NUTRIENT SUPPLEMENTATION AND SECONDARY METABOLITES IN MELANOMA CELLS

THESIS

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ABSTRACT

Considerable interest exists with regard to the putative therapeutic role of ascorbic acid in various conditions. A condition which has received much attention is cancer, as it is reported that ascorbic acid may be a prophylactic against cancer development. However, the actual involvement of ascorbic acid, an oxidizing/reducing agent, in the development and progression of tumours is presently a subject of much speculation.

This study initially addressed the effect of ascorbic acid supplementation over a nutritional concentration range (0 - 100 μg/ml) on the *in vitro* growth of non-malignant LLCMK and malignant B16 cells. Ascorbic acid supplementation of these two cell types resulted in an overall decrease in the growth of both types of cells. The actual inhibitory mechanism of ascorbic acid on cell growth was not clear. Further study attempted to define and explain a mechanism responsible for this effect.

Ascorbic acid has a role in the maintenance of tissue integrity and host defences, thus providing a rational basis for examining its relationship to cancer. Ascorbic acid is known to be essential for the structural integrity of the intercellular matrix of the cells, the latter being a complex aqueous gel containing, amongst other compounds, fats and prostaglandins. Fats and prostaglandins have diverse effects on membrane stability, enzyme activity and secondary messengers within cells. Hence, this study investigated the effect of ascorbic acid supplementation on certain enzymes and secondary metabolites within the cells, which had the potential to be involved in the control of cell growth.

Throughout this study, emphasis was placed on the B16 melanoma cells as ascorbic acid supplementation did not significantly affect levels of secondary metabolites within the non-malignant LLCMK cells.

Ascorbic acid supplementation of the B16 cells resulted in significant increases in adenylate cyclase activity and cyclic adenosine monophosphate levels, with a significant decrease in B16 cell growth in that particular experiment. As cyclic adenosine monophosphate has a regulatory role in the cell cycle, this study suggested that the inhibitory effect of ascorbic acid supplementation on cell growth was mediated through a final effect provided by the second messenger, cyclic adenosine monophosphate. However, clarification of the mechanism of the effect of ascorbic acid on adenylate cyclase activity was required.

Hence, a further study investigated prostaglandin E_2 levels, as these affect adenylate cyclase activity. Prostaglandin E_2 levels were also found to be inversely related to B16 cell growth with ascorbic acid supplementation. It thus appeared that adenylate cyclase activity was dependent on prostaglandin E_2 levels in the B16 cells, and further study showed that this was indeed the case. Here, higher levels of prostaglandin E_2 supplementation of the B16 cells inhibited cell growth significantly and also significantly increased adenylate cyclase activity.

Arachidonic acid is the precursor of prostaglandin E_2. In the presence of ascorbic acid supplementation, the percentage arachidonic acid composition of the B16 cells was inversely correlated with cell growth. Hence, prostaglandin E_2 levels in ascorbic acid supplemented B16 cells appeared dependent on the amount of precursor present. This was confirmed when B16 cells were supplemented with arachidonic acid. The latter had an inhibitory effect on B16 cell growth and also stimulated prostaglandin E_2 production.

The cause of the inverse relationship between B16 cell growth and arachidonic acid composition with ascorbic acid supplementation was further investigated and found to be dependent on the uptake of arachidonic acid and other essential fatty acids from the medium. The enzymes phospholipase A_2, delta-5 and delta-6-desaturase, and elongase which could influence arachidonic acid levels were not

affected to any extent by ascorbic acid supplementation and therefore did not influence the inverse relationship between B16 cell growth and arachidonic acid.

Hence, it can be concluded that the effect of ascorbic acid supplementation on the B16 cells is mediated, in part at least, by cyclic adenosine monophosphate. However, this is not the result of a direct effect of ascorbic acid supplementation. The initial effect of ascorbic acid supplementation concerns fatty acid - in particular arachidonic acid - uptake from the medium, with subsequent cascade effects on secondary metabolites, ultimately affecting the cellular levels of cyclic adenosine monophosphate.

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ABBREVIATIONS

AA	Arachidonic acid
AC	Adenylate cyclase
AMP	5′ Adenosine monophosphate
ANOVA	Analysis of variance
Asc	Ascorbic acid
ATP	Adenosine triphosphate
cAMP	Cyclic adenosine monophosphate
CDR	Calcium-dependent regulatory protein
DAG	Diacylglycerol
DGLA	Dihomo-gamma-linolenic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EFA	Essential fatty acid
ER	Endoplasmic reticulum
FA	Fatty acid
FCS	Foetal calf serum
G_1	Gap-1 phase
G_2	Gap-2 phase
G_i	Inhibitory guanine-nucleotide binding regulatory protein
G_s	Stimulatory guanine-nucleotide binding regulatory protein
GAG	Glycoaminoglycans
GDP	Guanosine diphosphate
GLA	Gamma-linolenic acid

GLC	Gas-liquid chromatography
GTP	Guanosine triphosphate
kDa	Kilodalton
LA	Linoleic acid
LLCMK	Monkey kidney cells
MEM	Minimum essential medium
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
PBS	Phosphate buffered saline
PDE	Phosphodiesterase
PG	Prostaglandin
PGE_1	Prostaglandin E_1
PGE_2	Prostaglandin E_2
PLA_2	Phospholipase A_2
PLAP	Phospholipase A_2 activating protein
PLC	Phospholipase C
PUFA	Polyunsaturated fatty acid
R_i	Inhibitory receptor
R_s	Stimulatory receptor
SEM	Standard error of the mean
TLC	Thin layer chromatography

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LITERATURE REVIEW

1.1 CANCER

Cancer is the uncontrolled growth of abnormal cells. The transformation of a normal cell into a neoplastic cell or abnormal cell is considered to proceed through three phases: initiation, promotion and progression (see Figure 1) (1,2,3,4,5).

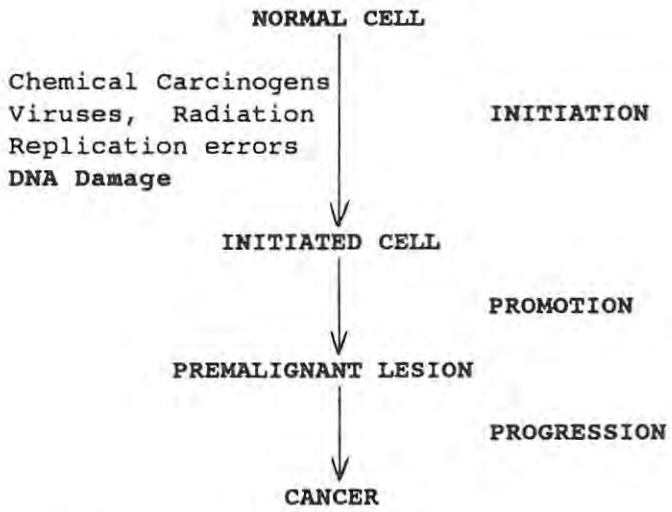


FIGURE 1: A multistep carcinogenesis model (1)

1.1.1 INITIATION

Initiation is the permanent alteration of the genetic information in the cell, *ie.* it is an irreversible step (6). Various stimuli lead to mutations in the cell deoxyribonucleic acid (DNA), but these mutations do not necessarily lead to cancer. They are merely an indication of the increased risk of neoplasia (1,6).

Ultraviolet light irradiation, X-rays and chemical carcinogens binding to DNA are stimuli that can

result in these irreversible mutations in the DNA (2).

1.1.2 PROMOTION

This is a reversible step and can be a very slow process (1,5). If the exposure of the promotor, *eg.* a chemical carcinogen, ceases before the cells gain the ability to multiply in its absence, then tumour formation can be avoided.

However, promotion can be carried to a stage at which the formation of premalignant tumours is inevitable (1,2). Premalignant tumours or lesions are defined by pathologists as the abnormal growth of tissue with altered cell nuclei (1). Exposure to chemical, environmental and viral carcinogens can result in the promotion of a normal cell to a tumour cell (3,4,7), due to damage done to the DNA of a critical target gene in a particular cell (8).

The mediators in this conversion are believed to be the oncogenes (9). Oncogenes, the existence of which was first postulated in the 1970s, are genes that are contained in normal cells. When activated by mutation or translocation, they contribute to tumour growth (8,9,10,11). In their natural state, the genes are called proto-oncogenes, with their expression being an important part of normal cell activity (8,12).

Cancer, in respect of oncogenes, is a problem of mutation and selection. The selective force, for example, could be the ability of cells to grow at a greater rate, *ie.* allowing for various mutations of proto-oncogenes into oncogenes, with the latter now the driving force for increased cell proliferation (11). Many types of oncogenes exist, *eg.* viral oncogenes, cellular oncogenes, etc. Within the different types of oncogenes, many different classes exist (9,11). Oncogenes, however, have been detected in only 15% to 20% of human tumours (8).

There is evidence (8,11,12) that anti-oncogenes exist, *ie.* growth suppressor genes. Evidence for this, is that a tumour cell often becomes aberrant through the loss of a critical growth-suppressing

gene or genes. If fusion of a tumour cell with a normal cell occurs, the tumour cell regains a growth-regulating gene it lost earlier in its evolution towards malignancy and growth control can be re-imposed on a cell that has temporarily lacked such control (8).

1.1.3 PROGRESSION

The concept of tumour progression has been distinguished from tumour promotion. The term progression usually refers to the multiple steps involved in the transition of the initiated or premalignant cell into a malignant neoplasm (2).

Groups of abnormally proliferating cells can arise in any part of the body. Those that do not invade surrounding tissue but that remain strictly local growths, are called benign tumours. The spreading of tumours from their site of origin or primary site, through the bloodstream and lymphatic system, results in secondary growths of malignant tumours (4). Tumours are lethal when they spread from their site of origin, a process known as metastasis (10).

In order for a tumour to form a secondary growth in a distant organ, its cells must not only enter the blood stream or lymphatic system by direct invasion from the primary site, but they must also survive and multiply in a relatively alien environment. An important fact is that tumour cells undergoing metastasis do not spread randomly (11), *eg*. tumours of the lung tend to colonize the brain and adrenal glands.

To conclude, with regard to the above three steps of the cancer process, it can be stated that cancer represents a fundamental problem in cellular behaviour, with many aspects of molecular biology being relevant to this behaviour and hence needing elucidation.

1.1.4 CHEMOPREVENTION

Chemoprevention, the use of non-toxic agents to block, arrest or reverse the development of

neoplasia, is becoming very important in cancer research (3). Substances are being sought that can block the initiation of a cell by a carcinogen, prevent the formation of a tumour subsequent to cell initiation or help elucidate the events that are important in the progression of an initiated cell (1,3,7).

Benedict *et al* (3) believe that ascorbic acid (Asc) may be able to play an important role in chemoprevention, as well as in isolating events necessary for oncogenic progression. Asc has been considered to be of possible chemopreventative use, mainly as a chemical to block the initiation of a cell (13), *eg*. it can either block nitrosamine formation or be involved in the immune response.

Since Asc and its role in the neoplastic process is the subject of this thesis, it is important to consider the general role of Asc in cell metabolism.

1.2 ASCORBIC ACID

1.2.1 HISTORY

Micronutrients, which include vitamins, major minerals and some trace elements, are dietary components essential to normal metabolic function (14). The term "essential" implies that the compound has to be acquired through the diet, as the body does not synthesize it. Asc, commonly known as Vitamin C, is a water-soluble vitamin and is increasingly being recognized as an agent with broad biological function and importance.

Asc is essential to the diet of man, other primates, the guinea pig, an Indian fruit-eating bat, the red-vented bulbul and some related species of Passeriform birds (cited by 15). All other species of animals synthesize their own Asc (cited by 15), as they do not lack the last enzyme in the Asc biosynthetic pathway, namely L-gulono-γ-lactone oxidase (EC 1.1.3.8) (16). It is estimated that primates lost the gene for this enzyme 70 million years ago (17).

Sailors, undertaking long voyages in the 15th to 19th centuries, experienced a defiency of Asc in their diet due to the lack of fresh fruit and vegetables (13,18). This condition was diagnosed as scurvy. In the mid 18th century, James Lind demonstrated that the juice of fresh citrus fruits cured the condition. The active agent in such juice is the enolic form of 3-keto-L-gulofuranlactone or hexuronic acid, later named ascorbic acid or Vitamin C. It was isolated from oranges, cabbages and adrenal glands in 1928 by Albert Szent-Gyorgyi. By the mid 1930s, the substance became widely available at low cost, as methods of synthesis were devised (13,18,19,20). Today, vitamin supplements have flooded much of society via the pharmaceutical industry.

1.2.2 STRUCTURE AND METABOLISM OF ASCORBIC ACID

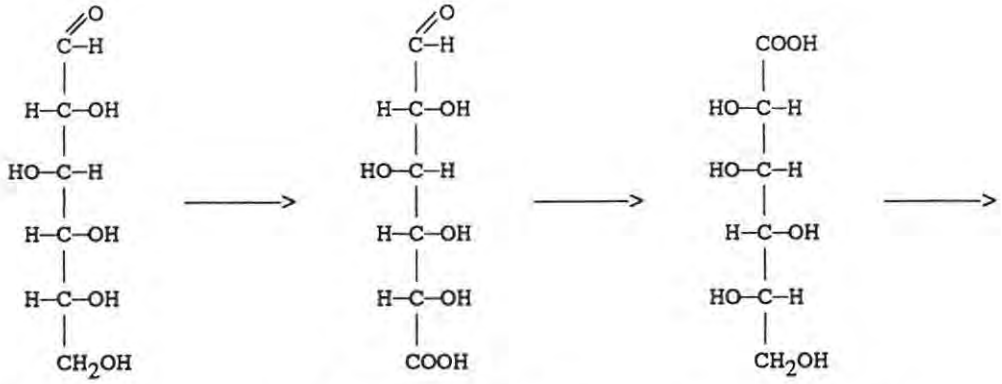
L-Asc-synthesizing mammals have L-gulono-γ-lactone oxidase present in the liver (17). In these animals, Asc is synthesized from glucose as depicted in Figure 2. However, in man, due to the absence of the enzyme L-gulono-γ-lactone oxidase, the oxidation of L-gulono-γ-lactone to L-Asc cannot occur (17,18).

Asc can act either as a reducing or an oxidizing agent, depending on the circumstances of the reaction. This is a result of its ability to exist as Asc (its hydrogenated form) or as dehydroascorbate (4,21). The latter form (see Figure 3) also has antiscorbutic activity (18). Both Asc and dehydroascorbate are excreted in the urine as oxalic acid (18,22).

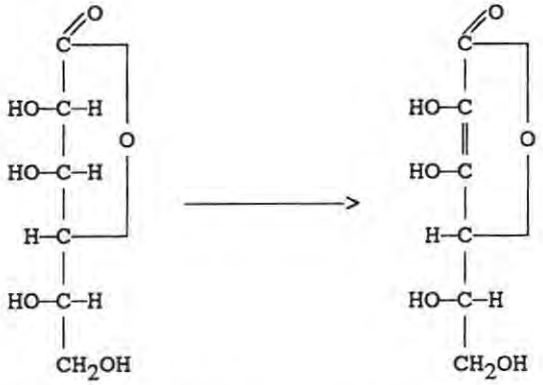
Chemical properties of Asc include high solubility in H_2O; insolubility in fat solvents; sensitivity to O_2 (especially in alkaline solutions); the absence of N; and the presence of a carboxyl group.

1.2.3 FUNCTIONS OF ASCORBIC ACID

Due to its strong oxidizing and reducing properties, Asc is believed to be involved in many biochemical processes within the body, but uncertainty exists regarding the levels of intracellular Asc. It is suggested that such concentrations could be as high as several millimolar (23,24). In

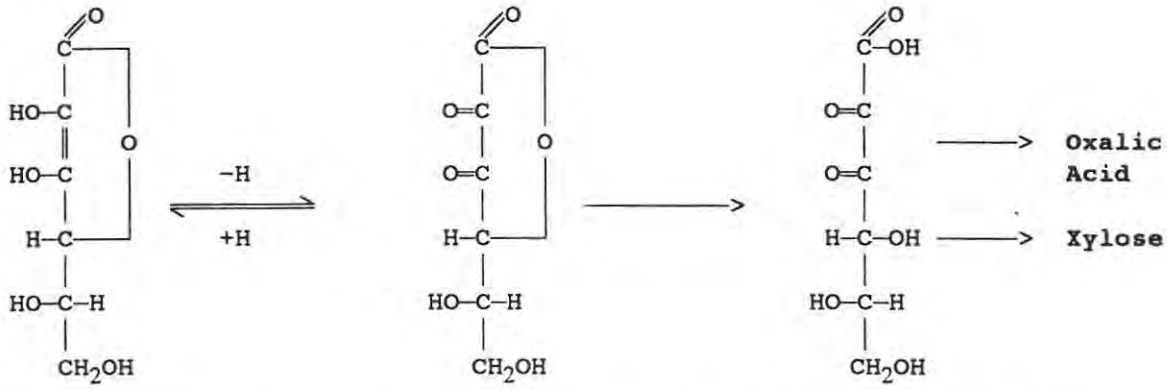


D-Glucose **D-Glucuronic acid** **L-Gulonic acid**



L-Gulono-γ-lactone **L-Ascorbic acid**

FIGURE 2: The metabolic pathway of L-ascorbic acid biosynthesis in animals



L-Ascorbic acid **Dehydro-L-ascorbic acid** **2,3 Diketo-L-gulonic acid**

FIGURE 3: Ascorbic acid metabolism in humans

man, the highest concentrations occur in the adrenals, ovaries, brain, pituitary gland, liver, spleen, blood cells and extracellular fluid surrounding the lung and eye (21,25).

1.2.3.1 Ascorbic acid in enzymatic reactions

Reactions specifically involving Asc are hydroxylations using molecular O_2. These reactions often require Fe^{2+} or Cu^{2+} as a cofactor (18), *eg.* dioxygenases containing prosthetic ferrous ions and monooxygenases with prosthetic cuprous ions (16). These enzymatic reactions are vital to many biochemical processes, for example in collagen synthesis (13,26).

Asc is thought to play either of two roles in these reactions:

(1) as a source of electrons for the reduction of O_2; *ie.* as a cosubstrate (see Figure 4), or

(2) as a protective agent, in that it provides electrons to keep prosthetic metal ions in the reduced form (16,18).

ENZYME-Fe^{3+} + ASCORBATE ——> ENZYME-Fe^{2+} + DEHYDROASCORBATE

FIGURE 4: The suggested role of ascorbic acid as a cosubstrate in an enzyme reaction

1.2.3.2 Ascorbic acid in the extracellular and intercellular matrix, and collagen biosynthesis

Severe Asc deficiency leads to the widespread disruption of collagen synthesis (13,16,18,26). The latter is an elaborate process of protein synthesis, posttranslational modification, protein secretion and extracellular matrix formation.

Collagen is a unique animal protein. Up to one third of its amino acid residues are glycine, with the remaining amino acids comprising an abundance of 4-hydroxyproline, a few residues of 3-hydroxyproline and hydroxylysine. The respective hydroxylases catalyzing the hydroxylation of these amino acids, require Asc for maximum activity (as described in 1.2.3.1). Failure of the

hydroxylation of proline and lysine is thought to be partially responsible for the disruption of the initial stage of collagen synthesis, which yields procollagen (4,16,18). Asc is also found to stimulate the secretion of procollagen from the endoplasmic reticulum (ER) for the formation of the extracellular matrix. In the extracellular matrix, procollagen is cleaved to tropocollagen and selected lysine residues are oxidized by the Cu^{2+} enzyme, lysyl oxidase, to form collagen (4,18,cited by 26). However, failure of the hydroxylation of proline and lysine alone, cannot explain the instability of the matrix resulting from Asc deficiency.

Upon completion of collagen synthesis, glycoaminoglycans (GAG) play a major role in stabilizing these collagen fibrils in the extracellular matrix and protecting them from degradation (4,27). The ground substance of the intercellular matrix is a complex aqueous gel containing electrolytes, metabolites, dissolved gases, trace elements, vitamins, hormones, enzymes, carbohydrates, fats and proteins. The important property of the intercellular matrix - extreme viscosity - depends upon the abundance of the already mentioned long-chained proteoglycans, GAG, as well as other related proteoglycans (13).

The chemistry of GAGs and other proteoglycans reveals that hyaluronic acid forms part of the long-chain polymers. The depolymerization of matrix GAG is brought about by the sequential action of hyaluronidase (13,28). The term hyaluronidase refers to a sequence of related degradative enzymes or an enzyme complex (13). Cells release hyaluronidase under normal conditions to permit cellular division, proliferation and migration. However, the continuous release of hyaluronidase will result in structural matrix disintegration and enhanced cell growth (13,28). This continuous release is observed in neoplastic cell proliferation, with enhanced GAG degradation resulting from abnormal hyaluronidase activity (27,28). It is suggested that Asc has the ability to inhibit the action of hyaluronidase (25,28,29) and hence has an inhibitory effect on the enhanced growth of tumour cells.

Cameron and Pauling proposed a mechanism to account for Asc-mediated inhibition of the growth and metastasis of malignant cells (28). GAG possesses glucuronic acid residues (4,13) and an Asc

residue is capable of replacing a terminal glucuronic acid unit. The Asc residue is termed the physiological hyaluronidase inhibitor, as it is resistant to hyaluronidase activity. Asc is therefore capable of blocking the whole process of GAG depolymerisation. The result is the stabilization of the inter- and extracellular matrices (13). This stabilization assists in preventing the migration of invasive malignant cells to other tissues, as normal tissue restraint and organization will be restored to a certain extent.

1.2.3.3 Ascorbic acid and immunocompetence

The effect of Asc on the immune response involves its effect on lymphoid organs, phagocytes, immediate and delayed hypersensitivity, antibody production, complement levels and interferon production. Asc appears to be necessary for the differentiation of lymphoid organs. It is capable of enhancing the regeneration of lymphoid tissue after X-irradiation (30). In the lymphoid tissue, Asc deficiency leads to a failure in T lymphocyte function, which results in excess activity of the B lymphocytes (31).

The phagocytic action of leucocytes, and the migratory behaviour of neutrophils and macrophages, is enhanced by Asc. Leucocyte motivity is dependent upon the activity of the hexose monophosphate shunt, which in turn is activated by Asc-mediated oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$ (13).

The supplementation of Asc in large amounts with immunizing doses of antigen, has been reported to increase antibody production in the humoral immune response, but these effects may be non-specific (30,31). Asc appears not to affect complement production or activity (30). However, the dietary Asc supplementation of mice has been reported to raise levels of circulating interferon (30) due to an increase in prostaglandin E_2 levels (PGE_2) (32).

Pauling (cited by 18) claimed that Asc would assist in preventing the common cold, due to a stimulated immune system. Leucocytes contain high concentrations of Asc. However, upon

infection these levels of Asc are depleted. Hence, Asc supplementation might maintain higher levels within the leucocytes and improve the immune response provided by the leucocytes.

1.2.3.4 Ascorbic acid and nitrosamines

Nitrosamines, products of the reaction of nitrite with a secondary amine, alkylurea, or an N-alkylcarbamate group, have been strongly implicated as a major group of environmental carcinogens (13,18). The inhibition of N-nitrosamine formation by Asc was suggested by Tannenbaum (33,cited by 34).

In an acidic environment within the body, for example the stomach, Asc reacts with nitrosating agents, *eg.* nitric acid (22). The latter are derived from dietary nitrite and Asc stimulates the conversion of nitrite to products which do not nitrosate. Thus, the conversion of secondary and tertiary amines to carcinogenic nitrosamines and nitrosamides is prevented (35,36).

1.2.3.5 H_2O_2 formation by, and antioxidant role of, ascorbic acid

It is suggested in the literature that Asc can generate H_2O_2 upon oxidation of Asc by molecular O_2 in biological systems (21,37). Asc oxidation involves single electron transfer reactions, which result in the formation of hydrogen peroxide - H_2O_2 (38).

This highly reactive hydrogen peroxide is termed a reactive molecule and is capable of causing cellular damage (39). The sites for the formation of this molecule include all cellular constituents such as the mitochondria, lysosomes, nuclear and ER membranes, as well as sites within the cytosol. Damage caused by these molecules involves H_2O_2 reactions with polyunsaturated fatty acids (PUFAs) in cellular membranes, nucleotides in DNA and critical sulfhydryl bonds in proteins (39).

Peterkofsky *et al* (40) proposed that the antitumour effect of Asc was mediated by H_2O_2, as the

latter molecule was found to be toxic to fibroblast cultures. Benade *et al* (37) found Asc to be toxic to Ehrlich ascites carcinoma cells *in vitro* and suggested this toxicity to be linked to H_2O_2 formation. Catalase is responsible for the detoxification of H_2O_2 with tumour cells having low catalase levels (41). Asc is an inhibitor of the activity of the H_2O_2-scavenging enzyme, catalase (42). Thus in tumour cells supplemented with Asc, the catalase levels will be less capable of effecting the detoxification of elevated H_2O_2 concentrations.

In contrast to this however, Asc is an antioxidant, in that it can react with and scavenge many free radicals, *eg*. singlet O_2, superoxide and hydroxy radicals (1,16,43,44,45). The latter cause cell damage and therefore have a tumourigenic character. In addition, Asc can regenerate the reduced form of α-tocopherol (Vitamin E), which is the major lipid-soluble antioxidant present in all cellular membranes (39). This general antioxidant role of Asc would suggest that it may prevent cellular oxidation and damage, thus assisting in the protection of cells.

1.2.3.6 Ascorbic acid and cancer

As mentioned earlier, substances are being sought that possess anti-carcinogenic properties. Anti-carcinogenic effects of Asc have both been suggested (15,34,36,46,47,48,49,50,51) and disputed (29,36,49,52,53,54,55,56). Antitumour activity of Asc is reported to be due to its chemical properties and not due to the metabolism of Asc as a vitamin (57).

Decreased levels of Asc in serum and plasma of cancer patients and tumour-bearing animals have been reported in several studies (25,48). Cameron and Pauling (58) reported the first large-scale trial of Asc supplementation in human cancer therapy. After one year of study, 22% of the Asc-treated patients were alive compared with only 0,4% of the control group. Cameron (59) has since published a protocol for the use of Asc in the treatment of cancer. Campbell (60) cautions against the use of mega Asc doses in cancer treatment, while nutritional remedies for cancer have been critically questioned by Dwyer (61).

Immunological studies show that Asc can induce immunity against some cancers in mice and that this immunizing action is accompanied by changes in the surface structure of the cancer cells (62). Reversion of transformed cells by Asc to normal morphology has been proposed (63,64,65). These studies suggest that low concentrations of Asc, in C3H/10T1/2/CL8 cells, can be effective in suppressing oncogenic progression but only prior to a stage where an initiated cell achieves the capacity to grow in semisolid medium and to produce tumours in immuno-suppressed animals (63,64).

Diet is very important with respect to the incidence of cancer. In Great Britain, in regions where the dietary Asc intake is low, a high overall cancer rate is experienced (13,36). The ingestion of Asc-containing foods appears to lower the risk of certain types of cancer (66,67). Japanese consume yellow and green vegetables daily and have a lower risk of lung cancer than people who rarely eat these types of food. Low total vegetable consumption is also associated with a high risk of laryngeal cancer (68).

The effect of Asc on tumour cell growth could possibly be mediated through various metabolic pathways, which influence cell growth. One such a pathway is the conversion of essential fatty acids (EFAs) to prostaglandins (PGs) and the effect of the latter on adenylate cyclase (AC) activity, which in turn influences the production of cyclic adenosine monophosphate (cAMP). This pathway is also influenced by exogenous EFAs, phospholipase A_2 (PLA_2) and subsequent mobilization of membrane fatty acids (FAs), as well as Ca^{2+} requirement of a number of the enzyme reactions. Since this study involves an investigation of the influence of Asc on the various metabolites in the above mentioned pathway, their interrelationships and possible association with tumour cell growth, a review of the pathway and its metabolites is necessary.

1.3 CELL MEMBRANES AND FATTY ACIDS

1.3.1 FLUID MOSAIC MODEL OF BIOLOGICAL MEMBRANES

The dynamic state of the lipids in the lipid bilayer of cell membranes was described by Singer and Nicolson in 1972 (4,69,70,71). They proposed a fluid mosaic model for the gross organization of biological membranes.

The essence of their model is that membranes are two-dimensional solutions of orientated globular proteins and lipids. Several forms of noncovalent and covalent forces are involved in the interaction of membrane protein with membrane lipid (72). The major features of the model include the following:

a) most of the membrane phospholipid and glycoprotein molecules are in a bilayer formation, with the central core of fatty acyl chains serving the dual purpose of being a solvent for intergral membrane proteins and a permeability barrier (4,69,70,73),

b) a small proportion of membrane lipids interacts specifically with particular membrane proteins and may be essential for the latters function (4,69,70,74,75,76),

c) membrane proteins are free to diffuse laterally in the lipid matrix unless restricted by special interactions, whereas they are not free to rotate from one side of a membrane to the other, *ie.* transverse diffusion (4,69).

Membrane fluidity is influenced by temperature, drugs and the nutrition of the organism (77). Thus, biological membranes are not of constant composition but are dynamic and responsive structures in terms of membrane constituents. This extensive diversity results in differences among cell types and among similar membrane types of different cells (72).

1.3.2 BIOSYNTHESIS OF FATTY ACIDS

Lipids, consisting of various FAs, are important constituents of cellular membranes (78). Most

saturated and monounsaturated FAs are derived either from the diet or from *de novo* synthesis by the condensation of acetate units followed by the direct oxidative desaturation of the long chain FAs (4,79,80). In contrast, PUFAs are derived only from dietary lipids, since mammalian tissues lack the enzymes necessary for synthesizing linoleic acid (LA) and gamma-linolenic acid (GLA). These are the precursors of all the PUFAs found in mammalian cells (79,80).

1.3.2.1 Saturated fatty acids

Saturated FAs are synthesized via a high molecular mass complex composed of two multi-active site polypeptide chains, known collectively as the fatty acid synthetase complex (4,79). This synthesis takes place in the cytosol of the cell (4). In mammalian systems the product is predominantly palmitic acid (16:0). The reaction has the following stoichiometry:

Acetyl CoA + 7 Malonyl CoA + 14 NADPH + 7 H^+ ——————>
Palmitic acid + 7 CO_2 + 14 NADP + 8 CoA + 6 H_2O

Palmitic acid can exert control over endogenous FA synthesis, either by inhibiting the activity of the FA synthetase complex or by reducing the availability of malonyl CoA (79).

1.3.2.2 Unsaturated fatty acids

In most animal systems, unsaturated FAs are synthesized by the direct oxidative desaturation of preformed long-chain saturated FAs. The enzymes responsible for the desaturation reactions are the delta-5, delta-6 and delta-9-desaturases, of which the delta-5 and delta-6-desaturase activities are under endocrine control (81). The desaturases are membrane-bound enzymes of the ER that require an electron-transport chain from nicotinamide adenine dinucleotide (NADH). This is catalysed by the desaturase complex consisting of NADH-cytochrome b_5 reductase (EC 1.6.2.2), cytochrome b_5 and the terminal desaturase enzymes (4,79,82,83,84). (See Figure 5). Of the three components, the terminal desaturase is the regulatory enzyme since it is the slowest step of the reaction sequence

(81).

In 1929, Burr and Burr (cited by 85,86,87) first recognized the fact that some unsaturated FAs could not be synthesized by mammalian tissues and needed to be acquired from the diet (4,88,89). These are referred to as "essential" FAs (4,79,89). Two groups of EFAs exist, the n - 6 and the n - 3 EFAs, with n representing the total number of carbon atoms in the FA chain. They are defined by the position of the first double bond in the fatty acid chain, starting from the carbon atom at the methyl terminus of the chain (88,90). The n - 6 EFAs have considerably more activity that the n - 3 EFAs, with debate as to whether the n - 3 group are actually essential FAs (87).

Mammals lack the enzymes to introduce double bonds at carbon atoms beyond C-9 in the FA chain (4), and therefore cannot synthesize the EFA, LA (18:2n-6) (4,80,91) or derivatives thereof. This EFA thus has to be taken up from the diet. The enzymes delta-6 and delta-5-desaturase are then able, respectively, to oxidatively desaturate LA to GLA (18:3n-6), and dihomo-gamma-linolenic acid (DGLA) (20:3n-6) to arachidonic acid (AA) (20:4n-6) (82,91,92). [Cats do not possess delta-8 or delta-6-desaturase activity (93,94).]

