTS:

The growth of the control cultures, in which the medium was supplemented with 1ml perchloric acid were found not to differ significantly from those controls which did not receive perchloric acid.

The results obtained are presented in Figure 2.1.

The cell proliferation of both the LLCMK cells and the B16 cells was found to decrease with an increase in the amount of zinc supplemented, when compared to the control cultures. This reduction in cell proliferation was significant ($p \leq 0.001$) for all the zinc concentrations of the B16 cells. In the case of the LLCMK cells, significant reductions were only obtained at zinc concentrations of 5 and 10μg/ml ($p \leq 0.01$ and $p \leq 0.001$, respectively).

It was also noted that there was a loss in the number of cells seeded in the respective zinc supplemented B16 cultures. A loss in the nett number of cells seeded in the case of the LLCMK cultures was only noted at a zinc concentration of 10μg/ml.

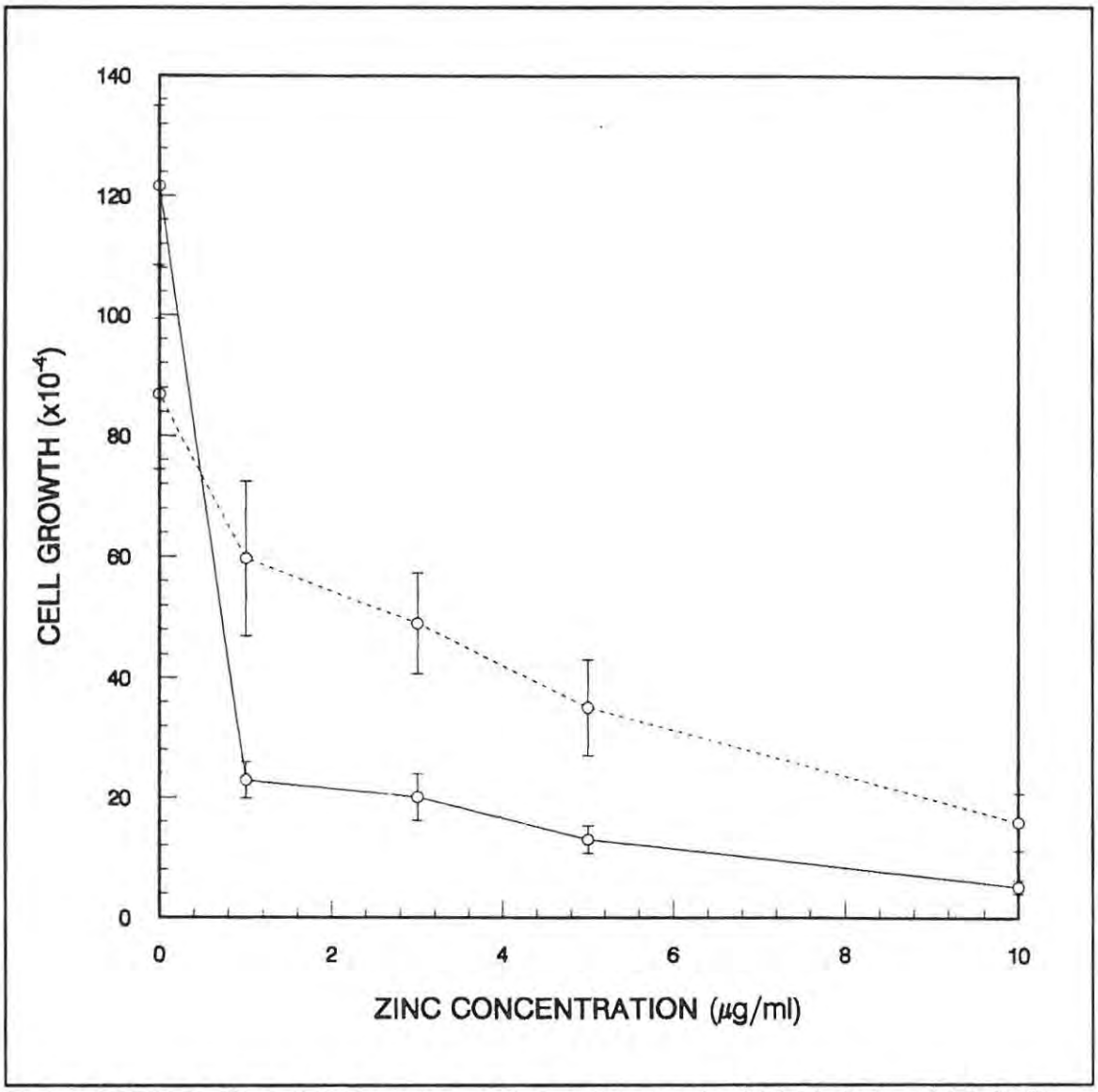

Figure 2.1: The effect of zinc supplementation on the growth of B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of 25 determinations ± SEM.

2.4 DISCUSSION:

Since perchloric acid supplementation had no effect on the growth of either the LLCMK cells or B16 cells, it can be concluded that any effect observed on the growth of the zinc supplemented cells should be directly due to the zinc itself.

The cytotoxic effect of increasing concentrations of zinc on the B16 murine melanoma cells is in agreement with similar findings in this and other laboratories (68-70).

Similar observations have also been made with regard to other malignant cell lines, both *in vivo* and *in vitro eg.* S-91 Cloudman murine melanoma cells, P51 melanoma cells and L1210 leukemia cells (44,64,71,72).

Although a reduction in cell growth was also observed for all the zinc supplemented non-malignant monkey kidney cells (LLCMK), this effect was only significant at high levels of zinc. It appears that zinc is only cytotoxic at a concentration of 10μg/ml. Results indicating a non-significant reduction in LLCMK cell growth supplemented with zinc have also been obtained by other researchers (69,70).

It can therefore be demonstrated that the supplementation of malignant B16 and normal LLCMK cells with increasing concentrations of zinc, results in a dose-dependent reduction in cell growth. Zinc has a cytotoxic effect at all concentrations in the B16 cells while being toxic only at a concentration of 10μg/ml in the case of the LLCMK cells.

The treatment of cancer requires an agent which is toxic to the tumour cells while having little or no effect on normal cells. The selective cytotoxicity of zinc, at low concentrations towards malignant B16 cells may, therefore, be indicative of its potential value in the treatment of cancer.

Further investigations in this study were designed to determine the possible biochemical mechanism of action of zinc responsible for the reduction in the growth of the malignant B16 cells.

CHAPTER 3: THE ESSENTIAL FATTY ACID COMPOSITION AND ACTIVITY OF THE Δ^6-DESATURASE, ELONGASE AND Δ^5-DESATURASE ENZYMES.

3.1 INTRODUCTION:

Biological membranes are made up of a lipid bilayer with a central core of fatty acyl chains. This hydrophobic core acts as a barrier, preventing the unregulated movement of ions and metabolic products across the membrane. Some proteins, including enzymes, receptors and transporters, diffuse into the membrane and interact with the fatty acyl chain (71,72).

The fatty acyl chains making up the lipid bilayer usually have between 16 and 22 carbon atoms. Thirty-five to forty percent are saturated with the remainder containing between 1 and 6 double bonds. A change in the composition of the fatty acyl chains can modify the structure of the lipid core and thus has the potential to affect its normal barrier function, as well as the responsiveness of the integral proteins with which the core interacts. Certain properties of the cell may change as a result of this (71,73,74).

Tumour cells obtain a large amount of fatty acids preformed from the host. This is supplied primarily from the circulating free fatty acids. After minor structural changes, these fatty acids are incorporated into all of the complex lipids formed by the neoplastic cells. If the composition of the circulating fatty acid mixture can be changed sufficiently, the phospholipids formed will have a different fatty acid composition and possibly different physical and functional properties. It is possible that the characteristics of the tumour cells may change sufficiently to cause an alteration in its growth pattern or increase its sensitivity to certain forms of therapy (71).

Most attempts to manipulate the fatty acid composition of cells have centred on the polyunsaturated fatty acids (PUFA's). The PUFA's present in animals cannot be synthesised *de novo* and are therefore derived from dietary lipids.

There are two main classes of PUFA's; the ω-6 or plant polyunsaturate class and the ω-3 or fish oil class. The PUFA's of the ω-6 class are referred to as essential fatty acids (EFA's) due to the serious deleterious effects which result from their deficiency (71,75). These deficiency symptoms include reduced growth, reproductive failure, acanthosis and hyperkeratosis of the skin. Other symptoms which have been observed in rats are: inflamed hind feet, dandruff, alopecia, skin lesions, increased water intake and susceptibility to bacterial infections (75-78).

The metabolism of EFA's in animals is outlined in Figure 3.1. The enzyme, Δ^6-desaturase introduces a double bond between the carbon atoms 6 and 7 of linoleic acid (LA) (18:2 ω-6) to form gamma-linolenic acid (GLA) (18:3 ω-6). GLA is in turn elongated by the addition of a 2-carbon unit to form dihomo-gamma-linolenic acid (DGLA) (20:3 ω-6), which gives rise to the 1-series prostaglandins. Desaturation of DGLA to arachidonic acid (AA) (20:4 ω-6) is catalysed by the enzyme Δ^5-desaturase. AA is released from esterified stores of phospholipids by the action of calcium-requiring phospholipases. It is then converted to the 2-series prostaglandins and thromboxanes by cyclo-oxygenase to leukotrienes and lipoxins by lipoxygenase and epoxyeicosatrienoic acid by cytochrome P_{450}. The rate limiting step in the conversion of LA to AA is Δ^6-desaturase (75,79-83).

The desaturases are membrane-bound enzymes and are considered to be a mixed function oxidase. NADH and NADPH donate electrons which are then transported to the enzyme by cytochrome b_5 reductase and cytochrome b_5. Two hydrogens from the reduced cofactors and 2 hydrogens from the fatty acids are combined to oxygen to form 2 molecules of water (79,84).

Some tumour cells have been shown to lack or have reduced Δ^6-desaturase activity. Stores of GLA and DGLA in most cells are strictly limited and very little GLA or DGLA is transported by the plasma. Cells which have lost their Δ^6-desaturase activity therefore lose their ability to synthesise the 1-series prostaglandins from DGLA (51). Supplementation of GLA to the cells can by-pass the block in Δ^6-desaturase activity and may selectively cause an alteration in the growth pattern and may inhibit the growth of cancer cell lines without affecting normal cells. Suppression of cell proliferation has been observed in some cancer cells, supplemented with GLA and has been ascribed to the normalisation of the intracellular levels of PGE_1 in the malignant cells (84-86).

A number of similarities have been noted between the symptoms of EFA deficiency and a deficiency in zinc. It has therefore been suggested that there may be a metabolic relationship between them. A positive correlation between plasma levels of zinc and AA has been noted while no significant correlation with LA has been observed. This suggests that zinc may play a role in Δ^6-desaturation of dietary LA (78,87). It has also been suggested that zinc is required for the activity of Δ^5-desaturase (31,51).

In addition to its proposed requirement for the activity of Δ^6- and Δ^5-desaturase, zinc has been shown to be necessary for the mobilisation of GLA and DGLA from the cell membrane (51,88).

Mobilisation of these fatty acids from the membrane could result in changes in the membrane fatty acid composition which may influence a number of cellular physiological activities. Membrane fluidity, macromolecule mobility, receptor availability, enzyme activity, prostaglandin and cyclic nucleotide (cAMP and cGMP) biosynthesis and degradation and amino acid and carbohydrate transport could be altered (89).

In order to ascertain what effect zinc has on the mobilisation of EFA's from the membrane of normal (LLCMK) and malignant (B16) cells, the EFA composition of the membrane and stroma fractions of the respective cells was determined. In addition, the effect of zinc on the activity of the enzyme Δ^6-desaturase was determined, as well as a possible effect on the activities of the elongase and Δ^5-desaturase enzymes.

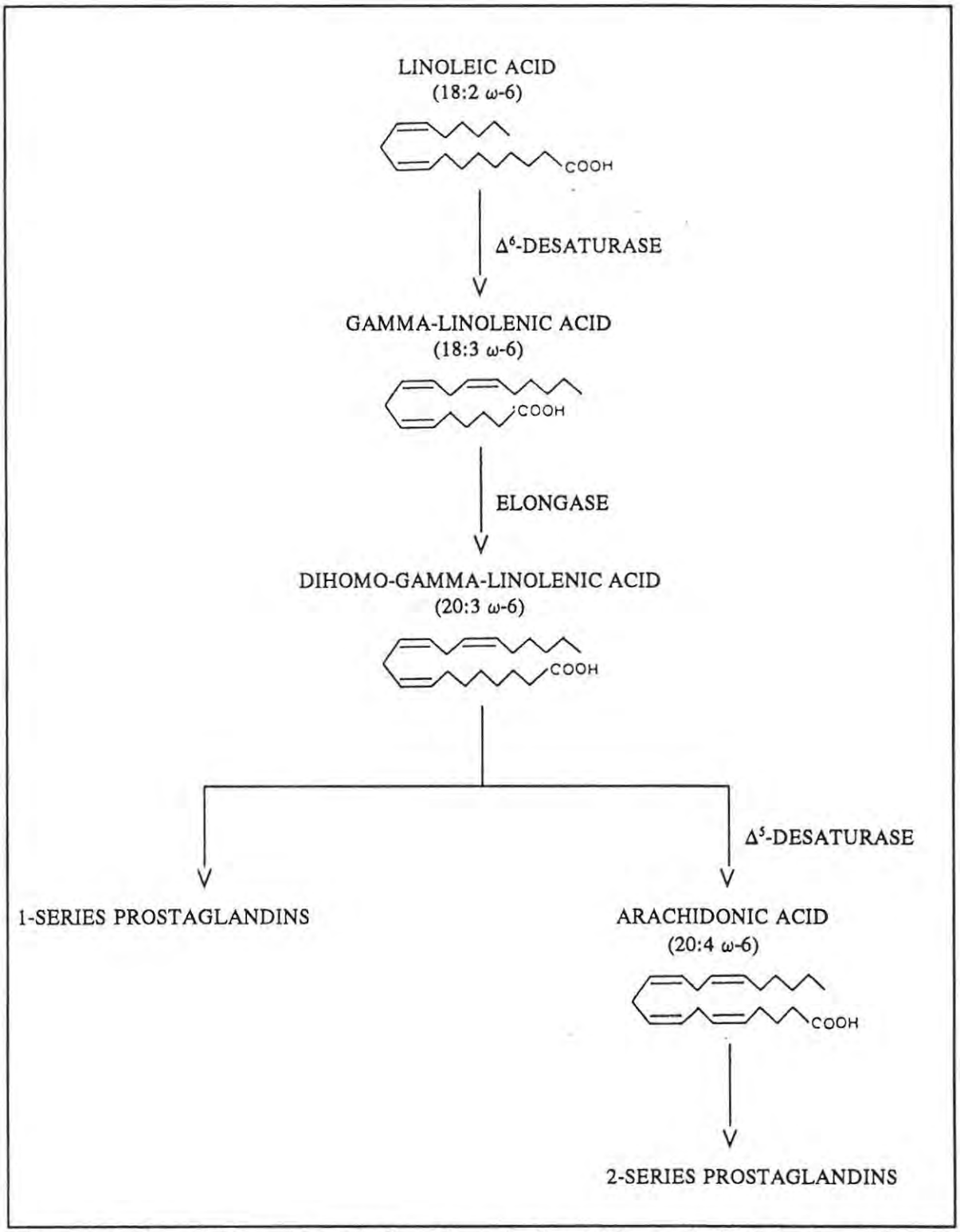

Figure 3.1: The ω6-fatty acid metabolic pathway leading to the synthesis of the 1- and 2-series prostaglandins.

3.2 MATERIALS AND METHOD:

3.2.1 Cell Culture Procedure:

The procedure followed for the preparation of media and culturing of cells for experimental work was as described in Chapter 2. After harvesting the cells, by trypsinisation, the cells were pelleted in a refrigerated bench-top centrifuge (Hettich Universal K2S) at 1000g.

3.2.2 Essential Fatty Acid Composition:

The cellular pellets were resuspended in 1ml of PBS and enumerated using a haemocytometer (Neubauer, W. Germany).

The resuspended cells were transferred to a Dounce homogeniser and 2ml of methanolic KOH was added. The cells were homogenised 25 times using the tight plunger. The suspensions were then transferred to thick-walled centrifuge tubes and the cells separated into their stroma and membrane fractions by differential centrifugation using a Beckman J2-21 centrifuge. The homogenates were centrifuged at 4000g for 20 minutes to remove cellular debris. The supernatant was retained and centrifuged at 20 000g for 30 minutes in order to obtain the respective membrane (pellet) and stroma (supernatant) fractions of the homogenised cells (90).

The lipids in each fraction were saponified by heating at 85°C with reflux, under nitrogen, for 45 minutes. The lipids were then esterified to free fatty acids by adding 1ml of 7N HCl.

Before each extraction the flask was placed on a vortex mixer for 2 minutes to ensure thorough extraction. The fatty acids were extracted twice with 3ml of distilled petroleum ether. The petroleum

ether extracts were pooled and evaporated to dryness at 60°C, under nitrogen.

Boron trifluoride-methanol-complex reagent (Fluka, Switzerland) (0.3ml) was added to the residual fatty acids which were methylated by heating at 100°C with reflux, under nitrogen, for 5 minutes. The fatty acid esters were extracted twice with 1ml distilled petroleum ether (vortex mixing for 2 minutes before each extraction). The pooled extracts were evaporated to dryness at 60°C, under nitrogen. The samples were then stored under nitrogen at -20°C until assayed.

Each sample was reconstituted to 20μl with distilled petroleum ether and 1μl of this was injected into a gas-liquid chromatograph (Hewlett-Packard 5890A) for separation of the fatty acids. The column used was a SP2330 fused silica capillary column. The program used in running the samples is shown in Appendix 1.

