METABOLIC RESPONSES IN MELANOMA CELLS TO COMBINED NUTRIENT SUPPLEMENTATION

THESIS

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ABSTRACT

This thesis examined the effect and biochemical mechanism by which combined vitamin E and C supplementation may influence tumour cell growth. The study initially addressed the effect of combined vitamin E succinate and Asc supplementation over a nutritional concentration range (5-20μg/ml) and (25-50μg/ml) respectively, on the *in vitro* growth of non-malignant LLCMK and malignant BL6 cells. Supplementation of BL6 and LLCMK cells with combined vitamin E succinate and ascorbic acid, resulted in no significant increasing or decreasing trend in LLCMK cell growth, while in BL6 cells a significant decrease in cell growth was observed at all combined vitamin concentrations. It has been suggested that these vitamins may act synergistically to inhibit tumour cell growth through their antioxidant properties in quenching free radicals and lipid peroxidation and furthermore through their modulation of the activities of various enzymes and metabolites in the eicosanoid pathway. This study consequently investigated the effects of combined vitamin E succinate and ascorbic acid supplementation on these parameters.

Throughout this study, emphasis was placed on the BL6 melanoma cells, as combined vitamin E succinate and ascorbic acid supplementation did not significantly affect growth or levels of secondary metabolites in the non-malignant LLCMK cells.

Combined vitamin E succinate and ascorbic acid supplementation of BL6 cells resulted in a marked but non significant increase in free radical and a significant increase in lipid peroxidation levels. This prooxidant effect was accompanied by a significant decrease in BL6 cell growth, suggesting that the growth inhibitory effects of combined vitamin E succinate and ascorbic acid on BL6 cells *in vitro* was not mediated through their synergistic antioxidant properties. Vitamin E succinate is a non-physiological antioxidant in its esterified form, hence cleavage of the succinate group must occur in order for ascorbic acid to interact with the free alcohol, vitamin E. The inability of combined vitamin E succinate and ascorbic acid to reduce free radicals and lipid peroxidation levels within BL6 cells may not be due to their ineffectiveness as antioxidants but rather the presence of other contributing factors which influence the oxidation state within the BL6 cells.

Vitamin E is believed to modulate membrane-bound enzymes through membrane stabilization. Furthermore, the stabilizing effect of vitamin E may be enhanced by the ascorbic acid-sparing effect of vitamin E. Hence, this study investigated the effect of combined vitamin E succinate and ascorbic acid in modulating the activity of various enzymes and secondary messengers in the eicosanoid

pathway. Supplementation with combined vitamin E succinate (5-20μg/ml) and ascorbic acid (25-50μg/ml) resulted in significant increases in phospholipase A_2, 5-lipoxygenase, cyclooxygenase and adenylate cyclase activity, with a significant decrease in BL6 cell growth. The possible synergistic action of these vitamins in terms of modulating membrane-bound enzymes was further substantiated by uptake and cellular distribution studies. Vitamin E succinate and vitamin E in the membrane fraction increased significantly compared to control cultures, while ascorbic acid levels were significantly higher in the stroma fraction when compared to membrane fractions. Consequently, another factor accounting for increased activities of phospholipase A_2, 5-lipoxygenase and adenylate cyclase activities as a result of vitamin supplementation in BL6 cells may be an increased availability of Ca^{2+}.

Supplementation of BL6 cells with combined vitamin E succinate and ascorbic acid resulted in significant increases in intracellular Ca^{2+} levels at all combined vitamin groups. Furthermore, this increase in intracellular Ca^{2+} was positively correlated with changes of the above-mentioned enzyme activities.