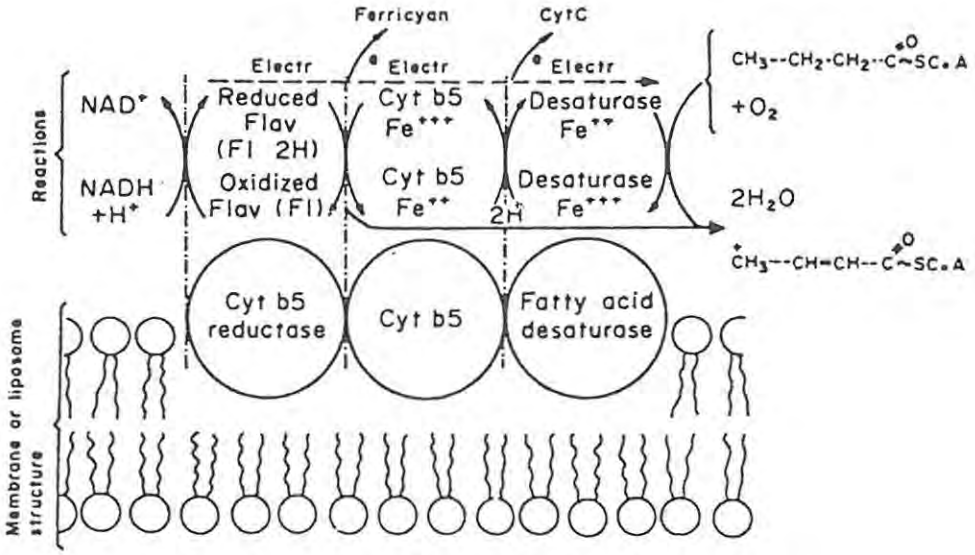


FIGURE 5: The structure of the fatty acid desaturating system in the lipid bilayer (83)

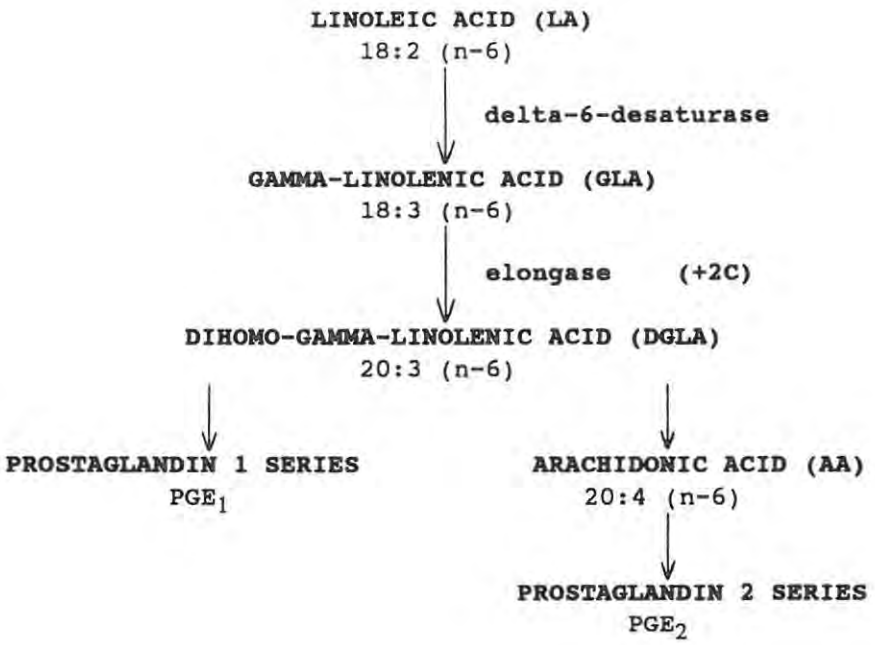


FIGURE 6: The linoleic acid series

Delta-6-desaturase, which converts LA to GLA, is reported to be the rate limiting enzyme in the linoleic acid series pathway (see Figure 6) (82,92,95,96,97). This enzyme appears important, since its activity determines the tissue levels of GLA and DGLA. GLA is elongated by an elongase enzyme to DGLA (cited by 98). The latter is the precursor for the 1 series eicosanoids, *eg.* prostaglandin E_1 (PGE_1). Stores of cellular GLA and DGLA are very low (26,99), with DGLA concentrations being a quarter of those of AA (100). DGLA is either converted to AA or PGE_1. DGLA is desaturated by delta-5-desaturase to form AA, which is the precursor of the 2 series eicosanoids, *eg.* PGE_2 (82). Rat liver is unusual in having large amounts of delta-5-desaturase, while little activity is detected in liver homogenates from rabbit, guinea pig and man (101).

1.3.3 THE FUNCTIONS OF FATTY ACIDS

The lipid requirement of all mammals is met by their dietary intake or by *de novo* synthesis of FAs. The FAs are then incorporated into triacylglycerols and phospholipids, the storage forms of FAs (79,102). Often these stored FAs serve as metabolic fuel for the cell (78), while AA in membrane phospholipids is structurally important (103). It is also suggested that free intracellular AA

represents a second messenger system in mammalian cells (87).

The n - 6 EFAs have at least four roles in the cell: a) modulation of membrane structure, b) formation of short-lived local regulating molecules such as eicosanoids, c) control of the water impermeability of the skin and possibly the permeability of other membranes such as the gastro-intestinal tract and the blood brain barrier and d) regulation of cholesterol transport and cholesterol synthesis (88). Thus, exclusion of these EFAs from the diet over a prolonged period results in such conditions as impaired growth, infertility (89), skin lesions (89,104,105), and caudal and kidney necrosis (89).

1.3.4 FATTY ACIDS IN BIOMEMBRANES

Membrane fluidity appears to be of general importance to processes ranging from passive permeability and lateral diffusion to cell recognition, differentiation and malignant transformation (73). This involves the nature of the relationship between membrane FAs, particularly the essential PUFAs, and the integral proteins that determine the functional properties of the membrane (75). The various organelle membranes in a eukaryotic cell differ widely in both protein and lipid composition (70,74,106).

EFAs within the body may be found as unesterified FAs or as components of cholesterol esters, triacylglycerols and phospholipids (88). The FA profiles of complex lipids vary with the type of lipid, position within a phospholipid, body organ, organ region, cell type, and even to selective domains within the plasma membrane (75). The influence that FAs exert on the fluidity and/or order of a membrane has widespread implications, *eg.* the hydrophobic FA core has the potential to stabilize and determine the thickness of cell membranes (75,107). Changes in membrane fluidity, due to FA content, could result in a change in the enzyme activity of related proteins, *eg.* AC (73,74). This is as a result of the membrane FAs directly affecting the dynamic properties of a protein (75), *ie.* its mobility within the plane of the membrane.

Modifications of membrane FA content, such as a change in the saturated/unsaturated FA ratio, can alter cellular functions such as a prostaglandin synthesis and cell growth (70). The incorporation of the saturated FA, stearate (18:0) into the membrane (74), results in a decrease in membrane fluidity and changes in AC activity (73,74,75).

1.3.5 CELL MEMBRANES IN TUMOUR CELLS

Neoplastic transformation leads to a dramatic change in the lipid structural organization within tumour membranes (108,109). Tumour plasma membranes generally exhibit decreased fluidity, in that they are structurally changed towards a higher rigidity (108,110). This loss of fluidity is often caused by free radical reactions on the unsaturated bonds in membrane lipids (39). PUFA enrichment in cells can cause an increase in plasma membrane fluidity (109), therefore tumour membrane rigidity could be due to the loss of PUFAs, as well as the free radical damage done to remaining PUFAs.

1.3.6 THE LINOLEIC ACID SERIES AND CANCER

A number of malignant cell types have been reported to lack (111,112,113,114,115) or have reduced activity of (91,116,117) delta-6-desaturase. Theoretically, due to the loss of delta-6-desaturase activity, these cells will not be able to synthesize GLA, DGLA, AA and the other long chain FAs from the precursor LA (26,118). Since DGLA and AA are the precursors for PGE_1 and PGE_2 (4), respectively, reduced levels of these precursors will also affect PGE formation. However, researchers (91,99) have reported that the loss of delta-6-desaturase activity appears to be specific and possibly an invariable concomitant of transformation. The activity of delta-5-desaturase, which converts DLGA to AA, is not impaired. As the latter enzyme is not impaired, AA stores may not be severely limited due to AA still being available from other dietary sources, in the form of DGLA or AA (99,119). Consequently, the 2 series PGs are less likely to be deficient in malignant cells (99).

Upon the addition of LA and GLA to cultured malignant cell lines, LA is reported both to stimulate (120,121,122,123) and to inhibit (124,125,126,127) cell growth of various different cell lines, while GLA generally results in an inhibitory effect on malignant cell growth of various tumour cells (98,124,125,128,129,130,131,132,133,134,135,136). GLA enrichment enhances the level of AA in phospholipids (137), but the exact mechanism of this GLA action in reducing tumour growth is still unclear (136). One hypothesis (13) suggests that GLA may exert its effects by increasing the production of prostaglandins, particularly PGE_1 (134,135,136,138). If this hypothesis is correct, the addition of DGLA to malignant cell cultures should also inhibit cell growth, but in one study DGLA supplementation produced effects opposite to those of GLA (138). An alternative suggestion accounting for the mechanism of GLA cytotoxicity is that supplemented GLA stimulates increased lipid peroxide formation in tumour cells (134).

In several rodent mammary tumour models (121,139,140,141,142,143,144,145,146,147), diets rich in fat have been shown to enhance the development of the tumours. The dietary fat appears to act as a promotor rather than affecting initiation of the mammary tumours (140,141). In general, it is found that PUFA diets yield more tumours than saturated FA diets (121,139,142,144,147). However, it is also reported that oleic acid (18:1) and LA can have inhibitory effects on the growth of Erhlich solid carcinoma in mice, while they prolong the life span of Ehrlich ascites carcinoma-bearing mice (148). LA-GLA combinations have also been reported to prolong the life span of tumour-bearing rats (125). No human study has shown LA in particular to be associated with cancer risk. It appears that in humans and animals that the effects of GLA and further metabolites are inhibitory to cancer growth (88).

1.4 PROSTAGLANDINS

PGs consist of a large group of cyclic derivatives of oxygenated unsaturated C_{20} FAs (149,150,151) and have been detected in virtually all cells and tissues of animals (149,152). They mediate a large variety of biological effects and appear to play a regulatory role in a number of systems (149).

1.4.1 PROSTAGLANDIN BIOSYNTHESIS

In 1930, Kurzrok and Lieb (cited by 86), studying artificial insemination found that on occasion human semen produced violent contractions of the human uterus. The active substance responsible for these contractions was believed to emanate from the prostate gland, hence the name prostaglandin (153). In 1957, Bergström isolated the first prostaglandins, PGE_1 and $PGF_{1\alpha}$ (cited by 86). Today, PG is a term applied to a series of compounds derived enzymatically and non-enzymatically from 20 carbon FAs (154) and which do not only emanate from the prostate gland.

PGs are not stored within the cell to any great extent (90,153,154,156,157), thus any stimulation of their release requires a prior mobilization of substrate precursors, the FAs (4,153). To fulfil a function, the PGs have to be synthesized at rates that overcome inactivation (158).

PGE_2 is of principle concern to this study. However, older literature does not always distinguish between PGE_1 and PGE_2, with the general term PGE being utilized. Therefore, in this introduction both PGE_1 and PGE_2 will be considered.

1.4.1.1 Biosynthesis of Prostaglandin E_1 and E_2

PGE_1, which has one double bond in its side chains (cited by 159), is synthesized from the FA precursor, DGLA (101,160,161,162,163), by lipoxygenase (164). However, DGLA can also be converted to an endoperoxide, PGG_2, by cyclooxygenase (4,cited by 165).

The principal substrate for PGE_2 formation is free AA. The release of this precursor FA is the regulatory step in this synthesis (90,151,153,157,166). AA bound to a phospholipid needs to be mobilized by PLA_2, which specifically removes the FA moiety from the carbon-2-position (153,167,168). The AA is then degraded by the so-called AA cascade which is comprised of two distinct pathways (153,169). The one pathway entails the hydroxylation of AA by FA lipoxygenase to form hydroxy acids (see Figure 7) (90,151,153). The alternative pathway (see Figure 7), which

is the one of concern in this study, is the interaction of AA with PG cyclooxygenase (PG endoperoxide synthetase (EC 1.14.99.1)) (90,150,151,153,170).

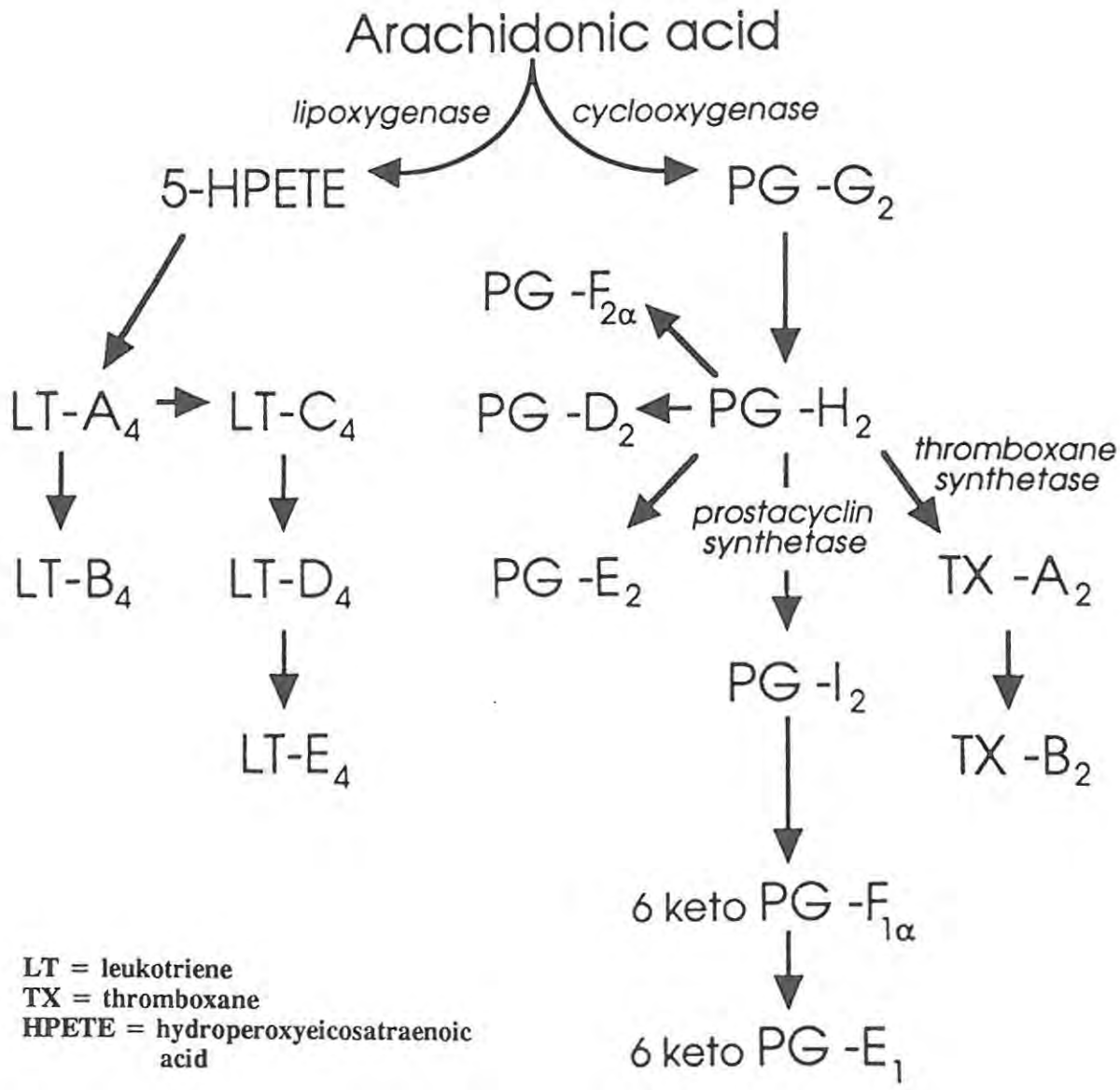


FIGURE 7: The formation of dienoic prostaglandins and thromboxanes from arachidonic acid (171).

PG cyclooxygenase is a membrane-bound multi-enzyme complex (150,153) on the smooth ER (4) which specifically catalyzes the oxygenation and cyclization of AA (150,153,170), thereby

generating 15-hydroxy-prostaglandin endoperoxides (PGH_2 and PGG_2) (150,153,171,172). PGE_2 isomerase, also known as endoperoxide E isomerase (EC 5.3.99.3), catalyses the isomerisation of PGH_2 to PGE_2 (150,170).

1.4.2 FUNCTIONS OF PROSTAGLANDIN E

PGs are cell-to-cell messengers produced by eukaryotic cells, often in response to extracellular stimuli (4). PG production is related to cell growth (173,174) and cells in the quiescent state are the most active PG producers (173). The lipophilic nature of the PGs may allow PGE to modify the mobility of membrane-associated receptor proteins by interacting with microfilaments in the vicinity (153).

There are regulatory interactions between the one and two series of PGE. PGE_1 inhibits AA mobilization, while PGE_2 stimulates DGLA mobilization and its conversion to PGE_1 (160).

1.4.2.1 Prostaglandin E_1 functions

PGE_1 is required for T lymphocyte function (26,159) and is important in the regulation of immune responses. PGE_1 is also important in the regulation of collagen synthesis and therefore matrix stabilization (26). These one series PGEs are reported to be important regulators of cell growth (175), while PGE_1 also enhances the activity of certain enzymes, *eg.* AC, in a dose-dependent manner (176,177).

1.4.2.2 Prostaglandin E_2 functions

Growth response is subject to alterations in hormone levels, with these changes in hormone levels also having an effect on PGE_2 concentrations, affecting the functions of the latter (68,178). PGE_2 is a regulator of the cell kinetics of gastrointestinal epithelial cells (179). PGE_2 is also capable of altering cAMP levels in certain cells, *eg.* in renal papillary collecting tubules it increases cAMP

levels (180,181), hence it also affects AC activity.

1.4.3 PROSTAGLANDINS AND CANCER

Eicosanoids can influence the carcinogenic process in many different ways, *eg.* they can either stimulate or inhibit tumour growth *in vivo*, influence tumour migration and metastatic potential, or act as tumour promotors by elevating levels of PGE_2 which may exert immunosuppressive effects and promote tumour growth (109,182,183), in various cell types.

Tumour cells secrete elevated levels of PGs, in particular the PGE series (149,154,158,183,184,185,186,187,188,189,190,191,192,193). It is not clear whether the increase in synthesis and release of PGs in tumours is the cause or the result of increased proliferation rates (194). It would be reasonable to assume that more than one event in the PG synthetic pathway is deranged by the malignant process (195). Interactions of PGs with growth factors have been described and these might contribute to the ability of the tumour cells to metastasize (186,188). PGE_2 is not causal of metastasis, but its presence contributes to the metastatic abilities of the cells which already possess the necessary functions (186,196,197,198).

PGE_1 and E_2 have been found to be both stimulatory (174,199,200) and inhibitory (178,186,201,202,203,204,205) to tumour cell proliferation, both *in vivo* and *in vitro* at physiological levels (206), approximately 100 pg/ml (174). Addition of exogenous PGEs to cells in culture, has demonstrated that PGE inhibits cell growth *in vitro* (154,157), in a dose-dependent fashion. The PGE effect is thought to be mediated through the activation of certain enzymes, *eg.* AC (154).

1.5 CALCIUM

Various metal ions, including Ca^{2+}, are essential to life. The unique role of Ca^{2+} in biology was first recognized in 1882 (cited by 207). The importance of Ca^{2+} to this study is through its

association with PLA_2 and AC activity, as discussed in sections 1.7 and 1.8.

1.5.1 CHARACTERISTIC PROPERTIES OF CALCIUM

Ca^{2+} is characterized by its charge, coordination number and unhydrated radius (208). Cells normally maintain very low concentrations of free Ca^{2+} ion, about 10^{-1} μM (207). This is due to Ca^{2+} ions being "sticky" and thus being able to bind to a wide variety of ligands such as proteins and phosphate groups. Ca^{2+} bound to the latter enables the ion to regulate metabolic processes (209). One such protein is the Ca^{2+}-dependent, regulatory protein calmodulin, which confers sensitivity to a number of enzyme systems (210).

Generally, most animal cell types have a very steep electrochemical gradient of Ca^{2+} across the plasma membrane. The electrical driving force for Ca^{2+} entry into cells is at a potential of about -60mV (209). Ca^{2+} is involved in electrical signalling processes, as well as transmembrane chemical signalling (209).

1.5.2 FUNCTIONS OF CALCIUM

1.5.2.1 Calcium and cell proliferation

Ca^{2+} availability is the key controller of cell proliferation in multicellular organisms and a defect in the Ca^{2+} system is the common determinant of unrestrained proliferation in cancer cells. Proliferation rates of both normal and cancer cells appear to be controlled by the availability of Ca^{2+} ions from inside cells at a particular time (211).

The proliferation of non-neoplastic cells seems to be controlled by extracellular Ca^{2+} ions as they require high-Ca^{2+} media, whereas neoplastic cells proliferate normally in low-Ca^{2+} media (208). Thus, the Ca^{2+} requirement of neoplastic cells is very low in comparison to that of normal cells (211). This is consistent with the observation that the Ca^{2+} content of hepatoma cells is 100%

greater than that of normal liver cells. The Ca^{2+} content of these tumour cells increases progressively with the increasing age of the tumour (212).

Ca^{2+} and calmodulin play significant roles in DNA synthesis and cell proliferation (213). cAMP has the ability to mediate this Ca^{2+} activity (208), with cAMP regulating the cell cycle (see section 1.8.7.1).

1.5.2.2 Calcium and enzymes

Many of the key enzymes involved in AA esterification or release are dependent on metal ions, particularly Ca^{2+}. Ca^{2+} stimulates acyl hydrolase activity (214), while membrane-bound PLA_2 shows an absolute requirement for Ca^{2+} (103,214). The role of Ca^{2+} in PLA_2 regulation of AA release is three-fold, in that Ca^{2+} is involved in the activation of protein kinase C and the subsequent phosphorylation of PLA_2. Ca^{2+} *per se* becomes directly stimulatory to PLA_2 through a Ca^{2+} requirement by the G proteins involved in PLA_2 activation (214,215).

Ca^{2+} also acts as a second messenger in eliciting a physiological response via Ca^{2+}-modulated proteins. AC forms a reversible Ca^{2+}-dependent complex with calmodulin, with calmodulin-Ca^{2+} interacting directly with the catalytic subunit of AC (207). Voltage-gated Ca^{2+} channels in atrial membranes, cardiac sarcolemmal, and skeletal muscle T tubule membranes, are positively modulated by a G protein. The latter is the stimulator of AC. However, G proteins are also involved in the action of certain receptors that inhibit Ca^{2+} channels (216). Thus G proteins have direct effects on plasma membrane Ca^{2+} channels (209).

1.5.3 INTRACELLULAR TRANSPORT OF CALCIUM

The major mechanism of Ca^{2+} entry into a cell from the outside is via voltage-sensitive Ca^{2+} channels. Two systems exist for the active outward transport of Ca^{2+} from the cell. Ca^{2+} can leave the cell by a Ca^{2+}-stimulated ATPase or a Na^{+}-Ca^{2+} exchange system (209). The detailed

mechanism of these transport systems has been described by Dawson (209).

1.6 G PROTEINS

It was first reported in 1971 that receptor-sensitive signal transduction systems are not only hormonally controlled, but that guanosine triphosphate (GTP) also regulates such systems (216,217). The evolution of hormonal signalling systems is reviewed in detail by Pertseva (218). These transmembrane signalling systems usually consist of two major components: the specific binding site (receptor) and the effector component (enzyme or channel proteins) (219).

1.6.1 PROPERTIES OF G PROTEINS

G proteins, also known as GTP-binding proteins, are heterotrimeric proteins involved in the transduction of a variety of external signals in all eukaryotic organisms. These signals must be transduced to generate the intracellular biochemical cascades of responses that will culminate with the physiological processes.

The three subunits of the heterotrimeric G proteins are designated α (MM = 39 - 46 kDa), ß (MM = 37 kDa) and γ (MM = 8 kDa) (220). On activation, G proteins dissociate into α-guanine nucleotide complexes and ßγ dimers (216,217). The α subunit can bind and hydrolyze GTP, *ie.* it has the guanine nucleotide-binding site and GTPase activity. It also defines the receptor and effector specificity of a G protein, and differs from one G protein to another (216). The ßγ dimer exists as a tightly associated complex that functions as a unit (220). The terms G_s and G_i describe the nucleotide binding sites responsible for stimulatory and inhibitory regulation of enzymes (221). Quantitative assessments of the amount of stimulatory G proteins in neoplastic human thyroid tissues show increased amounts of stimulatory G protein in these tumours (222).

Very low concentrations of GTP act not only to stimulate but also to inhibit the activity of some enzymes (221). The hydrolysis of GTP is irreversible, resulting in a unidirectional cycle (223).

1.6.2 SIGNALLING MECHANISM OF G PROTEINS

Hormone and transmitter competent systems have at least three types of transmembrane signal transduction mechanisms to achieve the final effect. These systems vary with the enzyme involved, *eg*. AC has a Type II signalling mechanism (218).

The G protein cycles between an inactive GDP form and an active GTP form (217). Hormone-receptor complexes activate the G protein by catalysing the replacement of bound GDP with GTP. Binding of GTP to the G protein α subunit causes it to dissociate from the $\beta\gamma$ subunits, thereby generating the two potentially active subunits, α-GTP and $\beta\gamma$ (224). The α-GTP subunit, in turn, alters the activity of the target effector system (217). Finally, the intrinsic GTPase activity of the α subunit converts the bound GTP to its inactive GDP-bound form, the latter binding the $\beta\gamma$ subunit with high affinity (217,224). Two enzymes controlled by G proteins, of interest to this study, are PLA_2 and AC.

1.7 PHOSPHOLIPIDS AND PHOSPHOLIPASE A_2

1.7.1 STRUCTURE AND SYNTHESIS OF PHOSPHOLIPIDS

Phospholipids are typical amphipathic compounds (71,73). They are derived either from glycerol or from sphingosine. If derived from glycerol, they are termed phosphoglycerides and consist of a glycerol backbone, two FA chains and a phosphorylated alcohol. Phosphatidate is the product if the phosphorylated alcohol esterifed at C-3 is phosphoric acid (4). The major intracellular site for the initial assembly of membrane lipids is the ER and they are transported from this organelle throughout the cell (84,106). Phosphatidate, the simplest of phosphoglycerides, is present in very small quantities in the membranes and is the key intermediate in the biosynthesis of the other phosphoglycerides (4).

Once synthesized, the phospholipids are modified by acyltransferases and phospholipases (84). FA

moieties bind to the sn-1 and sn-2 positions on the glycerol backbone during initial phospholipid synthesis. Phospholipid turnover and maintenance is dependent on deacylation and reacylation reactions concerning sn-1 and sn-2, while polar heads can be altered by base exchange (4,106). The mechanism determining which different membranes acquire a different FA composition is not known (87). However, unsaturated FAs are known to compete for binding to the sn-2 position of phospholipids (84). In natural membranes, the majority of the phospholipids contain one saturated and one unsaturated FA chain, the distinction being ensured by different acyltransferases (107). Both n-3 and n-6 EFAs are incorporated into phospholipids (73). The intracellular pool of free AA is very low (170,214), with most AA esterified to the sn-2 position of phospholipids such as phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol (170).

The major phosphoglycerides are derivatives of phosphatidate. The phosphate group of phosphatidate is esterified to the hydroxyl group of alcohol moieties such as serine, ethanolamine, choline, glycerol and inositol (4), forming phosphatidylcholine, phosphatidylserine, etc. The primary site for the synthesis of phosphatidylcholine and phosphatidylethanolamine by the choline- and ethanolamine-phosphotransferase pathways is located in the microsomal fraction of the rat liver and other mammalian tissues (106). Phosphatidylinositol phosphodiesterase is a cytosolic enzyme and it is thus necessary that its substrate be at the cytosolic face of the plasma membrane (84). The synthesis of phosphatidylserine in animals occurs almost exclusively through the exchange of ethanolamine in phosphatidylethanolamine with L-serine (106).

As many as forty different FAs can be incorporated into the sn-1 or sn-2 position of the phospholipid molecule (72). Studies with whole membranes indicate the importance of a roughly 50:50 balance between saturated and unsaturated FA chains (107). Whether the phospholipid is in a gel phase (equivalent to solid) or a liquid crystalline phase (a liquid-like state) is dependent on the FA content and the temperature of the phospholipids (71,73,107).

1.7.2 MODIFICATIONS TO PHOSPHOLIPIDS

There is considerable variation in the extent to which FA composition can be altered in phospholipids. The change of fatty acyl chains from laureate (12:0) to stearic acid (18:0) for phosphatidylcholine results in a change in the thickness of the hydrocarbon region of the lipid bilayer (107). These modifications are, however, not accompanied by any changes in phospholipid head group composition. Palmitic acid (16:0) supplementation of bovine pulmonary artery endothelial cells minimally increases the respective palmitic acid content and total saturated FA content of the phospholipids. However, oleic acid (18:1) supplementation of these cells increases oleic acid content of the phospholipids by 60%, while the saturated EFA content of the phospholipids decreases substantially (70). Conflicting reports exist concerning AA supplementation of phospholipids (70,225).

Membrane phospholipids of brain tumours and of cultured cell lines of neural-tumour origin have fatty acyl compositions that differ from corresponding normal tissues (cited by 226).

1.7.3 PHOSPHOLIPASE A_2

The last several years have broadened the knowledge concerning the diversity and functions of mammalian sn-2 acylhydrolases. The role of FA release from phospholipids, in particular that of AA, is now more defined.

1.7.4 PROPERTIES AND CLASSIFICATION OF PHOSPHOLIPASE A_2

Initially PLA_2 was determined to be strictly Ca^{2+}-dependent (103,227). However, it is now reported that different PLA_2s are either Ca^{2+}-dependent or Ca^{2+}-independent (228,cited by 229). Particular ions, such as Sr^{2+}, can completely inhibit both pancreatic and serum PLA_2 activities (230). PLA_2 activity is enhanced when acidic phospholipids are hydrolysed, as opposed to the hydrolysis of neutral phospholipids, while a negative surface charge also stimulates PLA_2 activity

(103). Polyunsaturated molecular species appear to be involved in making phospholipids accessible to PLA_2-mediated hydrolysis (231).

PLA_2 is competitively inhibited by unsaturated FAs, particularly AA (232), suggesting this might be a negative feedback mechanism to regulate free AA levels (214). A family of intracellular-phospholipid-binding proteins, thought to be induced by glucocorticoids, has been shown to inhibit PLA_2 activity *in vitro* (170). Human placenta reportedly also contains inhibitory proteins to PLA_2 activity (cited by 233). On the other hand, a PLA_2 activating protein (PLAP) has been identified (234). PLAP mediated stimulation of PLA_2 activity may involve an interaction between PLAP and the enzyme (229).

Formal nomenclature of PLA_2s is based solely on structure and sequence. A 100-kDa AA mobilizing PLA_2 has been purified from mouse spleen and the macrophage cell line J774 (235). The 14-kDa PLA_2 enzymes have been categorized into Types I, II and III. Type IV is an 85-kDa PLA_2 which is sn-2 acylhydrolase specific (168,172). The coexistence of different PLA_2s does occur (168).

1.7.5 FUNCTIONS OF PHOSPHOLIPIDS AND PHOSPHOLIPASES A_2

Phospholipids play an important role in the integrity of cell membranes, *ie.* lipid fluidity, membrane thickness, lipid phase and in the activity of membrane-bound enzymes (107). A few proteins may require specific phospholipid species (87,236). Another very important function of phospholipids is the storage of AA for eicosanoid synthesis (214). Some phospholipids also have very specific functions, for example phosphatidylinositol is important to the signalling systems in the cell (107).

The presence of PLA_2 in biological membranes is a prerequisite for phospholipid turnover in cellular metabolism and signal transduction (103). PLA_2 controls free AA levels within the cell to a certain extent (237), while some PLA_2s preferentially remove peroxidized FAs from

phospholipids (cited by 230).

An indirect function of PLA_2 is that of generating second messengers, which are involved in various metabolic pathways. The most commonly used pathway is that of the hydrolyses of membrane phosphatidylinositiol as a result of the activation of phospholipases following the stimulation of an appropriate receptor (87). Messengers generated here and in other systems include PGs, inositol phosphates and diacylglycerol (DAG) (87,237). Free intracellular AA also has the capacity to act as a second messenger, as it directly activates protein kinase C (168).

1.7.6 G PROTEINS AND PHOSPHOLIPASE A_2 MECHANISM

Two major routes of receptor-mediated regulation of PLA_2 activity have been implicated in the control of AA mobilization and release. One route is the activation of PLA_2 via a receptor-linked G protein not requiring phospholipase C (PLC) action (168), while the other involves indirect activation of PLA_2 through a PLC mediated pathway by increasing Ca^{2+} and activating protein kinase C (168,215). PLA_2 activated through the first route exhibits a direct link between G proteins and the enzyme (215,216,238), and hence is GTP-dependent. This PLA_2 activation leads to the direct release of AA (212).

G protein dependent PLA_2 activity exhibits characteristics which deviate from the typical G protein mechanism described earlier (see section 1.6.2). Only the α subunit regulates AC activity, whereas both the α and $\beta\gamma$ subunits regulate PLA_2 activity (217).

An important requirement of receptor-mediated signal transduction is its termination. The termination of the activity of PLA_2 linked to G proteins may occur by the reassociation of the inactive heterotrimer following the activation of the GTPase associated with the α subunit of the G protein. Other inhibitors include secondary messengers Ca^{2+} and cAMP, as well as secondary messenger systems of AA feedback or the activation of protein kinases (239).

1.8 ADENYLATE CYCLASE AND CYCLIC ADENOSINE MONOPHOSPHATE

AC (EC 4.6.1.1) was discovered by Sutherland and Rall in 1957 (240,241). AC mediates changes in cellular cAMP concentrations, as it is responsible for the conversion of adenosine triphosphate (ATP) to cAMP (241). Many hormones and neurotransmitters exert their effects on target cells by stimulating AC activity and altering the intracellular concentrations of cAMP (241,242).

1.8.1 STRUCTURE AND PROPERTIES OF ADENYLATE CYCLASE

AC occurs in most types of mammalian cells, in lower animals, in unicellular organisms, in bacteria and probably in plants (240). AC, a glycoprotein, is a single subunit enzyme of either 115 kDa or 150 kDa (216). However, this polypeptide exists within the AC system, which is ubiquitous, multi-component and membrane-bound (241,242,243). The AC system is composed exclusively of intrinsic membrane proteins and depends upon their proper integration in a membrane for hormonal regulation and hence adequate functioning (241,243).

Initially, the hormone-responsive AC system was reported to exhibit a three component model, comprising a receptor, a catalytic component and a guanine nucleotide-binding protein (236,241,242,243,244,245). However, further research has shown that the receptor and GTP-binding regulatory protein components are more complex. The hormonally regulated AC system has now been shown to consist of five main components: the stimulatory receptor (R_s), the stimulatory guanine-nucleotide binding regulatory protein (G_s) (itself composed of three subunits α, ß and γ), the catalytic moiety of AC, the inhibitory receptor (R_i) and the inhibitory GTP-regulatory protein (G_i) (also composed of three subunits α, ß and γ) (216,219,245).

AC stimulation appears to exhibit a transmembrane phenomenon, as the various components are asymmetrically disposed in the membrane (242,246). The catalytic site for the enzyme, which is responsible for the conversion of ATP to cAMP, is thought to face the inside of an intact cell. The receptor site, however, which interacts with specific circulating hormones, faces the external

medium (246). Like many integral membrane-bound enzymes, the activity of AC is modulated by the nature of its membrane-lipid environment (70,241,242).

1.8.2 PROPERTIES OF THE ADENYLATE CYCLASE COMPONENTS

1.8.2.1 The receptor component

Different hormones have their own specific receptor sites on the receptor component (cited by 247), but some receptors do recognise more than one hormone (243). The receptor-hormone complex acts by mediating the binding of purine nucleotides to a regulatory site (243).