The concentrations of the essential fatty acids, namely; linoleic acid (LA), gamma-linolenic acid (GLA), dihomo-gamma-linolenic acid (DGLA) and arachidonic acid (AA), were determined as percentages of the total free fatty acid content in the sample.

3.2.3 Enzyme Activity Determination:

[1-^{14}C]LA was added to each of the experimental cultures 18 hours prior to harvesting. The experimental cultures were then harvested, enumerated and homogenised.

After separation of the cells into their respective stroma and membrane fractions (as described above), the membrane fractions were assayed for protein concentration using the Folin - Lowry assay (91).

A 100μl aliquot of the membrane fractions was made up to 1ml with H_2O. Six millilitres of an

alkaline copper reagent, containing; 1ml of 1% $CuSO_4 \cdot 5H_2O$ solution, 1ml of 2% sodium tartrate solution and 98ml of 2% $NaCO_3$ in 0.1N NaOH was added to each of the tubes. These were incubated at room temperature for 10 minutes before adding and mixing in 0.3ml Folin and Ciocalteu's Reagent (SAARCHEM (Pty) Ltd., RSA). The tubes were then left for a further 30 minutes for the colour to develop whereafter their absorbance was read at 500nm using a Milton Roy Spectronic 301 spectrophotometer. A standard curve was produced using bovine serum albumin (Boehringer Mannheim, Germany) (Appendix 2).

After the protein determination, the fractions were saponified and esterified (as described above). After extraction with distilled petroleum ether, the samples were evaporated to dryness and stored under nitrogen at -20°C until assayed.

A $AgNO_3$ solution was prepared by dissolving 10g $AgNO_3$ in 20ml of water. Eighty millilitres of ethanol (95%) was then added to this solution. $AgNO_3$ is photosensitive and therefore the following methodology was carried out in a darkroom. Precoated thin layer chromatography (TLC) plates (silica gel 60F254) (Merck, W. Germany) were prepared for argentation TLC by immersing them in the $AgNO_3$ solution (for approximately 10 seconds) followed by air-drying. These were further dried for 1 hour in an oven at 80°C.

The extracted experimental fractions were reconstituted to 25μl with petroleum ether and together with individual fatty acid standards (LA, GLA, DGLA and AA), as well as a mixture of the standards, these were spotted on prepared argentation TLC plates. This was done 2cm from the bottom of the plates and 2cm apart. A solvent containing chloroform-methanol-acetic acid-water (90:7.5:7.5:0.8 v/v) was prepared for developing the plates. The plates were allowed to develop until the solvent front was 1cm from the top of the plates. The plates were then removed and air-dried before being sprayed with 0.4% 2,7-dichlorofluorescein in ethanol and visualised under ultra-violet

light.

The spots of the experimental samples, which corresponded to those in the standard mixture, were scraped off the TLC plate into scintillation vials containing 10ml Ready Value liquid scintillation cocktail (Beckman Inst. Inc., USA). The radioactivity in these samples was counted in a Beckman LS 3801 scintillation counter.

The activity of the enzymes: Δ^6-desaturase, elongase and Δ^5-desaturase were calculated as the % products formed per mg protein as follows:

A = cpm of [1-^{14}C]LA added to flask

B = cpm of [1-^{14}C]LA in the media after harvesting

C = A-B = Amount of [1-^{14}C]LA taken up by the cells

D = cpm of combined GLA, DGLA and AA spots

E = cpm of combined DGLA and AA spots

F = cpm of AA spot

Δ^6-desaturase activity:

$$\% \text{ products formed} = \frac{D}{C} \times 100 \text{ / mg protein}$$

Elongase activity:

$$\% \text{ products formed} = \frac{E}{C} \times 100 \text{ / mg protein}$$

Δ^5-desaturase activity:

$$\% \text{ product formed} = \frac{F}{C} \times 100 \text{ / mg protein}$$

Note: The elongase and Δ^6-desaturase activities determined are only estimates. In order to determine the actual activity of elongase, an experiment in which the cells had been supplemented with radioactively labelled GLA would have had to be done. Similarly, for Δ^5-desaturase activity, an experiment in which cells were supplemented with radioactively labelled DGLA would have had to be performed.

3.2.5 Statistical Analysis:

The results obtained were analyzed using a One-way ANOVA followed by the Student-Newman Keuls Multiple Range Test.

3.3 RESULTS:

3.3.1 Cell Growth:

The results obtained for the effect of zinc supplementation on the cell growth of the LLCMK and B16 cells were reported in Chapter 2.

3.3.2 Essential Fatty Acid Composition:

The results obtained are presented in Tables 3.1 - 3.3.

Results for the EFA composition of cells supplemented with 10μg/ml zinc were not obtained as the cell counts of these cultures were too low to give detectable values.

The percentage of EFA's, expressed per 10^6 cells (Table 3.1) indicated that the control membrane fractions of both cell types have consistently higher EFA levels than the respective stroma fractions. The B16 fractions were noted to have lower levels of the EFA's than the LLCMK fractions, particularly in the case of GLA and DGLA.

The percentage LA in the membrane fraction of the LLCMK cells decreased with an increase in zinc supplementation, except at a concentration of 5μg/ml zinc where the %LA was similar to that in the control cultures. The LA levels in the stroma fractions increased in the cultures supplemented with 1μg/ml zinc, but decreased to a level well below that of the control cultures in the 3μg/ml zinc supplemented cells. At a concentration of 5μg/ml, the %LA was similar to that observed in the control stroma fractions.

In the case of the B16 cells, the %LA increased with an increase in zinc supplementation in both the membrane and the stroma fractions. LA was not detected in the stroma fraction of the cells supplemented with 5μg/ml zinc.

The levels of GLA in the membrane fractions of the LLCMK cells decreased in those cells supplemented with 1 and 3μg/ml zinc. At a concentration of 5μg/ml zinc, the %GLA increased slightly again. In the stroma fractions, the %GLA increased markedly in the cells supplemented with 1μg/ml, but then decreased to levels well below that of the control stroma fractions in the 3μg/ml zinc supplemented cells. At a concentration of 5μg/ml zinc, the levels of GLA determined were similar to that of the control cultures.

The %GLA in the membrane fraction of the B16 cells decreased markedly with an increase in zinc supplementation in those cultures supplemented with 1 and 3μg/ml zinc, but increased again with 5μg/ml zinc. In the stroma fractions, the levels remained low except in the case of the cultures

supplemented with 5μg/ml zinc where the %GLA was considerably higher than in the control stroma fractions.

In both the membrane and stroma fractions of the LLCMK cells, the levels of DGLA increased substantially in the cultures supplemented with 1μg/ml zinc. These levels decreased markedly in the 3μg/ml zinc supplemented cells. Similar levels of DGLA were obtained in the cells supplemented with 5μg/ml zinc.

The %DGLA in the B16 membrane fractions remained fairly constant with an increase in zinc concentration, except for a decrease noted at 3μg/ml zinc. In the case of the stroma fractions, the %DGLA tended to increase in the cells supplemented with 1 and 3μg/ml zinc. The cultures supplemented with 5μg/ml showed similar levels of DGLA to those found in the control cultures.

The %AA in the LLCMK cells showed no specific trend in the membrane fractions. The levels decreased considerably in the cultures supplemented with 1μg/ml zinc but increased in the 3μg/ml zinc supplemented cells to levels well above those of the control cultures. The %AA decreased again in the cultures supplemented with 5μg/ml. In the stroma fractions the levels of AA increased with increased zinc supplementation, except at a concentration of 5μg/ml where the levels obtained were similar to those noted for the control cultures.

The membrane fractions of the B16 cells showed similar levels of AA for all concentrations of zinc, except at 1μg/ml zinc where the %AA was not detected. In the stroma fractions, the %AA increased slightly in the cultures supplemented with 1 and 3μg/ml zinc. Levels of AA were not detected in those cells supplemented with 5μg/ml zinc.

Table 3.1: The effect of zinc supplementation on the essential fatty acid composition per 10^6 cells, in the membrane and stroma fractions of the control cultures of the LLCMK and B16 cells. The results presented are the mean of 3 determinations ± SEM.

FRACTION: LLCMK	%LA	%GLA	%DGLA	%AA
MEMBRANE	0.050 ±0.015	0.030 ±0.006	0.070 ±0.002	0.030 ±0.012
STROMA	0.020 ±0.006	0.020 ±0.008	0.010 ±0.004	0.005 ±0.002
B16				
MEMBRANE	0.040 ±0.022	0.004 ±0.002	0.040 ±0.023	0.020 ±0.004
STROMA	0.020 ±0.008	0.004 ±0.002	0.010 ±0.004	0.005 ±0.001

Table 3.2: The effect of zinc supplementation on the essential fatty acid composition of the membrane and stroma fractions of LLCMK cells. The results presented are the mean of 3 determinations ± SEM.

ZINC (μg/ml)	MEMBRANE			
	%LA	%GLA	%DGLA	%AA
0	1.50 ±0.08	1.10 ±0.10	0.22 ±0.10	0.96 ±0.03
1	1.21 ±0.10	0.78 ±0.06	0.80 ±0.08	0.16 ±0.06
3	0.40 ±0.04	0.04 ±0.02	0.09 ±0.01	1.21 ±0.30
5	1.60 ±0.07	0.13 ±0.01	0.16 ±0.03	0.05 ±0.02
	STROMA			
0	0.50 ±0.10	0.14 ±0.01	0.47 ±0.02	0.29 ±0.01
1	0.79 ±0.06	0.63 ±0.08	0.60 ±0.10	0.61 ±0.01
3	0.20 ±0.05	0.04 ±0.01	0.09 ±0.02	0.85 ±0.12
5	0.53 ±0.17	0.13 ±0.01	0.10 ±0.03	0.30 ±0.10

Table 3.3: The effect of zinc supplementation on the essential fatty acid composition of the membrane and stroma fractions of B16 cells. The results presented are the mean of 3 determinations ± SEM.

ZINC (μg/ml)	MEMBRANE			
	%LA	%GLA	%DGLA	%AA
0	1.55 ±0.49	1.30 ±0.10	1.45 ±0.10	0.85 ±0.35
1	1.60 ±0.25	0.25 ±0.04	1.40 ±0.20	ND
3	1.70 ±0.85	0.20 ±0.04	0.90 ±0.03	0.60 ±0.03
5	2.00 ±0.31	0.60 ±0.02	1.50 ±0.42	0.80 ±0.22
	STROMA			
0	0.70 ±0.14	0.30 ±0.05	0.55 ±0.20	0.43 ±0.03
1	1.30 ±0.40	ND	1.00 ±0.30	0.60 ±0.14
3	1.80 ±0.40	0.20 ±0.03	0.95 ±0.04	0.70 ±0.12
5	ND	1.02 ±0.02	0.50 ±0.11	ND

3.3.3 Δ^6-Desaturase Activity:

The results obtained are presented in Figure 3.2.

The activity of the enzyme Δ^6-desaturase was significantly higher ($p \leq 0.05$) in the control cultures of the LLCMK cells than in the malignant B16 control cultures.

The Δ^6-desaturase activity in the LLCMK cells increased in a dose-dependent manner with increasing concentrations of zinc supplementation. This increase was however not significant for any of the zinc supplemented cultures.

In the case of the B16 cells, the Δ^6-desaturase activity decreased in the cultures supplemented with 1 and 3μg/ml zinc, when compared to the control cultures. At concentrations of 5 and 10μg/ml zinc, the activity of the enzyme increased markedly. This increase was significant ($p \leq 0.001$) for the cultures supplemented with 10μg/ml zinc.

3.3.4 Elongase Activity:

The results obtained are presented in Figure 3.3.

The activity of the enzyme elongase, was similar for both the controls of LLCMK and Bl6 cells.

The elongase activity in the LLCMK cells was noted to increase with increasing zinc supplementation. This increase was significant ($p \leq 0.001$) at concentrations of 3, 5 and 10μg/ml zinc.

In the case of the Bl6 cells, the enzyme activity decreased in the cultures supplemented with 1 and

3μg/ml zinc, when compared to the control cultures. The elongase activity was higher in the 5 and 10μg/ml zinc supplemented cultures. The increase was significant ($p \leq 0.001$) for those cells supplemented with 10μg/ml zinc.

3.3.5 Δ^5-Desaturase Activity:

The results obtained are presented in Figure 3.4.

The control cultures of the normal LLCMK cells and the malignant Bl6 cells exhibited the same amount of Δ^5-desaturase activity.

The desaturase activity in the LLCMK cells increased with an increase in zinc supplementation. This increase was significant ($p \leq 0.05$) at a concentration of 10μg/ml zinc.

In the case of the Bl6 cells, the enzyme activity increased only slightly. The increase was not significant for any of the zinc supplemented cultures.

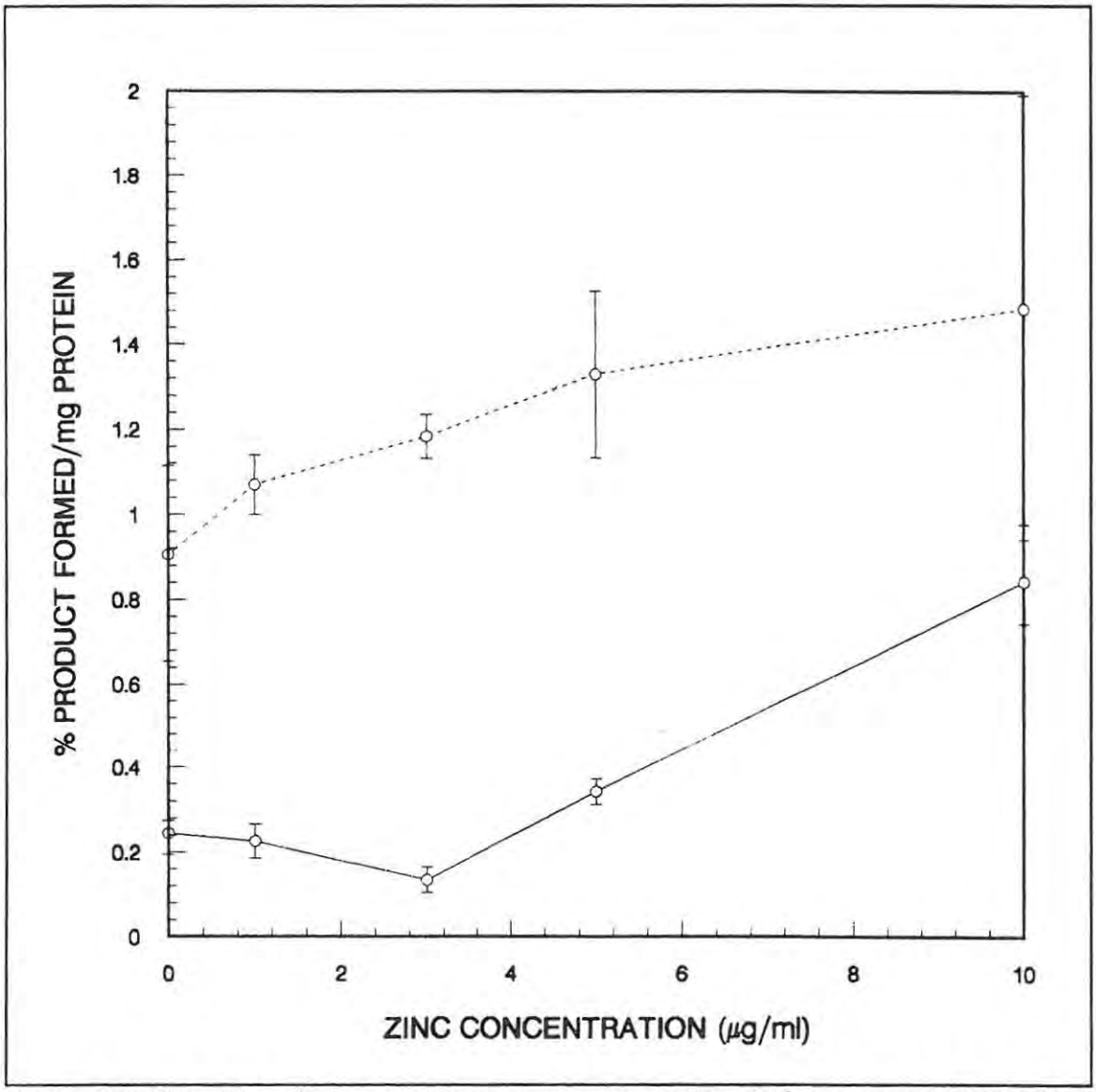

Figure 3.2: The effect of zinc supplementation on the activity of Δ^6-desaturase in B16 (—O—) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