Within the eicosanoid pathway, the rate of prostaglandin synthesis is regulated by phospholipase A_2 activity and arachidonic acid release, and the net prostaglandin production is dependent on cyclooxygenase activity, hence the effects of combined vitamin E succinate and ascorbic acid on arachidonic acid composition and prostaglandin production within BL6 cells was determined. The percentage arachidonic acid composition of the BL6 cells was elevated and inversely related to cell growth following combined vitamin E succinate and ascorbic acid supplementation. Prostaglandin E_2 and prostaglandin I_2 levels increased significantly, while those of prostaglandin D_2 and prostaglandin $F_{2\alpha}$ increased markedly following supplementation of combined vitamin E succinate and ascorbic acid. These increases in prostaglandin levels were inversely related to BL6 cell growth, suggesting that the prostaglandins were involved in negative regulation of BL6 cell growth. When comparing the levels of prostaglandins, prostaglandin E_2 levels were significantly higher when compared to prostaglandin D_2, prostaglandin $F_{2\alpha}$ and prostaglandin I_2 suggesting that vitamin E succinate and ascorbic acid effects were mediated primarily through an increase in prostaglandin E_2. Hence, prostaglandin E_2 levels in combined vitamin E succinate and ascorbic acid appeared to be dependent on the amount of precursor present and the activity of its synthetic enzymes. This was confirmed when BL6 cells were supplemented with arachidonic acid. Arachidonic acid had an inhibitory effect on BL6 cell growth and also stimulated prostaglandin E_2 production. Prostaglandin E_2 levels are in turn believed to modulate adenylate cyclase activity in BL6 cells, hence it is reasonable to conclude that adenylate

cyclase activity is dependent on prostaglandin E_2 levels. Combined vitamin E succinate and Asc supplementation to BL6 cells resulted in significant increases in adenylate cyclase and cyclic adenosine monophosphate, which again correlated with a significant decrease in cell growth. As cyclic adenosine monophosphate has a regulatory role in the cell cycle, this study suggested that the effect of combined vitamin E succinate and ascorbic acid supplementation was mediated through the final effect provided by the second messenger, cyclic adenosine monophosphate. This was confirmed when BL6 cells were supplemented with dexamethasone, a phospholipase A_2 inhibitor. This treatment resulted in combined vitamin E succinate and ascorbic acid having no inhibitory effect on BL6 cell growth. Cyclooxygenase activity, prostaglandin E_2 levels, adenylate cyclase activity and cyclic adenosine monophosphate levels were significantly lower in dexamethasone-treated cells compared to non-treated dexamethasone cultures.

The reason for the increased free radical and lipid peroxidation levels in BL6 cells was further investigated. Cyclooxygenase enzymes are believed to generate free radical species during catalytic activity. Analysis of free radical and lipid peroxidation levels following supplementation with dexamethasone revealed markedly lower free radical and significantly lower lipid peroxidation levels in comparison with control cultures and non dexamethasone-treated cultures. These results suggest that the observed increases in free radical and lipid peroxidation levels in BL6 cells supplemented with combined vitamin E succinate and ascorbic acid were indirectly due to the increase in cyclooxygenase activity in these cells.

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ABBREVIATIONS

α-TOH	Vitamin E = α-Tocopherol
AA	Arachidonic acid
AC	Adenylate cyclase
AMP	5' Adenosine monophosphate
ANOVA	Analysis of variance
Asc	Vitamin C = ascorbic acid
ATP	Adenosine triphosphate
BL6	Murine melanoma cells
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophospahte
CDR	Calcium-dependent regulatory protein
COX	Cyclooxygenase
CPM	Counts per minute
DHA	Dehydroascorbic acid
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
EFA	Essential fatty acid
FCS	Foetal calf serum
GAG	Glycoaminoglycan
GDP	Guanine diphosphate
Gi	Inhibitory guanine-nucleotide binding regulatory protein
GLC	Gas-liquid chromatography
Gs	Stimulatory guanine-nucleotide binding regulatory protein
GTP	Guanosine triphosphate
H_2O_2	Hydrogen peroxide
HDL	High density lipoprotein
HPLC	High performance liquid chromatography
kDa	Kilodallton
LDL	Low densiity lipoprotein
LLCMK	Monkey kidney cells
LOX	Lipoxygenase
LT	Leukotriene

MDA	Malondialdehyde
MEM	Minimum essential medium
Mg^{2+}	Magnesium
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NBT	Nitroblue tetrazolium
NO	Nitrous oxide
NO_2	Nitrite
NSAID	Non steroidal anti-inflammatory drug
PBS	Phosphate bufffered saline
PES	Prostaglandin endoperoxide synthase
PG	Prostaglandin
PGD_2	Prostaglandin D_2
PGE_2	Prostaglandin E_2
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGG_2	Prostaglandin G_2
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PHI	Physiological hyaluronidase inhibitor
PLA_2	Phospholipase A_2
PUFA	Polyunsaturated fatty acid
RDA	Recommended daily dietary allowance
Ri	Inhibitory receptor
Rs	Stimulatory receptor
SEM	Standard error of the mean
SOD	Superoxide dismutase
Sr^{2+}	Serium
TBA	Thiobarbituric acid
TBP	Tocopherol-binding protein
TCA	Trichloroacetic acid
TLC	Thin layer chromatography
TX	Thromboxane
VLDL	Very low density lipoprotein