1.8.2.2 The regulatory component

Nucleotides, especially guanine nucleotides, play an important role in regulating the function of hormone-sensitive AC systems (248). These G proteins are discussed in section 1.6. Two major subunits suggest two separate guanine regulatory sites for AC. One site mediates the inhibition of AC and is denoted the G_i site (245,249). Occupancy of the other site, G_s, has a stimulatory effect on AC (245,249,250).

The regulatory protein is necessary to functionally couple agonist occupancy of the receptors with activation of the catalytic moiety, hence modulating the catalytic activity of AC (244).

1.8.2.3 The catalytic component

This component has a relatively large hydrophobic surface area (242,243). The catalytic component must have a substrate site for ATP, as well as a binding site for the cation. Hence, this component presumably binds a divalent cation-ATP complex (242,251).

1.8.3 MECHANISM OF ADENYLATE CYCLASE ACTIVITY

GTP plays an important role in the regulation of AC activity, as the hormonal stimulation of AC requires the presence of a guanine nucleotide in addition to the substrate (243,245). Hence, AC has a Type II signalling mechanism, as GTP binding acts here as a transducer and coupling factor. Furthermore, AC is of a subtype within this type, where hormone receptor-enzymes generate secondary messengers (218).

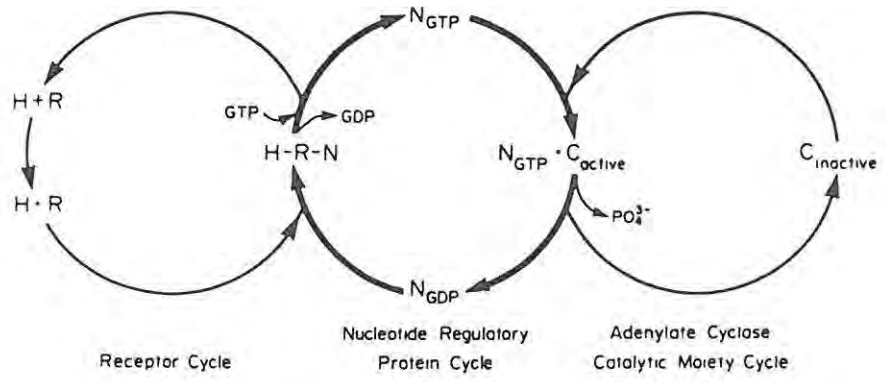


FIGURE 8: A model for the mechanism of adenylate cyclase activity (244).

The binding of an agonist to the receptor is followed by an interaction of this agonist-receptor complex with the stimulatory guanine-nucleotide binding regulatory protein (G_s) (219) (see Figure 8). G_s binds GTP and cations, as well as linking the receptor to the catalytic unit (242,245,250,252). Therefore it is a shuttle that conveys information from an agonist-occupied receptor to the catalytic moiety of AC (242,244,245). In the presence of high levels of GTP ($> 1\mu M$), the activated catalytic unit converts ATP to cAMP (219,242).

AC activity is inhibited by the stimulation of the inhibitory receptor (R_i) and the coupling to G_i (253). The latter results in the inactivation of the catalytic unit (219), due to the binding of GDP (217,218).

1.8.4 CYCLIC ADENOSINE MONOPHOSPHATE

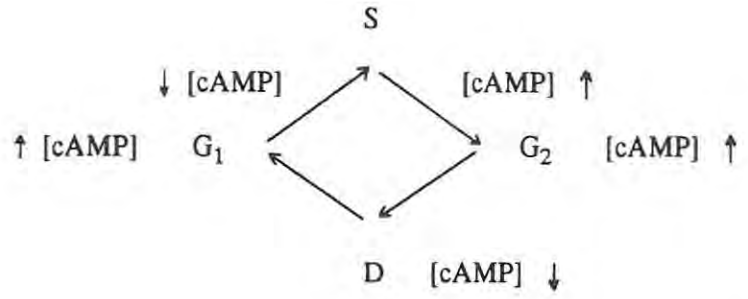
Many physiological activities are potentiated by hormonal actions which utilize cAMP as a "second messenger" or mediator (254,cited by 255). The extent of the activities of cAMP depends on its concentration levels (254). The discovery of the functions of cAMP was initiated in 1957 by Earl Sutherland (4,256), who at the time was investigating the mechanism of action of hormones. The conversion of ATP to cAMP by AC occurs in the presence of a divalent cation, such as Ca^{2+}, Mg^{2+} or Mn^{2+} (241,257,258). Phosphodiesterase (PDE) is the enzyme capable of destroying the biological activity of cAMP. cAMP is converted to 5' adenosine monophosphate (AMP) by PDE, in the presence of Mg^{2+} (241,257,259). Different types of PDE's exist, *eg.* calmodulin-dependent PDE (260).

1.8.5 BIOLOGICAL FUNCTIONS OF CYCLIC ADENOSINE MONOPHOSPHATE

A very interesting and important property of cAMP is that it affects the activity of different cell types in different ways (261). cAMP regulates a variety of functions in normal cells, which are of significance in transformed cells. Cell motility, morphology, growth, contact inhibition and the synthesis of proteoglycans (which are important in cell matrix synthesis) are controlled by cAMP (259,261,262,263). Regarding cancer, cAMP may be a modulator of the activity of several oncogenes (256).

The following evidence supports the role of cAMP in the control of cellular growth: a) cAMP levels are lower in transformed cells than in normal cells; b) agents which increase cellular cAMP levels generally suppress cell growth; and c) agents which decrease cellular cAMP levels generally stimulate cell growth (264,265).

cAMP concentrations also vary within the cell during the different phases of the cycle. These fluctuating levels of cAMP play a significant role in controlling the cell cycle (247,266).



G_1 = preparation for chromosome replication
S = DNA synthesis
G_2 = preparation for cytokinesis
D = cytokinesis or cell division

FIGURE 9: cAMP regulation of the cell cycle.

The cell cycle in eukaryotes can be divided into four phases (see Figure 9). It begins with the birth of the cell at cytokinesis or cell division. It then enters the G_1 (or gap-1) phase, during which it prepares to replicate its chromosomes; the next phase is the S phase (for DNA synthesis), where the cell replicates its chromosomes; it pauses in a second gap, or G_2 (or gap-2) phase, during which it prepares for mitosis and cytokinesis; and then finally functions as an individual cell, upon dividing into two new individuals during the D phase (267).

The G_1 phase is the most sensitive to inhibition by shortages of essential nutrients, but is also the most responsive to growth factors and hormones. There is a relatively prolonged cAMP surge in the G_1 phase of a large variety of cells (261,267). A second prolonged surge occurs in the late S phase or G_2 phase of some cells (261,266,267). These elevated levels of cAMP keep the cell in the quiescent state and only once the cAMP levels drop, is the cell then stimulated to resume cycling. Thus, high levels of cAMP in the S phase will prevent DNA synthesis (266,267). cAMP therefore functions as a negative regulator of DNA synthesis (267).

1.8.6 FACTORS INFLUENCING ADENYLATE CYCLASE ACTIVITY

1.8.6.1 Membrane lipid composition

AC is sensitive to changes in membrane fluidity. An increase in membrane fluidity augments enzyme activity, whereas activity is reduced when the membrane becomes rigid (242,268).

It has been suggested that AC exists in several different conformations. Varying activities and changes in membrane lipid composition can cause the enzyme to shift from one conformation to another (70). Changes in membrane lipid environment can thus exert profound effects on the activity of AC (74,269).

1.8.6.2 Prostaglandin E

PGs appear to be important regulators of AC activity (270), with the mechanisms of action of PGs in many biological systems having been shown to be associated with the activation of AC and an increase in cellular cAMP concentrations (271). PGE_1 must bind to membrane receptors before AC is activated (272) and this activation of AC is known to be dependent on exogenous GTP (273).

Initially, it was thought that the AC-linked receptors accepted both PGE_1 and PGE_2 (274). However, it is now known that these two PGs have their own receptor sites on the AC enzyme (100,275,276). PGE_1 causes a reversible, concentration-dependent stimulation of AC activity (241,261,272,277), while PGE_2 is reported to have stimulatory (184,276) and inhibitory (278) effects on AC activity. PGE_2 may produce a state of heterologous desensitization (278). Hence, the effects of all PGs are independent of each other, as separate receptors are present for each PG (276).

1.8.6.3 Calcium-dependent regulatory protein

The calcium-dependent regulatory protein (CDR), calmodulin, is recognized as the mediator of calcium-dependent control of a large number of enzymes. AC has displayed a requirement for CDR for the stimulatory effects of calcium on AC (243,279). CDR-calcium is however found to activate some ACs and not others (243,280).

1.8.7 CYCLIC ADENOSINE MONOPHOSPHATE AND FATTY ACIDS

cAMP evokes the phosphorylation of acetyl-CoA carboxylase that inhibits malonyl-CoA synthesis from acetyl-CoA, in normal cells. Therefore, the *de novo* biosynthesis of FAs is inhibited by elevated cAMP levels (83).

The first step in the biosynthesis of PUFAs involves delta-6-desaturase. The cAMP analogue, dibutyryl cAMP, has an inhibitory effect on delta-6-desaturase activity, resulting in a decrease in the oxidative desaturation of LA to GLA (82,83,281,282). However, cAMP has no effect on the elongase enzyme converting GLA to DGLA (281). A decrease in the conversion of DGLA to AA, in this case, is therefore due to lower levels of DGLA as a result of the loss of delta-6-desaturase activity and not as an effect on delta-5-desaturase activity (83,283).

In tumour cells, supplementation with PUFAs such as LA results in an increase in the levels of membrane PUFAs and cAMP levels. This may be a result of increased stability of the membrane, which will result in enhanced AC activity and hence the higher cellular cAMP levels (284,285,286).

In normal fat cells, lipolytic hormones such as ß-adrenergic agents stimulate AC and increase cAMP content. cAMP presumably activates cAMP-dependent protein kinase. The latter catalyzes the phosphorylation and subsequent activation of the hormone-sensitive triacylglycerol lipase and thus promotes the release of glycerol and FAs from stored triacylglycerols (260,287). FAs, therefore,

exhibit a relationship with cAMP.

1.8.8 CYCLIC ADENOSINE MONOPHOSPHATE AND PROSTAGLANDINS

As indicated in section 1.8.6.2, PGs are believed to act through cAMP in numerous systems (261), as they can alter the intracellular levels of cAMP and/or Ca^{2+} (174,181,274,288). PGE_1 and PGE_2 increase intracellular cAMP levels in renal papillary collecting tubule cells (180). In osteoblast-like cells, PGE_2 results in a greater stimulation of cAMP production than does PGE_1 (274). Cell proliferation shows an inverse relationship with these PG-induced changes in cAMP levels (289).

Since PGE_2 can activate cAMP formation in normal cells (160,290,291), elevated PGE_2 levels result in increased cAMP formation which suppresses DNA synthesis (292). Furthermore, cAMP acts as the second messenger for PGE_2-mediated modulation of biological functions in various types of cells (293,294). At concentrations greater than 10^{-6} M, PGE_2 stimulates platelet AC and results in raised cAMP. However, at concentrations of 10^{-10} M, PGE_2 has the opposite effect (cited by 295). In general, it appears that PGE_2 stimulates cAMP formation directly (296), with the latter acting as the second messenger for PGE_2-mediated modulation of biological functions in various types of cells (293,294).

Breast tumours and other *in vivo* tumours generally have elevated levels of PGE_2 and cAMP (291,297,298,299). PGE_1 is effective in elevating cAMP levels when it is added to tumour cells at the late and the early G_2 phase (300).

1.8.9 ADENYLATE CYCLASE ACTIVITY, CYCLIC ADENOSINE MONOPHOSPHATE AND CANCER

Malignant and transformed cells in culture generally have lower intracellular levels of cAMP than normal cells (264,301,302,303,304,305). Low intracellular levels of cAMP in transformed cells

may be due to alteration in the activity of one or both enzymes which control cAMP levels, *ie.* AC and PDE (303,306).

AC has two binding sites for Mg^{2+}: one at the catalytic site and the other at G_S. The latter is reported to be altered during transformation, resulting in the inactivation of this cation activation site (251). Increased G_s is present in thyroid tumours (222). Moreover, abnormalities at the cell surface receptor sites have also been correlated with a decrease of cAMP in tumours (251).

In transformed cells, several changes are known to occur in the plasma membrane. As AC is associated with the plasma membrane, it is conceivable that some of these changes will alter AC receptor sites, resulting in decreased AC activity and lower levels of cAMP (284,303,304,307). Lipid peroxidation can result in the polymerization and depletion of membrane PUFAs, and therefore, decreased membrane fluidity, thus inhibiting AC activity (308).

It has been reported (306) that during oncogenesis (the premalignant stage in cells) AC activity is always elevated, resulting in elevated cAMP levels in these cells. This alteration in AC metabolism could have a functional role in oncogenesis or it could be an accompaniment to other cellular changes (306,256). However, cAMP does not appear to function as a secondary messenger for oncogenes (306).

Numerous *in vitro* studies have shown an inverse correlation between intracellular cAMP levels and the rate of tumour cell proliferation (262,266,303,304,309,310,311). Therefore, it has been suggested that abnormal properties of transformed cells, *eg.* rapid growth rates, abnormal morphology, loss of contact inhibition, etc. can be reverted towards those of normal cells by treatment with agents which raise cAMP levels (26,262,312). This implicity assumes that many of the abnormal properties of transformed cells may be due to low levels of cAMP (303), as a result of reduced AC activity. However, several *in vivo* studies with experimental animal tumours have revealed elevated cAMP levels (299,309,313,314).

1.9 OBJECTIVES

The principle objective of this study was to investigate the effect and role of Asc supplementation, over a nutritional range, on the cellular functions of *in vitro* cultured normal and malignant cells.

The pathway (see Figure 10) that was studied in this regard, was that where FAs were converted to PGE_2, with subsequent possible effects of the latter on AC activity and cAMP production. Thus, the effect of Asc supplementation on the activity and metabolism of this pathway, as well on the metabolites which influence this pathway, was investigated.

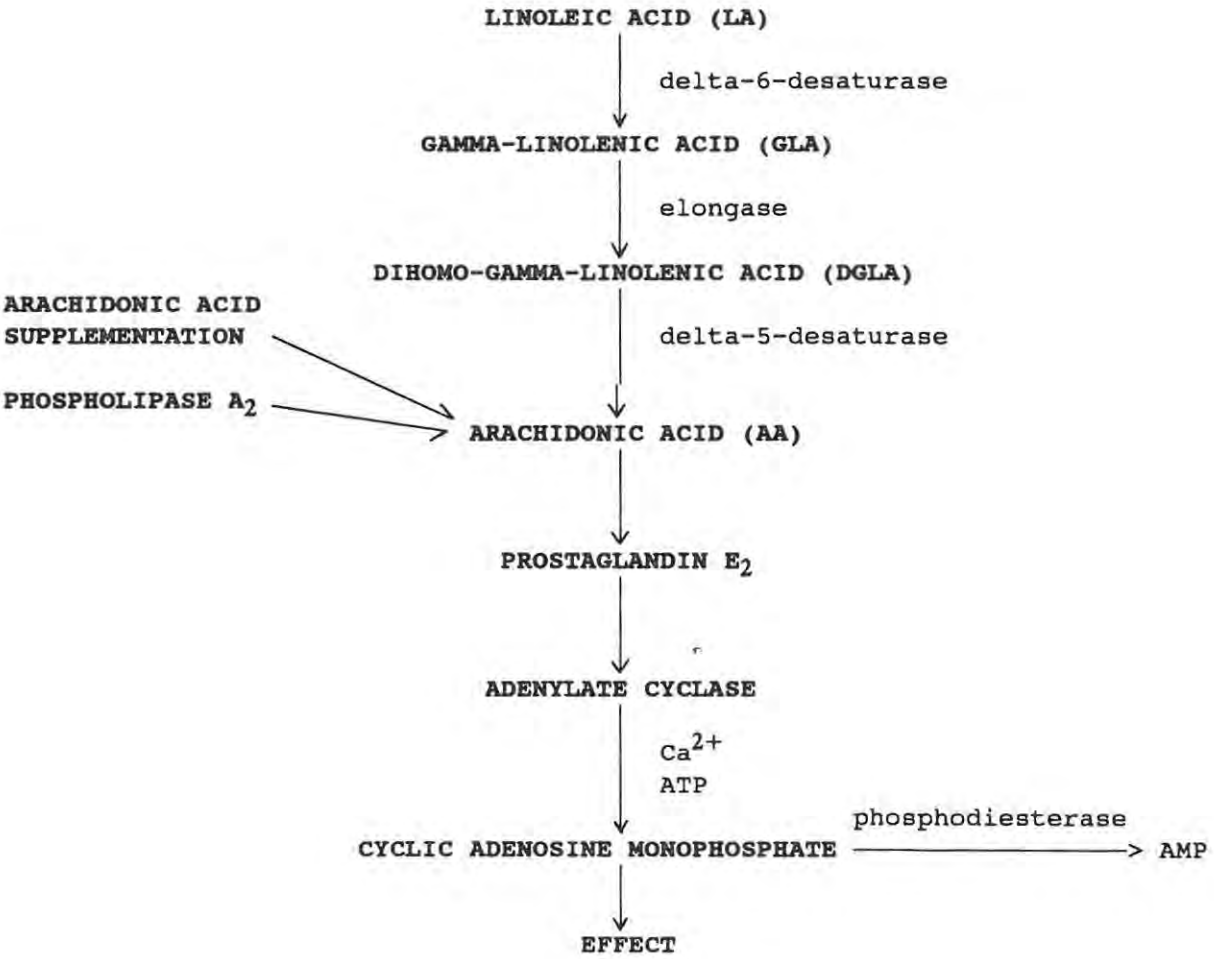


FIGURE 10: A schematic representation of the project outline

2

CELL GROWTH AND NUTRIENT SUPPLEMENTATION

2.1 INTRODUCTION

Animal cell culture has expanded considerably over the past few years. Once a purely experimental procedure, it is now an accepted technological component of many aspects of biological research and commercial exploitation. The development of continuous cell lines has attained great importance to research and is accompanied by many advantages over a finite cell line (315). Both cell culture techniques allow for the supplementation of the cultures with different nutrients and the monitoring of the effects of these on cell growth and cell metabolism.

In the race to find a cure for cancer, cultured malignant cell lines have found extensive use. These cells have been supplemented with various toxic chemical compounds, as well as with various natural biological substances, in an effort to find an effective inhibitor of tumour cell growth. The potential of the latter substances as a preventative measure to cancer development is also of importance, as a number of sources clearly indicate a relationship between diet and cancer incidence in human populations (66).

One of the dietary nutrients suggested to be important in malignancy is Asc. Ascitic lymphoma cells transplanted into mice led to decreased plasma Asc levels during malignancy, resulting in Asc deficiency of the hosts (25). This suggests that the inability of the malignant cells to maintain various biochemical processes may be partly due to the deficiency of an essential nutrient such as Asc. Tumour cells, for example, lose their ability to maintain the complex intra- and extracellular matrix (13), as well as the stability of the cell membrane (108). The administration of Asc to cancer patients as a therapeutic agent suggests that it could assist in overcoming Asc deficiency present and aid in stabilizing malignant cell development, thus preventing further metastasis (13,29,316).

The survival rate of mice bearing P388 leukaemia and Ehrlich carcinoma was increased after treatment with a mixture of vitamins C and B_{12} (317). Sodium Asc, in combinaton with some tumour therapeutic agents such as 5-fluorosil, bleomycin sulphate, cAMP-stimulating agents and X-irradiation, has been found to potentiate the growth inhibitory effect of these agents when applied to neuroblastoma cells *in vitro* (41). Asc has also been used in combination with other vitamins and chemotherapeutic measures, *eg* Asc and Vitamin K_3 (318).

Other nutrients which influence the fluidity and stability of all membranes in cells are the EFAs (4,70). In tumour cells, there may be substantially reduced quantities of 6-desaturated EFAs (88). Supplementation with EFAs, *eg* GLA (88,96,99,135), can overcome the deficiencies in these cells, thus elevating EFA levels and increasing eicosanoid production. Eicosanoid production, in turn, is essential in the generation of secondary metabolites, *eg* cAMP (see Chapter 1), which control many metabolic pathways including those associated with the control of cell growth.

In this study, cells were supplemented with Asc, a combination of Asc and AA, AA, and PGE_2, respectively. The influence of these compounds on the growth of non-malignant LLCMK (monkey kidney) and malignant B16 murine melanoma cell lines was monitored.

2.2 MATERIALS AND METHODS

MATERIALS

Basal Minimum Essential Medium (MEM), Foetal Calf Serum (FCS), LLCMK (monkey kidney) cells and B16 murine melanoma cells were purchased from Highveld Biologicals Ltd, South Africa. L-Serine and L-ascorbic acid (reduced form - 99%) were purchased from Sigma Chemical Co., England. Novo Streptomycin (1g/3ml) and Novo Penicillin (2 million U) were obtained from Novo Industries (Pharmaceuticals Ltd), South Africa. Unilab, SAARCHEM, South Africa supplied $NaHCO_3$ and Dimethyl Sulphoxide (DMSO). Glycine, D-glucose, Phenol Red, KH_2PO_4 and $Na_2HPO_4 \cdot 2H_2O$ were purchased from BDH Chemicals Ltd, England. Trypsin was purchased from

Boehringer Mannheim, Germany, while ethylenediaminetetraacetic acid (EDTA) was purchased from Holpro Chemical Co., South Africa and Cell-Cult sterile flasks ($75cm^2$ and $25cm^2$) were obtained from Sterilin, United Kingdom. Arachidonic Acid and Prostaglandin E_2 were obtained from Sigma Chemical Co., USA, while 15-^{3}H Arachidonic Acid was purchased from Amersham International, England. A haemocytometer was purchased from Neubauer, Germany (double ruling).

METHODS

2.2.1 PREPARATION OF CULTURE REAGENTS

2.2.1.1 Preparation of the medium

The following compounds were added to Basal MEM, which contained Hank's salts and glutamine but no $NaHCO_3$:

0.0005g/l Ascorbic acid	0.006g/l Glycine
0.01g/l Serine	0.75g/l $NaHCO_3$

5ml/l Novo-Strep and Novopen Mixture

(1 Vial of sodium benzylpenicillin (10^6U) and one vial of streptomycin sulphate (10^6 μg) were combined and then made up to 100ml with milli Q water)

Ten litres of medium was prepared at a time, using milli Q water.

2.2.1.2 Filtration of the medium

The medium was filtered through a millipore filtration unit (Millipore Corporation, USA), using the following filters: a prefilter, type SM 42 "Membr filter" 50K (Size 130); a 0.45μm type NA filter (HAWP 14250); and a 0.22μm type GS filter (GSWP 14250). The initial 200ml of medium pumped through was discarded, while the remaining medium was filtered into autoclaved bottles.

The bottles of medium were incubated at 37°C for a week, to test for contamination, before being used.

2.2.1.3 Preparation of growth and freezing media

Growth medium was prepared by filtering FCS through a 0.45μm Millipore filter using a Swinnex-25 holder (Millipore Corporation, USA), until the medium contained 10% (v/v) FCS. Once FCS had been added, all medium was incubated at 37°C for 48 hours to test for contamination. 15% (v/v) FCS medium was prepared for use in the first 24 hour growth period of vials of cells, thawed, from storage. Freezing medium was prepared by adding 10% DMSO and 20% FCS, before being frozen until required. If the pH of the growth medium was too acidic, a few drops of sterile 1M NaOH solution were added to return it to physiological pH.

2.2.1.4 Preparation of trypsin solution

The trypsin solution required was a 0.05% solution, therefore a 500 units/ml solution was prepared. The trypsin solution (1 litre constituted with milli Q water), contained the following:

8.0g	NaCl	0.2g	EDTA
0.4g	KCl	46.7mg	Trypsin
1.0g	D-glucose	0.02g	Phenol red
0.58g	$NaHCO_3$		
10ml	Novo-Strep and Novopen solution (prepared as in 2.2.1.1)		

The trypsin solution was filtered directly into the culture flask, through a 0.45μm Millipore filter using a Swinnex-25 holder.

2.2.1.5 Preparation of the ascorbic acid-containing media

A 10 mg/ml Asc stock solution was prepared. It was filtered into the medium, which already

contained 10% FCS, to yield four different batches of medium containing the following supplemented Asc concentrations: 25; 35; 50 and 100 μg/ml.

2.2.1.6 Preparation of phosphate buffered saline solution

Phosphate buffered saline (PBS), pH 6.6 in milli Q water, was prepared as follows:

8 g/l	NaCl	0.2 g/l	KCl
0.2 g/l	KH_2PO_4	0.15 g/l	$NaHPO_4 \cdot 2H_2O$

2.2.2 CELL CULTURE

2.2.2.1 Routine cell culture procedures

All work was conducted on a laminar flow bench which had previously been sterilised by exposure to ultra-violet light and all equipment used was either purchased sterile or autoclaved. All equipment was swabbed with 95% alcohol prior to use. Non-malignant LLCMK (monkey kidney) cells and malignant B16 (murine melanoma) cells were incubated at 37°C in 75cm^2 flasks containing 30ml 10% (v/v) FCS media, when they were not required for experimental purposes. The medium was changed frequently, approximately once a day.

To passage cells, the growth medium was discarded and 10ml of sterile trypsin solution was added to each flask. Thereafter, these flasks were incubated at 37°C for ten minutes in order to allow the cells to detach from the flask surface. The cells were then passaged into three or four flasks and medium containing 10% FCS was added.

2.2.2.2 Freezing of cells

The storage of a cell line involved near confluent flasks of cells being harvested and frozen in 2ml of freezing medium in cryogenic vials. Thereafter, these vials of cells were stored in liquid

nitrogen, until required.

2.2.3 THE EFFECT OF ASCORBIC ACID SUPPLEMENTATION ON CELL GROWTH

2.2.3.1 Experimental cell culture procedure

Large flasks of either LLCMK or B16 cells with near confluent cell growth were trypsinized with 10ml sterile trypsin. The flasks were incubated at 37°C until the cells had lifted off the flask surfaces. Once the cells had lifted off, they were poured into sterile tubes and centrifuged for 10 minutes at 3 000g (Hettich Universal Centrifuge). The trypsin solution was poured off and the pellet resuspended in 2ml 2% (v/v) FCS medium. The cells were counted using a haemocytometer. These cell counts allowed for the calculation of the volume of cell suspension required per flask in order to seed 500 000 cells per 75cm^2 flask, or 300 000 cells per 25cm^2 flask. Experiments 3.2*, 3.3*, 3.4* and 3.7* were set up using 75cm^2 flasks, while experiment 3.1* and 3.5* used 25cm^2 flasks. 30ml or 10ml medium, containing 10% (v/v) FCS and 0 - 100 μg/ml Asc, was added to the 75cm^2 or 25cm^2 flasks, respectively. Five flasks were set up at each Asc concentration, *ie.* 0, 25, 35, 50 and 100 μg/ml. The flasks were incubated at 37°C and the medium changed if required.

* The analytical work performed on these cultured cells is described in Chapter 3. Hence, the experiments here are denoted with numbering relevant to Chapter 3.

2.2.3.2 Harvesting of the experimental cell cultures

Upon the first flask reaching confluency, the cells in all the flasks were harvested under non-sterile conditions using 10ml or 5ml of trypsin solution, depending on the size of the flask. The cell suspensions were centrifuged for 10 minutes at 3 000g. The pellet was resuspended in 2ml PBS, unless otherwise stated, and the cells were counted using a haemocytometer. The cell counts were used as a reflection of cell growth, enabling the effect of Asc on cell growth to be determined. The counted cells were then used for further analysis.

2.2.4 THE EFFECT OF ASCORBIC ACID AND ARACHIDONIC ACID SUPPLEMENTATION ON CELL GROWTH

2.2.4.1 Experimental cell culture procedure

Non-malignant LLCMK or malignant B16 murine melanoma cells were seeded into six sets of five 25cm^2 flasks, at 3 x 10^5 cells/flask. Basal MEM, 10ml containing 10% FCS, was added to all the flasks. Asc was added to four sets of the flasks, to give concentrations of 25, 35, 50 and 100 μg/ml respectively. The six sets of flasks were incubated at 37°C, with one medium change. Twenty-four hours prior to confluency, 20μCi 15-^{3}H AA was added to one set of the Asc-free flasks and to all the Asc supplemented flasks. The flasks were incubated again. The sixth set was thus the control set, containing no Asc or 15-^{3}H AA.

The above methods were repeated for AA supplementation, with the following changes. Non-malignant LLCMK or malignant B16 murine melanoma cells were seeded into six sets of five 75cm^2 flasks, at 5 x 10^5 cells/flask. Basal MEM, 30ml containing 10% FCS, was added to all the flasks. Once again, Asc over the concentration range 25 - 100 μg/ml was added to four sets of the flasks. Twenty-four hours prior to confluency, these four sets of flasks plus one other, were supplemented with 2.5μM AA and the flasks incubated again. The control set was again the sixth set of flasks, with no Asc or AA supplementation.

The cells were harvested as described in 2.2.3.2, with the exception of 1ml PBS being used for the resuspension of the cells from both of the above experiments.

The above experimental procedure is relevant to experiment 3.6 in Chapter 3.

2.2.5 THE EFFECTS OF ARACHIDONIC ACID AND PROSTAGLANDIN E_2, RESPECTIVELY, ON CELL GROWTH

2.2.5.1 Experimental cell culture procedure

Non-malignant LLCMK or malignant B16 murine melanoma cells were seeded into five sets of five $75cm^2$ flasks, at 5×10^5 cells/flask. Basal MEM, 30ml containing 10% FCS, was added to all the flasks. The flasks were incubated at 37°C, with one medium change. Twenty-four hours prior to confluency, 1, 5, 10 or 50μM AA was added to the flasks and the flasks incubated again. Five flasks were set up at each concentration, as well as a control set of flasks to which no AA had been added.

The above methods were repeated for PGE_2 supplementation of the cells, with the following changes. Non-malignant LLCMK or malignant B16 murine melanoma cells were seeded into $25cm^2$ flasks, at 3×10^5 cells/flask. Basal MEM, 10ml containing 10% FCS, was added to all the flasks. Once again, the flasks were incubated at 37°C with one media change. Twenty-four hours prior to confluency, 1, 5, 10 or 100μM PGE_2 was added to the flasks and the flasks incubated again. Five duplicates were set up at each concentration, as well as a control set of five flasks.

The cells were harvested as described in 2.2.3.2, except for 1ml of PBS for the resuspension of the cells for PGE_2 analysis and 1ml of Tris-HCl buffer (4mM EDTA) for the cells harvested for AC activity analysis.

The above experimental procedure is relevant to the analytical assays performed in experiment 3.8 in Chapter 3.

2.2.6 STATISTICAL ANALYSIS:

The results obtained were analyzed using a one way analysis of variance (ANOVA) followed by the

Student-Newman Keuls Multiple Range Test.

2.3 RESULTS

2.3.1 THE EFFECT OF ASCORBIC ACID SUPPLEMENTATION ON LLCMK AND B16 CELL GROWTH

The LLCMK and B16 cell counts obtained in the relevant experiments, in which 0 - 100 μg/ml Asc supplementation was performed, are shown in Table 1. Relevant to discussion in this chapter, for both cell types, is the mean of the cell counts of all the experiments. Cell count results for each individual experiment will be discussed relative to cell metabolism in Chapter 4.

The overall mean of the cell count values, recorded in Table 1, indicate that Asc supplementation did not have a significant inhibitory or stimulatory effect on LLCMK cell growth. A general trend of decreased LLCMK cell growth, upon Asc supplementation, is apparent.

Asc supplemented B16 cells also exhibit an overall trend of decreased cell growth relative to the control cells. However, this decrease is not linear. Initial Asc supplementation, 25 μg/ml Asc, results in a decrease in B16 cell growth. At 35 μg/ml Asc supplementation cell growth increases slightly relative to that of the 25 μg/ml Asc supplemented cells, but is still lower relative to the growth of the control cells, although not significantly so. The greatest decrease in cell growth is detected at 50 μg/ml Asc supplementation. At the highest concentration of Asc supplementation, 100 μg/ml, B16 cell growth increases slightly relative to the cell growth recorded for the cells supplemented with 50 μg/ml Asc, but is again lower than that of the control cells.

The general decrease in B16 cell growth is not significant. However, B16 cell growth in certain individual experiments was significantly inhibited by Asc supplementation, *ie.* Exp 3.1 ($p \leq 0.001$), Exp 3.4 ($p \leq 0.01$) and Exp 3.5 ($p \leq 0.05$). This finding was not observed for the LLCMK cells.

TABLE 1: The effect of ascorbic acid supplementation on LLCMK and B16 cell growth, respectively. Values recorded are the mean of five cultures ± SEM.

[Asc] µg/ml	LLCMK CELLS							B16 CELLS						
	Cell No x 10^{-4} cells/ml							Cell No x 10^{-4} cells/ml						
	EXP 3.1	EXP 3.2	EXP 3.3	EXP 3.4	EXP 3.5	EXP 3.7	MEAN	EXP 3.1	EXP 3.2	EXP 3.3	EXP 3.4	EXP 3.5	EXP 3.7	MEAN
0	64.4 ± 8.1	558.7 ± 30.7	542.0 ± 22.2	255.7 ± 39.7	360.3 ± 14.8	252.9 ± 12.8	335.6 ± 32.5	105.8 ± 9.0	529.3 ± 51.7	549.0 ± 41.6	169.6 ± 29.2	736.2 ± 57.4	1213.6 ± 36.0	547.7 ± 70.0
25	60.8 ± 3.4	526.7 ± 31.5	521.6 ± 26.6	215.1 ± 14.5	374.3 ± 12.8	342.9 ± 73.3	338.5 ± 33.6	56.5 ± 7.5	666.7 ± 30.0	665.0 ± 30.8	40.8[b] ± 3.2	652.7 ± 31.4	1167.8 ± 17.5	517.5 ± 72.3
35	60.3 ± 9.2	459.3 ± 31.2	462.6 ± 27.4	192.3 ± 10.4	346.5 ± 15.3	286.9 ± 65.0	306.0 ± 30.1	8.5[a] ± 0.5	590.7 ± 41.5	611.0 ± 29.4	26.0[b] ± 2.9	644.1 ± 35.8	1182.3 ± 25.6	527.0 ± 75.8
50	87.1 ± 7.7	497.3 ± 39.7	514.4 ± 36.9	242.8 ± 14.8	347.7 ± 8.7	216.1 ± 59.4	316.7 ± 30.8	6.4[a] ± 1.4	597.3 ± 65.8	670.4 ± 75.5	16.8[b] ± 1.6	550.4[b] ± 15.5	1147.8 ± 13.5	492.0 ± 74.6
100	68.1 ± 5.2	414.0 ± 29.9	399.0 ± 32.3	311.5 ± 13.3	344.8 ± 3.6	277.4 ± 44.7	291.5 ± 23.9	6.0[a] ± 0.7	714.7 ± 55.4	725.6 ± 52.6	15.2[b] ± 1.1	590.8[c] ± 14.7	1081.2 ± 35.0	510.4 ± 71.3

a = $p \leq 0.001$
b = $p \leq 0.01$
c = $p \leq 0.05$

2.3.2 THE EFFECT OF ASCORBIC ACID AND ARACHIDONIC ACID SUPPLEMENTATION ON CELL GROWTH

Asc together with AA supplementation (radioactive and non-radioactive) resulted in an overall decrease in the cell growth of both the LLCMK and B16 cells (Table 2 and Table 3). The decrease in cell growth recorded at 25 μg/ml Asc and 15-^{3}H AA supplementation of the LLCMK cells was significant ($p \leq 0.05$) (Table 2). At 100 μg/ml Asc and 15-^{3}H AA supplementation, the decrease in B16 cell growth was significant ($p \leq 0.001$) (Table 2). Prior to Asc supplementation, AA and 15-^{3}H AA supplementation, respectively, did not affect the growth of the LLCMK or B16 cells to any significant extent.