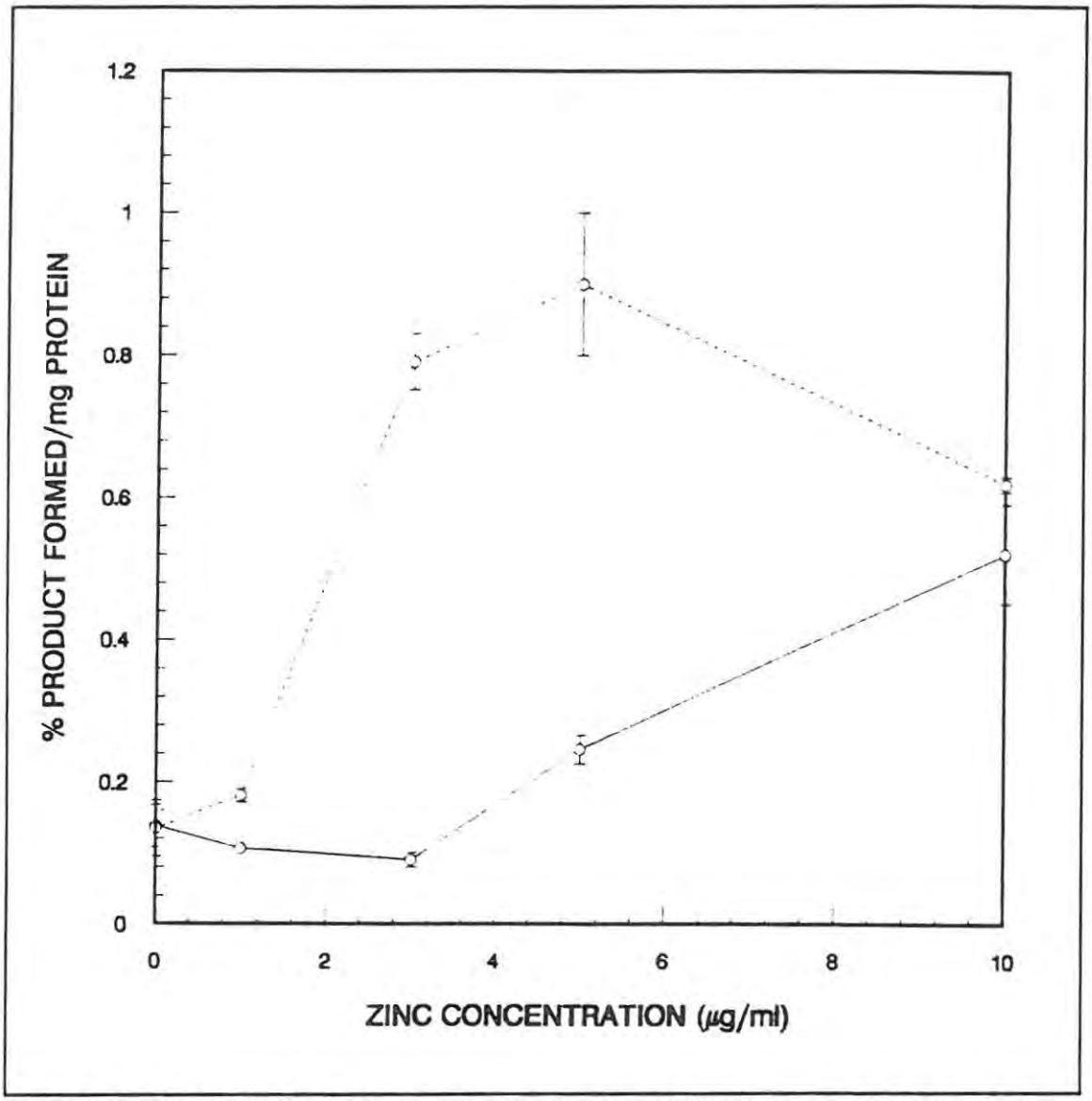

Figure 3.3: The effect of zinc supplementation on the activity of elongase in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

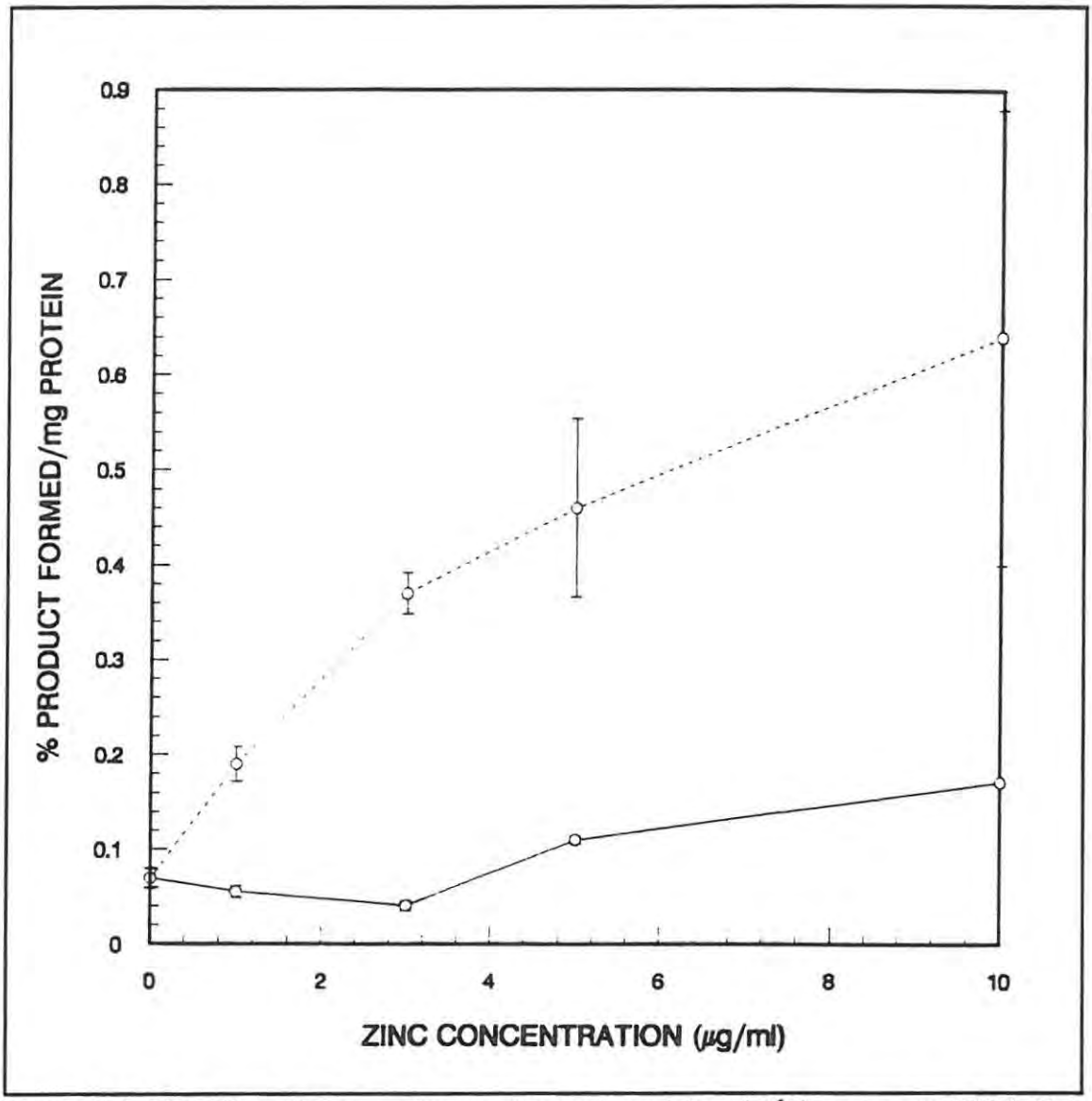

Figure 3.4: The effect of zinc supplementation on the activity of Δ^5-desaturase in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

3.4 Discussion:

3.4.1 Essential Fatty Acid Composition:

The B16 control cultures were found to have substantially lower levels of the EFA's per cell than the control cultures of the LLCMK cells. Reitz *et al.* (cited in 92) found that tumour microsomal membranes isolated from the Morris hepatoma 7777 contain about 50% less phospholipids than the controls. Galeotti *et al.* (93) also observed that the phospholipids in hepatoma plasma membranes are decreased to 60% of that of the controls. It would therefore follow that malignant cells, including the Bl6 melanoma cells, have lower concentrations of PUFA's than normal control cells.

The decrease in the %LA found in the membrane fractions of the LLCMK cells supplemented with 1 and 3μg/ml zinc may be due to an increase in the activity of the Δ^6-desaturase enzyme which converts LA to GLA. Zinc is thought to play a role in regulating the activity of Δ^6-desaturase (31,94). The %LA in the stroma fractions showed no trend and may reflect the uptake of LA at those zinc concentrations.

The levels of LA in the Bl6 cells were found to increase with increased zinc supplementation in both the membrane and the stroma fractions. This may be due to the findings that malignant cells lack or have reduced Δ^6-desaturase activity (51). This was found to be the case for the B16 cells as well (see Section 3.3.3). Zinc supplementation of 1 and 3μg/ml, resulted in a further decrease in the enzymes activity. The increase in the %LA observed in the stroma fractions reflect findings by Skeef (94) where zinc supplementation was found to have a stimulatory effect on the uptake of [1-^{14}C]LA by cells, with this effect being most marked for Bl6 cells. The noted increase in the levels of LA in the B16 cells may therefore be due to an increase in the uptake of LA and a reduction in Δ^6-desaturase activity.

The %GLA in both fractions of the LLCMK cells showed a similar trend to that observed for the %LA in the cell fractions *i.e.* the %GLA in the membrane decreased in the cells supplemented with 1 and 3μg/ml zinc and then increased again in the 5μg/ml zinc supplemented cultures. In the stroma fractions, the %GLA increased in the cells supplemented with 1μg/ml zinc and then decreased markedly in the cultures supplemented with 3μg/ml zinc. In the 5μg/ml zinc supplemented cells, the GLA levels increased slightly again. This suggests that the %GLA is dependent on the levels of LA in the cells, which is converted to GLA by the Δ^6-desaturase enzyme.

The levels of GLA in the membrane fractions of the B16 cells decreased most notably in the cells supplemented with 1 and 3μg/ml zinc and then increased again in the 5μg/ml zinc supplemented cells. In the stroma fractions of the B16 cells supplemented with 5μg/ml zinc, the %GLA was markedly higher than in the control stroma fractions. This increase in GLA levels in the stroma may be due to the mobilisation of GLA from the membrane phospholipid stores. Zinc has been shown to mobilise GLA and DGLA from the cell membrane lipid pools (20,50,87).

The marked increase in the %DGLA in the stroma fractions of the LLCMK cells supplemented with 1μg/ml zinc correlates with an increase in the %LA and %GLA at that concentration. This increase may be due to an increase in the substrate available for the enzyme elongase which converts GLA to DGLA. The activity of this enzyme did appear to increase with zinc supplementation in these cells (see Section 3.3.4).

Except for a slight decrease in %DGLA in the 3μg/ml zinc supplemented cells, zinc supplementation had no marked effect on the %DGLA in the membrane fractions of the B16 cells. Evidence of possible mobilisation of DGLA from the membrane is noted in that the %DGLA in the stroma fractions of the B16 cells supplemented with 1 and 3μg/ml zinc, increased substantially when compared to the control cultures.

The levels of AA in the stroma fractions of both the LLCMK cells and the B16 cells increased with zinc supplementation of 1 and 3μg/ml. This may be as a result of the increased substrate availability of the Δ^5-desaturase enzyme due to the mobilisation of DGLA from the phospholipid membrane stores. An increase in the activity of this enzyme was noted at the levels of zinc supplemented (see Section 3.3.5). At a concentration of 5μg/ml zinc, the levels of AA decreased again. In the membrane fractions, the %AA fluctuated with varying concentrations of zinc supplementation.

Although increased levels of all the EFA's, except GLA in the case of the B16 cells, have been found in the stroma fractions of both cell types supplemented with 1μg/ml zinc and also in some cases at other concentrations of zinc, these results are not conclusive in determining the effect of zinc supplementation on the mobilisation of the EFA's from the membrane stores. Zinc has been shown to facilitate increased uptake of LA (93) and further studies should be carried out in order to ascertain whether the effect observed in this study is not due to a possible increase in uptake of the other EFA's as well. The reduced activity of the Δ^6-desaturase and elongase enzymes found in the B16 cells at a concentration of 1μg/ml (Figures 3.2 and 3.3) precludes the assumption that the increased levels of EFA are due to increased activity of these enzymes in the presence of zinc.

3.4.2 Δ^6-Desaturase Activity:

The B16 control cultures were found to have significantly lower activity of the Δ^6-desaturase enzyme than the control cultures of the LLCMK cells. These results correspond with results obtained by other researchers who have reported that a number of malignant cells lack or have reduced Δ^6-desaturase activity (51,85,86,97).

The higher AA levels found after zinc supplementation by several researchers (78,87,96) suggests that zinc may have a role in Δ^6-desaturation of dietary LA. Dreosti *et al.* (99) however, found that GLA

was not effective in reversing zinc deficiency symptoms in young rats and that there was no difference in the Δ^6-desaturase activity in zinc-deficient and control rats. Driscoll *et al.* (100) have also implied that zinc is not involved in Δ^6-desaturation. In the present study, increased zinc supplementation did however, result in a dose-dependent increase of Δ^6-desaturase activity in the LLCMK cells.

Although the enzyme activity decreased in the B16 cells supplemented with 1 and 3μg/ml zinc, the activity increased markedly with the 5 and 10μg/ml zinc supplementation. This indicates that high concentrations of zinc may influence the activity of Δ^6-desaturase. Cunnane (1988) suggested that zinc may be involved in determining overall desaturase activity by influencing electron availability from NADH and NADPH (96).

3.4.3 Elongase Activity:

The results obtained in estimating the activity of elongase indicate that the B16 murine melanoma cells have the same level of activity as the normal monkey kidney cells.

The observed increase in elongase activity with zinc supplementation in the LLCMK cells may be due to an increase in the substrate availability of GLA, as a result of the increased Δ^6-desaturase activity noted, rather than a direct effect of the zinc.

The elongase activity in the B16 cells followed a similar trend to that observed for the Δ^6-desaturase enzyme. In these cells, the enzyme activity decreased in the cultures supplemented with 1 and 3μg/ml zinc. At concentrations of 5 and 10μg/ml supplementation, the elongase activity increased markedly. These results further substantiate the evidence that the activity of elongase is dependent on the levels of GLA and consequently on the activity of Δ^6-desaturase.

3.4.4 Δ^5-Desaturase Activity:

The Δ^5-desaturase enzyme appeared to have a similar activity in both the normal LLCMK and the malignant B16 control cultures. This suggests that these malignant cells do not have reduced Δ^5-desaturase activity.

The enzyme activity increased in the LLCMK cells with an increase in zinc supplementation. A slight, but non-significant increase in activity was also noted in the B16 cells. Although it appears as if this increase in activity may be due to increased substrate availability of DGLA rather than a direct consequence of zinc supplementation, research done in other laboratories has suggested that zinc does have a role in Δ^5-desaturase activity (31,101). The possibility that zinc may have a role in the activity of Δ^5-desaturase can therefore not be ruled out.

It can therefore be seen that the levels of EFA's tend to increase, particularly in the stroma fractions of both cell types when supplemented with zinc. The possibility that this may be due to an increase in the uptake of the EFA's should be investigated in order to ascertain whether the noted effect on the enzymes studied is due to increased substrate availability, or as a direct consequence of the zinc. Such a study would also confirm to what extent the mobilisation of GLA and DGLA has taken place. Studies should also be carried out in order to determine the actual effect of zinc on the elongase and Δ^5-desaturase enzymes, as the activities determined in this study are only estimates.

CHAPTER 4: FREE RADICAL FORMATION AND LIPID PEROXIDATION.

4.1 INTRODUCTION:

Molecular oxygen (O_2) is present as 20% of the atmosphere. It acts as the terminal oxidant during respiration which is the main source of energy in aerobic organisms. However, in addition to its beneficial roles, O_2, together with iron, possesses properties which can render it harmful to biological tissues (100).

During the reduction of O_2, reactive species such as the superoxide radical $O_2^{-}\cdot$ and hydrogen peroxide (H_2O_2) are readily formed. Iron can react with these species or with molecular O_2 itself to generate free radicals which can oxidise polyunsaturated fatty acids (PUFA) in the membrane. This process is known as lipid peroxidation. Cytotoxic metabolites such as malondialdehyde (MDA), 2-alkanals and hydroxy-alkenals are produced (101). These substances may react with biomolecules by reduction, oxidation, hydrogen abstraction, or addition across double bonds resulting in cellular damage, tissue damage, DNA modification and many pathological processes such as cancer, aging, inflammation and ischemia (100,102-104). Free radicals can be generated in living organisms by different pathological pathways as well, including; ultraviolet radiation, carcinogens, and oncogenes (105).

Defences against free radical damage include several enzymes whose main function is to decrease the amount of oxidants in the body thus providing a protective function against these reactive species.

Superoxide dismutase (SOD) scavengers superoxide radicals by catalysing the following reaction:

$$2O_2^{-} + 2H^{+} \rightarrow H_2O_2 + O_2.$$

In mammalian cells, two types of SOD have been identified which have different structures and metal contents. One contains copper (Cu) and zinc (Zn) and the other manganese (Mn). The CuZn SOD is associated with the cytosol while the Mn SOD is primarily confined to the mitochondria (106-109).

Catalase is another enzyme which protects the cell against lipid peroxidation by catalysing the following reaction:

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

Catalase is localised mainly in the peroxisomes (80%) and is responsible for removing large amounts of H_2O_2 generated within these organelles (100,102,107,108).

The most vital defense system against lipid peroxidation in the cell is dependent on reduced glutathione (GSH).

Glutathione peroxidase (GPx) is a selenium (Se) dependent enzyme which catalyses the oxidation of GSH to GSSG at the expense of H_2O_2:

$$H_2O_2 + 2GSH \rightarrow GSSG + H_2O.$$

Se-GPx may protect the cell by the removal of H_2O_2, which could be involved in the initiation of lipid peroxidation. GPx is present in both the cytosol (70%) and the mitochondrial matrix (30%) (100,102,107,109).

Glutathione reductase (GR) is also found in the cytosol and the mitochondria and catalyses the reaction:

$$GSSG + NADPH + H^+ \rightarrow 2GSH + NAD^+.$$

Non-enzymatic protection against lipid peroxidation involves several anti-oxidant nutrients. These include α-tocopherol (vitamin E), ascorbic acid (vitamin C) and β-carotene. Through their function with the above-mentioned metalloenzymes, the trace metals; copper, zinc, manganese (SOD), iron (catalase) and selenium (GPx) are considered to have "anti-oxidant" properties as well (100,102,109,110).

Free radicals are found to be involved in both the initiation and promotion of carcinogenesis. Altered anti-oxidant enzymes have been observed during carcinogenesis or in tumours. When compared to normal cells, tumour cells are found to be low in Mn SOD, usually low in CuZn SOD and almost always low in catalase activity. GpX and GR activities are variable. It could therefore be assumed that the malignant change may be able to be prevented by the addition of these enzymes, or by the increasing of low enzyme levels, during the early stages of carcinogenesis (93,102).