CHAPTER 1

LITERATURE REVIEW

1.1 CANCER

Cancer can be defined as a cellular malignancy in which loss of normal controls results in unregulated growth, lack of differentiation, and the ability to invade local tissues and metastasize (1). Two types of cancers have been identified. Clusters of abnormally proliferating cells can arise in any part of the body. Those which are unable to invade the surrounding tissues and so remain strictly local growths are called benign tumours. Those that spread from their site of origin, disseminating into the bloodstream and lymphatic systems are called malignant tumours (2). The transformation of a normal cell into a neoplastic or abnormal cell is thought to proceed through three phases: initiation, promotion and progression (3-7) (see Figure 1).

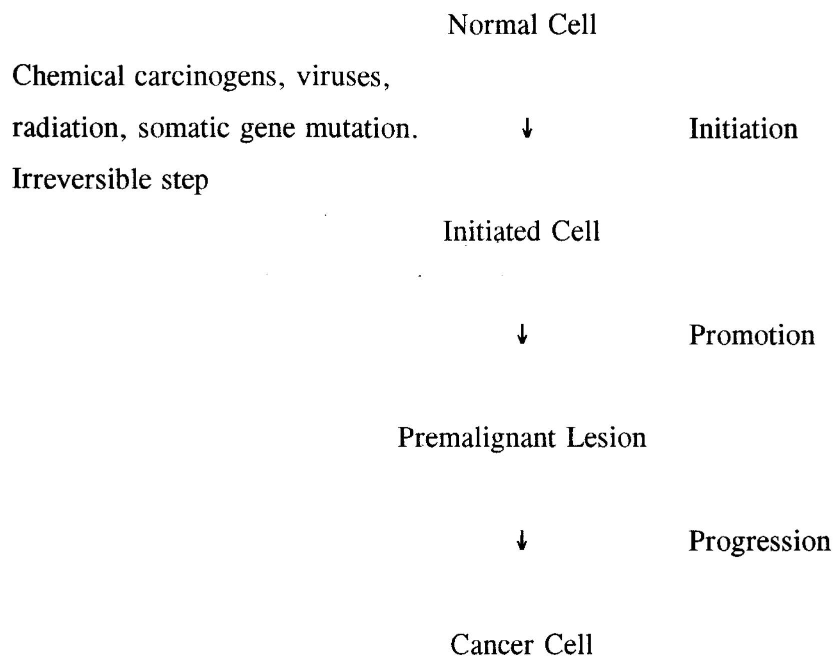


Figure 1 : A multistep carcinogenesis model (7).

1.1.1 INITIATION

The stage of initiation that occurs first in the natural history of neoplastic development reflects a permanent and irreversible change in the initiated cell. The efficiency of initiation is related to cellular replicative DNA synthesis and cell division (4). The transformation of a normal cell to a cancerous cell can be induced by a variety of agents that are generally chemical or physical in nature. These in turn have been grouped into four relatively distinct categories: chemical, physical, biological and genetic (4,8,9) (see Table 1) .

Table 1 : Representative examples of carcinogenic agents (4).

Class	**Examples**	**molecular mass range (Daltons)**
I. Chemical	polycyclic hydrocarbons, aromatic amines, halides, diet, hormones, metals and polymers surfaces	$5x10^{1}$ - $5x10^{4}$
II. Radiation	Ionizing (X and γ rays, α partical radiation) and UV	$\langle\langle\langle$ 1 - 1^{+}
III. Biological	Viruses (papova, herpes, retro and hepadna viruses)	$3x10^{6}$ - $170x10^{6}$
IV. Genetic	Transgenesis by enhancer - promoter- oncogene constructs, selective breeding	$\sim 10^{6}$ - 10^{8}

The primary action of each of these agents is either to generate or to be metabolically converted to electrophilic reactants which bind covalently to cellular DNA and other macromolecules (8). This in turn alters the information content or rate of expression of the genetic material. In the case of chemically induced cancers, unless the adduct is removed by repair processes, the lesion causes the introduction of a permanent genetic mutation. Initiation, the irreversible step, without the following steps, promotion and progression, rarely yields malignant tumours (8-10).