TABLE 2: The effect of ascorbic acid and 15-^{3}H arachidonic acid supplementation on the growth of the LLCMK and B16 cells, respectively. Results are the mean of five cultures $\pm$ SEM.

[Ascorbate] μg/ml	15-^{3}H AA μCi	Cell No x 10^{-4}/ml LLCMK	B16
0	0	36.0 ± 5.5	362.9 ± 22.4
0	20	28.8 ± 3.2	368.6 ± 12.9
25	20	14.3[b] ± 0.9	384.3 ± 40.3
35	20	29.1 ± 4.5	349.2 ± 34.2
50	20	26.0 ± 1.8	394.7 ± 10.9
100	20	27.2 ± 2.2	181.0[a] ± 35.4

a = $p \leq 0.001$: relative to the control and other B16 cultures supplemented with 15-^{3}H AA.
b = $p \leq 0.05$: relative to LLCMK control and 15-^{3}H AA supplemented cultures.

TABLE 3: The effect of ascorbic acid and arachidonic acid supplementation on the growth of LLCMK and B16 cells. The results are the mean of five cultures ± SEM.

[Ascorbate] μg/ml	[AA] μM	Cell No x 10^{-4}/ml LLCMK	B16
0	0	365.7 ± 7.8	823.0 ± 25.6
0	2.5	354.3 ± 14.1	805.0 ± 91.2
25	2.5	388.5 ± 11.4	631.0 ± 7.0
35	2.5	357.3 ± 22.5	619.3 ± 69.9
50	2.5	368.8 ± 2.6	728.3 ± 41.9
100	2.5	336.3 ± 1.7	767.7 ± 32.9

2.3.3 THE EFFECT OF ARACHIDONIC ACID AND PROSTAGLANDIN E_2 SUPPLEMENTATION ON CELL GROWTH

Separate AA and PGE_2 supplementation of the LLCMK cells resulted in a trend of slightly decreased cell growth (Table 4 and Table 5).

A similar effect was also observed with AA supplementation of the B16 cells (Table 4). However, this was not the case with PGE_2 supplementation of the B16 cells. Initially, 1μM PGE_2 supplementation of the B16 cells resulted in a significant ($p \leq 0.001$) increase in cell growth (Table 5). Further PGE_2 supplementation, 100μM PGE_2, reduced B16 cell growth significantly ($p \leq 0.001$).

TABLE 4: The effect of arachidonic acid supplementation on the growth of LLCMK and of B16 cells. Results are the mean of five cultures ± SEM.

[AA] μM	Cell No x 10^{-4}/ml LLCMK	B16
0	674.2 ± 64.8	916.8 ± 119.2
1	635.1 ± 46.1	699.3 ± 19.9
5	556.5 ± 54.3	725.9 ± 44.6
10	554.7 ± 39.1	809.6 ± 48.6
50	641.4 ± 48.0	763.4 ± 58.3

TABLE 5: The effect of prostaglandin E_2 supplementation on the growth of the LLCMK and B16 cells, respectively. Results are the mean of five cultures ± SEM.

[PGE_2] μM	Cell No. x 10^{-4}/ml LLCMK	B16
0	168.4 ± 16.5	171.1 ± 10.0
1	158.4 ± 21.8	226.0[a] ± 9.4
5	155.2 ± 8.7	147.0 ± 7.6
10	144.8 ± 12.5	137.0 ± 4.9
100	147.8 ± 11.1	60.0[b] ± 1.0

a = $p \leq 0.001$: relative to the B16 control and PGE_2 supplemented cells.
b = $p \leq 0.001$: relative to the B16 control and PGE_2 supplemented cells.

2.4 DISCUSSION

The Asc concentrations of the media used in this study were in the range 25 - 100 μg/ml. This is considered to be a physiological or nutritional concentration rather than a pharmacological level, as these levels are similar to normal Asc values *in vivo*. Plasma levels vary between 3 and 10 μg/ml Asc in the human body, while tissue levels can be as high as 400 μg/ml. The average Asc concentration in the body, when saturated, is approximately 50 μg/ml Asc (cited by 319). 100 μg/ml Asc supplementation, in this study, was considered to be between the nutritional and pharmacological Asc ranges. Interestingly, Asc supplementation of up to 200 μg/ml is considered not to cause any morphological changes in mouse melanoma (B16), mouse neuroblastoma, rat glioma and mouse fibrolasts in culture. Only higher Asc concentrations, 500 - 1000 μg/ml Asc supplementation, were lethal to these cultures (38).

Asc supplementation of normal and tumour cells has been shown to have either an inhibitory or a stimulatory effect on the growth of these cells (49,53,56,320,321,322). The effect appears to depend on the cell type and the level of Asc supplementation. The growth of Ehrlich ascites tumour cells (37,323), mouse melanoma, glioma and neuroblastoma cells (41) and human leukaemic cells (51) was inhibited by Asc supplementation at pharmacological levels. Melanoma cells have been found to preferentially incorporate Asc. *In vitro* studies have shown that Asc is more toxic to melanotic cells than to any others studied (47). *In vivo* treatment of a patient suffering disseminated reticulum cell sarcoma, who was dosed with pharmacological levels of Asc, resulted in complete spontaneous regression of his illness (324). On the other hand, Asc supplementation of mouse sarcoma 180 and mouse plasmacytoma cells (13), as well as human promyelocytic leukaemic cells (49), was reported to stimulate the growth of these cells. It has been reported that in non-malignant cells, Asc supplementation of osteoblast-like MC 3T3-E1 cells (325) resulted in enhanced proliferation, while Chinese hamster ovary cell proliferation (319) was inhibited by Asc supplementation.

Asc supplementation of the LLCMK cells resulted in a general decrease in cell growth. Asc

supplementation of the B16 cells in this study also resulted in an overall decrease in cell growth. In general, this decrease in cell growth was not significant. However what is noteable, and was not the case with the LLCMK cells, is the fact that B16 cell growth in certain experiments decreased significantly upon Asc supplementation. With reference to Exp 3.1, B16 cell growth at 100 μg/ml Asc supplementation was only 6% of the B16 control cell growth. The reason for the different effects of Asc supplementation on B16 cell growth is not clear. It appears that Asc supplementation of a certain cell line has varied effects within the same cell line. Certain data suggest that the inhibition of transformation by Asc may be associated with the regulation of redox potential, glycoproteins and lipids in these transformed cells (326). The antitumour activity of Asc is also suggested not to be due to the metabolism of Asc as a vitamin, but due to its chemical properties (57).

Concerning the uptake of Asc by cells, it appears that more than one transport mechanism is operative. Vitamins tend to cross cell membranes by simple diffusion and it is the concentration gradient within the membrane that determines the diffusion rate (327). The hydrophilic nature of the vitamin suggests that a carrier mechanism is needed for Asc entry into the cell (328). Asc-mediated transmembrane electron transport and Asc uptake are two different processes in leukaemic cell lines (329).

The supplementation of LLCMK and B16 cultures with both Asc and AA, again resulted in a general trend of decreased cell growth observed for both cell types. 100 μg/ml Asc and 20μCi 15-^{3}H AA supplementation had a significant inhibitory effect on B16 cell growth. As the supplemented level of radioactive AA remained constant, it is reasonable to conclude that this inhibitory effect on cell growth was due to the Asc supplementation. Results obtained for AA supplementation only, support the above conclusions, as this supplementation did not result in a significant inhibitory effect on either LLCMK or B16 cell growth. While not significant, this effect was greater in the B16 cells.

It has been reported that certain FAs in high concentrations can exert selective toxicity against

neoplastic cells, while these FAs can also lower the growth rate of cells without killing the cells (124,133). Human skin fibroblast growth was reduced by 25 to 50% when palmitic acid, LA or AA was added in concentrations of 50μM or above (330). Human breast, lung and prostate cancer cells were reported to be selectively killed when supplemented with n - 6 EFAs (98). It is suggested that the free carboxyl group of the free FAs plays a role in killing tumour cells (148). GLA has been found to both suppress and inhibit the growth of different tumour cells (99,131,132,134,135,138). However, it has been reported that dietary PUFA promotes the growth of transplantable rat and mouse mammary adenocarcinomas which are of different origin (140). Results also suggest that a high-fat diet rich in n - 6 PUFA can enhance the metastasis of human breast cancer cells grown in mice (331).

Since these FAs are the precursors to the PGs, the effects of PGs on cell growth were subsequently investigated. The PGE series, in particular PGE_2, has been shown to inhibit the rate of cell proliferation in several animal tumour cell lines (124,201,205) and in tumour-bearing rats (197). However, PGE_2 synthesized from supplemented LA had no effect on the growth of a metastatic mouse mammary tumour cell line (332). It is hypothesized that the growth-regulatory action of PGs could be mediated by cAMP (179,221). In this study, 100μM PGE_2 supplementation significantly decreased B16 cell growth. Another study (186) done on the B16 murine melanoma model also showed eicosanoid synthesis to be negatively correlated with metastatic potential. The concentration of supplemented PGE_2 to cultures appears to be important here, as PGE_2 levels below 0.1μM did not affect the proliferation rate of murine mammary tumour cells (288). These lower PGE_2 concentrations actually increased the growth of Raji lymphoid cells, while at higher concentrations PGE_2 inhibited this cell growth significantly (206). This effect was observed in the B16 cells supplemented with PGE_2. In this study, PGE_2 supplementation did not affect LLCMK cell growth to any significant extent.

In general, Asc, AA and PGE_2 can be classified as nutritional pharmacological agents with respect to cancer cells, since cell growth is affected by supplementation with these nutrients. In order to clarify this effect, the metabolic role of these nutrients in the LLCMK and B16 cells was further

investigated at a biochemical level. Certain metabolic pathways and their relevant metabolites, which are associated with cell growth, were selected to investigate the effects of nutrient supplementation in LLCMK and B16 cells.

3

METABOLISM OF SECOND MESSENGERS WITH NUTRIENT SUPPLEMENTATION

3.1 ADENYLATE CYCLASE ACTIVITY, CYCLIC ADENOSINE MONOPHOSPHATE FORMATION AND ASCORBIC ACID SUPPLEMENTATION

3.1.1 INTRODUCTION

The AC system is composed exclusively of intrinsic membrane proteins and is dependant upon their proper integration in a membrane in order to function adequately (241,243). Like many integral membrane-bound enzymes, the activity of AC is modulated by the nature of its membrane-lipid environment (70,74,241,242,268,333). Generally, it is thought that an increase in membrane fluidity augments enzyme activity, whereas the activity is reduced when the membrane becomes more rigid (236). However, evidence suggests that whereas increases in membrane PUFAs lead to a stimulation of AC activity in some membranes, such increases can also result in reduced activity in others (75).

Changes to the membrane intracellular signalling systems are frequently implicated in the development of neoplasia (280,334). Malignant and transformed cells in culture generally have lower intracellular levels of cAMP than normal cells (303,304,305). Numerous *in vitro* studies have shown an inverse correlation between intracellular cAMP levels and the rate of tumour cell proliferation (262,266,303,304,310,311), while elevated cAMP levels have been found in experimental animal tumours *in vivo* (299,309,313,314) during oncogenesis.

Low intracellular levels of cAMP in transformed cells may be due to an alteration in the activity of one or both enzymes which control cAMP levels, *ie.* AC and PDE (303). PDE hydrolyzes cAMP to form AMP (4). PDE activity in rat mammary carcinomas was 2.5 times lower than PDE activity in non-metastasizing mammary carcinomas (335). AC has two binding sites for Mg^{2+}, one at the

catalytic site and the other at G_i. The latter is reported to be altered during transformation, resulting in the inactivation of this cation activation site (251) and thus mediating changes in cAMP levels.

Furthermore, AC is stimulated by the hormones noradrenaline, adrenaline and serotonin. The cellular concentrations of these hormones are in turn influenced by Asc (254). This study was undertaken to determine whether Asc supplementation of non-malignant LLCMK cells and malignant B16 cells had any effect on AC activity and cellular cAMP levels.

3.1.2 MATERIALS AND METHODS

MATERIALS

Aluminum oxide 90, creatine-P-$Na_2 \cdot 4H_2O$, triethanolamine-HCl, $MgCl_2$ and creatine kinase were purchased from Merck, Darmstadt, Germany. [α-^{32}P] ATP (1 mCi/ml) and a cyclic AMP [^{3}H] assay kit were purchased from Amersham International, Amersham, United Kingdom. 3-Isobutyl-1- methylxanthine, cAMP·H_2O and ATP-$Na_2H_3 \cdot 3H_2O$ were obtained from Sigma Chemical Co., USA. $CuSO_4 \cdot 5H_2O$ was purchased from PAL Chemicals, South Africa, while Folin-Ciocalteu Reagent was purchased from SAAR CHEM, South Africa.

METHODS

3.1.2.1 Cell Culture

Refer to 2.2.3.1 and 2.2.3.2. The cell suspensions were centrifuged at 3 000g for 10 minutes and the pellet resuspended in 1ml Tris-HCl buffer containing 4mM EDTA.

3.1.2.2 Homogenization of the cells and separation of cellular components

The cell suspensions were poured into a Dounce homogenizer and were homogenized 30 times with the tight plunger. The homogenizer was rinsed with 1ml Tris-HCl buffer (4mM EDTA). The various homogenates were then centrifuged (Beckman Model J2-21 Centrifuge) at 480g for 20 minutes, to remove nuclei and non-disrupted cells. The supernantant was retained and centrifuged at 4 000g for 20 minutes, to remove the mitochondrial fraction. Once again, the supernatant was retained and centrifuged at 20 000g for 30 minutes, in order to obtain the respective stroma (supernatant) and membrane (pellet) fractions of the homogenized cells. The pellet was resuspended in 2ml Tris-HCl buffer containing 4mM EDTA.

3.1.2.3 Protein determination

The protein concentrations of the various membrane fractions were determined by the modified method of Lowry *et al* (cited by 336). The protein standard curve is recorded in Appendix 1.

3.1.2.4 Adenylate cyclase activity assay

AC activity of the membrane fraction was determined using the method of Schultz and Jakobs (240), and Salomon (337). It measured the rate of conversion of [α-^{32}P] ATP to [^{32}P] cAMP. Tris-HCl buffer, 20μl, was substituted for GTP and forskolin to give the desired final volume of the mixture. Time course assays were performed over a period of ten minutes. Optimal AC activity was detected after a 45 second incubation period of membrane homogenate and substrate solution. AC activity was calculated as catalytic activity relative to the mass of protein, expressed as the formation of 1 pmol cAMP per minute (1 unit) per mg protein.

3.1.2.5 Cyclic adenosine monophosphate extraction

The respective stroma fractions of the two cell types were heated in a boiling water bath for 5

minutes to coagulate protein. The protein was precipitated by centrifugation at 8 000g for 10 minutes and the supernatant then assayed for the presence of cAMP.

3.1.2.6 Cyclic adenosine monophosphate [^{3}H] assay

A commercial radio-immunoassay kit was used to determine the cAMP levels in the stroma fraction of the LLCMK and B16 cells, respectively. The assay was based on the competition between unlabelled cAMP and a fixed quantity of [^{3}H] cAMP for binding to a protein which had a high specificity and affinity for cAMP. The amount of labelled protein-cAMP complex formed was inversely related to the amount of unlabelled cAMP present in the assay sample. The cAMP standard curve is presented in Appendix 2.

3.1.2.7 Statistical Analysis

The results obtained were analyzed using a one way analysis of variance (ANOVA) followed by the Student-Newman Keuls Multiple Range Test. Data in all subsequent sections of this chapter were similarly analyzed.

3.1.3 RESULTS

3.1.3.1 The effect of ascorbic acid on adenylate cyclase activity

Asc supplementation did not result in a significant change in the AC activity of the LLCMK cells. The greatest level of AC activity determined for the LLCMK cells, was at 35 μg/ml Asc supplementation (Table 6).

AC activity in the B16 control cells was significantly lower ($p \leq 0{,}005$) than that detected in the LLCMK control cells. However, AC activity in the B16 cells was significantly increased ($p \leq 0.001$) by Asc supplementation. Increasing Asc levels resulted in an increase in AC specific

activity, with this activity being the greatest at 50 μg/ml Asc supplementation (Table 6).

TABLE 6: The effect of ascorbic acid supplementation on adenylate cyclase activity in the LLCMK and B16 cells, respectively. Values are the mean of five cultures ± SEM.

[Ascorbate] μg/ml	Adenylate Cyclase Activity (U/mg protein)	
	LLCMK	B16
Control	4.35[a] ± 1.30	0.92 ± 0.32
25	3.37 ± 0.37	2.09[b] ± 0.82
35	6.38 ± 0.47	3.12[b] ± 0.71
50	2.25 ± 0.57	5.12[b] ± 0.55
100	4.38 ± 0.93	4.33[b] ± 1.14

a = $p \leq 0.005$: relative to the B16 control cells
b = $p \leq 0.001$: relative to the B16 control cells

3.1.3.2 The effect of ascorbic acid on cyclic adenosine monophosphate levels

The LLCMK cells exhibited no significant trend in cAMP levels upon Asc supplementation of the cells (Table 7), although there was a significant decrease ($p \leq 0.01$) in cAMP levels of the cells supplemented with 25 μg/ml Asc.

cAMP levels in the LLCMK control cells and the B16 control cells were of a similar magnitude (Table 7). 25 μg/ml Asc supplementation of the B16 cells, as in the LLCMK cells, resulted in a significant decrease ($p \leq 0,01$) in cAMP levels. cAMP levels in the B16 cells detected at 35μg/ml Asc supplementation, however, were higher than the cAMP levels of the control and 25 μg/ml Asc supplemented B16 cells. With respect to the B16 control cells, a significant increase in cAMP concentration occurred at 50 μg/ml Asc supplementation. At 100 μg/ml supplemented Asc, the level of cAMP again decreased.

TABLE 7: The effect of ascorbic acid supplementation on cAMP concentrations determined in both the LLCMK and B16 cells. Values are the mean of five cultures ± SEM.

[Ascorbate] µg/ml	cAMP concentration (pmol/mg protein)	
	LLCMK	B16
Control	15.15 ± 3.24	17.94 ± 5.43
25	5.67[a] ± 1.51	7.28[a] ± 2.25
35	11.35 ± 1.87	20.88 ± 1.69
50	8.13 ± 1.49	79.10[b] ± 30.20
100	11.51 ± 3.98	12.81 ± 4.28

a = $p \leq 0.01$: relative to their respective control cells
b = $p \leq 0.025$: relative to the B16 control cells

3.1.4 DISCUSSION

cAMP is known to play a role in the control of cellular growth (264,335). A possible mechanism for the effect of Asc on cell growth could be mediated by an effect on AC activity and the resultant cAMP levels.

Neither the AC activity nor the cAMP levels of the non-malignant LLCMK cells were significantly affected by Asc supplementation. A study of the effect of Asc on guinea pig adrenal AC activity *in vivo*, however, revealed that the latter decreased with increasing Asc concentrations (338). This effect was not exhibited by the LLCMK cells in this study.

AC activity in the B16 control cells was significantly lower than that in the LLCMK control cells. This suggests an alteration in the AC mechanism of the tumour cells, which could be due to a change in membrane fluidity and receptor sites (242,268,284), hormonal stimulation (254) or an alteration of the enzyme (303,304). This result supports evidence that malignant cells generally

have impaired AC activity, which could lower the resultant cAMP levels within these cells (264,335).

In contrast, cAMP levels detected in the control cultures of the B16 and LLCMK cells used in this study, were virtually of the same magnitude. If related to AC activity of the respective cells, it would be expected that cAMP levels would be lower in the tumour cells, as has been reported previously (261). This could also be expected as it is reported that as B16 melanoma cells increase in metastatic potential, there is a significant loss in the ability of their cAMP system to respond appropriately to hormonal stimuli (339). However, it has also been reported (340) that the hormonal stimuli of AC did not increase cAMP levels, indicating that the expression of hormonal effects may be more complicated than merely stimulating AC and thereby generating cAMP. The variable nature of AC activity and cAMP levels in normal and tumour cells is further substantiated by a finding that the intracellular cAMP levels of breast carcinomas *in vivo* were significantly higher than cAMP levels detected in normal breast tissue (299).

The increase in AC activity in the B16 melanoma cells upon Asc supplementation was not only of statistical significance, but there was also a dose dependent increase in AC activity. Maximum AC activity was reached at 50 μg/ml supplemented Asc. At 100 μg/ml Asc supplementation, AC activity decreased slightly. This could indicate an over-saturation of AC receptors by regulatory compounds. However, it has also been reported that B16 clones of high and low metastatic potential differ in their capacity to desensitize AC (334,341). High levels of Asc supplementation could possibly be affecting the AC desensitization mechanism in the B16 cells of this study. Other reports indicate AC activity to be similar in B16 cells of high and low metastatic potential (334,339).

cAMP levels detected in the B16 cells exhibited a trend parallel with the AC activity in the cells, *ie.* increased cAMP levels at 35 and 50 μg/ml Asc supplementation. However, there was a significant decrease in cAMP concentration at 25 μg/ml Asc supplementation, which did not correlate with the AC activity measured at this point in the B16 cells. The highest AC activity in the B16 cells was

found at 50 μg/ml Asc supplementation, as was the highest cAMP levels. At 100 μg/ml Asc supplementation AC activity decreased though not significantly, while the cAMP concentration decreased significantly. These data suggest that a small change in AC activity, with Asc supplementation, affects cAMP production to a greater extent.

Whether Asc directly affects AC activity or acts through an effect on compounds which modulate AC activity is unclear. PGE_2 is one of many compounds that stimulates AC activity and it was therefore of interest to this study to determine the effect of Asc supplementation on PGE_2 levels in the LLCMK and B16 cells.

3.2 PROSTAGLANDIN E_2 LEVELS AND ASCORBIC ACID SUPPLEMENTATION

3.2.1 INTRODUCTION

PGs are cell-to-cell messengers produced by eukaryotic cells, often in response to extracellular stimuli. PGs are not stored within the cell to any great extent, thus any stimulation of their release requires a prior mobilization of substrate precursors such as FAs (4,90,153,155,156,157,342).

Tumour cells synthesize and secrete increased quantities of PGs, in particular the PGE series (PGE_1 and PGE_2) (139,149,154,182,183,186,187,188,189,190,192,193). It is not clear whether the increase in synthesis and release of PGs in tumours is the cause or the result of increased cellular proliferation rates (194). Elevated levels of PG have been found in the blood and/or urine of animals carrying neoplasms (157,198), as well as in transformed cells growing in tissue culture (157). PGs are suggested to mediate interactions between tumour cells and host defense elements, thus possibly contributing to the ability of the tumour cells to establish themselves at the metastatic site (cited by 186).

Studies conducted on human embryo lung fibroblasts indicate that Asc may be able to stimulate PGE_2 production (173). Asc, considered to be of therapeutic value as a possible prophylactic against cancer development, promotes a concentration-dependent (0.001M - 1M) synthesis of PGE_2 in human parenchyma (343). However, Asc supplementation in pig endometrium decreases PGE release (344). Hence, uncertainty exists concerning the role of Asc in PGE_2 metabolism and its metabolic effects. This study was undertaken to investigate the effect of Asc supplementation on PGE_2 levels in the two cell types used in this study, *ie.* the LLCMK and B16 cells.

3.2.2 MATERIALS AND METHODS

MATERIALS

Methyl formate was purchased from Sigma Chemical Co., USA. SEP-PAK C_{18} Cartridges were purchased from Waters Associated, Inc., Massachusetts, USA. The PGE_2 [^{125}I] assay kit with Amerlex-M^{TM} magnetic separation was obtained from Amersham International, Amersham, United Kingdom.

METHODS

3.2.2.1 Cell culture

The method recorded in 2.2.3.1 and 2.2.3.2 was utilized.

3.2.2.2 Homogenization of the cells and separation of cellular components

The procedure described in 3.1.2.2 was repeated, except that 2ml milli Q water was used to rinse the homogenizer. Therefore, the pellet (membrane fraction) was resuspended in 2ml PBS and 2ml milli Q water.

3.2.2.3 Extraction and isolation of prostaglandins

The PGs were extracted according to a modified method of Powell (345,346,cited by 347). The respective 4ml cellular fractions were added to 15ml cold ethanol (95%) and shaken for 10 minutes. The sample was diluted to 100ml with cold water and vortex mixed for 1 minute. The pH of the suspension was adjusted to 3.0 using 1N HCl. The sample was then passed through a SEP-PAK cartridge, which had previously been wet with 20ml of 80% aqueous ethanol and then washed with 20ml water to remove excess ethanol. The cartridges were washed with 10ml water and 10ml

petroleum ether, before the PGs were eluted with 5ml methyl formate. The eluant was dried under a stream of nitrogen at 25°C. The SEP-PAK cartridges were regenerated for re-use by washing with 20ml of 80% ethanol followed by 20ml water.

3.2.2.4 Prostaglandin E_2 [^{125}I] assay system

The extracted, dried PG fractions were reconstituted with 100μl of assay buffer, (pH 7,0). Methyl oximation reagent (100μl) was added to the reconstituted sample and vortex mixed. The resultant solution was incubated at 60°C for 1 hour, to allow methyl oximation of the sample to occur. Preparation of the samples for scintillation counting, using a Beckman Gamma 310 Scintillation counter, was as described in the assay kit. The PGE_2 standard curve is presented in Appendix 3.

3.2.3 RESULTS

3.2.3.1 The effect of ascorbic acid on prostaglandin E_2 metabolism

In the LLCMK and B16 cells, the level of PGE_2 in the membrane fraction was generally lower than that detected in the stroma fraction (Table 8). However, it is evident that the concentration of PGE_2 in some of the stroma fractions was also very low.

No PGE_2 was found in the stroma fractions of the B16 control cells with all of the PGE_2 being detected in the membrane fractions. The PGE_2 concentration in the membrane fraction of the B16 control cells was also significantly higher ($p \leq 0.05$) than that found in the membrane fractions of the Asc supplemented B16 cells. PGE_2 levels in the stroma fraction of the B16 cells supplemented with 25 μg/ml Asc were lower than those detected in the membrane fraction. Further Asc supplementation resulted in an increase in PGE_2 concentration in the stroma, particularly at 35 and 50 μg/ml supplementation (Table 8), while the PGE_2 concentration in the membrane was generally decreased by Asc supplementation.

TABLE 8: The effect of ascorbic acid supplementation on prostaglandin E_2 concentration in LLCMK cells and B16 cells, respectively. Values recorded are the mean of five samples ± SEM.

Cell Fraction	[Ascorbate] μg/ml	[PGE_2] (pg/10^6 cells)	
		LLCMK	B16
STROMA	0	10.60 ± 0.59	0.00 0.00
	25	2.03 ± 0.49	8.29 ± 3.19
	35	7.50 ± 1.45	63.59 ± 19.93
	50	121.97 ± 47.75	40.13 ± 15.01
	100	34.13 ± 7.76	9.99 ± 2.93
MEMBRANE	0	5.87 ± 1.45	100.66 ± 15.70
	25	4.99 ± 1.07	10.32[a] ± 3.05
	35	4.02 ± 0.70	7.42[a] ± 2.58
	50	4.80 ± 1.55	24.86[a] ± 7.829
	100	13.51 ± 2.88	3.42[a] ± 0.64

a = $p \leq 0.05$: relative to the B16 membrane control fraction

The stroma fraction of the LLCMK control cells, as well as the stroma fractions of the 35 - 100 μg/ml Asc supplemented LLCMK cells, had higher levels of PGE_2 than the respective membrane fractions (Table 8).

The PGE_2 concentration in the B16 control cells (combined fractions) was significantly higher ($p \leq 0.025$) than the PGE_2 concentration found in the LLCMK control cells (Table 9). Relative to the LLCMK control cells, Asc supplementation of the LLCMK cells resulted in an overall increase in the total PGE_2 concentrations of these cells. The opposite effect was found at 25 and 100 μg/ml

Asc supplementation of the B16 cells, as this supplementation resulted in a signficant decrease ($p \leq 0.025$) in total PGE_2 levels.

TABLE 9: The effect of ascorbic acid supplementation on total prostaglandin E_2 concentration in both the LLCMK and B16 cells. Values recorded are the mean of five cultures $\pm$ SEM.

[Ascorbate] µg/ml	Total [PGE_2] (pg/10^6 cells)	
	LLCMK	B16
0	16.47[a] ± 2.04	100.66 ± 16.61
25	7.02 ± 1.56	18.61[a] ± 6.24
35	11.52 ± 2.15	71.01 ± 22.51
50	126.77 ± 49.30	64.99 ± 22.83
100	47.64 ± 10.63	13.41[b] ± 3.56

a = $p \leq 0.025$: relative to the B16 control cells

3.2.4 DISCUSSION

In four types of normal endothelial and epithelial cells, Asc increased the release of AA from two of the cell types, but decreased the ability of three of the cell types to convert AA to PGs (342). The tissue Asc level in guinea pig tracheal tissue had no effect on PGE_2 generation (348). On the other hand, in fibroblasts, Asc stimulated PG production markedly, with the conversion of free AA to PGE_2 also being markedly increased (173,349). A similar effect, that of increased PGE_2 levels with Asc supplementation, was found with the non-malignant LLCMK cells used in this study.

In this study, a comparison of the PGE_2 concentrations found in the LLCMK and B16 control cells, respectively, indicated significantly higher PGE_2 concentrations in the malignant cells. This has been found previously with analogous non-malignant and malignant tissue (190). It is suggested that the increase in eicosanoids can influence the carcinogenic process in many different ways. The

increased eicosanoids may stimulate tumour growth *in vivo* (109) or act as tumour promotors by exerting immunosuppressive effects and promoting tumour growth (109,350). They also have the ability to influence tumour migration and metastatic potential of various cell types (350), as well as inhibit tumour growth.

PG synthesis occurs in the endoplasmic reticulum (4). In the B16 control cells, no PGE_2 was found in the stroma fraction, with all of it assumed to remain bound to the membrane. However, upon Asc supplementation, PGE_2 levels in the stroma of these cells increased relative to those in the membrane, suggesting PGE_2 may have been mobilized from the membrane by Asc supplementation and transported to the stroma. This release appears to be concentration dependent, with PGE_2 levels in the stroma and membrane fractions of the B16 cells being similar upon 25 μg/ml Asc supplementation. As the supplemented Asc concentration was increased, there was a simultaneous increase of the relative PGE_2 levels in the stroma fraction, with the exception of stromal PGE_2 levels at 100 μg/ml Asc supplementation.

The precise role of PGE_2 in metastasis of transplanted tumour cells is still unclear (351). Some researchers suggest that in the B16 melanoma model, PGE_2 is negatively correlated with metastatic potential (186,203,352). In most cells, PGE_2 is not causal of metastasis, but its presence contributes to the abilities of the cells to metastasize (186,196,197,198). Asc treated (10^{-4}M) bone marrow-derived macrophage cultures, however, demonstrated substantial increases in PGE_2 production compared with untreated controls (32). However, Asc supplementation of the B16 cells decreased total PGE_2 levels. This was also observed in squamous carcinoma cells, in which Asc supplementation inhibited PGE_2 synthesis (349). Asc supplementation of the LLCMK cells stimulated PGE_2 synthesis, as was the case with the Asc supplementation of human fibroblasts (349).

PGE_2 is the major metabolite synthesized from AA in cells and consequently the effect of Asc supplementation on AA and other EFA levels within both the LLCMK and B16 cells needed to be determined.

3.3 ESSENTIAL FATTY ACID COMPOSITION AND ASCORBIC ACID SUPPLEMENTATION

3.3.1 INTRODUCTION

FAs are important components of the intra- and extracellular matrix (13), and are essential in maintaining the stability of the cell membrane (70,75,107). FAs also act as storage forms of energy (107). These properties are characteristic of the EFA, AA (20:4), in addition to which AA can act in a number of ways as an intracellular or intercellular messenger in normal cells (87,353). Since AA has such a key regulatory role in cellular metabolism, it is necessary that the level of free AA (237), as well as the general FA content and composition of the membrane and stroma of cells, be maintained (97).

Neoplastic transformation of cells leads to a dramatic change in the lipid structural organization within the tumour cell membrane (108,109). The tumour plasma membranes generally exhibit decreased fluidity and can be structurally changed towards a higher rigidity (108,110). Enrichment of tumour cells with PUFAs, including the EFAs, has been shown to cause an increase in plasma membrane fluidity (109). Therefore, tumour membrane rigidity could be due to reduced levels of PUFAs.

Asc, an oxidizing/reducing agent, is also essential for the maintenance of the cellular matrix in normal cells (13,16,18,354). There are suggestions in the literature (26,92) that the effects of Asc on tumour cell growth may be mediated through an association of Asc with enzymes regulating EFA synthesis, with resultant effects on cellular EFA composition and possibly membrane structure.

Objectives of this study were to compare the EFA composition of the non-malignant LLCMK cells and the malignant B16 murine melanoma cells, as well as to determine the effect of Asc supplementation, over a physiological range, on this EFA composition.

3.3.2 MATERIALS AND METHODS

MATERIALS

A SP 2330 gas-liquid chromatography (GLC) column manufactured by Supelco was supplied by Anatech Instruments, South Africa. Methylated fatty acid standards were purchased from Nu Chek Prep, Inc., Minnesota, USA. BF_3 methanol reagent (14%) was purchased from Merck, Darmstadt, Germany.