Zinc supplementation was found to increase the mobilisation of GLA and DGLA from phospholipid stores as well as to have a stimulatory effect on the activity of the enzyme Δ^6-desaturase, and possibly also on Δ^5-desaturase, in malignant B16 cells (Chapter 3). Unsaturated fatty acids have been shown to augment free radical generation (101). In addition, GLA has been found to stimulate the initiation of its own peroxidation by increasing the amount of superoxide radicals and by increasing the substrate available for lipid peroxidation (111). However, zinc is considered to protect cells against lipid peroxidation through its requirement in the activity of the enzyme superoxide dismutase. The following experiments were carried out in order to ascertain what effect zinc supplementation has on the formation of free radicals and lipid peroxidation in normal (LLCMK) and malignant (B16) cells.

4.2 MATERIALS AND METHOD:

4.2.1 Cell Culture Procedure:

The procedure followed for the preparation of media and culturing of cells for experimental work was as described in Chapter 2. After harvesting the cells by trypsinisation, the cells were pelleted in a refrigerated bench-top centrifuge (Hettich Universal K2S) at 1000g.

4.2.2 Free Radical Formation:

The cellular pellets were resuspended in 1ml phosphate buffered saline (PBS) (pH 7.4) and enumerated using a haemocytometer (Neubauer, W. Germany)

Free radical formation was determined by using the NBT-assay as described by Sangeetha, *et al.* (112). The assay is based on the reduction of nitroblue tetrazolium (NBT) to the insoluble diformazan by the superoxide anion. A 0.6ml aliquot of a 0.1% NBT solution (Sigma Chemical Co., USA) in phosphate buffered saline (PBS) (pH 7.4), was added to each of the resuspended cultures. These were incubated for 4 hours at 37°C. After incubation, the reactions were terminated by adding 1.2ml glacial acetic acid. The absorbance was read at 560nm in a spectrophotometer (Milton Roy Spectronic 301). A standard curve was produced using nitroblue diformazan (Sigma Chemical Co., USA) (Appendix 3).

4.2.3 Lipid Peroxidation:

The pellets obtained from centrifuging the cultures were resuspended in 1ml of 0.9% NaCl and enumerated using a haemocytometer (Neubauer, W. Germany).

Lipid peroxidation was measured by the thiobarbituric acid (TBA) assay which measures the malondialdehyde (MDA) equivalents which are products of lipid peroxidation. A modified method of that described by Sangeetha, *et al.* was used (112). A 0.5ml aliquot methanolic butylated hydroxytoluene (BHT) (Sigma Chemical Co., USA) (0.5g/*l*) was added to each of the cultures to reduce oxidation of polyunsaturated fatty acids (113). Two millilitres of 24% trichloroacetic acid (TCA) (Sigma Chemical Co., USA) was added and the samples were centrifuged at 2000g for 15 minutes, at 4°C, to remove any TBA-MDA complex which may have adsorbed to the precipitated protein. A 0.5ml aliquot of 0.33% TBA (Sigma Chemical Co., USA) was added to 2ml of the protein-free supernatant and all of the tubes were boiled for 1 hour at 95°C. The tubes were cooled and the MDA was extracted with 2ml of N-butanol. The absorbance was then read at 532nm in a spectrophotometer (Milton Roy Spectronic 301). A standard curve was produced using 1,1,3,3-tetramethoxy-propane (Fluka, Switzerland) (Appendix 4).

4.2.4 Statistical Analysis:

The results obtained were analyzed using a one-way ANOVA followed by the Student-Newman Keuls Multiple Range Test.

4.3 RESULTS:

4.3.1 Cell Growth:

The results obtained for the cell growth of the LLCMK and B16 cultures with increasing zinc supplements are reported in Chapter 2.

4.3.2 Free Radical Formation:

The results obtained are presented in Figure 4.1.

The B16 control cultures were found to have higher concentrations of diformazan when compared to the concentrations in the control cultures of the LLCMK cells.

In the case of the B16 cells, the concentration of diformazan determined increased with an increase in the concentration of zinc supplemented to the media. However, this increase was not significant at any of the zinc concentrations when compared to the control. The diformazan concentrations determined for the LLCMK cultures showed no specific trend. The diformazan concentration increased initially in the cultures supplemented with 1μg/ml zinc ($p \leq 0.05$), returned to control concentrations at 3μg/ml zinc and increased again at 5μg/ml zinc ($p \leq 0.001$). Cultures supplemented with 10μg/ml zinc showed diformazan concentrations only marginally higher than those observed for the control cultures.

4.3.3 Lipid Peroxidation:

The results obtained are presented in Figure 4.2.

The control cultures of the LLCMK cells showed higher concentrations of MDA than those of the B16 controls.

The levels of MDA remained fairly constant at the added zinc concentrations of 1 and 3μg/ml zinc in both the LLCMK and the B16 cells, when compared to the controls. At an added zinc concentration of 5μg/ml the MDA concentration increased significantly ($p \leq 0.001$) in both cell types and again at 10μg/ml zinc ($p \leq 0.001$).

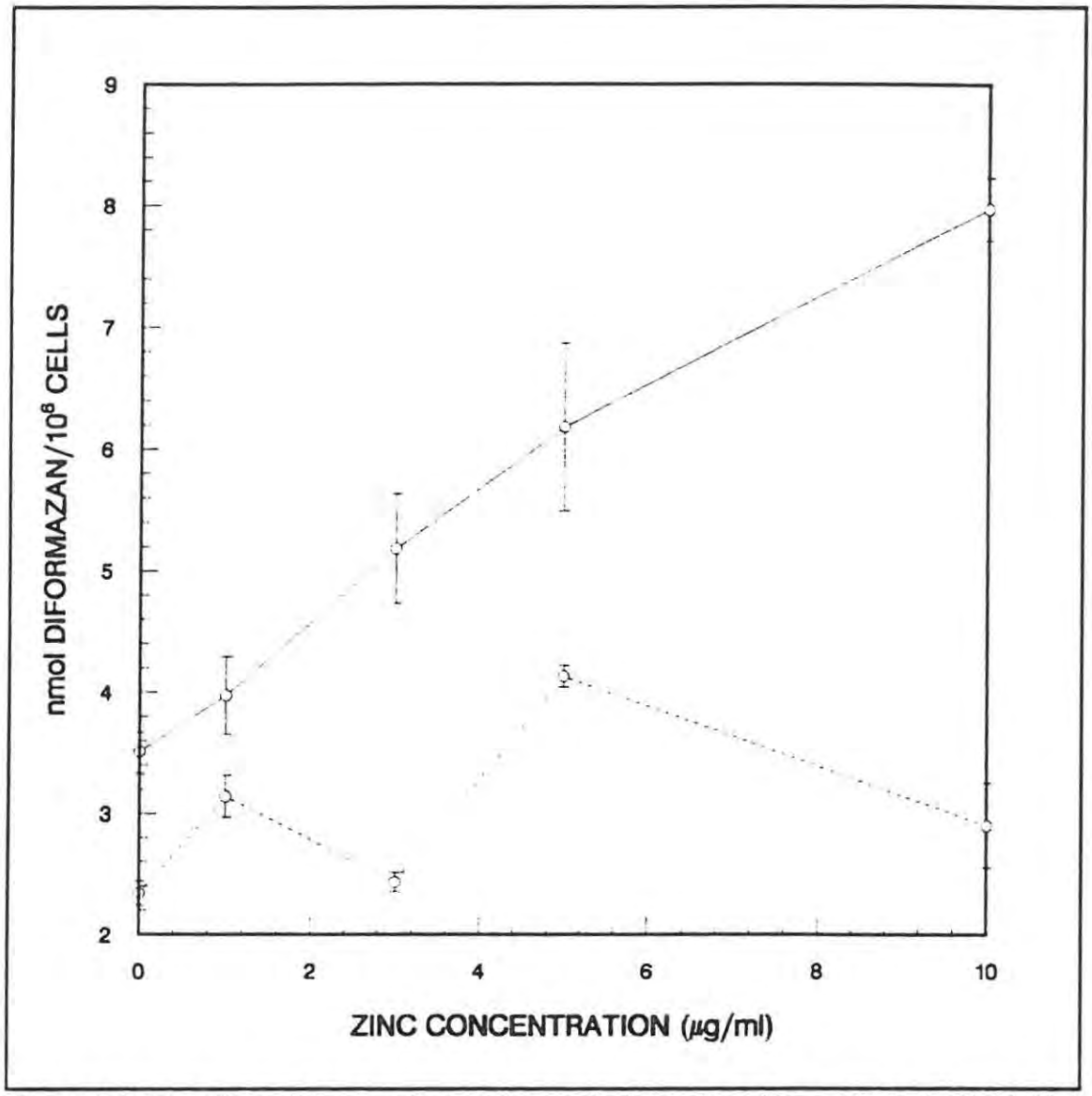

Figure 4.1: The effect of zinc supplementation on the formation of free radicals in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

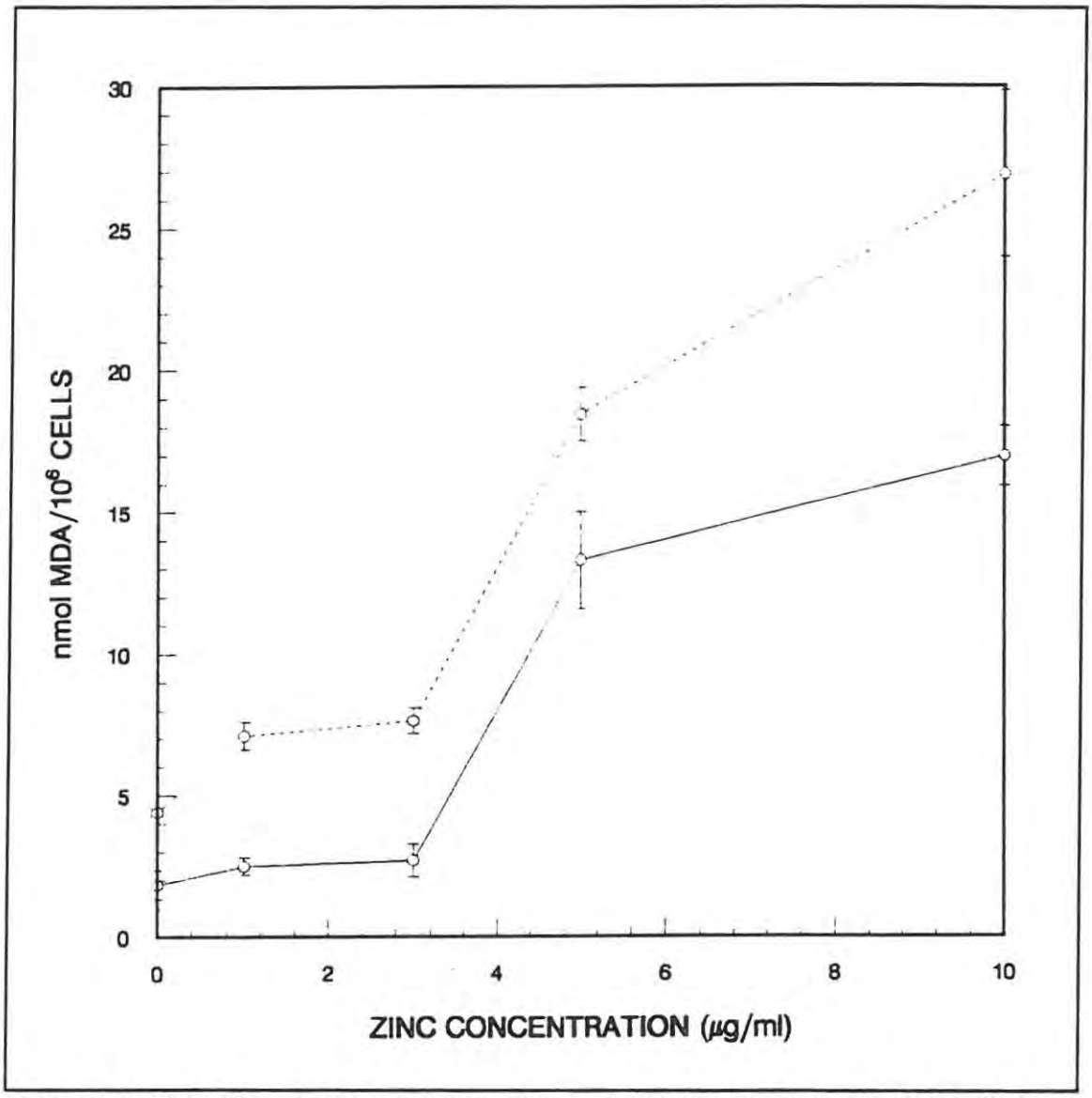

Figure 4.2: The effect of zinc supplementation on the lipid peroxidation in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

4.4 DISCUSSION:

4.4.1 Free Radical Formation:

The control cultures of the B16 cells contained higher concentrations of diformazan (indicative of higher levels of the superoxide anion radical) than the control cultures of the non-malignant LLCMK cells. The diformazan concentration, in the B16 cells, increased in a dose-dependent manner with an increase in zinc supplementation. The LLCMK cultures showed no specific trend, with the cells supplemented with 10μg/ml zinc having only marginally higher concentrations of diformazan than the control cultures.

Tumour cells have been found to have reduced activity of the anti-oxidant enzymes *viz.* CuZn SOD, Mn SOD and catalase. Low levels of these enzymes results in the accumulation of free radicals, which can result in damage to DNA, RNA, lipids and proteins (102).

With the requirement of zinc in the activity of the enzyme CuZn SOD, and it thus being referred to as having "anti-oxidant" properties (100,102,108,110), it may have been expected that zinc supplementation would result in an increase in the enzymes activity and thus a decrease in free radical formation. However, the results obtained may be explained in that high zinc intakes have been found to induce a relative copper deficiency by interfering with copper absorption (28). Superoxide dismutase contains 2 atoms of copper and 2 atoms of zinc per molecule. No active replacement of copper has been found, but almost any transition metal will substitute for zinc (26).

Cao *et al.* (114) found that mice supplemented with 1000% of the recommended amount of dietary zinc for mice, exhibited a significant reduction in blood and hepatic copper levels as well as reduced blood and hepatic CuZn SOD activities. This was accompanied by an increase in free radical

formation.

In addition, unsaturated fatty acids have been found to increase the formation of free radicals (101). The observed increase in the mobilisation of GLA and DGLA and activity of the Δ^6-desaturase enzyme found in zinc supplemented B16 cells (Chapter 3) may account for some of the noted increase in free radical formation with increasing zinc supplementation.

It is therefore possible that the increase in free radical generation found in the B16 cells is due to the reduced CuZn SOD activity in malignant cells. The activity of the enzyme may be further impaired by an effective copper deficiency induced by zinc supplementation. The increase in PUFA's in the cells due to mobilisation of GLA and DGLA and increased Δ^6-desaturation may also account for some of the free radical formation.

4.4.2 Lipid Peroxidation:

At all concentrations of zinc supplementation, the concentration of MDA was consistently higher in the LLCMK cells than the B16 cells.

Galeotti *et al.* (93) have shown that membranes isolated from hepatomas catalyse relatively low rates of lipid peroxidation when compared to control liver membranes. It has also been observed that the hepatoma membranes contained 60% less phospholipids than the control. The rate-limiting step of lipid peroxidation is substrate availability.

The levels of PUFA's were found to be lower in the malignant B16 cells than the normal LLCMK cells (Chapter 3). The lower levels of lipid peroxidation in the B16 cells may therefore be due to reduced substrate availability.

Both the superoxide radical ($O_2^{-}\cdot$) and H_2O_2 have been found to oxidise unsaturated fatty acids of the membrane resulting in the accumulation of lipid peroxidation by-products (101,114). The increase in lipid peroxidation may therefore be as a result of increased free radical generation and increased substrate availability with zinc supplementation.

CHAPTER 5: CYCLO-OXYGENASE ACTIVITY AND PROSTAGLANDIN E_2 SYNTHESIS.

5.1 INTRODUCTION:

Prostaglandins (PG's) are 20-carbon hydroxy polyunsaturated fatty acids. All PG's are comprised of a cyclopentane ring with two side chains. Primary PG's contain a C15-hydroxyl group and a 13,14-trans double bond. There are 9 groups of PG's. Each group of PG has a group letter based on the functionality of the cyclopentane ring. A subscript number follows the group letter and indicates the number of double bonds present in the side chains *i.e.* the degree of unsaturation. A further classification index, either α or β, refers to the stereochemistry of the C9-hydroxyl group. The precursors to the PG's are the C20 ω-6 polyenoic acids. The 1-series PG's are derived from DGLA while the 2-series PG's are derived from AA (55,115,116).

Fatty acid oxidation, catalysed by cyclo-oxygenase, is the initial rate-limiting step in the conversion of fatty acids to PG's. Cyclo-oxygenase is a membrane-bound multi-enzyme complex which specifically catalyses the incorporation of molecular O_2 into the PUFA's, thereby generating 15-hydroxyprostaglandin endoperoxides (PGG_2 and PGH_2) which are intermediates in AA metabolism. Evidence that glutathione peroxidase inhibits cylco-oxygenase activity indicates that cyclo-oxygenase catalysis requires some form of peroxide (55,117,118).

PG's have been found to induce a number of biological actions in various tissues at extremely low concentrations. They play a regulatory role in a number of systems, including the reproductive, renal, neural and cardiovascular systems. They are not stored within the cell and their release depends on the mobilisation of precursors such as free fatty acids (55,82,119).

PG's, especially those of the E-series, have been shown to be elevated in a large number of tumours, in comparison to normal cells and may play a role in tumour growth and spread. There are at present two ideas as to the role of PG's in the cell proliferation of tumours. The first claims that a response to increased PG synthesis is a limiting of tumour growth and therefore, by adding exogenous PG's, tumour growth can be inhibited. The alternative theory claims that PG's are involved in the initiation and promotion of tumour growth and therefore inhibition of PG synthesis will inhibit tumour growth. Evidence exists which supports both theories (6,119-121).