1.1.2 PROMOTION

The primary characteristic of promotion that distinguishes it from the stages of initiation and progression is its operational reversibility (4). Promotion is a phenomenon of gene activation in which the latent altered phenotype of the initiated cell becomes expressed through selection and clonal expansion. Tumour promoters are not carcinogenic themselves, but must operate in succession with initiators. In the absence of initiators, promoters can generate reversible changes in cell proliferation and phenotypic expression. The molecular mechanisms of tumour promoters are not fully understood. However, it is suggested that various promoters override the homeostatic regulatory mechanisms utilized by the growth factors (10,11). Proliferation of cells in culture is regulated predominantly by extracellular agents of which the polypeptide growth factors are among the most important. These factors interact with specific cell-surface receptors which in turn deliver intracellular signals ultimately leading to DNA synthesis. The characterization of oncogenes associated with tumourigenesis has prompted intensive research into the molecular mechanisms underlying the loss of growth control which is characteristic of tumour cells (12,13).

Oncogenes are modified variants of normal cellular genes which, once activated by a mutation, an oncogene promotes excessive or inappropriate cell proliferation. Their products are presumed to function at least in part by mimicking the products of the cellular genes from which they arose (12,13). When isolated oncogenes from tumours are inserted into normal cells, the latter take on many traits of cancer cells. Such gene transfer experiments demonstrate that oncogenes function to deregulate the growth of cells into which they are introduced, and so must be accountable at least in part for the irregular functioning of the tumour cells from which they were extracted (13). Numerous studies have reported that oncogenes are altered versions of normal genes called proto-oncogenes that function as focal regulators of growth in normal cells. Various mutations in the course of the normal cells existence can convert one of these normal genes into a malignant oncogene (13,14). To date, oncogenes have been detected in only 15 or 20% of human tumours. The evolution of a line of tumour cells appears to hinge on the accumulation of mutations transforming a number of genes, among them oncogenes (15). Cellular antigens encoded by tumour viruses and some antigens encoded by cellular oncogenes offer advantages as targets for immunotherapy by being inextricably associated with the neoplastic phenotype (16).

The levels of free radicals may represent an additional or accompanying mechanism for mediating some of the actions of tumour promoters. Numerous promoters of carcinogenesis act by the

generation of oxygen radicals, resulting in lipid peroxidation processes. Lipid peroxidation cross-links proteins and affects all aspects of cell organization, including membrane and surface structure in addition to the mitotic apparatus (17,18). A number of cellular defences against these transient but damaging radical species exist and can be grouped into enzymic and non-enzymic antioxidant defence mechanisms. The non-enzymic agents include minerals as well as the antioxidant vitamins such as vitamin E, β- carotene and vitamin C (16,17). α-Tocopherol (α-TOH) quenches singlet oxygen, reduces radicals formed from hydroxyl radicals, and protects the lipids from peroxidation by trapping peroxide radicals. Ascorbic acid (Asc) scavenges superoxides and in addition helps keep α-TOH from being completely inactivated. Hence these two vitamins are most effective in combination (5,16-18).

1.1.3 PROGRESSION

Progression is fundamentally typified by its karyotypic instability and evolution, and the formation of irreversible aneuploid malignant neoplasms. Modifications in the structure of the genome of the neoplastic cells during this stage are directly related to the growth rate, invasiveness, metastatic capability, and biochemical alterations in the malignant cell (4).

The bulk of cancer deaths are not caused by the primary tumour, but rather by secondary tumour growth elsewhere in the body. The ability of malignant tumour cells to disseminate can be considered the most lethal aspect of cancer. The spread of cancer from primary to secondary sites is called metastasis (19). It is convenient to view the process of metastasis in three phases: i) entrance of cells into the blood stream, ii) circulation of cells to distant organ sites via the blood or lymphatic system, iii) arrest and proliferation of tumour cells in a distant organ or regional lymph node (19,20).

The formation of a metastasis represents the end stage of several highly selective processes. By virtue of invasive properties or juxtaposition to the circulatory system in the primary tumour, some cells are "selected" for entry into the circulation. In the blood or lymph most tumour cells are destroyed. A few resilient cells adhere to capillary endothelium and after passing through other selective steps an even smaller number of cells initiate a metastasis. Studies by Boseman et al (cited in 20) showed the super metastatic strains exhibited changes in their electrophoretic mobility and, increased activity of degradative enzymes, surface glycoproteins and proteases. Immunoselection may also be an important aspect of cell selection for metastasis.