Methanolic KOH (10%): A 40% (w/v) KOH solution was prepared. Methanolic KOH (10%) was composed of 25% of the 40% stock solution and 75% methanol (v/v).

METHODS

3.3.2.1 Cell culture

The relevant methods are referred to in 2.2.3.1 and 2.2.3.2.

3.3.2.2 Homogenization of the cells and separation of cellular components

Refer to the methods described in 3.1.2.2, and note the following change: the pellet was resuspended in 2ml PBS.

3.3.2.3 Saponification, esterification and extraction of fatty acids

The method used was that of Skeef (355). The various cell fractions were transferred into round-bottomed flasks and 2ml of 10% methanolic KOH was added to each flask. The lipids were saponified by heating with reflux under nitrogen for 45 minutes at 85°C. On completion of saponification, the free FAs were acidified by adding 1ml of 7N HCl. The FAs were extracted twice with 3ml of petroleum ether and vortex mixed for 2 minutes each time. The petroleum ether

extracts were pooled and evaporated to dryness under nitrogen at 60°C. The residual FAs were methylated by heating with 0.3ml BF_3-methanol reagent, with reflux and under nitrogen for 5 minutes at 100°C. The FA esters were again extracted, twice, using 1ml petroleum ether and vigorously shaken for 2 minutes each time. The pooled extracts were evaporated to dryness under nitrogen and reconstituted with 20μl petroleum ether. They were stored at -20°C under nitrogen and protected from light until used.

3.3.2.4 Free fatty acid analysis by gas-liquid chromatography

GLC, with a SP 2330 fused capillary column in the Hewlett-Packard 5890 Gas-liquid Chromatograph, was used to achieve separation of the free FAs which were reconstituted in the petroleum ether. The temperature programme involved an initial heating of the column at 130°C for 15 minutes, followed by an increase in temperature of 4°C/minute to 220°C, at which temperature the column was held for the final 7.5 minutes of the run. A 1μl sample of the reconstituted FAs was loaded onto the SP 2330 column. Only FAs which form part of the n - 6 linoleic acid series, namely LA (18:2), GLA (18:3), DGLA (20:3) and AA (20:4), were considered in this study. Results are expressed as percentage composition of a particular FA relative to the total amount of FA in that sample.

3.3.3 RESULTS

3.3.3.1 The effect of ascorbic acid on cellular essential fatty acid composition

The combined stroma and membrane percentage composition of the respective EFAs in the LLCMK control and Asc treated cells was generally higher, although not significantly so, than that obtained for the same EFAs in the B16 control and Asc treated cells (Figures 11 and 12). Levels of GLA in the LLCMK cells were more detectable than GLA levels in the B16 cells.

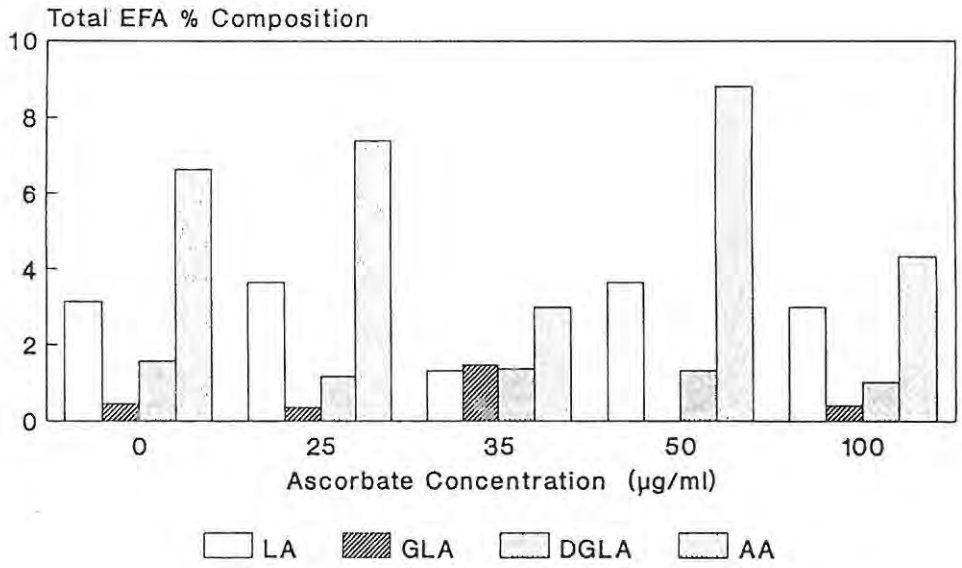


FIGURE 11: The effect of ascorbic acid supplementation on the total essential fatty acid percentage composition of the LLCMK cells. The results are the mean of five cultures.

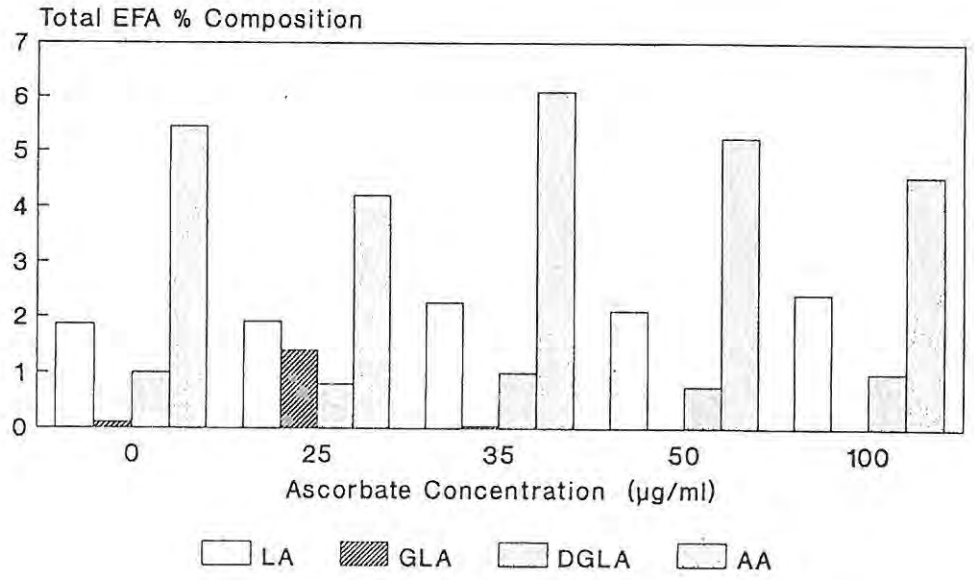


FIGURE 12: The effect of ascorbic acid supplementation on the total essential fatty acid percentage composition of the B16 cells. The results are the mean of five cultures.

The relative EFA percentage composition in the stroma and membrane fractions of the respective LLCMK and B16 cells are shown in Table 10 and Table 11. Generally, the percentage composition of the various EFAs measured in the stroma fractions of both cell types was higher than that determined for the membrane fractions. In addition, the percentage compositions of GLA and AA in the 35 and 100 μg/ml Asc supplemented LLCMK stroma fractions, respectively, were significantly higher ($p \leq 0.005$ and $p \leq 0.05$) than the same EFAs in the corresponding membrane fractions (Table 10). Generally, AA levels in the LLCMK cells were higher than any of the other EFA levels in these cells, with the values recorded at 25 and 50 μg/ml Asc supplementation of the stroma fraction being significantly higher ($p \leq 0.05$). The percentage LA composition in the stroma fraction of the control B16 cells was significantly higher ($p \leq 0.05$) than that in the membrane fraction of these cells (Table 11).

Asc supplementation had no significant effect on the total (combined stroma and membrane fractions) EFA percentage composition in the LLCMK cells. 35 and 100 μg/ml Asc supplementation of the LLCMK cells corresponded to an increase in total (combined stroma and membrane fractions) GLA composition, together with a decrease in total (combined stroma and membrane fractions) AA content, although that was not statistically significant (Figure 11).

LA percentage composition of the B16 control membrane fraction was lower than the LA composition determined for the Asc treated membrane fractions. GLA levels in both the control and Asc supplemented B16 membrane fractions were not detectable (Table 11). No GLA was detected in the B16 stroma fractions supplemented with 50 and 100 μg/ml Asc. The percentage GLA composition of the B16 stroma fraction supplemented with 25 μg/ml Asc was significantly higher ($p \leq 0.05$) than any of the other GLA levels. AA levels recorded in the stroma fraction at 0 and 35 μg/ml Asc supplementation were significantly higher ($p \leq 0.05$) than the percentage EFA composition in any of the other stroma or membrane fractions. 100 μg/ml Asc supplementation of the B16 cells resulted in a decrease in the percentage composition of AA in the membrane fraction, compared with AA levels in the other Asc-treated membrane fractions. The percentage composition of total (combined stroma and membrane fractions) AA in the B16 cells, was generally higher than

the total percentage composition of any of the other EFAs present in these cells (Figure 12).

TABLE 10: The effect of ascorbic acid supplementation (0 - 100 μg/ml) on the percentage essential fatty acid composition in the stroma and membrane fractions of LLCMK cells. The results are the mean of five cultures ± SEM.

[Ascorbate] μg/ml	%LA 18:2	%GLA 18:3	%DGLA 20:3	%AA 20:4
STROMA				
0	4.2 ± 0.5	0.4 ± 0.1	1.8 ± 0.3	7.8 ± 1.2
25	4.5 ± 0.5	0.4 ± 0.1	1.2 ± 0.1	8.6[c] ± 0.9
35	1.9 ± 0.5	2.2[a] ± 0.6	1.5 ± 0.4	5.4 ± 1.9
50	4.1 ± 0.4	ND ND	1.6 ± 0.1	11.0[c] ± 0.5
100	3.6 ± 0.3	ND ND	1.1 ± 0.2	7.8[b] ± 2.0
MEMBRANE				
0	2.1 ± 0.5	0.5 ± 0.1	1.3 ± 0.2	5.5 ± 0.6
25	2.8 ± 0.1	0.3 ± 0.1	1.1 ± 0.1	6.2 ± 0.7
35	0.7 ± 0.2	0.7 ± 0.1	1.2 ± 0.1	1.7 ± 0.8
50	3.2 ± 0.5	ND ND	1.0 ± 0.1	6.6 ± 0.9
100	2.4 ± 0.3	0.8 ± 0.1	0.9 ± 0.2	1.5 ± 0.6

a = $p \leq 0.005$: relative to the GLA percentage composition in the 35 μg/ml Asc supplemented membrane fraction.
b = $p \leq 0.05$: relative to the AA percentage composition in the 100 μg/ml Asc supplemented membrane fraction.
c = $p \leq 0.05$: relative to the percentage composition of any of the other essential fatty acids detected in either stroma or membrane fractions.
ND = Not detectable

TABLE 11: The effect of ascorbic acid supplementation (0 - 100 μg/ml) on the percentage essential fatty acid composition in the stroma and membrane fractions of B16 cells. The results are the mean of five cultures ± SEM.

[Ascorbate] μg/ml	%LA 18:2	%GLA 18:3	%DGLA 20:3	%AA 20:4
STROMA				
0	2.6[a] ± 0.2	0.2 ± 0.1	1.0 ± 0.2	7.2[c] ± 0.1
25	1.8 ± 0.4	2.8[b] ± 1.0	0.8 ± 0.1	2.8 ± 1.4
35	2.4 ± 0.1	0.1 ± 0.0	1.0 ± 0.2	6.9[c] ± 1.1
50	2.0 ± 0.5	ND ND	0.7 ± 0.2	5.5 ± 1.2
100	2.8 ± 0.4	ND ND	1.0 ± 0.1	5.5 ± 0.8
MEMBRANE				
0	1.1 ± 0.3	ND ND	1.0 ± 0.4	3.7 ± 1.1
25	2.0 ± 0.2	ND ND	0.8 ± 0.1	5.6 ± 1.5
35	2.1 ± 0.2	ND ND	1.0 ± 0.1	5.3 ± 0.6
50	2.2 ± 0.2	ND ND	0.8 ± 0.1	5.0 ± 0.5
100	2.0 ± 0.4	ND ND	1.0 ± 0.1	3.6 ± 0.7

a = $p \leq 0.05$: relative to the respective percentage LA composition in the control membrane fraction.
b = $p \leq 0.05$: relative to the percentage composition of GLA in the other stroma and membrane fractions.
c = $p \leq 0.05$: relative to the percentage composition of any of the other essential fatty acids detected in either stroma or membrane fractions.
ND = Not detectable

3.3.4 DISCUSSION

The observation that the overall (stroma and membrane) EFA percentage compositions in the

LLCMK cells were generally greater than corresponding EFA percentage compositions in the B16 cells, supports earlier reports that tumour cells have a lower PUFA content, including the EFAs, than non-malignant cells (108,110,356). This was especially true with regard to the membrane fraction, as has been suggested by an earlier report (109).

The percentage LA in both the non-malignant and malignant *in vitro* cultured cells can be influenced by the availability of EFAs from FCS in the culture medium (89). The percentage LA composition of the total FA content of the FCS was 6,4% (78). Of the FA content in the LLCMK control cells, the total LA percentage composition was approximately half of this value and that of the B16 control cells approximately a third. This suggests that the cells were either not absorbing the LA effectively or that the absorbed LA was being converted to other n- 6 EFAs and their metabolites. Asc supplementation of the B16 cells resulted in higher levels of LA in the membrane fraction relative to the amount of LA in the membrane fraction of the control cells. This suggests that Asc supplementation could thus be enhancing the uptake and storage of LA by the membranes of these cells.

A lower percentage composition of AA was detected in the B16 control cells in this study, in comparison with the LLCMK control cells. Low AA levels have also been reported in Novikoff hepatoma cells, while the content of its precursor, LA, was reported not to differ much from that in normal liver cells (118). However, relative to the LA content found in the respective non-malignant and malignant control cells, AA concentrations were greater in the B16 cells. This may be due to LA conversion to AA in the B16 control cells. 3T3 cells are reported to convert supplemented LA to AA, with the AA content of the phospholipids increasing (70).

The levels of GLA in the B16 cells were generally very low in comparison with those detected in the LLCMK cells. This was probably due in part to the lower LA content of the B16 cells. No GLA was detected in the membrane fractions of either the control or Asc supplemented B16 cells, or at 50 and 100 μg/ml Asc supplementation in the stroma fractions. Malignant cells have been reported to lack delta-6-desaturase activity (111,112,113,114,115) or, as in the case of B16 cells, to

have reduced delta-6-desaturase activity (91,116,117). Thus, the possibility exists that the reduced GLA composition in B16 cells may arise from an impairment in delta-6-desaturase activity in the B16 cells. However, in an earlier study, the addition of Asc was found to increase delta-6-desaturase activity in B16 cells but not in the LLCMK cells (175). Therefore, it is more likely that low GLA levels are a result of rapid conversion of GLA to DGLA, and subsequently to other metabolites, since DGLA in turn has been reported to be stored in relatively small amounts compared to AA (26). However in this study, while total DGLA levels in the B16 cells were found to be lower than the total AA levels, there was still a fairly large store of DGLA. This suggests a possible greater rate of conversion of GLA to DGLA and a slower conversion of DGLA to AA and other metabolites.

In summary, Asc supplementation over a nutritional range did have some influence on the levels of the EFAs that comprise the n - 6 linoleic acid series pathway in the two cell types used in this study although their effects were not significant. A possible effect of Asc on the enzymes regulating n - 6 FA synthesis could not be discounted since Asc has previously been shown to influence delta-6-desaturase activity (175). It was thus relevant to this study to determine the effect of Asc supplementation on delta-5-desaturase activity in these two cell types.

3.4 DELTA-5-DESATURASE ACTIVITY AND ASCORBIC ACID SUPPLEMENTATION

3.4.1 INTRODUCTION

Dunbar *et al* (114) and Maeda *et al* (91) have demonstrated that the delta-5 and delta-6-desaturases, involved in the synthesis of AA from LA, are two different enzyme systems. Relative reaction rates of the delta-5 and delta-6-desaturases are equal in most normal animal cells, which limits the accumulation of DGLA in the cells (cited by 83). The rat appears to be unusual in having a very active hepatic delta-5-desaturase enzyme system compared with that of humans, so some species differences do occur in the capacity of animals to metabolize DGLA to AA (88,101).

Delta-5-desaturase does not respond to cellular changes as rapidly as does delta-6-desaturase (97). However, delta-5-desaturase activity can be modified by hormonal and dietary changes to the cell (83), although this enzyme will be activated only when sufficient substrate is present. Stores of GLA and DGLA in most cells are strictly limited, with very little of either being transported by the plasma (301).

A number of reports suggest that delta-6-desaturase activity is absent or impaired in tumour cells (91,111,112,116). It is reported that delta-5-desaturase activity is unaffected in both normal and transformed cells (91), while it is also claimed that metastatic cells have attenuated delta-5-desaturase activity and predominantly use DGLA to synthesize PGE_1 (357).

The aim of this part of the study was to determine the relative uptake of DGLA, the substrate of delta-5-desaturase, by the respective control and Asc supplemented LLCMK and B16 cells. Subsequently, an attempt was made to establish the effect of Asc supplementation on the delta-5-desaturase activity in these cells.

3.4.2 MATERIALS AND METHODS

MATERIALS

^{14}C-DGLA (specific activity = 47 mCi/mmol) was purchased from New England Nuclear Products, Boston, USA. Ready-Solv^{TM} EP scintillation cocktail was purchased from Beckman, Ireland. $AgNO_3$ and 2,7-dichlorofluorescein were purchased from BDH Chemicals Ltd., England. Silica Gel $60F_{254}$ aluminium thin layer chromatography (TLC) plates, precoated, 20 x 20cm, 20mm were obtained from Merck, Darmstadt, Germany. Arachidonic acid and dihomo-gamma-linolenic acid standards were purchased from Nu Chek Prep, Inc., USA.

10% $AgNO_3$ solution: 10g $AgNO_3$ was dissolved in 20ml distilled water. 80ml of 95% ethanol was added. This solution was re-usable and stored protected from light.

0.4% 2,7-Dichlorofluorescein: 0.4g 2,7-dichlorofluorescein was dissolved in 100ml of 95% ethanol.

METHODS

3.4.2.1 Cell Culture

The procedures of 2.2.3.1 and 2.2.3.2 were used with the following changes. Twenty-four hours prior to harvesting, 0.2μCi of ^{14}C-DGLA was added to each flask (control and Asc treated flasks) and these flasks were again incubated at 37^{o}C.

Prior to harvesting the cells, 1ml aliquots of growth medium were collected for the determination of ^{14}C-DGLA uptake by the cells. The medium was then discarded and the cells trypsinized with 10ml trypsin. The harvested cells were resuspended in 1ml PBS.

3.4.2.2 Homogenization of the cells and separation of cellular components

The method described in 3.1.2.2 was repeated, with the pellet being resuspended in 1ml PBS.

3.4.2.3 Saponification, esterification and extraction of fatty acids

Part of the procedure outlined in 3.3.2.3 was repeated. FAs were only extracted twice with 3ml of petroleum ether and these pooled fractions evaporated to dryness under nitrogen at 60°C. These FA extracts were then stored in 20μl petroleum ether under nitrogen and protected from light. The methylation and subsequent second extraction of the FAs was not required.

3.4.2.4 DGLA and AA separation by argentation thin layer chromatography

Precoated aluminium TLC sheets were immersed in the 10% $AgNO_3$ solution for ten seconds, removed and blow-dried. The plates were then further dried in an oven at 80°C for 30 minutes after which they were ready for use.

Various 20μl sample and standard FA aliquots were spotted 2cm from the bottom of the plate and 1.5cm apart. The plates were developed in a chloroform:methanol:acetate:water system (90:4.75:4.75:0.5), until the solvent front was 3cm from the top of the plate. Approximate development time was 1.5 hours. The entire procedure outlined above was performed in the dark. Once developed, the plates were removed, blow-dried and sprayed with the 0.4% 2,7-dichlorofluorescein solution. Plates were heated at 80°C for ten minutes to fix the spots, before being visualized under ultra-violet light.

The R_f values of the DGLA and AA standard spots were used to identify sample DGLA and AA spots, together with the ultra-violet light visualization of the spots. The radioactively labelled DGLA and AA spots were scraped into scintillation vials containing 10ml scintillation cocktail. Radioactivity was determined by liquid scintillation counting in the Beckman LS 3801 scintillation

counter.

3.4.2.5 Delta-5-desaturase activity determination

Delta-5-desaturase activity was measured as ^{14}C-AA found in the different fractions of each cell type. Since no significant differences in substrate (^{14}C-DGLA) uptake into each cell type was found, the amount of ^{14}C-AA determined for each fraction was not corrected for substrate uptake.

3.4.3 RESULTS

3.4.3.1 The effect of ascorbic acid supplementation on cellular ^{14}C-DGLA uptake

A comparison of ^{14}C-DGLA upake by the LLCMK and B16 cells indicates a significantly higher ($p \leq 0.025$) ^{14}C-DGLA uptake by the B16 cells (Table 12).

TABLE 12: The effect of ascorbic acid supplementation on the uptake of ^{14}C-DGLA by both the LLCMK and B16 cells. The results are the mean of five cultures ± SEM.

[Ascorbate] µg/ml	^{14}C-DGLA uptake (cpm/10^4 cells)	
	LLCMK	B16
0	420.6 ± 57.0	1155.8[b] ± 209.1
25	631.0 ± 96.7	2138.8[b] ± 524.9
35	677.9[a] ± 59.1	3254.0[b] ± 957.7
50	468.2 ± 40.7	4352.7[b] ± 1808.8
100	363.3 ± 24.1	952.1[b] ± 198.1

a = $p \leq 0.025$: relative to the control LLCMK cells
b = $p \leq 0.025$: relative to the corresponding LLCMK control and Asc supplemented cells

^{14}C-DGLA uptake by the LLCMK cells increased with Asc supplementation, until significance ($p \leq 0.025$) at 35 μg/ml Asc supplementation (Table 12). Thereafter, further Asc supplementation resulted in a decrease of ^{14}C-DGLA uptake by these cells. Asc supplementation of the B16 cells resulted in a general increase in ^{14}-C DGLA uptake. However, at 100 μg/ml Asc supplementation, ^{14}-C DGLA uptake by these cells decreased.

3.4.3.2 The effect of ascorbic acid supplementation on ^{14}C-DGLA and ^{14}C-AA levels, as well as on delta-5-desaturase activity

R_f values (the distance travelled by each compound from the origin relative to the solvent front) were determined for the DGLA and AA standards and are recorded in Table 13.

TABLE 13: R_f values for the DGLA and AA standards were determined by argentation thin layer chromatography.

Fatty Acid	R_f value
DGLA	0.90
AA	0.35

Table 14 indicates that the percentage ^{14}C-DGLA and ^{14}C-AA did not differ significantly between the stroma and membrane fractions within the two cell types. However, ^{14}C-DGLA and ^{14}C-AA percentages were lower in the control and Asc supplemented B16 cells in comparison with the corresponding LLCMK cells.

Asc supplementation of the LLCMK cells did not significantly affect the ^{14}C-DGLA and ^{14}C-AA percentages. The highest percentage ^{14}C-DGLA and ^{14}C-AA in the B16 cells was detected in the B16 control cells. Only 35 μg/ml Asc supplementation significantly decreased ($p \leq 0.025$) the levels of these two ^{14}C-FAs in the B16 cells. Delta-5-desaturase activity was similar in each cell type but was lower in the B16 cells when compared with the LLCMK cells, as substrate uptake was greater in the B16 cells.

TABLE 14: The effect of ascorbic acid supplementation on the percentage ^{14}C-DGLA and ^{14}C-AA recorded in the respective stroma and membrane fractions of both the LLCMK and B16 cell types. Values are recorded as a percentage of total ^{14}C-DGLA added to 10^4 cells and are the mean of five cultures ± SEM.

[Asc] µg/ml	% Fatty Acid in Stroma and Membrane Fractions							
	LLCMK cells				B16 cells			
	%DGLA$_s$	%DGLA$_m$	% AA$_s$	% AA$_m$	% DGLA$_s$	% DGLA$_m$	% AA$_s$	% AA$_m$
0	0.23 ± 0.01	0.24 ± 0.02	0.24 ± 0.01	0.22 ± 0.01	0.013 ± 0.004	0.012 ± 0.004	0.009 ± 0.003	0.011 ± 0.003
25	0.21 ± 0.07	0.22 ± 0.06	0.20 ± 0.06	0.20 ± 0.05	0.007 ± 0.003	0.007 ± 0.004	0.006 ± 0.003	0.005 ± 0.003
35	0.19 ± 0.01	0.17 ± 0.02	0.18 ± 0.02	0.17 ± 0.02	0.001[a] ± 0.0001	0.001[a] ± 0.0001	0.001[a] ± 0.0001	0.001[a] ± 0.0001
50	0.19 ± 0.01	0.19 ± 0.01	0.18 ± 0.01	0.19 ± 0.01	0.005 ± 0.002	0.008 ± 0.004	0.006 ± 0.003	0.003 ± 0.002
100	0.18 ± 0.04	0.19 ± 0.01	0.19 ± 0.02	0.19 ± 0.01	0.011 ± 0.002	0.009 ± 0.002	0.010 ± 0.002	0.009 ± 0.002

a = $p \leq 0.025$: relative to the control and other Asc supplemented B16 cells
s = stroma; m = membrane

3.4.4 DISCUSSION

The results of this study indicate that the ^{14}C-DGLA uptake by the control and Asc supplemented B16 cells was significantly greater than that of the corresponding LLCMK cells. Since tumour cells are reported to have a decreased PUFA content (108,109), it is possible that due to a decreased DGLA content in the B16 cells (Exp 3.3), these cells take up more DGLA in an attempt to stabilize the EFA content of the cells.

Levels of ^{14}C-DGLA and ^{14}C-AA in the respective stroma and membrane fractions, of each cell type, were essentially the same. This indicates that there was no preferential uptake of DGLA by either fraction of the B16 or LLCMK cells.

Relative to the amount of ^{14}C-DGLA taken up by both the cell types, the levels of ^{14}C DGLA and

^{14}C-AA within the cells were very small. DGLA supplementation of cultured human breast carcinoma cell lines did increase PG production significantly (136). Hence, the question arises as to the whereabouts of the ^{14}C-DGLA within the cells. A few possibilities exist. Firstly, DGLA is converted to AA in the linoleic acid series (4). However, the percentage ^{14}C-AA in the cells did not increase dramatically. It is possible that ^{14}C-AA levels could have increased and as AA is the precursor to the two series PGs (171), ^{14}C-DGLA could have been converted to these PGs. The second possibility is that DGLA is the direct precursor to the one series PGs (4) and could have been converted accordingly.

Asc supplementation of the LLCMK cells did not affect the ^{14}C-DGLA or ^{14}C-AA content of these cells. However, 35 μg/ml Asc supplementation of the B16 cells resulted in a significant decrease in the levels of ^{14}C-DGLA and ^{14}C-AA in these cells. As Asc supplementation is known to enhance the conversion of DGLA to PGE_1 (160), this could account for the decrease in ^{14}C-DGLA in the cells and the subsequent decrease in the conversion of DGLA to AA. 35 μg/ml Asc would appear to be optimal for the conversion, with further Asc supplementation leading to the reduced conversion of DGLA to PGE_1, and hence higher DGLA and subsequently AA levels.

The fact that there was a conversion of ^{14}C-DGLA to ^{14}C-AA indicates that delta-5-desaturase is present in both the LLCMK and B16 cells. The presence of delta-5-desaturase was also reported in human lung mucoepidermoid carcinoma grown in mice (115) and in two murine mammary tumour cell lines, with delta-5-desaturase activity in the one cell line being eight-fold higher than that in the other (183). Asc supplementation did not affect the relative proportions of ^{14}C-DGLA and ^{14}C-AA. It can therefore be speculated that Asc supplementation does not have an effect on delta-5-desaturase activity within these cells.

In conclusion, Asc supplementation appears not to affect delta-5-desaturase activity, as the latter did not affect AA levels in the LLCMK and B16 cell types. Another enzyme which generates AA, and could possibly affect AA levels, is PLA_2. The effect of Asc supplementation on PLA_2 activity is investigated in the next section.

3.5 PHOSPHOLIPASE A_2 ACTIVITY AND ASCORBIC ACID SUPPLEMENTATION

3.5.1 INTRODUCTION

Phospholipids are the major structural components of cell membranes (70). The FAs, such as AA, present in the one and two positions on the phospholipid molecule determine and maintain fluidity of these membranes (87). The existence of PLAs in biological membranes is a prerequisite of normal phospholipid turnover in cellular metabolism and signal transduction (231).

PLA_2 removes FAs, mostly AA, from the sn-2 position on the phospholipid glycerol backbone (170,358). Four types of PLA_2 exist, with the membrane-bound 85-kDa PLA_2 (168) being important to this study. PLA_2 activity can be triggered by the activation of cell surface receptors with the consequent release of intracellular as well as extracellular AA (87).

In disease states such as neoplastic transformation, it has been reported that lipid structures are altered and hence the membrane fluidity of a number of tissues is changed (108,109). This change can affect membrane bound enzyme activity and therefore result in the possible impairment of PLA_2 activity in malignant cells. Uncertainty exists as to whether there is a difference in the PLA_2 activity of normal and tumour cells. Regarding the substrate of PLA_2, phospholipids were found to be increased in transformed C3H/10T1/2 cells (326). Asc supplementation, which is important to this study, reduced the above mentioned increase by 30% (326).

The aim of this part of the study was to determine the PLA_2 activity of the non-malignant LLCMK and the malignant B16 cells, as well as the effect of Asc supplementation on this activity.

3.5.2 MATERIALS AND METHODS

MATERIALS

1-palmitoyl-2-[1-^{14}C]arachidonyl-sn-glycerol-3-phosphatidyl choline was purchased from New England Nuclear Products, Boston, USA. Taurocholic acid, fatty acid-free bovine serum albumin, arachidonic acid, bovine serum albumin and phosphatidyl choline were purchased from Sigma Chemical Co., USA. The 250 μM silica gel G plates were obtained from Macherey-Nagel, Düren, Germany, while $CaCl_2$ was supplied by Merck, Darmstadt, Germany. Sodium tartrate was obtained from BDH Laboratory Supplies, England.

METHODS

3.5.2.1 Cell culture

The methods in 2.2.3.1 and 2.2.3.2 were repeated with the exception that the harvested cells were resuspended in 1ml Tris-HCl buffer (0.1M), pH 7.5.

3.5.2.2 Homogenization of the cells and separation of cellular components

The method described in 3.1.2.2 was repeated with one change. The pellet, *ie.* the membrane fraction, was retained and resuspended in 1ml Tris-HCl buffer (0.1M), pH 7.5.

3.5.2.3 Protein analysis

Refer to 3.1.2.3 for the assay procedure.

3.5.2.4 Determination of phospholipase A_2 activity

Membrane PLA_2 activity was assayed by using a modification of the methods of Ballou and Cheung (359,360), and the method of Krumhardt and Dupont (361). PLA_2 activity was determined by measuring the amount of [1-^{14}C] AA released from the substrate 1-palmitoyl-2-[1-^{14}C]arachidonyl-sn-glycerol-3-phosphatidyl choline (specific activity = 52 mCi/mmol).

A 225μl aliquot of membrane suspension was added to 25μl of substrate and incubated at 37°C for 10 minutes. The substrate contained in Tris-HCl buffer, 50mM taurocholic acid, 20mM calcium chloride, 0.05g% fatty acid-free bovine serum albumin and 0.03μCi of radioactively labelled substrate. At membrane suspension addition and after a 10 minute incubation period, 100μl aliquots of reaction mixture were removed and added to 100μl of 100% ethanol containing 2% glacial acetate and 330μM AA, in order to terminate the reaction. This solution was vortex mixed and 100μl applied to 250μM silica gel G plates, which separated the [1-^{14}C] AA released from the labelled substrate in a solvent system of ethyl acetate:acetic acid (99:1, vol/vol). After the development of the TLC plates, bands of the silica gel were scraped into scintillation vials containing scintillation cocktail. Radioactivity was determined by liquid scintillation counting. PLA_2 activity is determined as the nett release of [1-^{14}C] AA from labelled substrate and is expressed as pmol of AA released per minute per mg membrane protein.

3.5.3 RESULTS

3.5.3.1 The effect of ascorbic acid supplementation on phospholipase A_2 activity

A comparison of the PLA_2 activity in the two cell types reveals that the PLA_2 activity of the B16 control and Asc supplemented cells is lower than that of the corresponding non-malignant LLCMK cells (Table 15). This lower PLA_2 activity was significant in the 100 μg/ml Asc supplemented B16 group.

TABLE 15: The effect of ascorbic acid supplementation on phospholipase A_2 activity detected in the respective LLCMK and B16 cells. Values are the mean of five cultures ± SEM.

[Ascorbate] μg/ml	PLA_2 activity (pmol AA/min x mg protein)	
	LLCMK	B16
0	0.288 ± 0.090	0.187 ± 0.051
25	0.183 ± 0.067	0.144 ± 0.014
35	0.263 ± 0.082	0.109 ± 0.020
50	0.232 ± 0.027	0.107 ± 0.032
100	0.165 ± 0.021	0.047^{ab} ± 0.005

a = $p \leq 0.05$: relative to the corresponding LLCMK fraction
b = $p \leq 0.05$: relative to the B16 control cells

Increasing Asc supplementation of the LLCMK and B16 cells resulted in a general trend of decreased PLA_2 activity. A significant decrease ($p \leq 0.05$) in PLA_2 activity occurred at 100 μg/ml Asc supplementation of the B16 cells.

3.5.4 DISCUSSION

It has been demonstrated that there are Ca^{2+}-dependent and Ca^{2+}-independent pathways of PLA_2 activation (228). The membrane-bound PLA_2 of interest in this study shows an absolute requirement for Ca^{2+} (103). Ca^{2+} ions were also necessary for optimal PLA_2 activity of porcine pancreas (358). In the present study, a predetermined concentration of Ca^{2+} ions was delivered by the $CaCl_2$ solution used in the assay procedure. The 85-kDa PLA_2 shows preference for sn-2-arachidonyl-containing phospholipid substrates (168), with 1-palmitoyl-2-[1-^{14}C] arachidonyl-sn-glycerol-3-phosphatidyl choline being utilized during the PLA_2 assay performed in this study. It must be stressed that PLA_2 activities measured in cell homogenates are not necessarily always absolutely accurate. This is reportedly due to inhibitory factors in the homogenates that interfere

with the optimal measurement of 85-kDa PLA_2 activity, as well as the activity of other PLA_2s (168).