The addition of prostaglandins to cell cultures derived either from normal or malignant cells have been shown to result in a dose-dependent inhibition of cell growth. However, reports are also found which indicate that supplementation of certain PG's can enhance cell proliferation. The action of the PG's on cell proliferation therefore appears to be cell-type specific (6,38,121-123).

PG's are reported to regulate specific cellular functions by influencing cAMP levels, thus enhancing glycosaminoglycan synthesis and reducing proliferation. Their action on cAMP is thought to be mediated through the membrane-bound enzyme, adenylate cyclase (38,124).

Based on the results obtained in Chapter 4, which indicate that normal (LLCMK) and malignant (B16) cells produce higher levels of lipid peroxides when supplemented with zinc, the following experiments were carried out in order to ascertain whether these elevated levels of lipid peroxides had any effect on the activity of cyclo-oxygenase, as suggested by Hemler (117) and Fujimoto (125), and consequently on prostaglandin E_2 synthesis.

5.2 MATERIALS AND METHODS:

5.2.1 Cell Culture Procedure:

Experimental cell cultures were set up as described in Chapter 2. After the first flasks reached confluency, the cells were harvested and centrifuged at 1000g in a bench-top centrifuge (Hettich Universal K2S). The pellets were resuspended in 1ml of PBS and the cells enumerated.

The resuspended cells were transferred to a Dounce homogeniser and homogenised 25 times using the tight plunger.

5.2.2 Cyclo-oxygenase Activity Determination:

The method used for the determination of cyclo-oxygenase activity was a modified version of that used by Ohki, *et al.* (126).

A mixture containing: 50μl 0.2M Tris-HCl buffer (pH 8.0), 10μl 40μM Manganese protoporphyrin IX (Sigma Chemical Co., USA) and 5μl (0.46pmol) [5,6,8,9,11,12,14,15-^{3}H]Arachidonic acid (Amersham Int. plc., UK) was prepared for each sample. This mixture was vortex mixed before adding 10μl homogenated cell suspension.

The reaction mixture was mixed, using a vortex mixer, at 24°C for 2 minutes whereafter the reaction was terminated by adding 0.4ml of an ethyl ether-Methanol-0.2M citric acid (30:4:1 v/v) solution, precooled to -20°C.

The tubes were mixed using a vortex mixer and kept in an ice-bath. Anhydrous sodium sulphate

(0.5g) was added, for the dehydration of the organic phase, and the samples were mixed gently.

A 25μl aliquot of the upper organic phase was removed and spotted on pre-coated silica gel 60F254 TLC plates (Merck, W. Germany). The samples were spotted 2cm from the bottom of the plate and 2cm apart. Arachidonic acid and prostaglandin B_2 (PGB_2) (Sigma Chemical Co., USA) were spotted as markers. Prostaglandin G_2 (PGG_2) is said to be located between these markers (128) (PGG_2 is not available commercially). The plates were developed in ethyl ether-petroleum ether-acetic acid (85:15:0.1 v/v), at 4°C, to 1cm from the top of the plates. They were then air-dried and the spots were visualised using iodine vapour.

Regions corresponding to arachidonic acid and PGG_2 were scraped off the plates into scintillation vials containing 10ml Ready Value liquid scintillation cocktail (Beckman Inst. Inc., USA). The remaining silica gel, for each sample run, was also scraped into scintillation vials in order to determine the total cpm's recovered. The cpm's were determined by reading the samples in a Beckman LS 3801 scintillation counter.

The cyclo-oxygenase activity was determined as the amount of PGG_2 formed (pmoles) from the arachidonic acid added.

The amount of PGG_2 formed was calculated as follows:

$$A = \% \text{ Radioactivity of AA} = \frac{\text{CPM (AA)}}{\text{TOTAL CPM}} \times 100$$

$$B = \% \text{ Radioactivity of } PGG_2 = \frac{\text{CPM } (PGG_2)}{\text{TOTAL CPM}} \times 100$$

$$\text{Amount of } PGG_2 \text{ (pmol)} = \text{AA added (0.46pmol)} \times \frac{B}{A}$$

5.2.3 Prostaglandin Extraction and Isolation:

The prostaglandins (PG's) were extracted according to a modified method of Powell (127).

The homogenised cells were centrifuged at 1000g for 20 minutes to remove precipitated proteins. The supernatant was added to 15ml of cold ethanol (95%) and shaken for 10 minutes. The samples were diluted to 100ml with cold water, and vortex mixed for 1 minute. The pH of the solutions was adjusted to 3.0 using 1N HCl. Sep-Pak C18-octadecylsilyl (ODS) silica cartridges (Waters Associated Inc., USA) were moistened by passing 20ml of 80% ethanol through them, followed by 20ml water, to remove excess ethanol. The samples were then passed through the cartridges. The cartridges were washed with a further 10ml water and 10ml petroleum ether before eluting the PG's using 5ml methyl formate. The eluent was dried, under nitrogen, at 25°C and stored at -20°C until assayed.

The samples were reconstituted to 25μl with acetonitrile. The concentration of PGE_1 in the samples was assayed by injecting a 20μl aliquot of the sample into a high performance - liquid chromatograph (Beckman, System Gold). This however, proved to be unsuccessful due to the concentrations of the PG's in the samples being too low to be detected. In order to obtain a measure of the synthesis of PG's in the cells supplemented with zinc, a highly sensitive Prostaglandin $E_2[^{125}I]$ assay system (Amersham Int. plc., UK) was used. No equivalent assay for the detection of PGE_1 is available.

Prior to analyzing the extracted PG fractions using the assay system, the PG's were converted to their methyl oximate derivatives as follows: the dried PG fractions were reconstituted with 100μl PBS (pH 7.0). A 100μl aliquot of methyl oximation reagent (supplied with the PGE_2 assay kit) was added and the samples vortex mixed. The resulting solutions were incubated at 60°C for 1 hour to allow methyl oximation of the samples to take place. Samples were then diluted to a final volume of 500μl with PBS (pH 7.0) and assayed using the radioimmunoassay procedure as described in the assay protocol.

A 100μl aliquot of sample was pipetted into appropriately labelled tubes. One hundred microlitres of tracer was added to each vial followed by 100μl of antiserum. All the tubes were vortex mixed and incubated for 2 hours at 25°C in a water bath. After incubation, 250μl Amerlex-M second antibody reagent was added to each tube. The tubes were incubated for a further 15 minutes at room temperature before separating the antibody bound fraction by centrifuging at 1500g for 10 minutes at 4°C. After centrifugation, the supernatant was poured off and discarded and the radioactivity present in each tube was determined by counting for 1 minute in a Packard auto-gamma scintillation counter.

The normalised percent bound for each sample was calculated using the following relationship:

%B = Percent of tracer bound

Bo = Zero standard

NSB = Non-specific binding

$$\%B/Bo = \frac{\text{Sample cpm - NSB cpm}}{\text{Bo cpm - NSB cpm}} \times 100$$

The pg PGE_2/tube was read directly from the standard curve generated by plotting the normalised percent bound as a function of the $\log_{10}$ prostaglandin E_2 concentration (Appendix 5).

5.2.4 Statistical Analysis:

The results obtained were analyzed using a one-way ANOVA followed by the Student-Newman Keuls Multiple Range Test.

5.3 RESULTS:

5.3.1 Cell Growth:

The results obtained for the growth of the LLCMK and B16 cultures at different levels of zinc supplementation are reported in Chapter 2.

5.3.2 Cyclo-oxygenase Activity:

The results are presented in Figure 5.1.

The activity of cyclo-oxygenase was found to be slightly higher in the control cultures of the B16 cells than in those of the LLCMK cells. For all the zinc supplemented cultures, the cyclo-oxygenase activity was markedly higher for the B16 cultures when compared to the corresponding supplemented LLCMK cultures.

The cyclo-oxygenase activity in the B16 cells increased sharply at an initial concentration of 1μg/ml zinc, when compared to that of the control cultures. This level of activity then remained fairly constant for cultures supplemented with 3 and 5μg/ml zinc. The activity of the enzyme again increased significantly ($p \leq 0.001$) in those cultures supplemented with 10μg/ml zinc.

The LLCMK cultures supplemented with 1 and 3μg/ml zinc showed only a marginal increase in enzyme activity in relation to the control cultures. Supplementation with 5μg/ml and 10μg/ml zinc resulted in a significant increase in cyclo-oxygenase activity ($p \leq 0.025$ and $p \leq 0.001$ respectively).

5.3.3 Prostaglandin E_2 Synthesis:

The results obtained are presented in Figure 5.2.

The control cultures of the malignant B16 cells had lower concentrations of PGE_2 than those determined for the LLCMK cells.

The concentrations of PGE_2 determined for both the LLCMK and the B16 cells increased significantly in those cultures supplemented with 1μg/ml and 3μg/ml zinc ($p \leq 0.01$) when compared to their respective controls.

At a concentration of 5μg/ml zinc, the B16 cells showed no further increase in PGE_2 levels than those observed at the lower zinc supplements. However, at a concentration of 10μg/ml zinc, the PGE_2 concentrations once again increased significantly ($p \leq 0.001$).

The concentration of PGE_2 decreased to a level just above that determined for the control cultures of the LLCMK cells when supplemented with 5μg/ml zinc. Similar concentrations of PGE_2 were found in the cultures supplemented with 10μg/ml zinc.

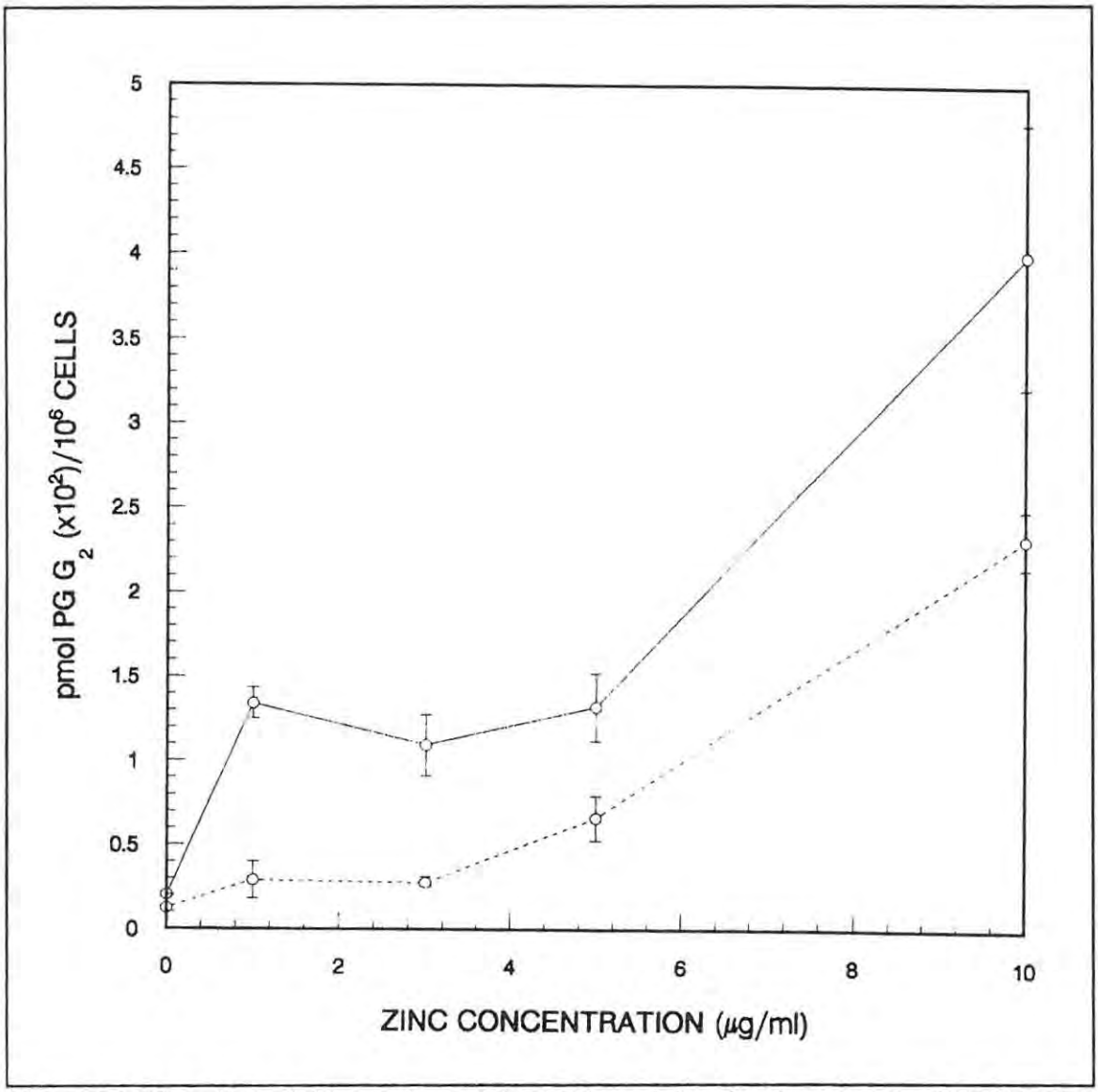

Figure 5.1: The effect of zinc supplementation on the activity of cyclo-oxygenase in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

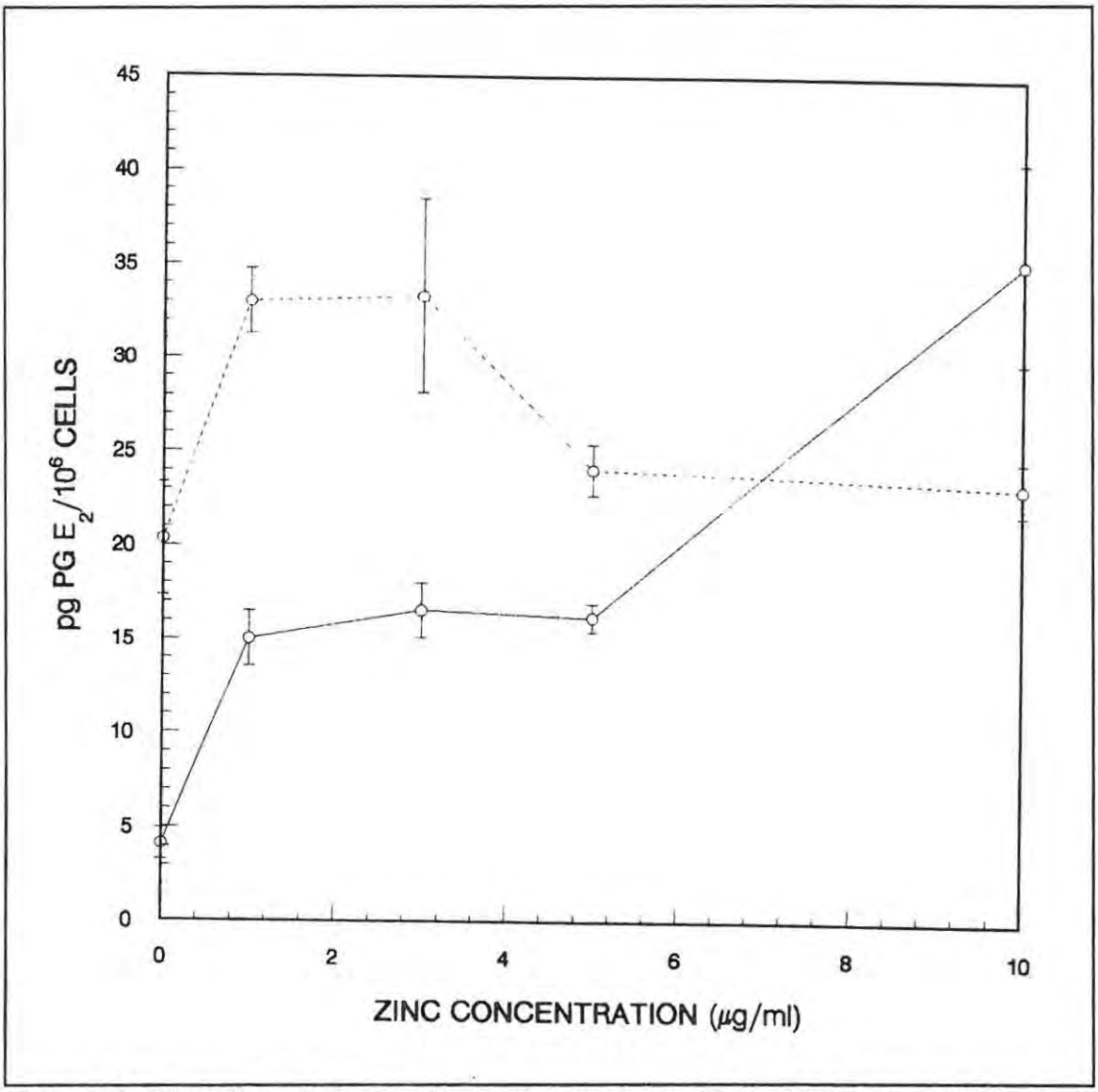

Figure 5.2: The effect of zinc supplementation on the synthesis of PGE_2 in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

5.4 DISCUSSION:

5.4.1 Cyclo-oxygenase Activity:

The B16 cultures, at all concentrations of zinc supplementation, showed higher levels of cyclo-oxygenase activity than the normal LLCMK cells. This may be due to an increase in substrate availability as a result of the increased mobilisation of GLA and DGLA in the B16 cells with certain zinc concentrations (Chapter 3).

The cyclo-oxygenase activity in the B16 cells increased markedly at 1μg/ml and again at 10μg/ml zinc. In the LLCMK cells the enzyme activity increased significantly at concentrations of 5 and 10μg/ml zinc.

Hemler (117) and Fujimoto (125) have suggested that the cyclo-oxygenase enzyme may require peroxides to trigger its activity. The polyunsaturated fatty acids in the membrane phospholipids generate hydroperoxide intermediates during lipid peroxidation. This leads to stimulatory levels of peroxidised lipids which results in the synthesis of the endoperoxide, PGG_2. Once the reaction is triggered by small amounts of any hydroperoxide, an accelerated production of peroxide results in continued activation (117,125).

The increase in cyclo-oxygenase activity noted in both the malignant B16 and the normal LLCMK cells with zinc supplementation may be due to the increased lipid peroxidation observed with increasing concentrations of zinc supplementation (Chapter 4). The resulting peroxides formed may lead to the increased activation of the enzyme observed.

5.4.2 Prostaglandin E_2 Synthesis:

Although the E-series prostaglandins have been shown to accumulate in a number of tumours in comparison to normal cells, in this study the B16 melanoma control cultures were found to have substantially lower concentrations of the PGE_2 than the normal LLCMK controls. These findings are in contrast with those of other researchers. Favalli *et al.* (128) found that B16 melanomas synthesised 7 times as much PGE as did adjacent normal tissues. Stoll *et al.* (90) found that the malignant B16 cells had significantly higher PGE_2 concentrations than the non-malignant LLCMK cells. The reason for the higher PGE_2 concentrations noted in the LLCMK cells in this study is unknown.

The cyclo-oxygenase activity increases gradually with an increase in zinc supplementation in the LLCMK cells. However, the PGE_2 levels increase significantly at a concentration of 1μg/ml zinc but decrease again to levels only slightly higher than in the control cultures in those cultures supplemented with 5μg/ml zinc. Similar concentrations were determined in the cultures supplemented with 10μg/ml zinc. It is known that PG synthesis is dependent on the mobilisation of the PUFA precursors to the PG's (55). In the results obtained in Chapter 3, it was determined that GLA and DGLA are mobilised from the phospholipid stores in the B16 cells, at certain zinc concentrations, to a greater extent than in the LLCMK cells.

Cunnane *et al.* (96) found that zinc caused the release of phospholipids by the hydrolytic action of the enzyme phospholipase A_2, which facilitates the release of PUFA's from the membrane. Conflicting results were obtained by Stossel *et al.* (cited in 64) who reported that zinc inhibits the activity of phospholipase A_2 and therefore also the mobilisation of PUFA's from the membrane. These results suggest that the action of zinc on phospholipase A_2 activity may be cell-type specific.

It is therefore possible that at a concentration of 5μg/ml zinc the phospholipase A_2 activity is inhibited in the LLCMK cells resulting in a lack of precursor availability. These results are consistent with those obtained for the levels of AA. The effect of zinc supplementation on the activity of the enzyme phopholipase A_2 would have to be studied in both cell types before conclusive deductions can be made.

In the B16 cells, the concentration of PGE_2 increased significantly in the cultures supplemented with 1μg/ml zinc. These levels remained fairly constant for the 3 and 5μg/ml zinc supplemented cultures and then increased again with 10μg/ml zinc. These results mimic the trend observed for the cyclo-oxygenase activity in the B16 cells, suggesting that these PGE_2 levels are directly related to the activity of the cyclo-oxygenase enzyme in the presence of elevated levels of the precursor AA. In addition, The concentrations of PGE_2 noted in the B16 cells are inversely related to the growth of the cells (Chapter 2).

PGE is an exogenous regulator of normal and malignant cell proliferation. This effect of PGE is thought to be mediated by its action on cAMP either through the activity of adenylate cyclase or cAMP phophodiesterase (19,118,119,122). The possibility of this is examined in Chapter 6.

CHAPTER 6: ADENYLATE CYCLASE ACTIVITY AND CYCLIC-AMP LEVELS.

6.1 INTRODUCTION:

The principle role of adenosine 3´,5´-cyclic monophosphate (cAMP) is as an intracellular "second messenger" which mediates the effect of several hormones, which in turn control specific physiological processes at a cellular level. These hormones are considered as potential regulators of cellular growth and differentiation in normal tissues (130-132).

Evidence that cAMP is involved in the regulation of cell division is well documented. Bürk (132) found that cAMP inhibits cell division. This finding was supported by Ryan and Heidrick (133) who observed inhibition of HeLa and Strain L cell growth by cAMP. This inhibition was found to be reversible on the removal of the cAMP (132).

Further evidence for the regulation of cell proliferation by cAMP has arisen from studies done on a variety of cultured cell lines. In general, this inhibition of cell proliferation is dose-dependent with increasing concentrations of cAMP resulting in increased inhibition of cell proliferation (134,135).

The inhibition of cell proliferation by exogenous cAMP suggests that low levels of cAMP would be found in rapidly dividing cells and high levels in cells which are in a stationary phase of growth. Likewise, transformed or malignant cells would be expected to have lower levels of cAMP. It has been shown that transformed cells contain less cAMP than normal cells and that an inverse relation exists between cAMP levels and cell growth (136-140).

Malignant and transformed cells in culture have several of the abnormal properties of transformed cells *e.g.* loss of contact inhibition, abnormal morphology, decreased adhesiveness and rapid growth

rate. These properties can be reverted back towards those of normal cells by treatment with agents which raise cAMP levels (136).

The intracellular level of cAMP is mainly determined by the balance between its formation from 5´-ATP by the enzyme adenylate cyclase and its catabolism to 5´-AMP by cAMP-phosphodiesterase (PDE). Activation or inhibition of either of these enzymes could result in marked changes in the metabolism of cAMP (132,136).

Sutherland and Rall discovered adenylate cyclase in 1957. It is found to be present in most types of mammalian cells, lower animals, unicellular organisms, bacteria and possibly also in plants. In the case of animal cells, the majority of the enzyme is bound to the plasma membrane (141).

Owing to the close association of adenylate cyclases with the plasma membrane, modification of the enzymes' activity could be expected with changes in the membrane lipids due to, for example, dietary fat intake. Individual tissues vary widely in their response to essential fatty acid deficiency. Essential fatty acid-deficient diets result in reduced fluidity of the membrane which is shown by a lower double bond index. This has been associated with an increase in the activity of adenylate cyclase in submandibular salivary glands (142). In contrast, results obtained from work done on hearts of rats fed essential fatty acid-deficient diets showed lower cAMP levels, which are consistent with lower adenylate cyclase activity (143). These results have however, been ascribed to reduced prostaglandin activity. The adenylate cyclase system can be stimulated by one or more of a large number of hormones, including PGE_1 as well as other PG's (130,144). Treatment of neuroblastoma cells with PGE_1 results in an increase in both cAMP levels and PDE activity (136).

There is a great diversity in the forms of PDE. A broad division into low Km (Approximately $1 moll^{-1}$) and high Km (approximately $100 moll^{-1}$) forms may be made. The concentration of cAMP

within the cell is between 0.1 and 1moll^{-1}, it therefore seems apparent that the low Km (high affinity) form is normally the physiologically significant fraction (131).

Manganiello *et al.* and D'Armiento *et al.* (cited in 145) found that cells incubated for 24 hours with PGE_1 showed a gradual increase, up to 30%, in PDE activity in L-929 cells. Bourne *et al.* (cited in 145) reported that the S49 line of lymphoma cells also showed increased PDE activity following stimulation by PGE_1.

Although the PDE activity increases in cells incubated with PGE_1, this increase appears to be regulated by the intracellular levels of cAMP and not as a direct consequence of the PG (136,145).

Owing to the results obtained in Chapter 5, which indicate that treating normal (LLCMK) cells with increasing concentrations of zinc results in an increase in the PGE_2 levels at low zinc concentrations and that malignant B16 cells show a dose-dependent increase in PGE_2, the activity of the enzyme adenylate cyclase was consequently determined, as well as the levels of cAMP in the cells. Evidence in the literature that PDE is only indirectly dependent on PG's, through the activation of adenylate cyclase, resulting in increased intracellular levels of cAMP, precluded an investigation of the enzymes' activity in response to zinc treatment.

6.2 MATERIALS AND METHOD:

6.2.1 Cell Culture Procedure:

The procedure followed for the preparation of media and culturing of cells for experimental work was as described in Chapter 2. Once the first flasks had reached confluency, the cells were harvested, enumerated, homogenised and separated into their membrane and stroma fractions (as described in Chapter 4).

6.2.2 Adenylate Cyclase Activity Determination:

The membrane pellets were taken up in cold Tris-HCl buffer (1M) (pH 7.5) and stored at -20°C until used.

The membrane fractions were assayed for their protein concentration using the Folin - Lowry assay (91) (as described in Chapter 4).

The adenylate cyclase activity was determined using the method described by Bergmeyer *et al.* (141).

A standard component mixture was prepared by mixing; 250μl 1M Triethanolamine buffer (Merck, Germany), 250μl 100mM $MgCl_2$ solution, 500μl 10mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma Chemical Co., USA) solution, 50μl 10mM cyclic-adenosine monophosphate (cAMP) (Sigma Chemical Co., USA) solution, 50μl 10mM adenosine triphosphate (ATP) (Merck, Germany) solution, 8.2mg creatine-P-$Na_2 \cdot 4H_20$ (Boehringer Mannheim,Germany), 2mg creatine kinase (Boehringer Mannheim, Germany), 5mg bovine serum albumin (BSA) and 400μl water, to give a final volume sufficient for 50 assays.

A 30μl aliquot of the standard component mixture was added to 20μl Tris-HCl buffer (1M) (pH 7.5) and 20μl [α-^{32}P]ATP (Amersham Int. plc., UK). After mixing thoroughly, the tubes were allowed to equilibrate thermally at 37°C for 5 minutes. A 30μl aliqot of the membrane suspensions was added to the tubes, mixed and incubated for a period of one minute. Initially, the reactions were stopped at 15 second intervals by adding 400μl 125mM $ZnCl_2$ solution and 500μl 125mM Na_2CO_3 solution, to determine the optimum reaction time.

The samples were centrifuged for 5 minutes at 10 000g in a Beckman J2-21 centrifuge. After centrifugation, 800μl of the supernatant fluid was transferred to alumina columns which had been prepared by pouring dry neutral alumina (Merck, Germany) into plastic syringes to a height of 2cm. These columns were then washed with 10ml 0.1M Tris-HCl buffer (pH 7.5) before the sample was applied. After the sample had drained, 2ml of the Tris-HCl buffer was applied, followed (after draining) by another 2ml of this buffer. The entire eluent was collected in scintillation vials containing 10ml of Ready Value liquid scintillation cocktail (Beckman Inst. Inc., USA). The columns were regenerated by washing with 10ml of the same Tris-HCl buffer.

For the determination of the blank value of the labelled ATP used, samples containing [α-^{32}P]ATP and denatured adenylate cyclase were prepared. Denatured adenylate cyclase containing membrane preparations were prepared by heating the membranes for 5 minutes at 100°C.

In order to determine the specific activity of [α-^{32}P]ATP added per assay tube, 20μl of the [α-^{32}P]ATP solution was added to a scintillation vial containing 10ml Ready Value liquid scintillation cocktail. The radioactivity present in each eluate was determined by counting in a Beckman LS 3801 scintillation counter.

The adenylate cyclase activity was calculated as follows:

C_p = cpm of the individual sample measured.

C_{bl} = cpm of samples with denatured adenylate cyclase.

R = Recovered fraction of [^{32}P]cAMP in the $ZnCO_3$ supernatant applied to the alumina column.

X = Specific radioactivity of total ATP added per tube (cpm/pmol)

m = Protein added per tube (mg)

t = Incubation time (min)

$$\text{AC activity} = \frac{C_p - C_{bl}}{R \times X \times m \times t} \text{ pmol} \times \text{min}^{-1} \times \text{mg}^{-1} \text{ (U/mg)}$$

6.2.3 Cyclic-AMP Levels:

The stroma fractions were assayed for their protein concentration using the Folin - Lowry assay (91) (as described in Chapter 4).

The samples were heated in a boiling water bath for 5 minutes to coagulate protein. After centrifugation, at 3000g, in a Beckman J2-21 centrifuge, the cAMP in the supernatant was assayed using a Cyclic AMP[^{3}H] assay system (Amersham Int. plc., UK). The tubes, as required, were placed in racks in an ice bath which was kept at 0°C. Fifty microlitres of the samples were added to the appropriate assay tubes. Labelled cAMP (50μl) was added to every assay tube followed by 100μl of the binding protein. All the tubes were vortex mixed for 5 seconds. The ice bath containing the tubes was placed in a cold room at 4°C and left for 2 hours. At least 15 minutes before the end of the incubation time, 20ml of ice cold distilled water was added to the charcoal reagent which was

stirred continuously during use. At the end of the 2 hour incubation period, 100μl of the charcoal suspension was added to each of the tubes. The tubes were centrifuged at 3000g for 4 minutes in a Beckman J2-21 centrifuge. A 200μl aliquot of the supernatant from each tube was removed, without disturbing the sediment, and placed in scintillation vials containing 10ml of Ready Value liquid scintillation cocktail (Beckman Inst. Inc., USA). The radioactivity present in each sample was determined by counting in a Beckman LS 3801 scintillation counter.

The results were calculated as follows:

C_o = cpm bound in the absence of unlabelled cAMP

C_x = cpm bound in the presence of unlabelled cAMP

$\frac{C_o}{C_x}$ was calculated for each level of standard cAMP and the unknowns.

$\frac{C_o}{C_x}$ was plotted against pmoles of inactive cAMP/tube.

From the $\frac{C_o}{C_x}$ value for the sample, the pmoles of inactive cAMP was determined from the standard curve (Appendix 6).

6.2.4 Statistical Analysis:

The results obtained were analyzed using a one-way ANOVA followed by the Student-Newman Keuls Multiple Range Test.

6.3 RESULTS:

6.3.1 Cell Growth:

The results obtained for the effect of zinc on the growth of normal LLCMK cells and malignant B16 cells are reported in Chapter 2.

6.3.2 Adenylate Cyclase Activity:

The results obtained are presented in Figure 6.1.

The adenylate cyclase activity in the control cultures of the LLCMK cells was higher than that determined for the malignant B16 cells.

At a concentration of 1μg/ml added zinc, the activity of the enzyme decreased significantly in both the LLCMK ($p \leq 0.001$) and the B16 cells ($p \leq 0.025$).

In the LLCMK cells, the activity of adenylate cyclase increased slightly with increasing zinc concentrations of 3, 5 and 10μg/ml but, remained significantly lower than the control levels ($p \leq 0.001$ for all concentrations of zinc).

The adenylate cyclase activity in the B16 cells remained essentially at the levels obtained at 1μg/ml zinc for all of the other zinc supplements ($P \leq 0.025$).

6.3.3 Cyclic-AMP Levels:

The results obtained are presented in Figure 6.2.

The B16 control cultures were shown to have significantly lower levels of cAMP than the LLCMK control cultures ($p \leq 0.001$).

The cAMP levels in the B16 cells supplemented with 1μg/ml zinc increased significantly ($p \leq 0.005$) compared to the control cultures. The levels were even higher in those cells treated with 3μg/ml zinc ($p \leq 0.001$) but decreased again in cells supplemented with 5 and 10μg/ml zinc to levels approximating those found in the 1μg/ml zinc supplemented cells ($p \leq 0.005$).

At a concentration of 1μg/ml zinc, the cAMP levels in the LLCMK cells decreased sharply. Similar levels were recorded in those cells treated with 3μg/ml zinc. The concentration of cAMP determined in the cells supplemented with 5 and 10μg/ml zinc showed a highly significant decrease ($p \leq 0.001$) in comparison to those cells treated with 3μg/ml zinc.