A difference exists between soluble PLA_2 and membrane-bound PLA_2 activity (231). PLA_2 activity measured in the LLCMK and B16 cells is that of membrane-bound PLA_2 activity. Upon comparison of the PLA_2 activity found in the control cells of the two cell types used in this study, lower PLA_2 activity was found in the malignant B16 cells. Tumour cells have been reported to have altered EFA composition in their membranes which in turn alters the activity of membrane enzymes, such as adenylate cyclase (87). The presence of polyunsaturated phosphocholine and phosphoethanolamine is required to maintain the accessibility of membrane phospholipids to PLA_2 (231). Thus, the lower PLA_2 activity in the B16 cells may be due to an alteration in the polyunsaturated EFA content of the B16 cell membrane.

While PLA_2 activity decreased with Asc supplementation in the LLCMK cells, the decrease was not significant. A trend of decreased PLA_2 activity was also found in the B16 cells, upon Asc supplementation. However, the decrease in PLA_2 activity in these cells was significant at 100 μg/ml Asc supplementation. Asc supplementation at higher concentrations thus had a significant inhibitory effect on PLA_2 activity. However, the mechanism of Asc inhibition of PLA_2 activity is not clear. A factor that should be considered here, is the effect of Asc supplementation on endogenous Ca^{2+} levels in the cell which is described later in this chapter.

In summary, these experiments revealed that relative to the non-malignant cells, PLA_2 activity was lower in the malignant cells. It was also found that Asc supplementation of the B16 cells significantly decreased PLA_2 activity at higher Asc concentrations, which may in turn affect the AA levels in the cells. A further factor to consider with regard to changes in AA levels is the possible effect of Asc on AA uptake into the cells and the influence that exogenous AA in the media may exert on this.

3.6 ARACHIDONIC ACID LEVELS WITH ASCORBIC ACID AND ARACHIDONIC ACID SUPPLEMENTATION

3.6.1 INTRODUCTION

Most AA is esterifed to specific phospholipids, such as phosphatidylcholine, and therefore cells usually contain only small amounts of free AA (214). FA supplementation of cells *in vitro* or changes in dietary fat intake can lead to marked changes in the FA composition of membrane phospholipids (75,362), without disrupting basic membrane or cellular integrity (109). However, these modifications are extensive enough to alter membrane fluidity and to affect a number of cellular functions such as cell growth (330).

Tumour cells are reported to have an altered membrane PUFA content (70). The relative level of LA in tumour tissue increases with an increase in dietary LA (84,363). Cultured neuroblastoma cells are also able to rapidly incorporate exogenous FAs from the medium (102,285,364). The exogenous FAs are incorporated into membrane phospholipids as a result of the action of acyltransferases (364).

In this study the ability of the non-malignant and malignant cells to incorporate AA (as 15-^{3}H AA) from the culture medium was determined, together with the effect of Asc supplementation on this uptake. The effect of AA supplementation, with or without Asc supplementation, on AA levels in the different cellular fractions of these cells was also investigated.

3.6.2 MATERIALS AND METHODS

MATERIALS

The materials used are referred to in 2.2 and 3.3.2.

METHODS

3.6.2.1 Cell culture

Refer to the procedure outlined in 2.2.4.1.

3.6.2.2 Homogenization of the cells and separation of cellular components

The methods in 3.1.2.2 were repeated, with the exception that the final pellet obtained was resuspended in 1ml PBS.

3.6.2.3 Determination of 15-^{3}H arachidonic acid uptake

The respective stroma and membrane fractions of the two cell types supplemented with 15-^{3}H AA, were placed into scintillation vials which contained 5ml of scintillation cocktail. The amount of 15-^{3}H AA in each fraction was determined by scintillation counting.

3.6.2.4 Saponification, esterification and extraction of fatty acids

The procedure detailed in 3.3.2.3 was repeated.

3.6.2.5 Free fatty acid analysis by gas-liquid chromatography

Refer to 3.3.2.4, with the percentage composition of AA being the only EFA of interest here.

3.6.3 RESULTS

3.6.3.1 The effect of ascorbic acid supplementation on 15-^{3}H arachidonic acid uptake by the cells

The uptake of 15-^{3}H AA by the LLCMK and B16 cells (combined stroma and membrane fractions) is recorded in Figure 13. 15-^{3}H AA levels determined for the B16 control cells are significantly higher ($p \leq 0.025$) than the 15-^{3}H AA levels of the LLCMK control cells. Asc supplementation of the LLCMK cells did not result in any significant trend in the uptake of total 15-^{3}H AA by these cells. However, with respect to the B16 cells, Asc supplementation (with the exception of 100 μg/ml) resulted in a decrease in total 15-^{3}H AA uptake.

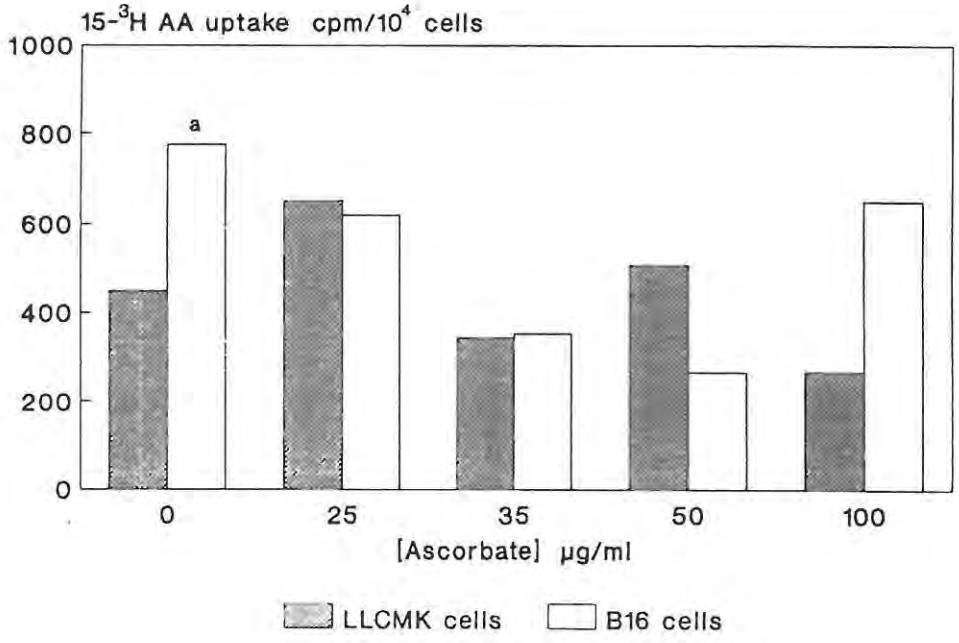


FIGURE 13: The effect of ascorbic acid supplementation on total 15-^{3}H arachidonic acid uptake by the LLCMK and B16 cells. a = $p \leq 0.025$ relative to control LLCMK cells.

The uptake of 15-^{3}H AA in the LLCMK control and Asc supplemented stroma fractions was significantly greater ($p \leq 0.05$) than the uptake of 15-^{3}H AA in the corresponding membrane fractions (Table 16). The level of 15-^{3}H AA in the LLCMK stroma fraction without Asc supplementation was significantly lower ($p \leq 0.05$) than the amount of 15-^{3}H AA detected in those stroma fractions supplemented with 25 and 50 μg/ml Asc. In contrast, the level of 15-^{3}H AA in the LLCMK membrane fraction, not supplemented with Asc, was significantly higher ($p \leq 0.025$) than the amount of 15-^{3}H AA detected in the membrane fractions supplemented with 35, 50 and 100 μg/ml Asc (Table 16). A significant decrease ($p \leq 0.025$) in 15-^{3}H AA uptake in these membrane fractions occurred with increasing Asc supplementation.

TABLE 16: The effect of ascorbic acid supplementation on the uptake of 15-^{3}H arachidonic acid by the LLCMK and B16 cells, respectively. Values recorded are the mean of five cultures ± SEM.

[Asc] μg/ml	15-^{3}H AA (μCi)	15-^{3}H AA in stroma (cpm/10^4 cells)		15-^{3}H AA in membrane (cpm/10^4 cells)	
		LLCMK	B16	LLCMK	B16
0	20	264.2[a] ± 59.8	324.3 ± 20.4	185.0[d] ± 67.4	451.9[e] ± 26.4
25	20	558.2[b] ± 74.4	247.1 ± 42.8	94.5 ± 22.1	372.6[e] ± 41.4
35	20	309.3[b] ± 32.8	162.1 ± 40.3	33.5 ± 5.2	190.1 ± 77.4
50	20	460.5[b] ± 48.0	171.4 ± 11.9	47.3 ± 3.8	93.6 ± 10.5
100	20	253.9[ab] ± 45.7	465.8[c] ± 103.4	12.8 ± 1.0	184.4 ± 23.5

a = $p \leq 0.05$: relative to the 25 and 50 μg/ml Asc supplemented LLCMK stroma fractions.
b = $p \leq 0.05$: relative to the corresponding LLCMK membrane fractions
c = $p \leq 0.05$: relative to the 25, 35 and 50 μg/ml Asc supplemented B16 stroma fractions.
d = $p \leq 0.025$: relative to the 35, 50 and 100 μg/ml Asc supplemented LLCMK membrane fractions.
e = $p \leq 0.01$: relative to the 35, 50 and 100 μg/ml Asc supplemented B16 membrane fractions.

A comparison of the B16 stroma and membrane fractions supplemented only with 15-^{3}H AA,

revealed that the level of 15-^{3}H AA in the membrane fraction was greater than that in the stroma fraction. The 15-^{3}H AA detected in the 100 μg/ml Asc supplemented B16 stroma fraction was significantly higher ($p \leq 0.05$) than the 15-^{3}H AA detected in other Asc supplemented stroma fractions (Table 16). The level of 15-^{3}H AA in this stroma fraction was now greater than the 15-^{3}H AA detected in the corresponding membrane fraction. This latter effect was also found for the stroma and membrane fractions of the B16 cells supplemented with 50 μg/ml Asc. The level of 15-^{3}H AA detected in the 0 and 25 μg/ml Asc supplemented B16 membrane fractions was significantly higher ($p \leq 0.01$) than the 15^{3}H AA found in the 35, 50 and 100 μg/ml Asc supplemented membrane fractions.

3.6.3.2 The effect of ascorbic acid and arachidonic acid supplementation on the percentage composition of arachidonic acid in the cells

The percentage AA composition in the LLCMK stroma fractions of the control, the 2.5μM AA supplemented, as well as the 50μg/ml Asc and 2.5μM AA supplemented cells was significantly higher ($p \leq 0.01$) than the percentage AA composition in the other stroma fractions (Table 17). The percentage AA composition in the LLCMK control stroma fraction was considerably higher than that detected in the corresponding membrane fraction.

This latter observation was also made concerning the control stroma and membrane fractions of the B16 cells. Generally, however, the percentage AA composition in the B16 membrane fractions was higher than that in the stroma fractions. Upon 25 μg/ml Asc and 2.5μM AA supplementation, the percentage AA composition in the B16 membrane fraction increased significantly ($p \leq 0.05$) relative to the percentage AA composition in all the other B16 membrane fractions. Further increases in Asc supplementation resulted in a decrease in membrane percentage AA composition of these cells.

The LLCMK control cells contained a higher percentage AA composition than the corresponding B16 control cells (Table 17).

TABLE 17: The effect of ascorbic acid and arachidonic acid supplementation on the percentage arachidonic acid composition in the respective stroma and membrane fractions of the LLCMK and B16 cells. Results are the mean of five cultures ± SEM.

[Asc] μg/ml	[AA] μM	%AA in stroma		%AA in membrane	
		LLCMK	B16	LLCMK	B16
0	0	6.47[a] ± 1.17	3.09 ± 0.62	2.58 ± 0.84	1.01 ± 0.39
0	2.5	5.03[a] ± 0.25	0.84 ± 0.01	4.77 ± 0.70	1.57 ± 0.56
25	2.5	1.61 ± 0.61	0.99 ± 0.09	5.52 ± 1.18	5.32[b] ± 0.09
35	2.5	0.86 ± 0.18	2.43 ± 0.21	2.36 ± 0.19	3.57 ± 0.99
50	2.5	6.12[a] ± 0.66	1.76 ± 0.18	4.14 ± 0.87	2.35 ± 0.46
100	2.5	1.97 ± 0.79	2.02 ± 0.40	6.45 ± 2.15	1.01 ± 0.19

a = $p \leq 0.01$: relative to the 25, 35 and 100 μg/ml Asc supplemented LLCMK stroma fractions.
b = $p \leq 0.05$: relative to all the B16 membrane fractions, except for that at 35 μg/ml Asc supplementation.

3.6.4 DISCUSSION

Human gastric cancer cells supplemented with [^{14}C]AA are able to incorporate this AA into the cells. This AA is preferentially esterified to phosphatidyl choline before being transferred to phosphatidylethanolamine (365). The uptake of 15-^{3}H AA by the LLCMK and B16 cells in this study indicates that these cells are able to incorporate exogenous AA. However, the mechanism of PUFA permeation into cells is still not clear. The AA entry step appears to be insensitive to the intracellular metabolism of the FA and is therefore not driven by transmembrane concentration gradients (366,367). Present evidence suggests that a common entry path exists for AA and other long-chain free FAs, and that the entry is mediated by a mechanism of facilitated diffusion driven by transmembrane potential difference (367). FA uptake is also possibly mediated by a carrier-protein common to long-chain FAs (368,369,370). However, a consistent characteristic of AA

transport is that of a unidirectional influx of AA, exhibiting features of a saturable process (366,467). It is also suggested that the process is energy independent (371).

^{14}C-LA uptake studies in normal and tumour cells indicated that the uptake was almost the same, whereas ^{14}C-AA uptake was substantially less in the tumour cells (127). The B16 cells supplemented only with 15-^{3}H AA in this study incorporate a significantly greater amount of 15-^{3}H AA than the corresponding LLCMK cells, which contradicts the previous observation. Due to an altered PUFA content in tumour cells (70), it seems possible that these cells incorporate more AA in order to stabilize the membrane. However, on examining the percentage AA composition in the 2.5μM AA supplemented LLCMK cells, it was found to be greater than that in the corresponding B16 cells. The reason for these opposite effects is not clear, although it could be due to the fact that the AA content of LLCMK cells is generally higher than that in the B16 cells (section 3.3).

Significantly less 15-^{3}H AA was taken up into the LLCMK membrane fractions supplemented with increasing Asc. A similar effect was observed in the B16 membrane fractions, with Asc supplementation above 25 μg/ml. Upon Asc supplementation, the 15-^{3}H AA content of the B16 membrane fraction also decreased relative to the 15-^{3}H AA levels in the stroma fraction. At 100 μg/ml Asc supplementation the increase in 15-^{3}H AA in the B16 stroma fraction was in fact significant relative to that found in the other stroma fractions at lower levels of Asc. Thus, upon Asc supplementation there is a reduced uptake of 15-^{3}H AA by the B16 membrane accompanied by an increased uptake of the FA by the stroma. This is supported by the finding of significantly high percentage AA composition in the membrane fraction at 2.5 μM AA and 25 μg/ml Asc supplementation, and a lower percentage AA in the 2.5μM AA and 100 μg/ml Asc supplemented membrane fraction. At 100 μg/ml Asc supplementation the percentage AA composition in the B16 stroma fraction was again higher than that of the membrane fraction.

It can thus be concluded that AA supplementation together with increasing Asc supplementation of the B16 cells results in a higher stromal AA content than membrane AA content.

3.7 CALCIUM LEVELS AND ASCORBIC ACID SUPPLEMENTATION

3.7.1 INTRODUCTION

Ca^{2+} binds to a variety of ligands such as proteins and phosphate groups (372). Such binding, in excess, can lead to a decrease in membrane fluidity if these groups form part of the membrane (236). The function of this Ca^{2+} binding to these groups enables Ca^{2+} to regulate metabolic processes (211,372), as well as result in the cells containing a very small proportion of free intracellular Ca^{2+} (372).

The understanding of Ca^{2+} levels in cells is very complex, as many factors influence these levels. It has been reported that unsaturated FAs produced by mitochondrial PLA_2 can be essential in the regulation of Ca^{2+} retention in, and release from, the mitochondria (373). Intracellular Ca^{2+} concentrations are controlled by second messenger AA and its metabolites in a pituitary cell line, as AA results in intracellular Ca^{2+} mobilization within, and stimulation of Ca^{2+} entry into, these cells (374). AA may therefore serve as both a positive-or-negative feedback regulator of the effects of intracellular Ca^{2+} (87). cAMP also has the ability to mediate Ca^{2+} activity, especially during cell proliferation (208).

Malignant cells undergo various cellular changes, with the malignancy of a rat osteosarcoma cell line being associated with hypercalcaemia (298). Subcutaneously-implanted hepatoma cells contain at least 100% more Ca^{2+} than the normal liver of the animal (212). Soluble Ca^{2+} represents less than 50% of the total Ca^{2+} in most tumours, with the insoluble Ca^{2+} being present as cytoplasmatic particles (372). A general similarity exists between the distribution pattern of these two Ca^{2+} types within tumours and other normal tissues such as liver and kidney (372), despite the fact that the total Ca^{2+} content is considerably lower in the normal organs (212,372).

The dependence of PLA_2 (358) and AC (207) activity on Ca^{2+} concentrations within the cell, led to the investigation of Ca^{2+} levels in the LLCMK and B16 cells, respectively. While PLA_2 and

AC activity in Asc supplemented cells had been determined, it was also necessary to examine the effect of Asc supplementation on Ca^{2+} levels within these two cell types.

3.7.2 MATERIALS AND METHODS

MATERIALS

A Varian Techtron Model 1000 Atomic Absorption Spectrophotometer was used for Ca^{2+} detection. $CaCl_2$ was purchased from Merck, Darmstadt, Germany. All glassware was acid washed in a 25% HNO_3 solution at 60°C overnight. Thereafter it was rinsed with milli Q water.

METHODS

3.7.2.1 Cell Culture

The method described in 2.2.3.1 and 2.2.3.2 was repeated, with the exception that the cells harvested from this experiment were resuspended in 1ml Tris-HCl buffer, pH 7.4.

The cells were not homogenized and fractionated, as problems were envisaged with detection of Ca^{2+} in separate fractions.

3.7.2.2 Acid digestion of the cells and calcium detection

On completion of the cell counts, the cell suspensions were centrifuged at 3 000g for 10 minutes and the supernantant discarded. The cells were washed with 1ml Tris-HCl buffer and again centrifuged at 3 000g for 10 minutes. The supernantant was discarded and the centrifuge tubes were drained. The pellet was resuspended in 0.2ml concentrated HCl and boiled at 100°C for two hours. After the acid digestion, a final volume of 1ml was constituted using milli Q water. The acid digested fractions were once again centrifuged at 3 000g for ten minutes to remove any debris. The

supernatant was assayed for Ca^{2+} content by flame atomic absorption spectroscopy. The fuel source was air-acetylene; the slit width was 0.50nm; the wavelength was set at 422.7nm; and the current was 3mA.

Standard $CaCl_2$ solutions were assayed using flame atomic absorption spectroscopy. The Ca^{2+} standard curve is recorded in Appendix 4.

3.7.3 RESULTS

3.7.3.1 The effect of ascorbic acid supplementation on calcium levels within the cells

Ca^{2+} levels in the LLCMK cells were generally higher than those levels found in the B16 cells (Table 18). Ca^{2+} levels in the 25 and 50 μg/ml Asc supplemented LLCMK cells were significantly higher ($p \leq 0.05$) than any of the Ca^{2+} levels detected in either the B16 control or Asc supplemented cells (Table 18).

TABLE 18: The effect of ascorbic acid supplementation on calcium levels in the LLCMK and B16 cells, respectively. The values recorded are the mean of five cultures ± SEM.

[Ascorbate] μg/ml	[Ca^{2+}] (nM/10^4 cells)	
	LLCMK cells	B16 cells
0	92.6 ± 16.5	13.8 ± 2.7
25	153.0[a] ± 49.1	24.8 ± 8.1
35	39.8 ± 15.2	18.2 ± 1.0
50	123.1[a] ± 47.0	24.0 ± 4.4
100	33.6 ± 9.3	26.2 ± 2.3

a = $p \leq 0.05$: relative to the B16 control and Asc supplemented cells

Asc supplementation of the LLCMK cells resulted in a very erratic effect on Ca^{2+} levels within these cells relative to the control LLCMK cells. 25 and 50 μg/ml Asc supplementation increased Ca^{2+} levels in these cells relative to the control cells, while 35 and 100 μg/ml Asc supplementation decreased Ca^{2+} levels in these cells relative to the control cells.

Asc supplementation of the B16 cells increased the Ca^{2+} concentration in these cells relative to the control cells, although not significantly so (Table 18). The highest level of Ca^{2+} was found in the B16 cells supplemented with 100 μg/ml Asc. Levels of Ca^{2+} in the 35 μg/ml Asc supplemented cells were somewhat lower than the values recorded for Ca^{2+} levels in the other Asc supplemented B16 cells.

3.7.4 DISCUSSION

The level of free Ca^{2+} in pancreatic acinar carcinoma cells has been reported to be lower than in normal acinar cells (cited by 334). In this study, Ca^{2+} levels in the B16 control cells were lower than Ca^{2+} levels in the LLCMK control cells, which supports the above finding. However, as reported earlier in this section, some tumour cells do have a higher Ca^{2+} content than that of normal cells (212,298). Ca^{2+} levels of different tumour cells also vary relative to one another. It would appear that B16 melanoma cells are characterised by low Ca^{2+} levels, as it is reported that levels of free intracellular Ca^{2+} in B16 melanoma cells are lower than Ca^{2+} levels in a transformed murine melanocyte cell line, Melan A (334). B16 melanomas, however, reportedly do have a higher Ca^{2+} content than normal liver and kidney cells (372). Despite the reports of very low Ca^{2+} levels in B16 melanoma cells, it is also reported that nine human melanoma cell lines were able to proliferate in medium depleted of Ca^{2+}, whereas normal cells were unable to proliferate (375). This implies either that these tumour cells have high levels of intracellular Ca^{2+} and do not require more, or that they are well adapted and normal metabolic processes can continue at lower Ca^{2+} levels. Thus, changes in free intracellular Ca^{2+} could be relevant to the development of neoplasia (334), as for example, human ovarian tumour cells have altered cellular Ca^{2+} regulatory processes associated with the defective down-regulation of protein kinase C (376).

The effect of Asc on Ca^{2+} levels in Asc supplemented cells has not previously been reported. Extremely high levels of Ca^{2+} were detected in LLCMK cells supplemented with 25 and 50 μg/ml Asc, respectively. These levels of Ca^{2+} represented an approximate four-fold increase of Ca^{2+} levels compared with those found in the 35 and 100 μg/ml Asc supplemented LLCMK cells, and a 1.5-fold increase on control LLCMK Ca^{2+} levels. The reasons for this inconsistent trend are unclear.

With respect to the B16 cells, Asc supplementation increased Ca^{2+} levels within these cells relative to the control B16 cells. Initially, 25 μg/ml Asc supplementation of the B16 cells resulted in an increase of Ca^{2+} levels within these cells, whereafter the level of Ca^{2+} determined for these cells essentially remained constant. The effect of Asc supplementation on Ca^{2+} levels in these cells could be the result of its property as an oxidizing/reducing agent (13). It is possible that this property could be affecting the electron potential within these cells, thus affecting Ca^{2+} levels. As Ca^{2+} cannot be broken down, the only mechanisms available for causing large-scale changes in Ca^{2+} concentration involve movements either across the plasma membrane or across intracellular membranes (209).

Unfortunately, due to detection limitations, the LLCMK and B16 cells could not be separated into the respective stroma and membrane fractions. Of interest would have been the amount of Ca^{2+} bound to the membrane proteins and phosphate groups (209). Nevertheless, this study did reveal an increase in Ca^{2+} levels in the B16 cells upon Asc supplementation.

Since the majority of this study has involved an investigation of the effect of Asc on secondary messengers and subsequent potential effects of these secondary messengers on cell metabolism and growth, the final aspect of this study examined the influence of certain of these messengers on other metabolites in the cascade pathway described in Figure 10 (page 41).

3.8 PROSTAGLANDIN E_2 LEVELS AND ADENYLATE CYCLASE ACTIVITY, WITH ARACHIDONIC ACID AND PROSTAGLANDIN E_2 SUPPLEMENTATION

3.8.1 INTRODUCTION

As most AA is esterified to specific phospholipids, *eg.* phosphatidylcholine, cells usually contain only small amounts of free AA (214). FA supplementation of cells *in vitro* or changes in dietary fat can lead to marked changes in the FA composition of membrane phospholipids (75,362). These modifications are extensive enough to alter membrane fluidity and affect a number of cellular functions such as cell growth (330). Tumour cells are reported to have altered membrane fluidity due to changes in the membrane PUFA content (70). The influences that FAs exert on the fluidity and/or order of a membrane have widespread implications for the functioning of those proteins, the actions of which depend on mobility within the plane of the membrane (75).

One of the major intracellular signalling systems is that regulated by AC, which generates cAMP from ATP and is in turn regulated by guanine nucleotide regulatory proteins (4,334). An example of this signalling system is the action exerted by PGs in many biological systems, which has been shown to be associated with the activation of AC (152,155,261). Changes to the membrane intracellular signalling systems are frequently implicated in the development of neoplasia (334). As the transformation of cells leads to a dramatic change in the lipid structural organization within the tumour cell membrane (108,109) and the activity of many integral membrane-bound enzymes is modulated by the nature of its membrane lipid environment (236,242), this in turn can affect AC activity.

The aim of this investigation was to determine the effect of AA supplementation on PGE_2 levels in the LLCMK and B16 cells, in the absence of Asc supplementation. Further investigation involved the analysis of AC activity in these two cell types, with PGE_2 supplementation only. The purpose of this study, more generally, is to clarify the role of AA and PGE_2 in various metabolic systems. This will then later aid in the elucidation of the role and effect of Asc supplementation concerning

second messenger metabolism in these two cell types.

3.8.2 MATERIALS AND METHODS

MATERIALS

Arachidonic acid and Prostaglandin E_2 were purchased from Sigma Chemical Co., USA.

METHODS

3.8.2.1 Cell culture

Refer to the methods in 2.2.5. The cells harvested for the determination of PGE_2 levels were resuspended in 1ml PBS buffer. Those cells harvested for AC activity determination were resuspended in 1ml Tris-HCl buffer containing 4mM EDTA.

3.8.2.2 Homogenization of the cells and separation of cellular components

The procedure of 3.1.2.2 was repeated. The pellet for PGE_2 determination was resuspended in 1ml PBS, while the pellet for the determination of AC activity was resuspended in 1ml Tris-HCl containing 4mM EDTA.

3.8.2.3 Extraction and isolation of prostaglandins

The method of Powell (345), described in 3.2.2.3, was repeated.

3.8.2.4 Prostaglandin E_2 [^{125}I] assay system

Refer to 3.2.2.4. This assay only detects PGE_2 levels up to 160 pg/tube, which was exceeded in

the B16 stroma fraction supplemented with 50μM AA.

3.8.2.5 Protein determination

As outlined in 3.1.2.3.

3.8.2.6 Adenylate cyclase activity assay

Refer to the method of Schultz and Jakobs (240) in 3.1.2.4.

3.8.3 RESULTS

3.8.3.1 The effect of arachidonic acid supplementation on the prostaglandin E_2 levels within the cells

In both the LLCMK cells and the B16 cells, the levels of PGE_2 in the stroma fractions were significantly higher ($p \leq 0.005$) than the PGE_2 levels in the respective membrane fractions (Table 19). PGE_2 levels in the B16 stroma fractions were significantly higher ($p \leq 0.05$) than those of the LLCMK stroma fractions.

PGE_2, detected in the AA supplemented stroma fractions of the LLCMK cells, was greater than the PGE_2 found in the control stroma fraction of the LLCMK cells. At 5μM AA supplementation, this increase was significant ($p \leq 0.05$). In the LLCMK membrane fractions, the PGE_2 levels in both the control and AA supplemented cells were similar (Table 19).

The levels of PGE_2 in the B16 stroma fraction supplemented with 1μM AA decreased relative to that of the control stroma fraction. Further AA supplementation resulted in an increase in PGE_2 levels, especially in the B16 stroma fraction suppplemented with 50μM AA (Table 19). An increase in PGE_2 was found in the membrane fraction of B16 cells supplemented with 1μM and 50μM AA.

This increase was only significant ($p \leq 0.05$) in the 50μM AA supplemented B16 cells (Table 19).

TABLE 19: The effect of arachidonic acid supplementation on the prostaglandin E_2 levels in the stroma and membrane fractions of both the LLCMK and B16 cells. Results are the mean of five cultures $\pm$ SEM.

[AA] μM	PGE_2 (pg/tube) in stroma		PGE_2 (pg/tube) in membrane	
	LLCMK	B16	LLCMK	B16
0	$12.04^{d} \pm 2.00$	$141.80^{bd} \pm 18.20$	3.55 ± 1.30	5.80 ± 1.27
1	$16.66^{d} \pm 2.12$	$98.42^{bd} \pm 24.19$	2.48 ± 0.06	11.79 ± 3.86
5	$45.82^{ad} \pm 9.70$	$137.20^{bd} \pm 19.27$	5.71 ± 1.26	4.57 ± 1.44
10	$19.38^{d} \pm 0.69$	$124.80^{bd} \pm 18.71$	4.04 ± 1.07	2.70 ± 0.44
50	$17.36^{d} \pm 2.63$	$160.00^{bd} \pm 0.00$	3.79 ± 0.85	$14.22^{c} \pm 1.87$

a = $p \leq 0.05$: relative to any other LLCMK stroma fractions
b = $p \leq 0.05$: relative to the LLCMK stroma fractions
c = $p \leq 0.05$: relative to the B16 control, 5μM AA and 10μM AA supplemented membrane fractions.
d = $p \leq 0.005$: relative to their corresponding membrane fractions

3.8.3.2 The effect of prostaglandin E_2 supplementation on adenylate cyclase activity in the cells

AC activities detected in both the control and PGE_2 supplemented LLCMK cells were significantly higher ($p \leq 0.005$) than AC activity in the B16 control cells.

PGE_2 supplementation did not appear to affect AC activity in the LLCMK cells to any extent. However, with regard to the B16 cells, AC activity in the control cells was lower than that in the PGE_2 supplemented cells, although not significantly so (Table 20).

TABLE 20: The effect of prostaglandin E_2 supplementation on adenylate cyclase activity in the membrane fractions of the respective LLCMK and B16 cells. Results are the mean of five cultures ± SEM.

[PGE_2] μM	Adenylate cyclase activity (U/mg)	
	LLCMK cells	B16 cells
0	3.34 ± 0.51	0.59[a] ± 0.07
1	3.78 ± 0.64	1.47 ± 0.30
5	4.09 ± 0.51	1.94 ± 0.67
10	3.28 ± 0.42	1.69 ± 0.30
100	3.06 ± 0.57	1.69 ± 0.31

a = $p \leq 0.005$: relative to AC activity in the control and PGE_2 supplemented LLCMK cells.

3.8.4 DISCUSSION

PGE_2 levels in the control B16 cells were significantly higher than those detected in the control LLCMK cells. This difference in PGE_2 levels was also apparent between the LLCMK and B16 control cells analysed in Exp 3.2. PGs, especially of the E series, have been shown to be elevated in a large number of human and experimental tumours, and may play a role in the growth and spread of tumours (149,174).

AA supplementation (100μM) resulted in a significant increase in cell supernatant PGE_2 levels in a human melanoma cell line (377). Results for the B16 cells supplemented with 50μM AA also revealed an increase in PGE_2 levels in the stroma fraction, although not significantly so. However, PGE_2 levels in the membrane fraction of B16 cells supplemented with 50μM AA were significantly greater than those of B16 control fractions.

AC activity in rat liver has been shown to be stimulated by PGE_2, but this stimulation was

dependent on the presence of GTP, using substrate ATP concentrations from 0.2 to 2.0mM (378). Intracellular levels of cAMP in primary cultures of mouse embryo palate mesenchyme cells and rat hypothalamus synaptic membrane preparations were also elevated by the administration of exogenous PGE_2 (379,380). However, the AC activity in the LLCMK cells used in this study was only somewhat stimulated by levels of up to 5μM PGE_2, relative to the control LLCMK AC activity.

Supplemented FAs are incorporated into phospholipids in significant amounts, and will therefore have an effect on membrane environment, in turn affecting AC activity (246). PGE_2 receptor stimulation of murine mammary tumour cell lines also led to elevated cAMP levels in these cell lines (288). This, therefore, does not rule out the possibility that PGs such as PGE_2 directly stimulates AC. In this study, PGE_2 supplementation of the B16 cells did increase AC activity three-fold, with the greatest AC activity occurring at 5μM PGE_2 supplementation. This was a saturable process, as shown by the fact that further PGE_2 supplementation did not to increase AC activity.

It is apparent from this study that AC activity in the B16 cells is stimulated by PGE_2 and that these PGE_2 levels are in turn influenced by the amount of AA in these cells.

The effect of Asc on the interrelationship between the secondary messengers and metabolites described in this chapter and the possible influence this may have on cell growth and metabolism will now be discussed.

4

SECOND MESSENGERS AND CELL GROWTH

Cell metabolism is a collective term for a highly intergrated network of chemical reactions within the cell. The fundamental motifs of metabolism are to extract energy and reducing power from the cell environment and to synthesize the building blocks of macromolecules (4). The pathway outlined in Figure 10 (repeated on page 113) is just one of many reactions in this intricate network. It is, however, a pathway that is essential to metabolism, as it produces secondary messengers which control many metabolic processes, as well as the cell cycle.

Foodstuffs are vital energy and metabolite suppliers, the latter including vitamins, fats, proteins, etc. Epidemiological studies over the last few years reveal that there are links between food consumption patterns and the growth of different types of cancers (36,66,68,156,357). These food consumption patterns can either be protective of tumour growth or enhance tumour occurrence and growth. However, compounds such as vitamins, fats and others are not necessarily only involved in the cancer process as they also have a host of other functions to fulfil in the cell.

Of main concern in this study has been the effect of Asc supplementation on the metabolism of the LLCMK and B16 cells. The particular pathway considered in these cells is that outlined in Figure 10. This study does consider other nutrient supplementation, but the emphasis is on Asc supplementation. Chapter 2 investigated the effect of nutrient supplementation on both LLCMK and B16 cell growth, while Chapter 3 determined the effect of nutrient supplementation on certain metabolites within these cells. This chapter will relate the LLCMK and B16 cell growth to the second messengers associated with the above pathway, as well as to factors influencing this pathway.