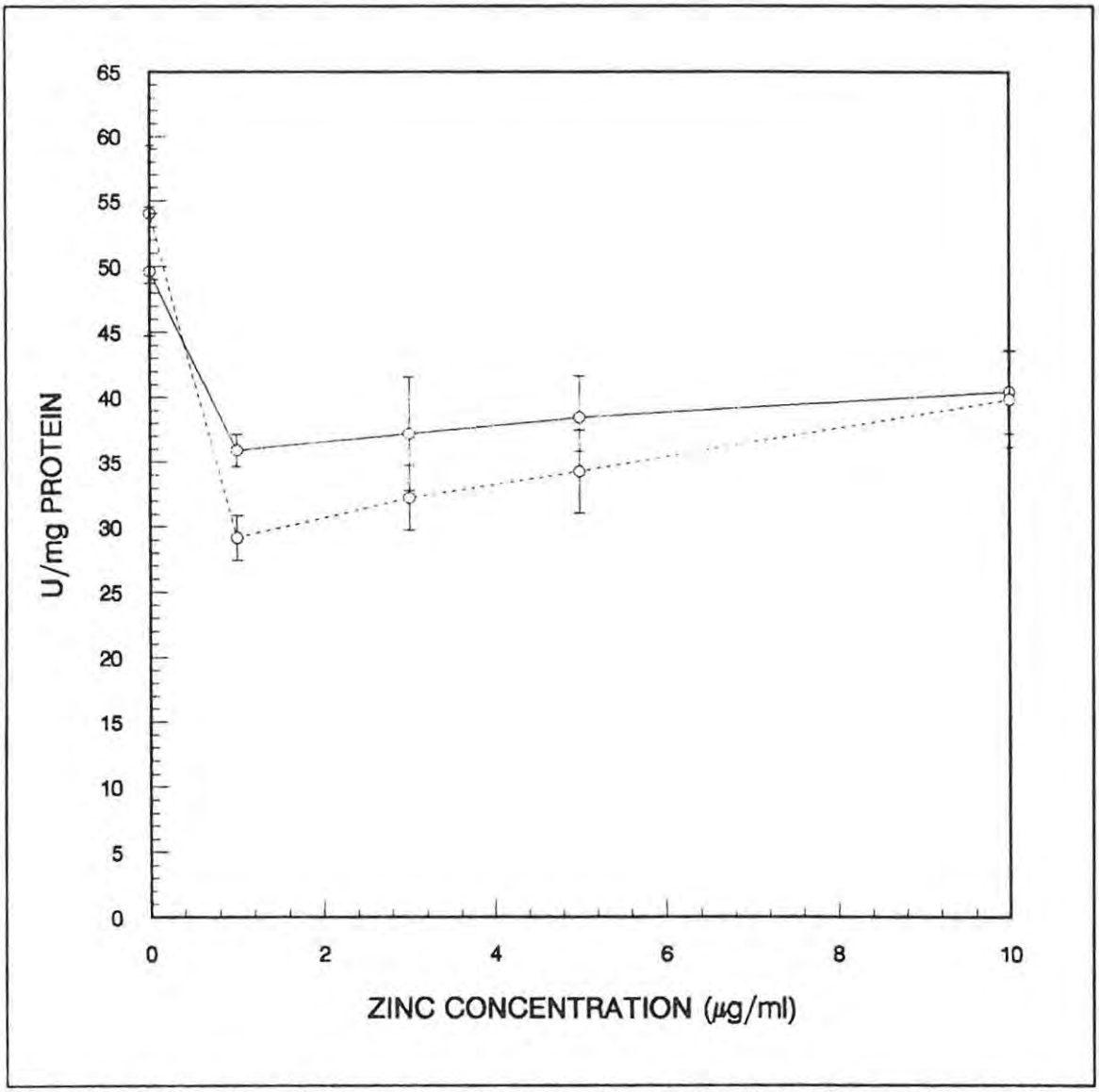

Figure 6.1: The effect of zinc supplementation on the activity of adenylate cyclase in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

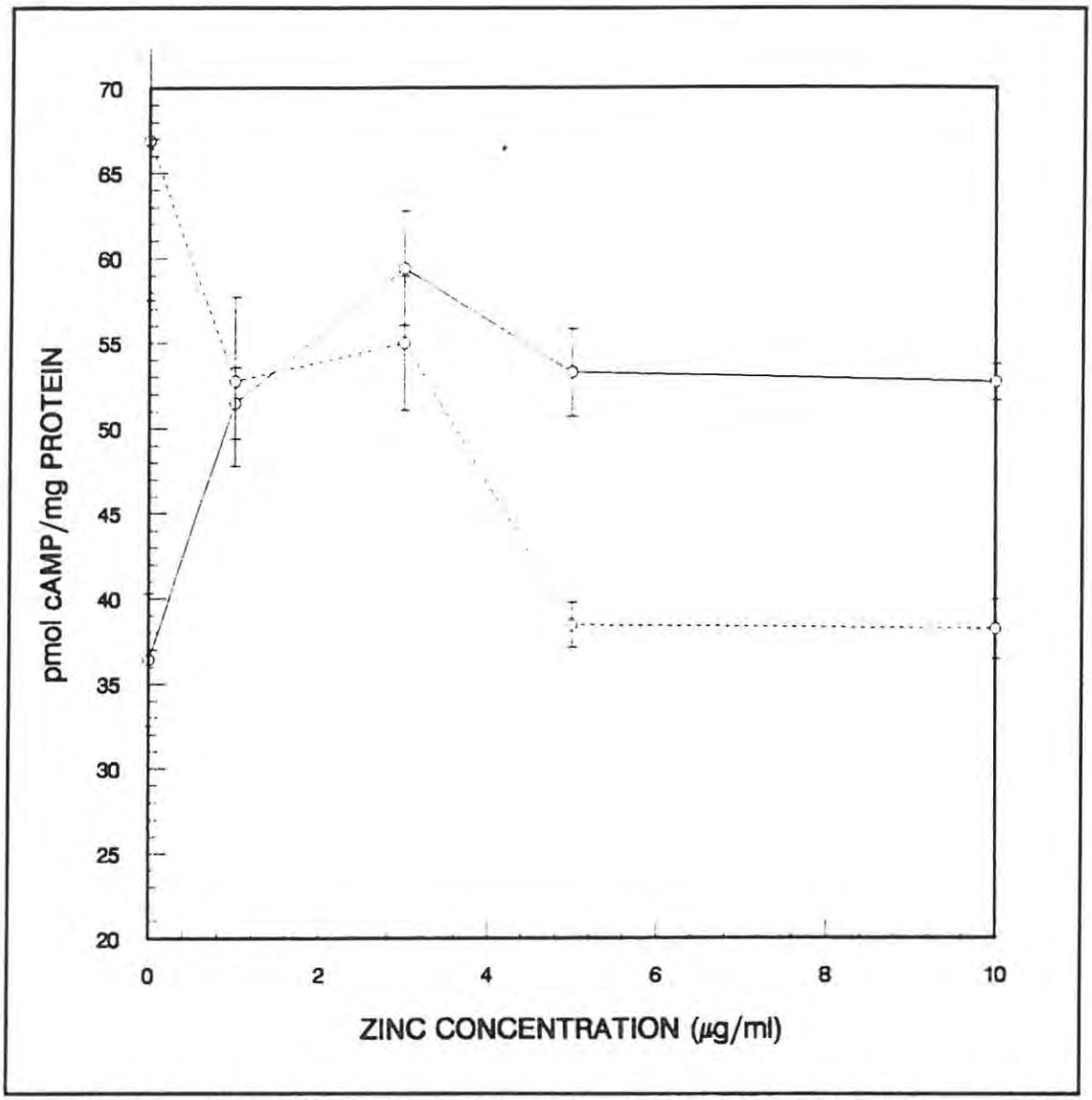

Figure 6.2: The effect of zinc supplementation on the cAMP levels in B16 (–O–) and LLCMK (- O -) cells. Each point represents the mean of five determinations ± SEM.

6.4 DISCUSSION:

6.4.1 Adenylate Cyclase Activity:

The enzyme adenylate cyclase is activated by the prostaglandins (146,147). The higher activity of the enzyme in the LLCMK control cultures when compared to that in the B16 control cultures, may be due to the substantially higher concentrations of PGE_2 noted in the control cultures of the LLCMK cells than in the B16 cells (Chapter 5).

Although the PGE_2 levels increased significantly in both cell types when supplemented with 1μg/ml zinc, the adenylate cyclase activity decreased significantly in the 1μg/ml zinc supplemented cells. This indicates that despite stimulatory levels of PGE_2, zinc has an inhibitory effect on the activity of adenylate cyclase. This enzyme inhibition was noted for all concentrations of zinc supplementation.

Haesungcharen *et al.* (148) have found that the metal ion zinc showed pronounced inhibition of adenylate cyclase from human spermatozoa. Other researchers have also reported that zinc inhibits the activity of adenylate cyclase in the human liver and the rat brain (149,150).

6.4.2 Cyclic-AMP Levels:

The concentration of cAMP determined in the LLCMK control cultures was markedly higher than the cAMP levels obtained for the B16 control cultures. Malignant cells have been shown to have lower levels of cAMP than normal cells by a number of researchers (136-140).

The cAMP concentration in the LLCMK cells supplemented with 1μg/ml zinc decreased significantly in comparison to the control cultures. Similar levels were obtained in those cells supplemented with

3μg/ml zinc. With 5μg/ml zinc supplementation, the cAMP levels decreased further. These levels remained constant in the 10μg/ml zinc supplemented cells. The decrease in cAMP levels at zinc concentrations of 1 and 3μg/ml can be correlated to the inhibition of the adenylate cyclase by zinc. The further decrease in the cAMP concentrations with 5 and 10μg/ml zinc may be as a result of the zinc-induced inhibition of the enzyme as well as the decrease in PGE_2 levels in these cultures when compared to those in the cultures supplemented with 1 and 3μg/ml zinc (Chapter 5).

In the case of the B16 cells, despite the decrease in adenylate cyclase activity, the concentration of cAMP increased significantly in the cultures supplemented with 1 and 3μg/ml zinc. The results obtained at zinc concentrations of 1, 3 and 5μg/ml mimic the trend obtained for the PGE_2 levels at these concentrations. The evidence that zinc inhibits the activity of adenylate cyclase and that PGE_2 is therefore not acting through this enzyme, leads to the suggestion that PGE_2 may be acting by inhibiting the enzyme cAMP phosphodiesterase, which is responsible for the catabolism of cAMP. This is in contrast to reports by Chlapowski (145) who claimed that PDE activity increased in cells incubated with PGE_1 due to increased intracellular levels of cAMP as a consequence of the PGE activating adenylate cyclase. However, the action of PG's on PDE has not been studied *per se*. Paoletti *et al.* (cited in 116) suggests that PG's may be exerting some of their effect on cAMP accumulation indirectly through inhibiting PDE activity. In addition to this proposed effect of PG's on PDE, zinc has been shown to be a potent inhibitor of PDE activity (141). The activity of the enzyme PDE in response to PG's and zinc supplementation in the LLCMK and B16 cell lines would have to be studied in order to obtain conclusive evidence regarding these proposals.

CHAPTER 7: GENERAL DISCUSSION AND CONCLUSION.

The treatment of metastatic cancer with chemotherapeutic drugs has always presented problems owing to the non-specificity of the drugs used. Researchers are therefore continuing to search for agents which are selectively toxic to tumour cells. Some of the attention has been focused at nutritional compounds which are not toxic to normal cells, even in high doses, but which interfere with the proliferation of cells. Zinc has been determined to be essential for cell growth, and experiments have shown that both a zinc deficiency and zinc supplementation can result in a reduction in cell proliferation. Owing to the severe effects associated with zinc deficiency, this study has been directed towards the effect of zinc supplementation on the cell growth of malignant murine melanoma (B16) cells and non-malignant monkey kidney (LLCMK) cells. In addition, certain metabolic responses to zinc supplementation were studied.

The effect that zinc supplementation was found to have on the proliferation of both cell types used in this study, supported the findings of other researchers. Zinc had a cytotoxic effect at all concentrations used in the B16 cells while being toxic only at a concentration of 10μg/ml in the case of the LLCMK cells. This selective toxicity of zinc at low concentrations towards malignant B16 cells may, therefore, be indicative of its value in the treatment of cancer.

It has been suggested that cell proliferation can be regulated by cAMP levels mediated through the activation of the enzyme adenylate cyclase by the prostaglandins which are in turn synthesised from precursor fatty acids (pathway outlined in Figure 1.1). The present study therefore investigated the metabolic responses of zinc supplementation in the ω6-fatty acid pathway which leads to the synthesis of the 1- and 2-series prostaglandins.

A study of the EFA composition of the membrane and stroma fractions indicated that zinc supplementation may result in the mobilisation of the EFA's from membrane phospholipid stores. An investigation of the Δ^6-desaturase enzyme suggests that zinc may also play a role in the enzymes' activity, particularly at high concentrations. In estimating the activity of the elongase and Δ^5-desaturase enzymes, it was concluded that zinc had little or no effect on the activity of these enzymes. Zinc supplementation has been shown to facilitate the uptake of [1-^{14}C]LA (94) and further investigations should be carried out in order to ascertain whether the above observations are possibly due to an increase in the uptake of LA and the other EFA's in the presence of zinc rather than an effect on EFA synthesis and/or mobilisation.

Zinc is considered to have "anti-oxidant" properties through its requirement in the activity of superoxide dismutase. Unsaturated fatty acids have been shown to augment free radical generation and the initiation of lipid peroxidation (103,113). The levels of certain fatty acids were found to increase with zinc supplementation. For this reason, the effect of zinc on the formation of free radicals and levels of lipid peroxidation was studied. The B16 cells showed a dose-dependent increase in free radical formation with an increase in zinc supplementation. No effect was observed in the LLCMK cells. The increase in free radical formation found in the B16 cells may be due to reduced CuZn SOD in the malignant cells. The observed increase of PUFA's in the cells may also account for some of the free radical formation.

The LLCMK cells were found to show higher levels of lipid peroxidation than the B16 cells. The rate-limiting step of lipid peroxidation is substrate availability (93) and the B16 cells were found to have lower levels of PUFA's than the non-malignant LLCMK cells. The increase in the levels of the lipid peroxidation in both cell types, may be due to an increase in substrate availability and increased free radical generation with zinc supplementation.

Fatty acid oxidation, catalysed by cyclo-oxygenase, is the initial rate-limiting step in the conversion of fatty acids to PG's. It has been suggested that peroxides are required to trigger its activity. The increase in cyclo-oxygenase activity found in both the LLCMK and the B16 cells may be as a result of the increased lipid peroxidation in the cells in the presence of elevated levels of the PUFA's, with increasing zinc supplementation.

The levels of PGE_2 determined in both cell types indicate that PGE_2 synthesis is directly dependent on the levels of AA in the cells available for conversion to the PG's by cyclo-oxygenase.

It has been suggested that the PG's are responsible for the activation of the adenylate cyclase enzyme which catalyses the conversion of ATP to cAMP. A study of the activity of the adenylate cyclase enzyme showed that zinc inhibited its activity at all the concentrations used. This effect has also been observed by other researchers (150-152).

The inhibition of adenylate cyclase by zinc resulted in a decrease in cAMP levels at low concentrations of zinc in the LLCMK cells. A further decrease in the cAMP levels at 5μg/ml zinc, which corresponded to a similar decrease in PGE_2 levels, and the fact that the cAMP levels in the B16 cells mimic the trend observed for the PGE_2 levels, suggests that the PG's are still responsible for producing an effect on the cAMP levels. It is therefore possible that the PG's may exert part of their effect on cAMP accumulation indirectly through inhibiting PDE activity. In addition, zinc has been found to be a potent inhibitor of PDE activity (142). The activity of the enzyme PDE, in response to PG's and zinc supplementation would have to be studied in order to obtain conclusive evidence regarding these proposals.

From the result obtained in this study, it would appear that zinc may exert its effect on cell proliferation at two possible areas in the metabolic pathway examined. Firstly, zinc together with a

possible additional effect of the PG's may result in the inhibition of the enzyme PDE preventing the catabolism of cAMP to 5´AMP. However, due to the inhibition of adenylate cyclase by zinc, the levels of cAMP are unlikely to be elevated to the point that this would result in a significant effect on cell proliferation.

It is therefore proposed that zinc may exert its effect on cell growth through increasing the substrate available for lipid peroxidation by possibly increasing the uptake of the PUFA's, mobilising GLA and DGLA from the phospholipid store and increasing Δ^6-desaturase activity. Lipid peroxidation is thought to result in the formation of hydroperoxy fatty acids (HPETE's) from AA. HPETE's are not directly responsible for the cell death observed but appear as precursors to toxic secondary degradation products (82,111).

In addition, the transition metals; iron and copper have been found to catalyze HPETE decomposition and to accelerate the rate of destruction of the cells supplemented with PUFA's (111,152,153). It is therefore possible that zinc, which is also a transition metal, may have a similar mode of action.

In conclusion, it is therefore possible that zinc may exert its effect via the ω6-fatty acid metabolic pathway but not as a consequence of PG's exerting their action through the cyclic nucleotide mechanism. Instead, it would appear that the elevated levels of lipid peroxides may result in the formation of HPETE's from AA. These in turn are degraded to toxic secondary peroxidation products, possibly catalyzed by zinc. It is these degradation products which are likely to be toxic to the cells resulting in cellular death. The pathway outlined in Figure 7.1 is therefore given as a possible alternative to that of Figure 1.1.

Further studies on the effect of zinc supplementation on the generation of secondary products such as HPETE's in tumour cells are required in order to substantiate the above suggestion.