It is important to note that, as discussed in Chapter 2, there was considerable variation in the effect of Asc supplementation on cell growth between experiments. Consequently, interrelationships

between cell growth and metabolites will be discussed on the basis of the results of each of the individual studies which made up this investigation. An attempt will then be made to draw together these individual cell growth and metabolite relationships with respect to the pathway under consideration.

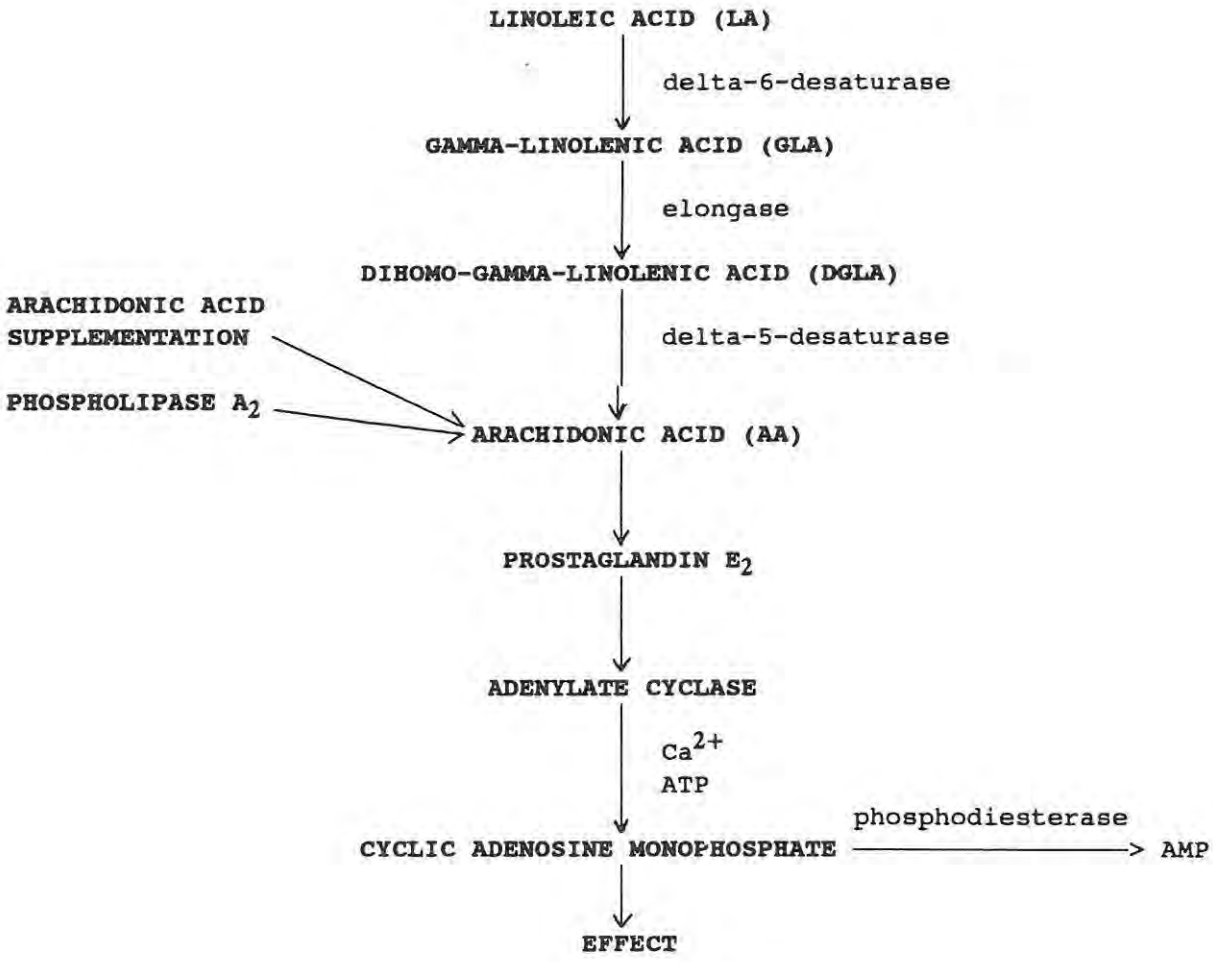


FIGURE 10: A schematic representation of the project outline

cAMP is the initial second messenger that will be considered in this discussion. The AC system converts ATP to cAMP, with cAMP influencing the cell cycle (267). Thus, of concern here is the relationship between cell growth, AC activity, cAMP levels and Asc supplementation. The responsiveness of B16 melanoma cell clones to hormones does not result from alterations in cyclic

nucleotide transport mechanisms but probably reflects intrinsic differences in the ability of each clone to synthesize and accumulate cAMP (341).

Asc supplementation of the LLCMK cells did not significantly affect cell growth or AC activity. cAMP levels in the LLCMK cells reduced slightly with Asc supplementation, which was signficant at 25 μg/ml supplemented Asc. Asc supplementation of the B16 cells, however, resulted in a significant decrease in cell growth, together with a significant increase in AC activity (Figure 14). A similar inverse relationship was also observed to a certain extent when comparing cAMP levels and cell growth in B16 cells (Figure 15). Hence, it would appear that as more ATP is converted to cAMP, the latter in turn regulates cell growth negatively (247,267). The inhibition or stimulation of tumour cell growth by cAMP depends on the cell type, the oncogene regulating its growth, the dose of cAMP and the environment of the cell (256). The cAMP signalling system of an osteosarcoma cell line was found to be antiproliferate (276). High concentrations of cAMP inhibited the proliferation of HeLa human cervical carcinoma cells, introducing the notion that cAMP is a negative regulator of cell proliferation (267). Thus, Asc appears to be an inducer of cAMP as a negative regulator of B16 cell growth.

As PGE_2 stimulates AC activity (170), an investigation of the relationship between PGE_2 levels and cell growth, with Asc supplementation, was thus necessary. LLCMK cell growth and PGE_2 levels of these cells were affected in a similar way by Asc supplementation, with both cell growth and PGE_2 levels either simultaneously increasing or decreasing. The only exception was that recorded at 25 μg/ml Asc supplementation where cell growth increased while PGE_2 levels decreased. Hence, no trend was apparent. Asc stimulated PG production in human lung embryo fibroblasts during quiescence and this was due to an increase in PG synthetase activity (173). A decrease in endogenous PGE_2 levels in small intestinal crypts led to an increase in proliferative activity (179). However, this was not the case with the LLCMK cells in this study.

The precise role of PGE_2 in metastasis is still unclear (351). Some researchers suggest that in the B16 melanoma model, PGE_2 is negatively correlated with metastatic potential (186,203,352).

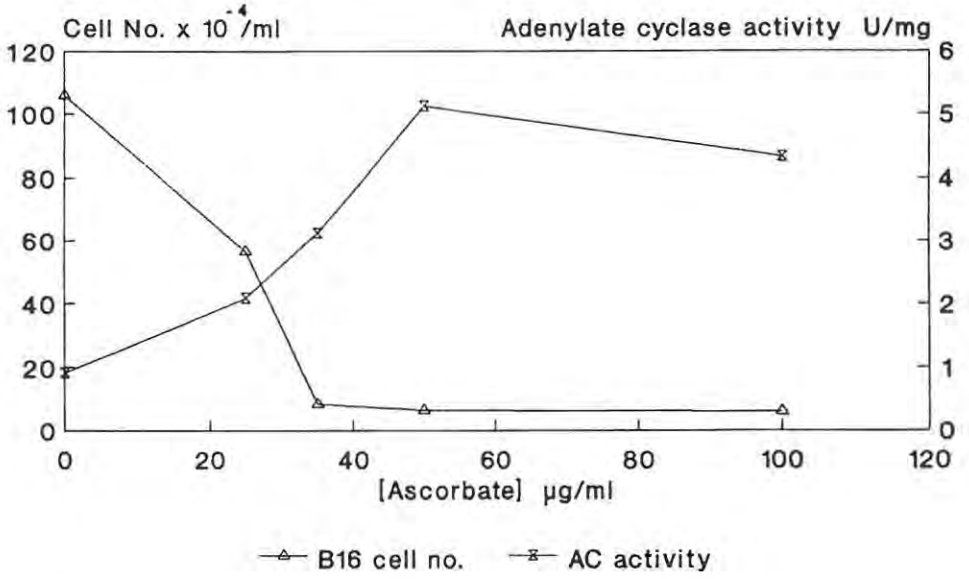


FIGURE 14: The effect of ascorbic acid supplementation on B16 cell growth and adenylate cyclase activity.

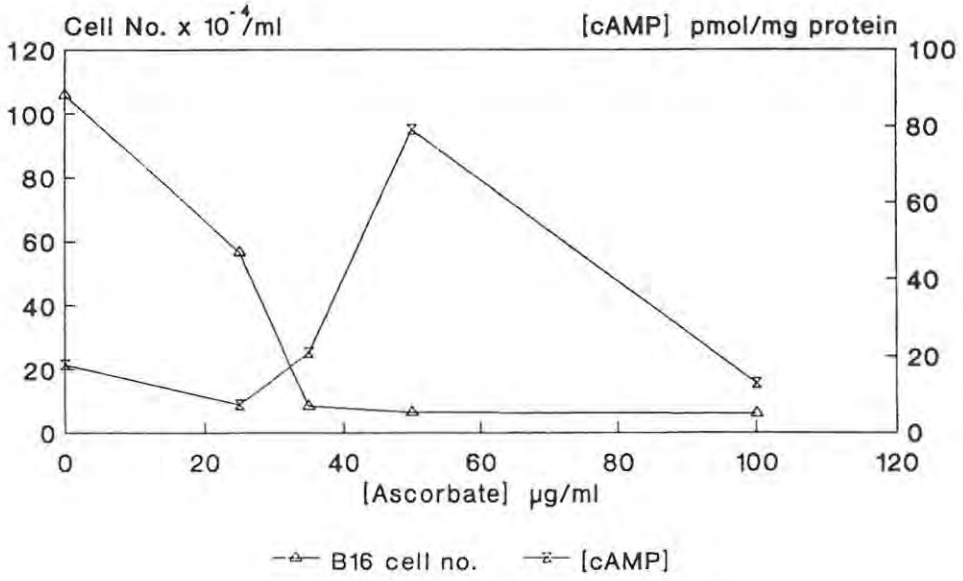


FIGURE 15: The relationship observed between B16 cell growth and cyclic adenosine monophosphate levels with ascorbic acid supplementation.

Upon Asc supplementation, the PGE_2 levels found in the B16 cells in this study were inversely related to B16 cell growth (Figure 16) (381). It has been reported that in the absence of Asc supplementation, an inverse relationship exists between HeLa cell proliferation and PGE production during control and altered (indomethacin supplemented) growth cycles (204). There is thus evidence for the role of PGs in the control of cell proliferation (174).

Since AA is the precursor of PGE_2 (4), the relationship between Asc supplementation and EFA composition of the LLCMK and B16 cells was investigated. Of particular interest was the relationship between Asc supplementation and AA composition. In this experiment, increasing Asc supplementation of the LLCMK cells resulted in an overall decrease in cell growth. The total percentage AA composition of these cells followed the same trend, with increasing Asc supplementation. An exception occurred at 25 μg/ml Asc supplementation, as total percentage AA composition increased as LLCMK cell growth decreased.

In the experiment concerning the effect of Asc on AA composition in the B16 cells, 25 to 100 μg/ml Asc supplementation increased the growth of these cells to a certain extent. The general trend that was apparent was that as B16 cell growth increased, total percentage AA composition decreased and *vice versa* (Figure 17). Hence, an inverse relationship existed between B16 cell growth and total percentage AA composition, with Asc supplementation (381). Of the two B16 subcellular fractions, the percentage AA composition determined for the B16 stroma fractions was the fraction which tended to be inversely related to B16 cell growth. In studies on the effect of PUFAs on cell growth, the proliferation rate of human osteogenic sarcoma cells was suppressed by PUFAs such as LA, GLA and AA (124), while neuroblastoma cells exhibited the opposite effect with oleate, LA, GLA and AA stimulating cell growth (364). In general, *in vivo* studies have shown that n - 6 FA diets may promote tumour growth and metastasis (357).

At this point, the four major components of Figure 10, *ie.* the FAs, PGE_2, AC and cAMP, have been considered. With respect to Asc supplementation they are all, to a certain extent, inversely related to B16 cell growth. The levels of the second messengers also appear dependent on and

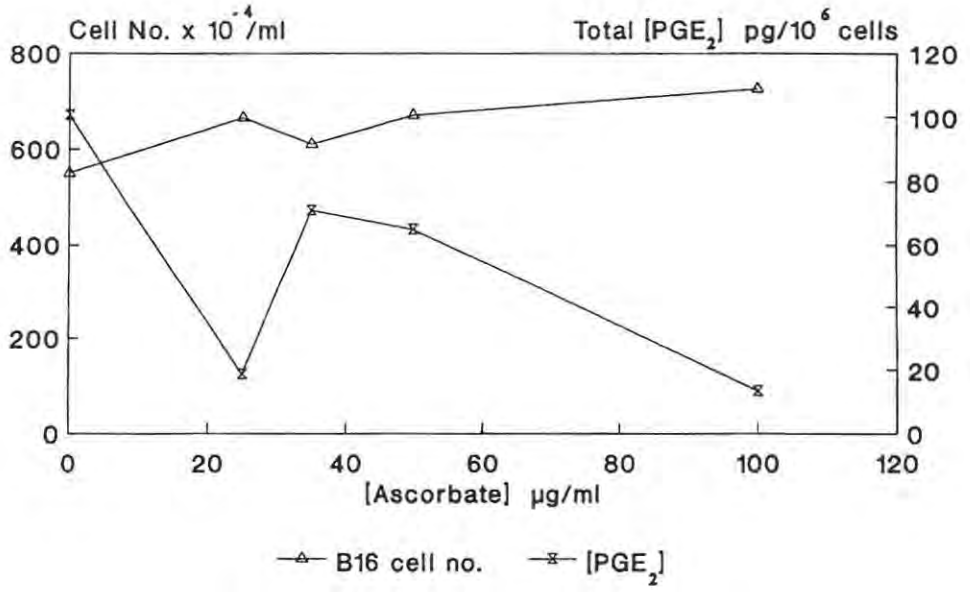


FIGURE 16: The effect of ascorbic acid supplementation on B16 cell growth and prostaglandin E_2 levels.

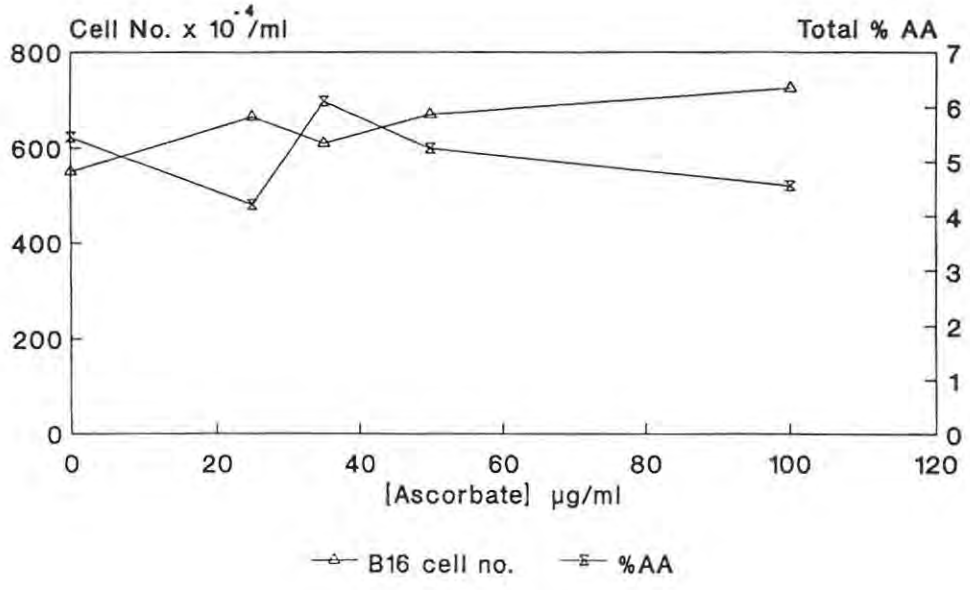


FIGURE 17: B16 cell growth and total percentage arachidonic acid composition with ascorbic acid supplementation.

affected by their precursor levels. This is substantiated by the data reported in section 3.8 in which it was shown that AA supplementation at high levels increased PGE_2 concentrations in B16 cells and PGE_2 supplementation in turn increased the activity of AC in these cells. Thus, the factors influencing AA composition in the B16 cells need to be considered in an attempt to clarify the possible reason for the inverse relationship between AA composition and B16 cell growth with Asc supplementation. This is necessary, since it is this relationship which in turn influences the inverse correlation between its resultant secondary metabolites and B16 cell growth. In Chapter 3, the linoleic acid series pathway was examined and it is apparent from this study that Asc supplementation did not affect the percentage composition of these EFAs. Three additional factors which may influence AA levels were consequently investigated. Firstly, delta-5-desaturase activity was determined in both cell types in the presence of Asc, as was PLA_2 activity. Finally, the uptake of AA by these two cell types, with Asc supplementation, was investigated.

Asc supplementation of the LLCMK cells supplemented with ^{14}C-DGLA resulted in an overall increase in cell growth, with an overall decrease in ^{14}C-DGLA uptake by the cells. This was the first inverse relationship found with LLCMK cell growth and one of the metabolites indicated in Figure 10. With respect to the B16 cells, Asc and ^{14}C-DGLA supplementation significantly decreased cell growth, while ^{14}C-DGLA uptake increased substantially in these cells. Thus, ^{14}C-DGLA uptake was also inversely related to B16 cell growth, with an exception at 100 μg/ml Asc supplementation (Figure 18). The decrease in ^{14}C-DGLA uptake at 100 μg/ml Asc supplementation could mean that at this concentration Asc inhibits DGLA uptake by the B16 cells. As discussed earlier, the percentage DGLA uptake in the two fractions of both cell types was equivalent to the percentage AA composition in the cells. If delta-5-desaturase activity was affected by Asc supplementation then the percentage AA composition would be expected to be much lower or higher. Thus, it was concluded that delta-5-desaturase is not affected by Asc supplementation and hence is not responsible for the inverse relationship between B16 cell growth, AA composition and the subsequent metabolites.

Since PLA_2 releases AA from phospholipids, it was possible that PLA_2 was being affected by Asc

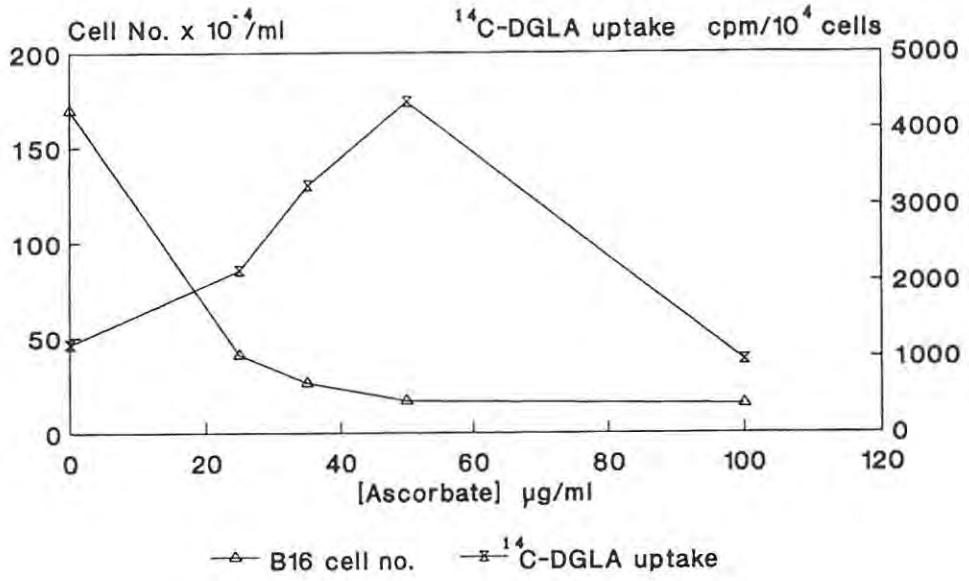


FIGURE 18: B16 cell growth and the uptake of ^{14}C-DGLA with ascorbic acid supplementation.

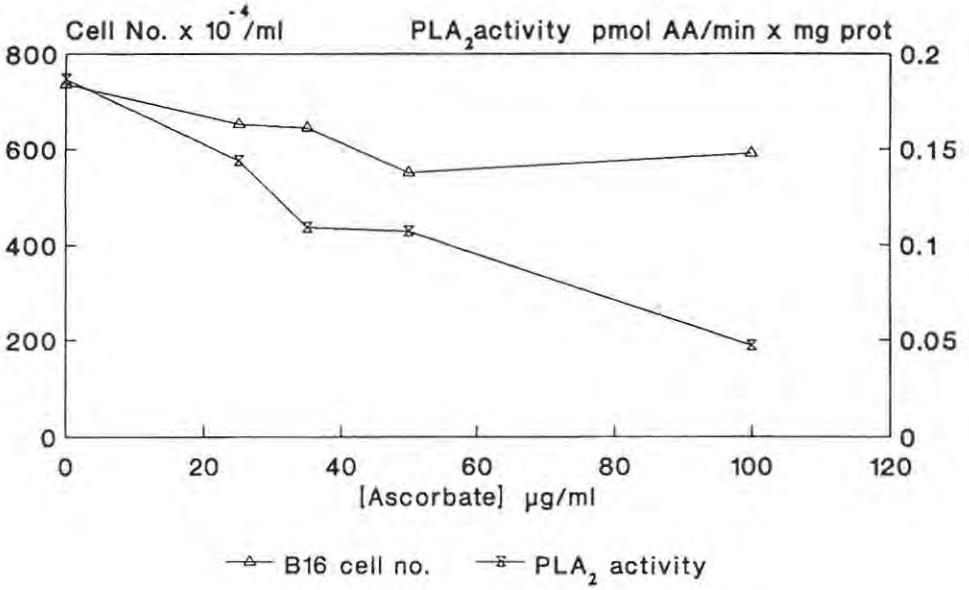


FIGURE 19: The effect of ascorbic acid supplementation on B16 cell growth and phospholipase A_2 activity.

supplementation. PLA_2 activity of the LLCMK cells was inversely related to cell growth, with the exception of activity measured at 100 μg/ml Asc supplementation. In the relevant experiment, Asc supplementation of the B16 cells significantly inhibited cell growth and, interestingly, Asc supplementation of these cells also significantly decreased PLA_2 activity (Figure 19). This, therefore, could not explain the inverse relationship between B16 cell growth and the other metabolite concentrations referred to earlier in this study. If PLA_2 activity was involved in the inverse relationship between B16 cell growth and total percentage AA composition, one would expect an increase in PLA_2 activity with the decreased B16 cell growth.

LLCMK cell growth was not affected by Asc and 15-^{3}H AA supplementation. An exception was that at 35 μg/ml Asc and 15-^{3}H AA supplementation, as LLCMK cell growth was signficantly inhibited. 15-^{3}H AA uptake was inversely related to the LLCMK cell growth. The combination of Asc and 15-^{3}H AA supplementation significantly inhibited B16 cell growth at the highest concentration. 15-^{3}H AA uptake was inversely correlated to B16 cell growth, except at 35 μg/ml Asc and 15-^{3}H AA supplementation (Figure 20). Further investigation on the effect of Asc and AA supplementation on AA composition and cell growth of the two cell types revealed that AA and Asc supplementation also resulted in an overall decrease in B16 cell growth. The addition of AA alone to the cells resulted in a decrease in B16 cell growth and an accompanying decrease in total percentage AA composition. Combined Asc and AA supplementaion resulted in a decrease in B16 cell growth at low concentrations of Asc and then increased slightly at higher concentrations but was still lower than that of the control B16 cells. Furthermore, total percentage AA composition was inversely related to B16 cell growth since increases and decreases in percentage AA composition of the Asc and AA supplemented LLCMK cells were correlated with increases and decreases in cell growth (Figure 21). The relationship between total percentage AA composition of the Asc and AA supplemented B16 cells versus cell growth was in fact similar to that of the Asc supplemented B16 cells (Figure 17). This suggests that effects of Asc supplementation on AA uptake into the B16 cells could be responsible for the inverse relationship between metabolite levels and cell growth.

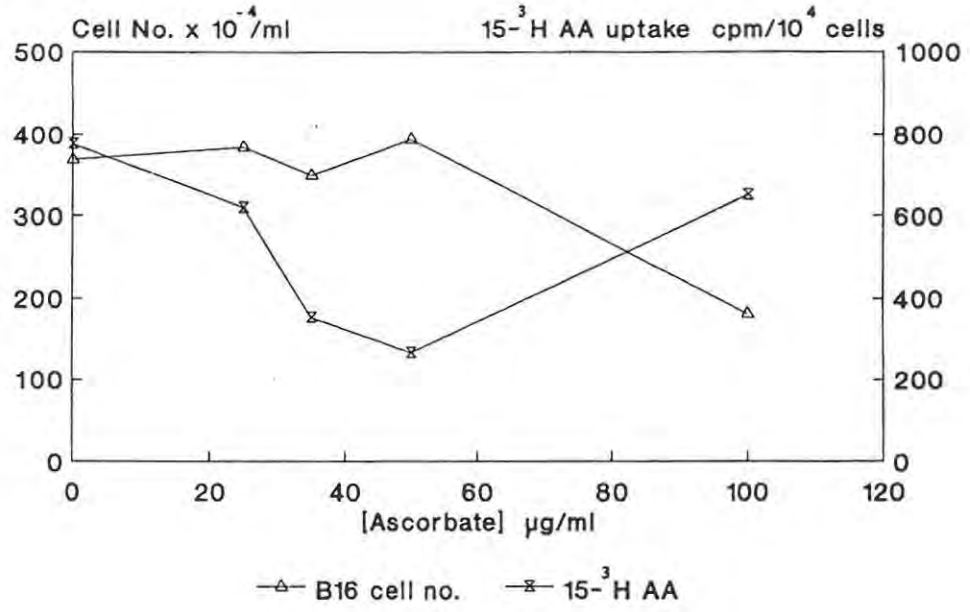


FIGURE 20: The effect of ascorbic acid supplementation on 15-^{3}H arachidonic acid uptake and B16 cell growth.

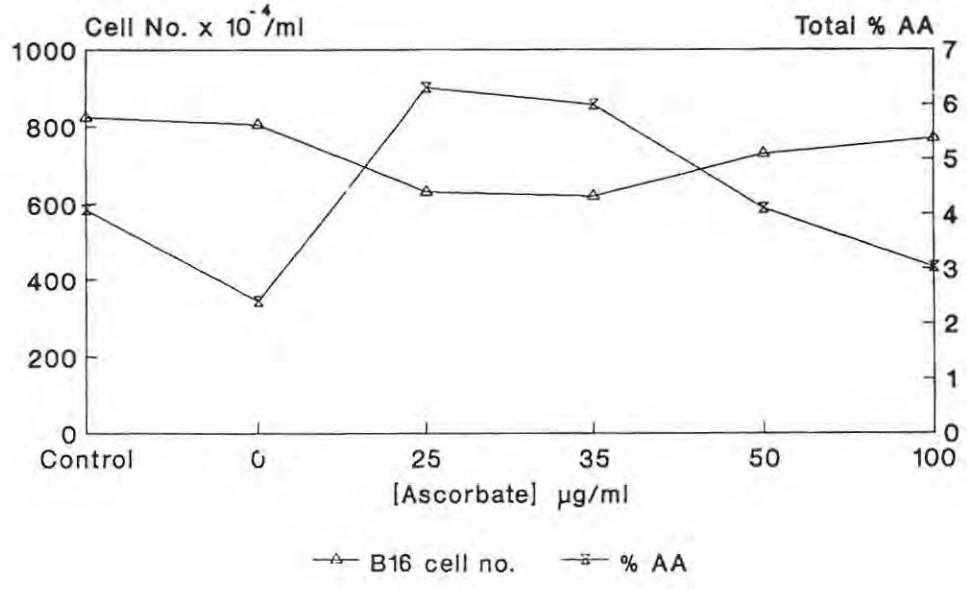


FIGURE 21: The effect of ascorbic acid and arachidonic acid supplementation on B16 cell growth and total percentage arachidonic acid composition.

An additional factor which needed to be considered was the effect of Asc supplementation on cellular Ca^{2+} levels since it is suggested that Ca^{2+} controls cell proliferation (211) and it is known that Ca^{2+} affects AC and PLA_2 activity. In the LLCMK cells, Asc supplementation did not result in any trend between cell growth and Ca^{2+} levels. The only exceptions were in the cells supplemented with 50 and 100 μg/ml Asc in which Ca^{2+} concentration varied inversely with cell growth. In the studies examining Ca^{2+} levels, B16 cell growth decreased with Asc supplementation, although this effect was minimal. Ca^{2+} levels increased with increasing Asc supplementation and at high Asc concentrations were inversely related to the B16 cell growth (Figure 22). Ca^{2+} has been reported in another study to control the proliferation of nontumourigenic epithelial and mesenchymally derived cells *in vitro*, but it had little or no effect on corresponding tumour cells (208). However, it is also reported that small tumours have considerably increased total Ca^{2+} concentrations (372).

The LLCMK and B16 cells were also exposed to AA and PGE_2 supplementation, respectively, for two reasons. Firstly, it was of interest to determine the effect of AA or PGE_2 supplementation on the cell growth. Secondly, it was necessary to verify the previous observations that PGE_2 levels and AC activity were dependent on precursor availability.

AA supplementation of the LLCMK and B16 cells resulted in an overall decrease in cell growth. PGE_2 levels generally increased slightly in the LLCMK cells and significantly so at 5μM AA supplementation. In the B16 cells, Figure 23 shows that there is a significant increase in PGE_2 levels at 50μM AA supplementation and that this corresponds to a decrease in B16 cell growth. Thus, greater AA levels result in greater PGE_2 levels.

In examining the effects of PGE_2 supplementation on the LLCMK and B16 cells, a slight decrease in LLCMK cell growth was found, while the decrease in B16 cell growth was significant at PGE_2 levels greater than 1μM. AC activity in the LLCMK cells was slightly decreased, while AC activity in the B16 cells increased. AC activity was thus inversely related to B16 cell growth (Figure 24) and was enhanced by increasing PGE_2 levels.

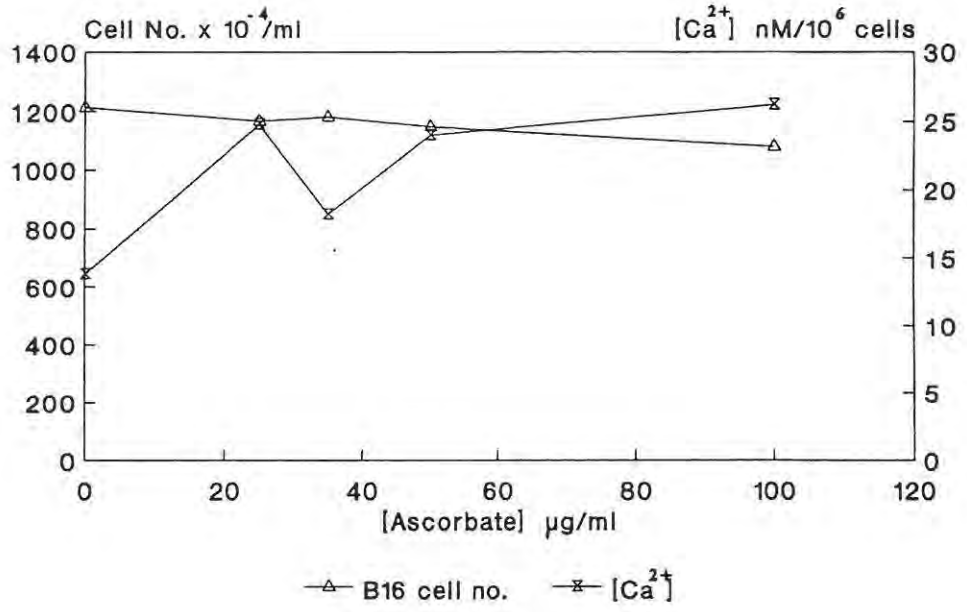


FIGURE 22: Calcium levels and B16 cell growth with ascorbic acid supplementation.

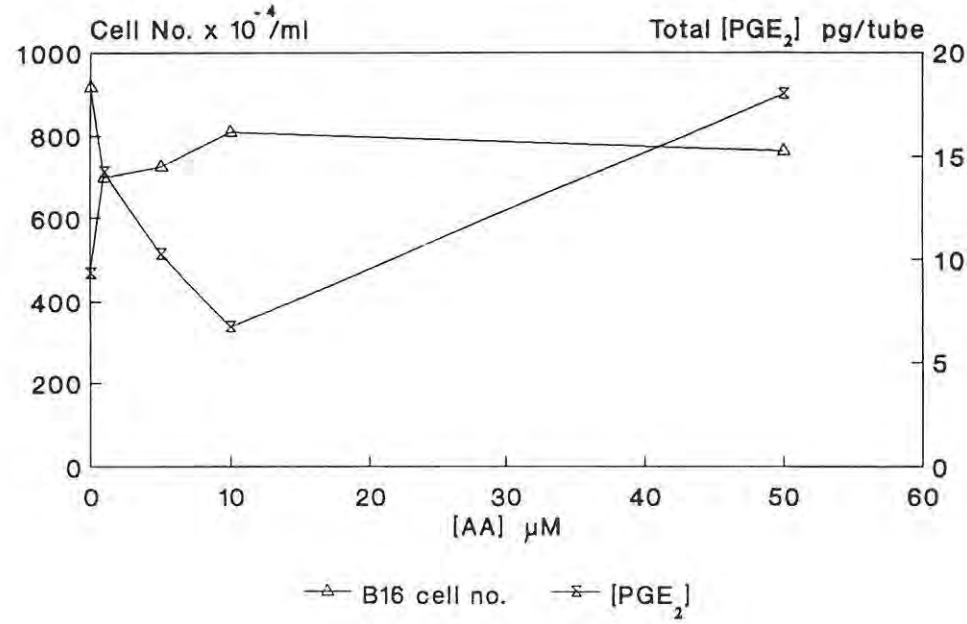


FIGURE 23: The effect of arachidonic acid supplementation on B16 cell growth and prostaglandin E_2 levels.