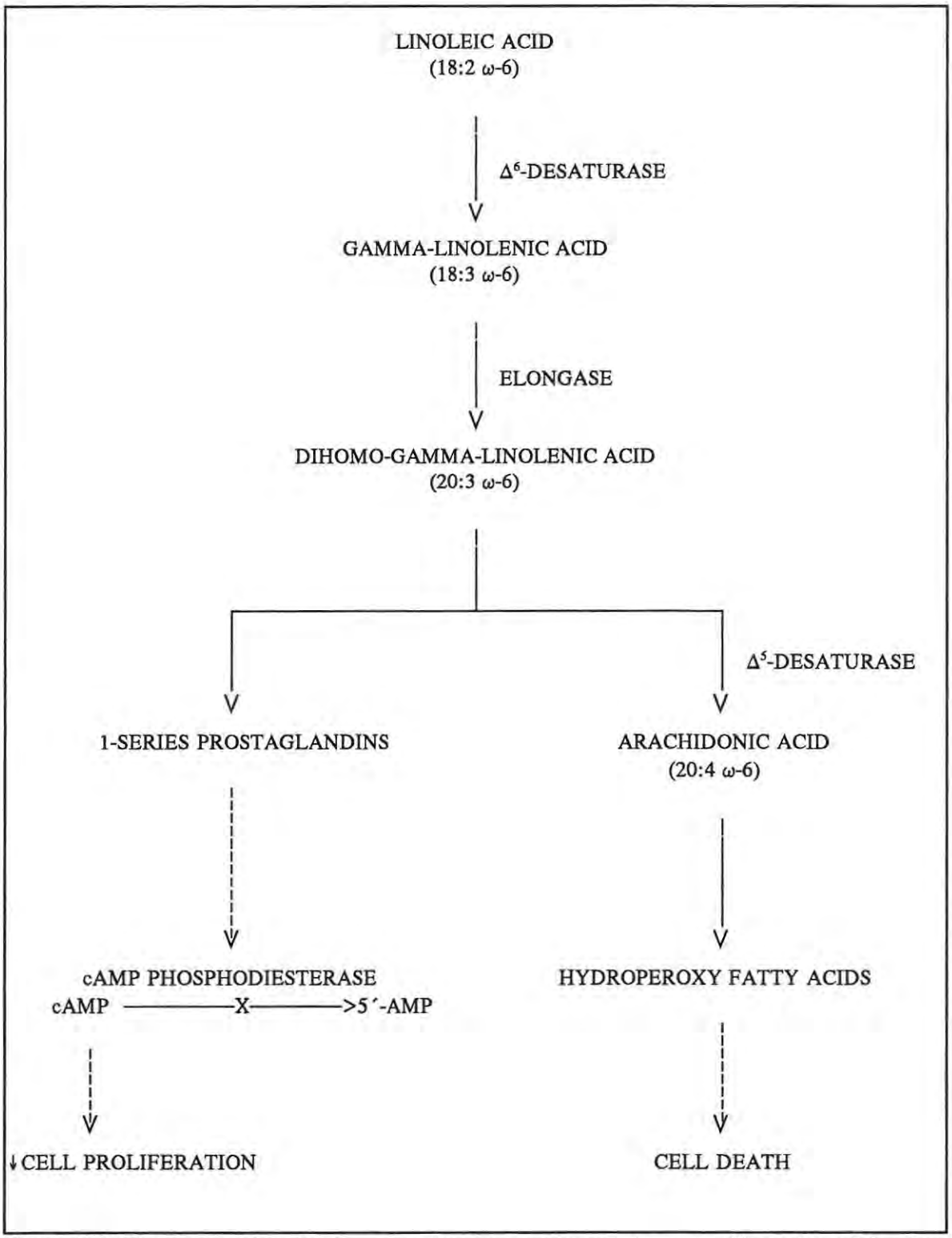

Figure 7.1: The proposed mechanism whereby zinc supplementation may affect cell proliferation.
————> Metabolic pathway
- - - - - - > Suggested pathway

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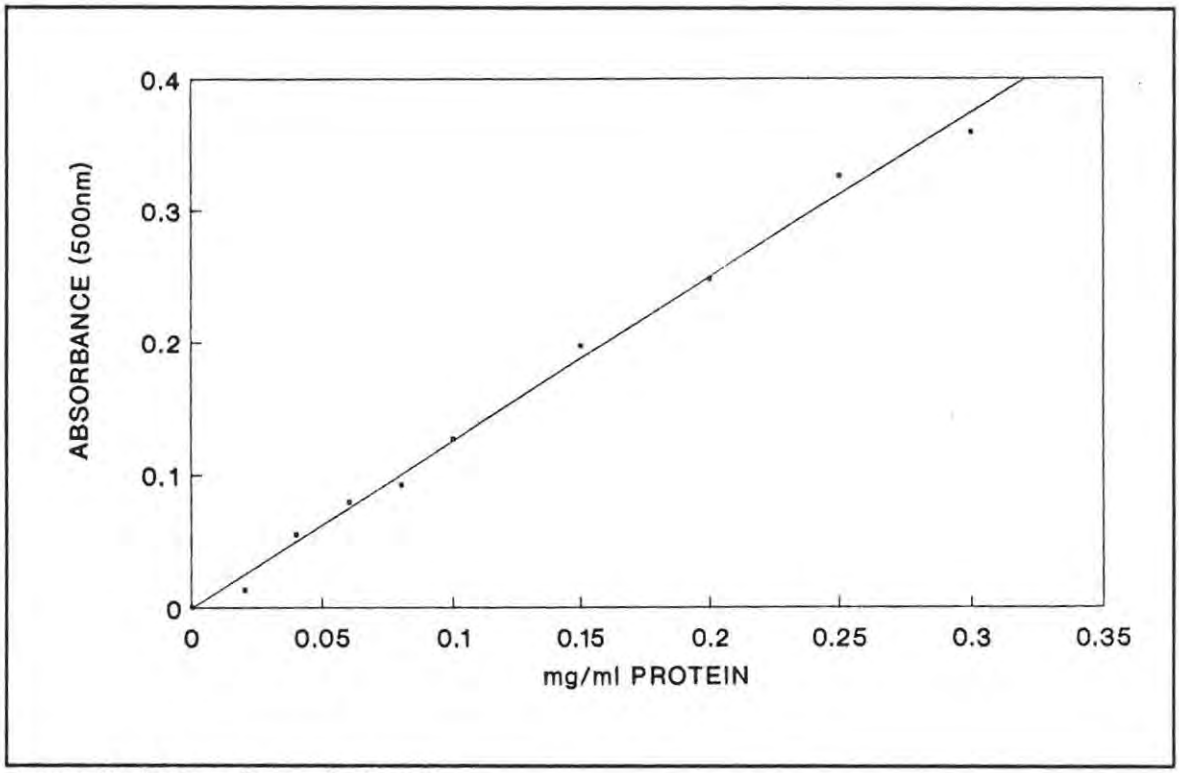
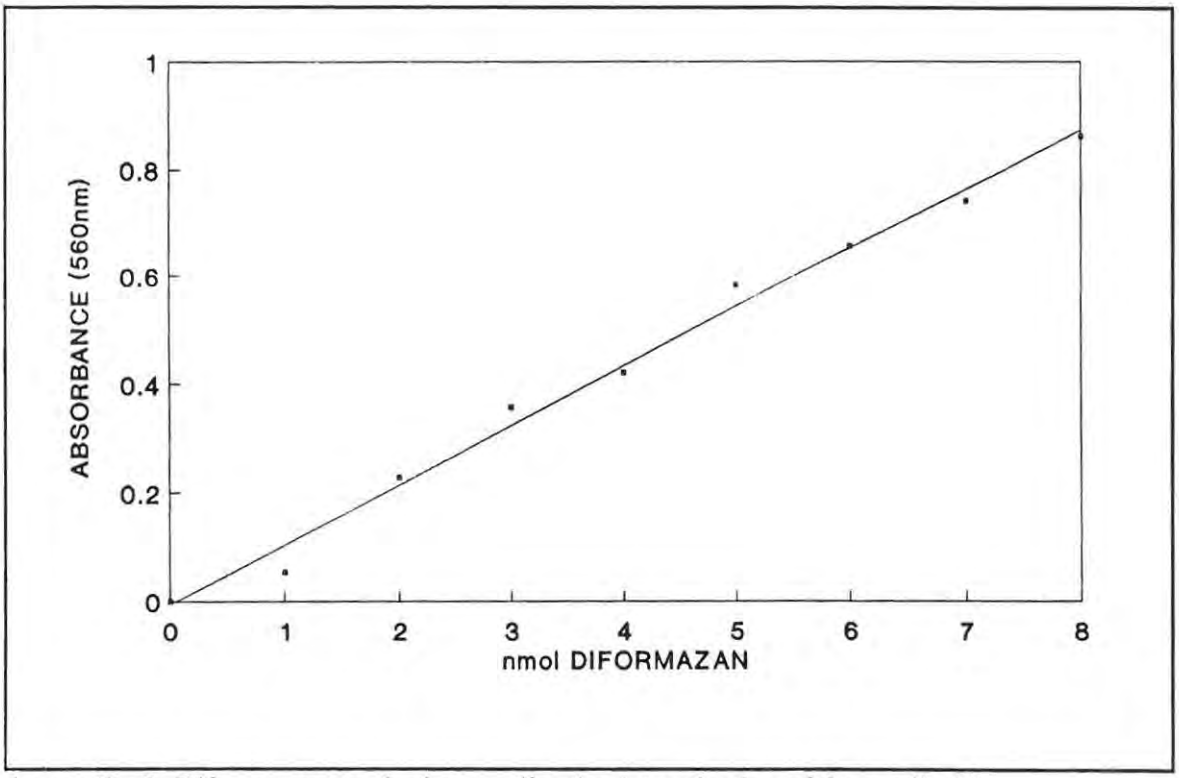
APPENDIX 1: Programme used for the Gas - Liquid Chromatograph in assaying for the essential fatty acid composition of the membrane and stroma fractions of the B16 and LLCMK cells.

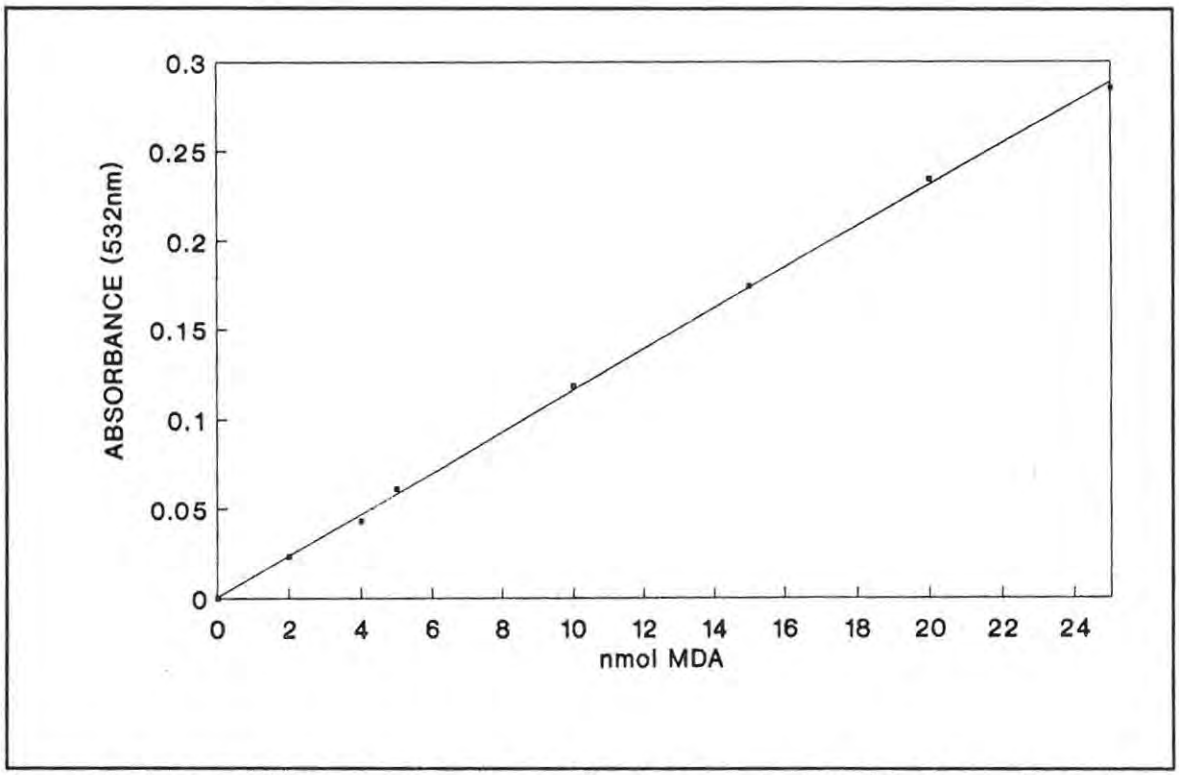
Initial temperature	: 130°C
Initial time	: 15.00 minutes
Rate	: 4°/minute
Final temperature	: 220°C
Final time	: 7.5
Injector B temperature	: 220°C
Detector A temperature	: 220°C
Oven maximum	: 250°C
Signal	: ± 20
Flow B	: ON
Purge B	: ON
Column head pressure	: 64kPa



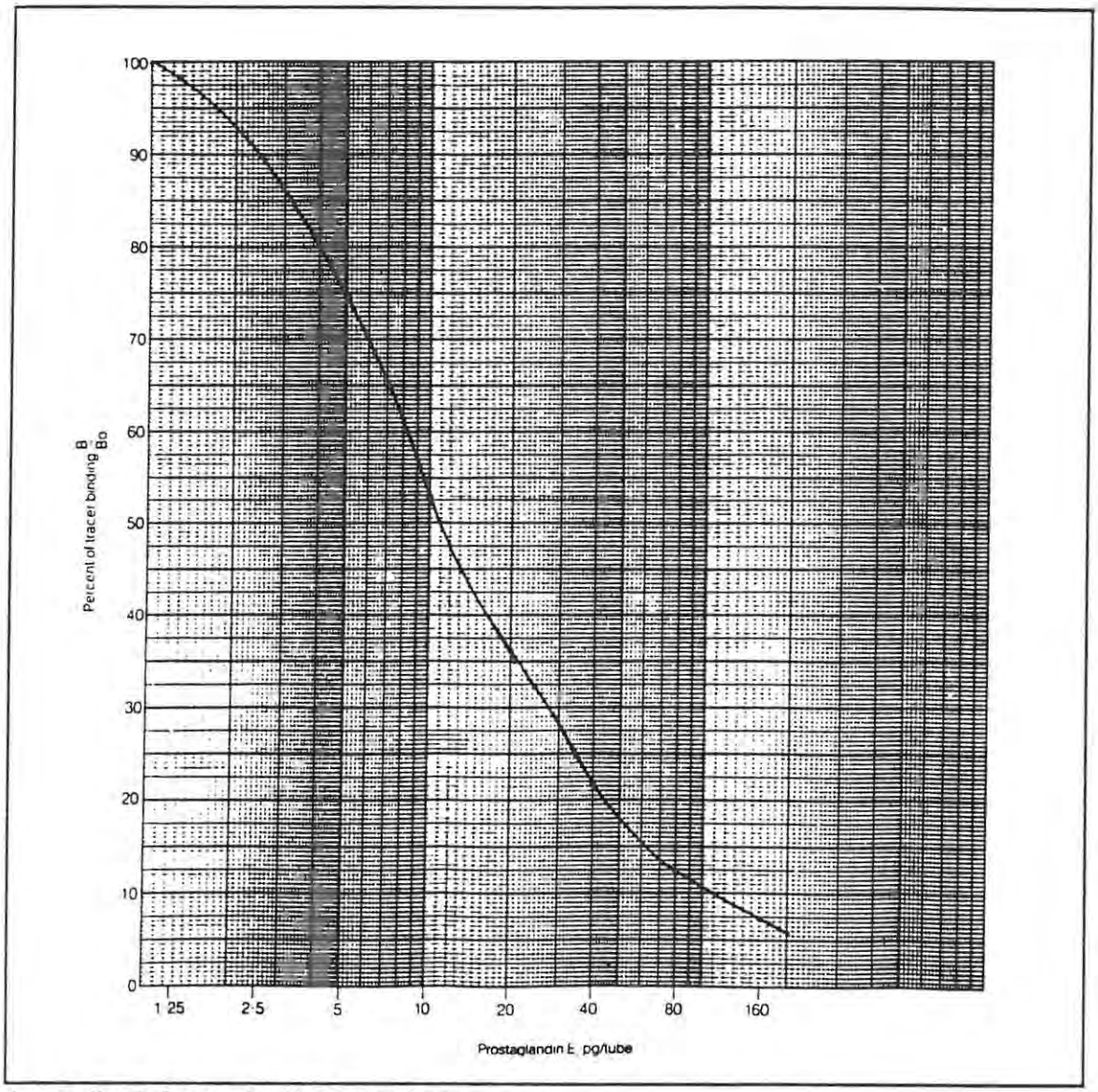
Appendix 2: Protein standard curve.



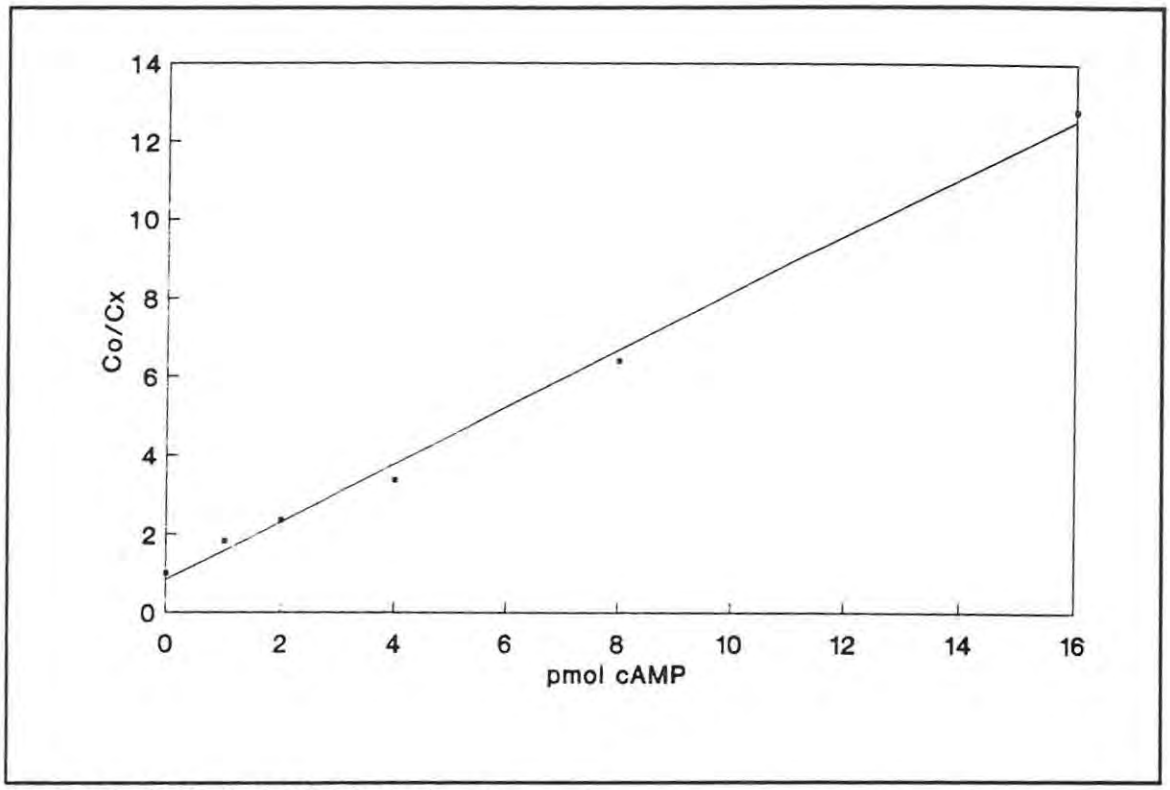
Appendix 3: Diformazan standard curve (for the determination of free radicals).



Appendix 4: Malondialdehyde standard curve (for the determination of lipid peroxidation).



Appendix 5: Prostaglandin E_2 standard curve.



Appendix 6: Cyclic-AMP standard curve.