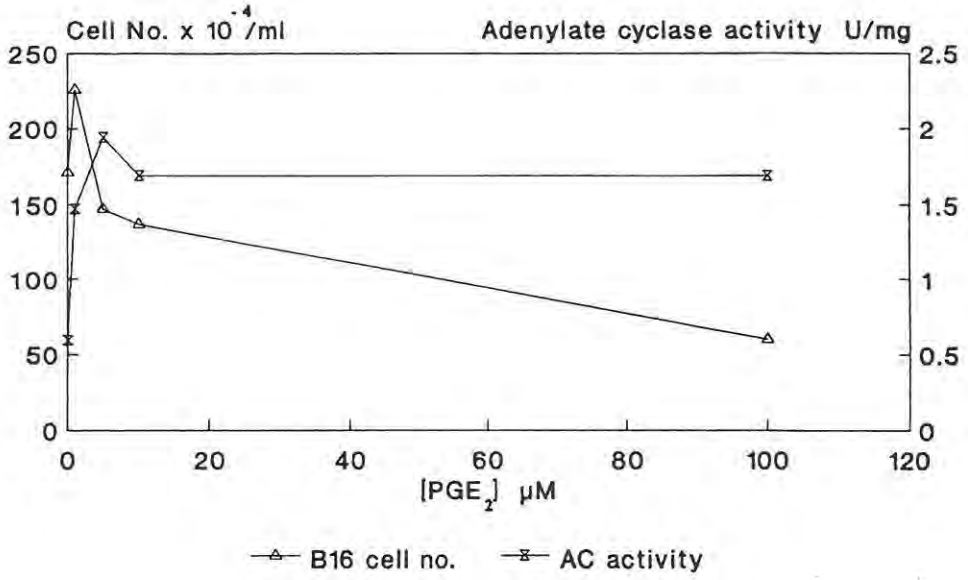


FIGURE 24: The effect of prostaglandin E_2 supplementation on B16 cell growth and adenylate cyclase activity.

In conclusion, it must be noted that no definite relationship was determined between the LLCMK cells and the second messengers shown in Figure 10, with Asc supplementation. However, the Asc supplemented B16 cells generally exhibited an inverse relationship between cell growth and the second messengers of the pathway, with the magnitude of this relationship varying somewhat between the second messengers. The direct relationship between B16 cell growth and PLA_2 activity was the only exception recorded, with respect to the inverse relationships. A primary effect of Asc supplementation concerning the B16 cells, is on the uptake of AA and its precursor DGLA from the medium. Hence, the percentage AA composition of the B16 cells is affected and this effect in turn appears to influence the cellular levels of the products of AA.

5

GENERAL DISCUSSION

Fundamental differences regarding cellular function and compostion are reported between non-malignant and malignant cells (see Chapter 1). The normal and tumour cells referred to in these reports usually originated from the same tissue type. In contrast, in this study the non-malignant and malignant cells were derived from monkey kidney cells and murine melanoma cells, respectively. Despite originating from different tissue types, the non-malignant and malignant cells in this study did display considerable differences with respect to certain enzyme activities and secondary metabolite levels determined for these two cell types. A characteristic physiological feature which generally differentiates tumour and normal cells is that of cell growth. This is due to the fact that tumour cells usually have less control of proliferation rates (1). Generally, the growth rate of the B16 melanoma cells used in this study was greater than that of the LLCMK cells, in both the experimental control cultures and the maintenance cultures of these cells.

Regarding the metabolites of the LLCMK and B16 cells, the total percentage composition of the respective EFAs of the B16 control cells were lower than those determined for the LLCMK control cells. Of particular interest was the lower percentage AA composition determined for the B16 cells relative to that of the LLCMK cells. These findings support reports that tumour cells have decreased levels of PUFAs, which could be responsible for the decreased membrane fluidity of tumour cells (74,75,246,382). When specifically investigating the percentage EFA composition of the B16 control cell membranes, they were found to be lower than the percentage EFA composition of the LLCMK cell membranes. The membrane percentage EFA composition of the B16 control cells was also lower than that of the stroma percentage EFA composition of these cells.

The difference in EFA content observed between tumour and normal cells has wider implications than just affecting membrane fluidity and stability. The membrane fluidity of cells affects the activity of enzymes in the membrane (70,242), with decreased membrane fluidity usually

resulting in decreased enzyme activity (70). AC activity determined in the B16 control cells was significantly lower than that of the LLCMK control cells. This difference could possibly be attributed to the lower PUFA content of the B16 cells. It is also reported (269) that changes in dietary fat induces alterations in AC activity in the rat liver plasma membrane *in vivo* by changing the surrounding lipid environment. Membrane PUFAs also have the ability to affect interactions between adenosine receptors and AC, as in neuroblastoma cells increasing PUFA supplementation modified adenosine receptor function and stimulated AC activity (285). The alteration in AC activity undoubtedly has important consequences for the functioning of the B16 cells, in that cAMP levels could be affected and cAMP is an important regulator of cell growth (267). cAMP levels found in the B16 and LLCMK control cells, respectively, were virtually of the same magnitude.

The two other membrane bound enzymes investigated in this study were PLA_2 and delta-5-desaturase. PLA_2 activity was substantially lower in the B16 control cells relative to the LLCMK control cells, while delta-5-desaturase activity was also somewhat lower in the B16 control cells than in the LLCMK control cells. Platelet PLA_2 is regulated by the availability of intracellular Ca^{2+} (103), as were the PLA_2 and AC activities analyzed in the LLCMK and B16 control cells. The Ca^{2+} levels of the B16 control cells were substantially lower than the Ca^{2+} levels of the corresponding LLCMK cells. Ca^{2+} levels of tumour cells have previously been reported to be altered (cited by 375). Hence, together with PUFA alteration of the membrane, the lower Ca^{2+} concentration of the B16 control cells could be another factor resulting in the reduced activity of the AC and PLA_2 enzymes. In a normal system, *ie.* the dense tubule system of human platelets, AC activity was proposed to be regulatory of intracellular Ca^{2+} levels and AA metabolism (383). Hence, it can be concluded that AC activity can be affected by the AA and Ca^{2+} content of the cells, but these levels are in turn influenced by AC activity. This is thus a possible feedback system, which could also exist in the LLCMK and B16 cells.

Tumour cells characteristically produce excessive amounts of the two series PGs (301). PGE_2 levels were in fact significantly higher in the B16 control cells than in the LLCMK control cells used in this study. B16 cells reportedly synthesize PGE_2 as the major AA metabolite (154), with

B16 melanoma tissue synthesizing more PGE than analagous normal tissue (cited by 190). As PG metabolism is generally disturbed in cancer cells (155), inversed PG synthesis could also account for the decrease in AA levels in tumour cells, *ie.* in the B16 cells of this study, relative to those of normal cells. A highly positive correlation has been noted between high levels of PGE, high tumourigenicity and metastatic potential in mammary murine adenocarcinomas (198). That PG levels are abnormally high in the rapidly growing tumour cells is supported by the fact that in normal fibroblasts PGs only increase in the quiescent state, which is indicative of a reduced growth rate (173). The proliferation of smooth muscle cells *in vitro* was also inhibited by increased PGE_2 (165). The PGE_2 levels in the LLCMK cells were significantly lower than those of the B16 cells, indicative of a more normal growth rate. As these LLCMK cells were still multiplying, it is assumed that they were not in the quiescent state.

It is estimated that up to 80 - 90% of neoplasms are predominantly caused by environmental factors such as diet, smoking, infectious agents and industrial exposures (384). This study considered the role of diet in cancer formation, in particular the role of Asc. Asc, a water-soluble vitamin, is an oxidizing/reducing agent which plays a role in many biochemical reactions, as it has a broad biological function (27,354). A very important feature involves its role in maintaining the intracellular matrix of cells, which contains compounds such as fats and PGs (13). Asc supplemented at high doses, *ie.* pharmacological levels (greater than 100 μg/ml Asc) reportedly inhibits the growth of various tumour cells (34,47,51). This study considered Asc supplementation at nutritional levels since numerous reports have linked cancer epidemiology with diet (357,385). Epidemiological studies have shown that diets rich in one or more antioxidant nutrients may reduce the risk of cancers of the lung, uterine cervix, mouth and gastrointestinal tract (1). The nutritional range of Asc supplemented in this study was 0 - 100 μg/ml Asc. This nutritional range of Asc supplementation had an overall inhibitory effect on both the LLCMK and B16 cell growth, but the effect of Asc supplementation on cell growth did vary with each experiment.

Many proposals have been made in an attempt to explain the toxic effect of Asc on tumour cells, *eg* that of an antioxidant, immunocompetence, etc. This study considered the possibility that the toxic

effect of Asc supplementation on tumour growth was mediated to a certain extent through an effect of this vitamin on the AA composition in the B16 cells, resultant PGE_2 levels and the final cAMP concentration (a known regulator of cell proliferation). It was also important to establish whether any such effects on these metabolites were the result of a direct influence of Asc supplementation or whether this supplementation was indirectly related.

It was established in Chapter 4 that the inverse relationship between B16 cell growth and percentage AA composition upon Asc supplementation was due to the effect of Asc supplementation on the uptake of AA by the B16 cells from the medium (386). Since bovine serum albumin binds AA, LA and oleic acid (387) it is possible that Asc affected the binding of EFAs, including AA, to the bovine serum albumin of FCS, thereby influencing their uptake. LA was also found to increase proportionally with AA. Asc supplementation therefore generally resulted in a greater uptake of EFA by the B16 cells. AA levels were more pronounced as Asc supplementation stimulated not only its uptake, but that of LA and DGLA too, which in turn could be converted to AA. However, it must be emphasized that Asc supplementation did not affect the conversion of LA to AA (388), *ie.* it did not stimulate or inhibit delta-6 (175) or delta-5-desaturase activity. *In vivo*, one could postulate that Asc in the diet would enhance AA uptake from the diet. The changes in AA were then able to affect further secondary metabolism within the B16 cells.

Relevant to the stroma percentage AA composition of the B16 cells, Asc supplementation increases the membrane percentage AA composition in these cells. The B16 cells probably incorporate most of this AA into phospholipids. Human gastric cells reportedly predominantly incorporate ^{14}C-AA into phosphatidylcholine (365). This in a sense could lead to the stabilization of the B16 cell membrane and a possible increase in membrane fluidity as a result of Asc supplementation. The antitumour effect of GLA in neuroblastoma cells is suggested to be due to cellular dysfunction caused by FA modification after GLA incorporation (132). AA supplementation of the B16 cells resulted in an overall decrease in cell growth, which could be due to toxicity of the FA or its resultant metabolites. Basal AC activity in neuroblastoma cells cultured with exogenous FAs, depended on FA type, in that LA formed more cAMP than GLA supplemented and control cells

(308,384). Thus, FA toxicity of the cells is a reduced possibility in the B16 cells in this study, as it is more likely that the antitumour effect was due to subsequent FA metabolites such as cAMP, the final product in the pathway considered here.

In the B16 control cells, no PGE_2 was found in the stroma fraction, with all of it assumed to still be bound to the membrane. However, upon Asc supplementation, PGE_2 levels in the stroma of these cells increased relative to those in the membrane, suggesting PGE_2 may have been mobilized by Asc from the membrane and transported to the stroma. This release increased gradually with Asc supplementation. Total PGE_2 levels were inversely related to B16 cell growth with Asc supplementation, as was total percentage AA composition. Hence, AA levels and PGE_2 levels were proportionately related, *ie.* the level of PGE_2 synthesized was thus dependent on the level of its precursor (381). Therefore, Asc did not enhance the conversion of AA to PGE_2. This was also found to be the case in human platelets in which Asc supplementation (10 - 100 μg/ml) had no effect on the conversion of ^{14}C-AA to PGE (161). In squamous carcinoma cells Asc supplementation in fact inhibited PGE_2 synthesis from exogenous AA (349). In an attempt to confirm that greater PGE_2 levels were simply due to greater AA levels rather than a direct effect of Asc supplementation, the cells were supplemented with AA. This was indeed the case, as at high AA supplementation PGE_2 levels in the B16 cells increased significantly.

PGE_2 stimulates AC activity in a variety of cells, including tumour cells, although tumours can vary in the degree of cAMP response (170, cited by 389). Asc supplementation of the B16 cells significantly decreased cell growth in experiments done in this study, while AC activity and cAMP levels increased significantly. As PGE_2 levels were also inversely related to B16 cell growth upon Asc supplementation, it is reasonable to conclude that AC activity was stimulated by greater PGE_2 levels. This was indeed so, as increasing PGE_2 supplementation of the B16 cells stimulated AC activity in these cells. PGE_2 has also been found to inhibit the growth of human carcinoma and osteogenic osteosarcoma cells via the stimulation of cAMP production (62,205,390). The hormonal control of cAMP levels was also present in Ewing sarcoma cells and PGs stimulated cAMP production in these cells with an efficacy order of $PGE_1 > PGI_2 > PGE_2$ (310). cAMP

concentrations are therefore influenced by PGE_2 levels, although the reverse has also been found. In HeLa cells, cAMP addition increased PGE_2 levels and inhibited cell growth, which suggested PGE_2 toxicity. This is a possibility, as PGE_2 supplementation at high levels also significantly inhibited B16 cell growth in this study. In other studies PGs of the E and A series have been found to be potent inhibitors of two murine tumours, B16 melanoma and the Friend virus-induced erythroleukaemia (cited by 201).

The inhibitory effect of Asc supplementation on B16 cell growth is complex. Many secondary metabolites are affected and they themselves exhibit toxic properties, *eg* AA and PGE_2. However, the most effective inhibitor of cell proliferation is probably cAMP, which is increased in B16 cells with decreased cell growth, as it is directly involved in the cell cycle. cAMP thus has an important role in cell growth, with intracellular cAMP levels rising coincident with the entrance of cells into the quiescent state (266). The entrance of the B16 cells into the quiescent state, upon Asc supplementation, is thus possible. However, this is an indirect effect of Asc supplementation, as it originally directly affects AA levels in the B16 cells, with this effect then being expressed through PGE_2 levels and the effect of the latter on AC activity. Another possible growth inhibitory mechanism of cAMP is to stimulate GAG synthesis in cells, thus modifying the extra- and intracellular matrices and contributing to growth inhibition (380). This was the case in human breast carcinoma cell lines (391) and the elevated cAMP levels in the Asc supplemented B16 cells could be responsible for increased GAG synthesis in these cells.

PDE is the enzyme which converts cAMP to AMP, thus terminating any regulatory effect of cAMP (4). The decrease in cAMP levels at 100 μg/ml Asc supplementation in the B16 cells was accounted for in section 3.1 by the slight reduction in AC activity of these cells. This study did not investigate the effect of Asc supplementation on PDE activity. The question does arise, however, as to whether PDE possibly hydrolyzed cAMP to AMP in this instance or not. This possibility is unlikely, as it has been found that cAMP and Asc compete for active sites on PDE. cAMP and Asc each possess a ring-structure with a negatively charged oxygen, which when hydrolysed produces an acid group. It is this structure that competes for the active site. L-Asc, in concentrations as low as

0.1mM, is effective in inhibiting the breakdown of cAMP (254).

With regard to another possible effector of AA level variations in cells, and the consequent effects on cell growth already described, Asc supplementation of the B16 cells resulted in a decrease in PLA_2 activity. This effect, associated with decreased cell growth, was contrary to the expected effect. If PLA_2 activity was responsible for the enhanced release of AA from phospholipids, one would have expected PLA_2 activity to be inversely related to cell growth, as AA levels were. However, this was not the case and PLA_2 activity in Asc supplemented B16 cells could therefore not be responsible for the inverse relationship between AA composition in B16 cells with cell growth.

The release of AA from prelabelled [^{3}H]-AA pig aortic endothelial cells closely followed changes in intracellular Ca^{2+} (228). This indicates that the PLA_2 activity here was Ca^{2+}-dependent (228). PLA_2 activity may be modulated by G protein-dependent secondary messengers such as Ca^{2+}, cAMP and/or the kinases they activate. PLA_2 activity determined in the B16 cells was also Ca^{2+}-dependent. The B16 cells supplemented with Asc had increased AA levels with decreased cell growth, while PLA_2 activity decreased with the same cell growth. Hence, it is possible that greater AA levels in B16 cells with decreased growth had an inhibitory effect on PLA_2 activity. This indicates PLA_2 to be susceptible to a negative feedback system, as has been proposed before (214,232).

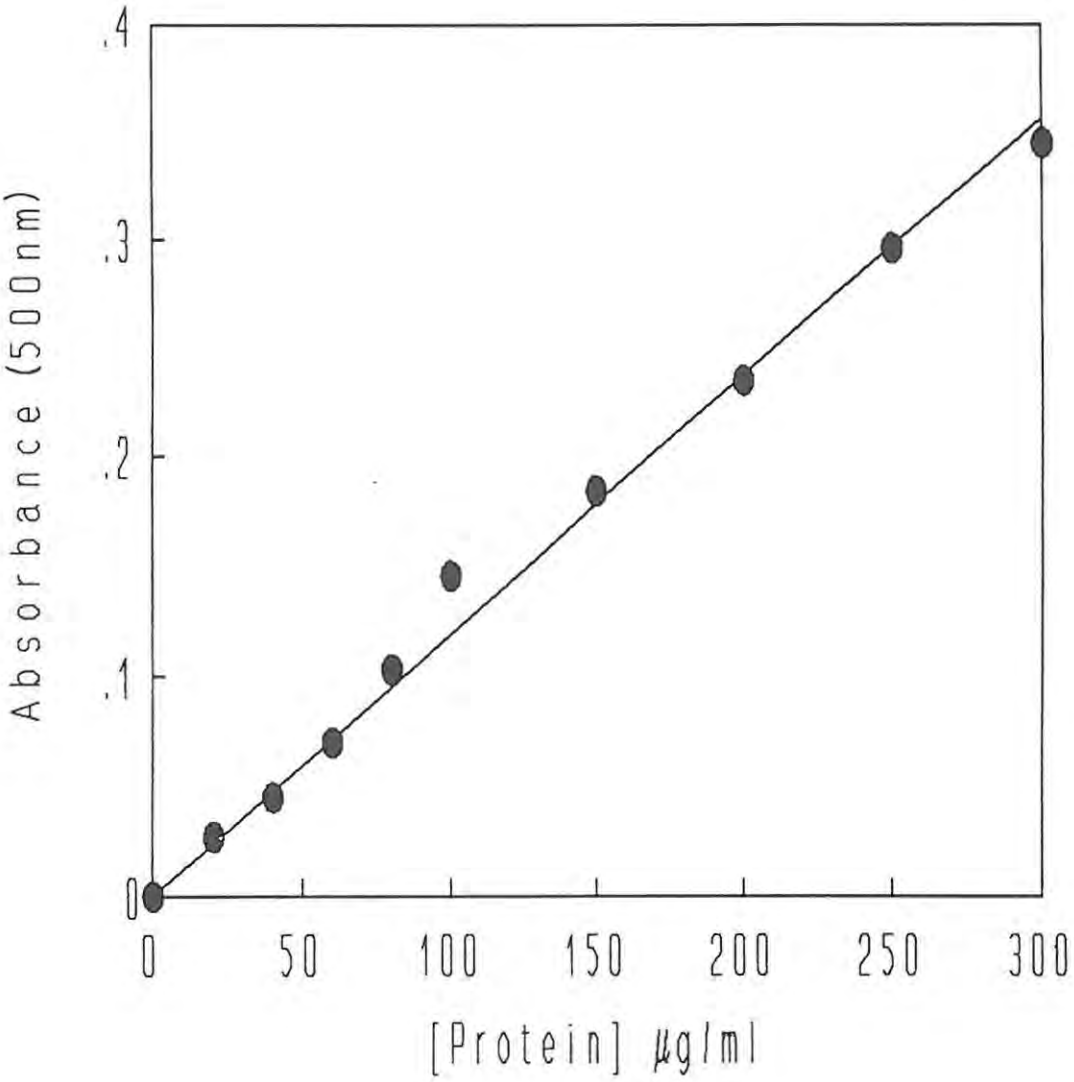
The passage through G_1 and G_2 of the cell cycle is not only dependent on cAMP concentrations, but is also Ca^{2+} dependent (267). The cycle can become blocked in either phase under a variety of conditions and can be altered in tumour cells (cited by 375). It is claimed that tumour cells do not regulate cytoplasmic Ca^{2+} levels (301). Interestingly, Ca^{2+} levels in the B16 cells were increased with Asc supplementation and were correlated with decreased cell growth. Increased Ca^{2+} and cAMP levels are therefore present in Asc supplemented B16 cells which have a reduced growth rate. In pituitary cells (374) there is control by secondary messenger AA of intracellular Ca^{2+} levels, with higher AA levels resulting in Ca^{2+} mobilization. The higher AA levels in the Asc

supplemented B16 with reduced growth rate could have resulted in higher Ca^{2+} levels in the cells, *eg* influx into the cells from the medium. This increased Ca^{2+} was then, in turn, able to affect many other processes in the cell.

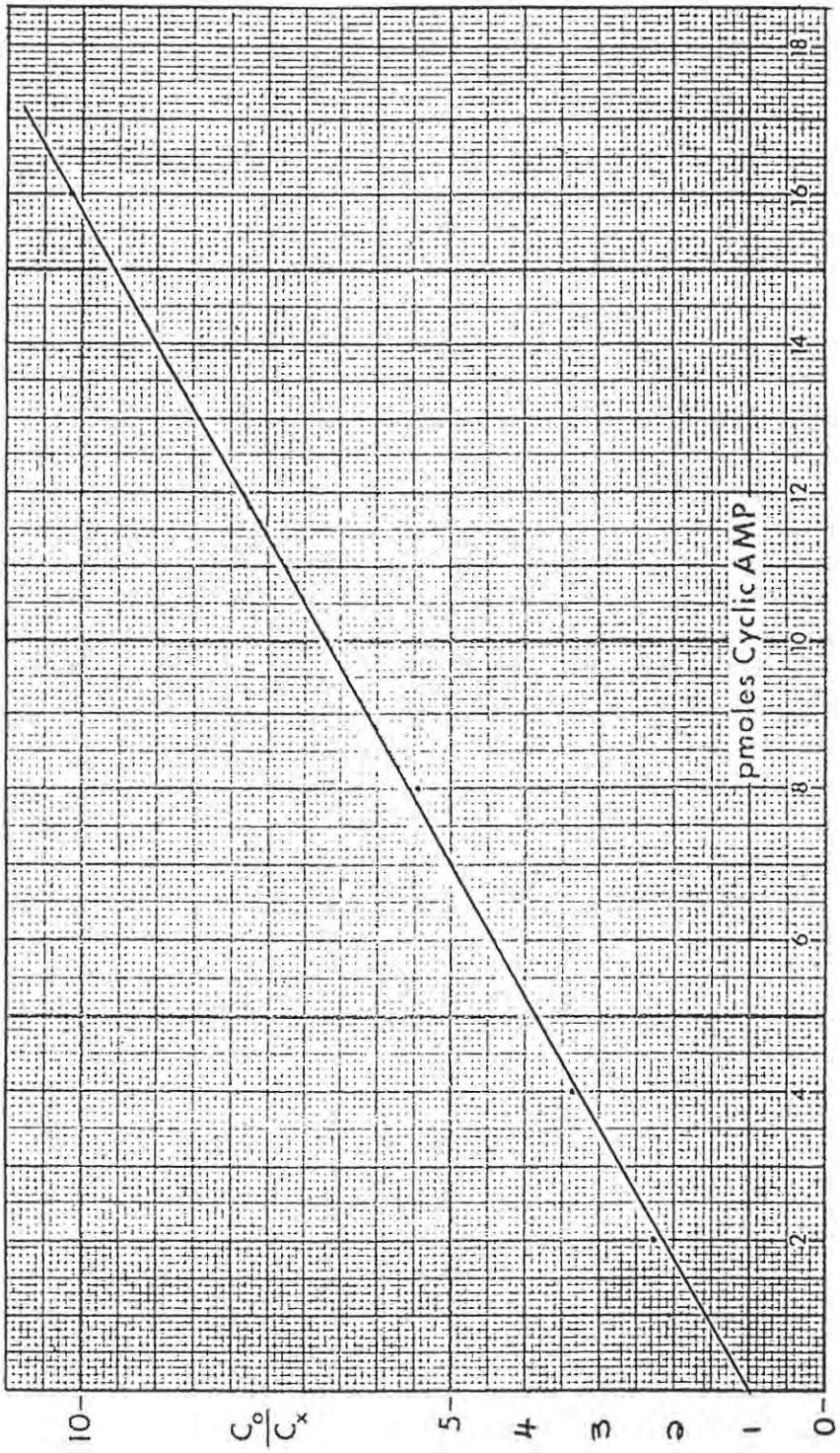
The normal cell line used in this study, the LLCMK monkey kidney cells, did not exhibit any significant trends or relationships with Asc supplementation. Most changes in enzyme activity or metabolites were related to changes in cell number. Hence, this discussion has mainly concerned the B16 melanoma cells. The only exception was the inverse relationship between LLCMK cell growth and PLA_2 activity as a result of Asc supplementation. The percentage AA composition in the LLCMK stroma fractions supplemented with the higher Asc concentrations did show an increase relative to the percentage AA composition of the membrane fractions of these cells. This suggests an increase in AA release possibly due to enhanced PLA_2 activity. However, this was the only notable feature detected in these cells.

It has previously been hypothesized (cited by 348) that the protective effect of Asc in tumour cells is mediated by an alteration of AA metabolism within these cells, where Asc results in the increased production of PGE_2. One of the conclusions of this thesis is that in B16 murine melanoma cells, Asc supplementation does mediate an alteration in AA metabolism. The observed alteration was initiated by Asc supplementation of these B16 cells, affecting the uptake of AA and other EFAs by the cells, thereby affecting cell growth. Increased PGE_2 levels in the B16 cells were also correlated with decreased growth after Asc supplementation. However, this study has shown that the increased PGE_2 levels detected in the B16 cells were due to the increased AA levels already present in the cells and not due to Asc supplementation stimulating the conversion of AA to PGE_2. Furthermore, these increased PGE_2 levels in the Asc supplemented B16 cells were able to stimulate AC activity and increase cAMP production. The increased cAMP levels are believed to be responsible for the inhibitory effect of Asc supplementation on B16 cell growth, possibly through facilitating the entry of the B16 cells into the quiescent state of cell growth. cAMP, however, is not the sole factor involved in the process, since it is aided by other second messengers such as Ca^{2+}, AA, PGE_2, etc. The action of these second messengers, as influenced by Asc supplementation,

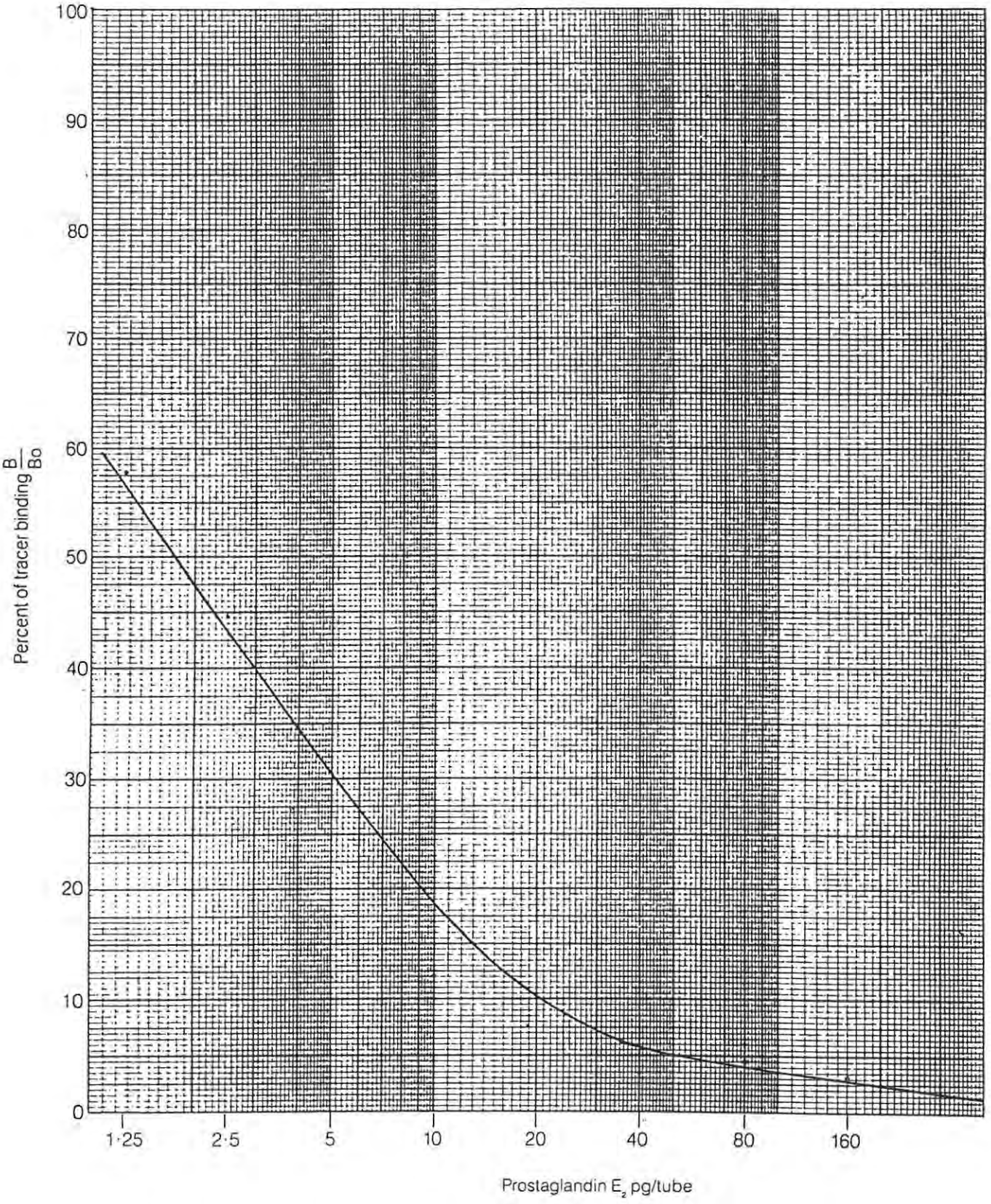
also affect B16 cell growth to a certain extent, which could explain the variable effects of Asc supplementation on B16 cell growth in different experiments. It is therefore clear that the effect of Asc supplementation on malignant B16 murine melanoma cell growth is an extremely complex process.



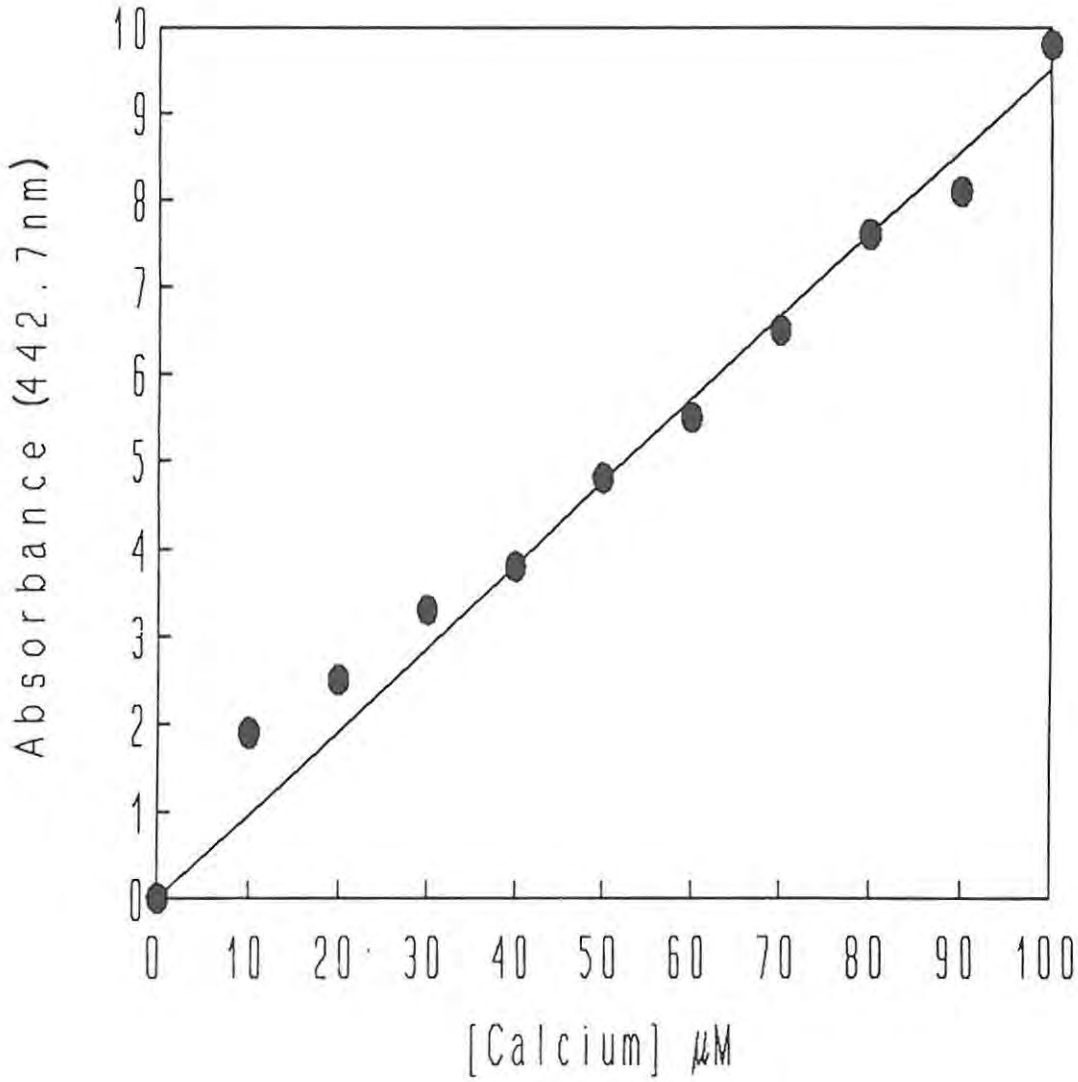
APPENDIX 1: Protein standard curve



APPENDIX 2: Cyclic adenosine monophosphate standard curve



APPENDIX 3: Prostaglandin E_2 standard curve



APPENDIX 4: Calcium standard curve

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