Organ selection by metastatic cells is not random: experimental observations indicate that metastasizing cells "refer" some organs to others. Not much is known about the mechanisms involved in organ selection by metastatic cells, however the hypothesis of Green and Harvey (cited in 20) suggests that a tumour cell-endothelial cell bond is important in organ selection.

1.1.4 CHEMOPREVENTION

Established prevention and therapy approaches have proven unsuccessful in controlling the increased incidence of, and low survival rates related to, many solid tumours. Numerous new approaches are in progress; one of the foremost is chemoprevention. Chemoprevention is defined as the systemic use of specific natural or synthetic chemical agents to reverse or suppress the progression of a premalignancy to invasive cancer (6,21). A group of such agents is the antioxidants.

Many studies have sought to improve our understanding of the role of antioxidants in protecting the human body against cancer. Consequently, cancer chemoprevention through supplementation and fortification of the diet with micronutrient antioxidants may become an effective strategy for cancer treatment before the close of the century (22). Benedict *et al* (6) believe that Asc may play an important role in chemoprevention, in addition to identifying events necessary for oncogenic progression. Asc has been considered to be of possible chemopreventive use, mainly as a chemical block to cancer cell initiation. Furthermore, Asc exhibits antioxidant and antiviral activity, and has the ability to block nitrosamine formation (6). Vitamin E (α-TOH) has also generated much attention as a protective agent against chemically-induced toxicity to cells (23,24). The principal role of vitamin E in enhancing immune responses and phagocytosis is the prevention of lipid peroxidation of cell membranes. The rapidly proliferating cells of the stimulated immune and phagocytic systems are particularly prone to peroxidative damage by free radical, peroxides and superoxides. The antioxidant effect also modulates the biosynthesis and activity of important cell regulators, prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs). These studies suggest that vitamin E and vitamin C interact positively in enhancing immune responses and protection from infectious diseases and cancer (24).

Since Asc and α-TOH and their role in the neoplastic process is the subject of this thesis, it is important to consider the general role of both Asc and α-TOH in cell metabolism.

1.2 ASCORBIC ACID

1.2.1 HISTORY

Micronutrients, which include vitamins, minerals and some trace elements, are dietary components essential to normal metabolic function (25). The term "essential" implies that Asc has to be acquired in the diet as it cannot be synthesized *de novo* in *Homo sapiens*. 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 (25).

Asc is essential in the diet of man, other primates, the guinea pig, an Indian fruit- eating bat, the red-vented bulbul and some related species of the Passeriform birds (cited in 26). All other animal species are able to synthesize Asc (26) because they possess the enzyme L-gulono-γ-lactone oxidase (EC 1.1.3.8). This enzyme catalyzes the conversion of L-gulono-γ-lactone to Asc (27). It is estimated that primates lost the gene for L-gulono-γ-lactone oxidase 70 million years ago (28).

Scurvy, a disease known since antiquity, is a consequence of an Asc deficient diet which was particularly noted in sailors on long voyages in the 15th through to the 19th Centuries (29,30). Sir Richard Hawkins in the 16th Century discovered that the juice of fresh citrus fruits cured scurvy. This discovery was again used by James Lind in the 18th Century for curing British sailors (29). The active agent, the enolic form of 3-keto-L-gulofuranlactone or hexuronic acid, was later named Asc or vitamin C. It was first isolated from oranges, cabbages and adrenal glands in the late 1920s by Albert Szent-Györgi (29,31,32).

1.2.2 STRUCTURE AND METABOLISM OF ASCORBIC ACID

Asc is synthesized from D-glucose as shown in Figure 2. In man, due to the absence of the enzyme L-gulono-γ-lactone, the last step in the Asc biosynthetic pathway involving the oxidation of L-gulonolactone to Asc cannot occur (28,29).

Thus Asc is shown to be a γ-lactone, although other tautomeric forms may exist in small quantities. The configuration at C-5 is L- (or S-, using the Cann-Ingold-Prelog system). The acid nature in aqueous solution derives from the ionization of the enolic OH on C-3, with the resulting Asc anion being localized (33). It has been estimated that about forty thousand tonnes of the vitamin are now

manufactured annually. In addition to the precursor D- glucose, the synthesis of Asc from C-5 sugars is now well established with xylose, lyxose, and recently arabinose being used as precursors (33). Vitamin C is the most commonly available single nutrient supplement used in the US (30).

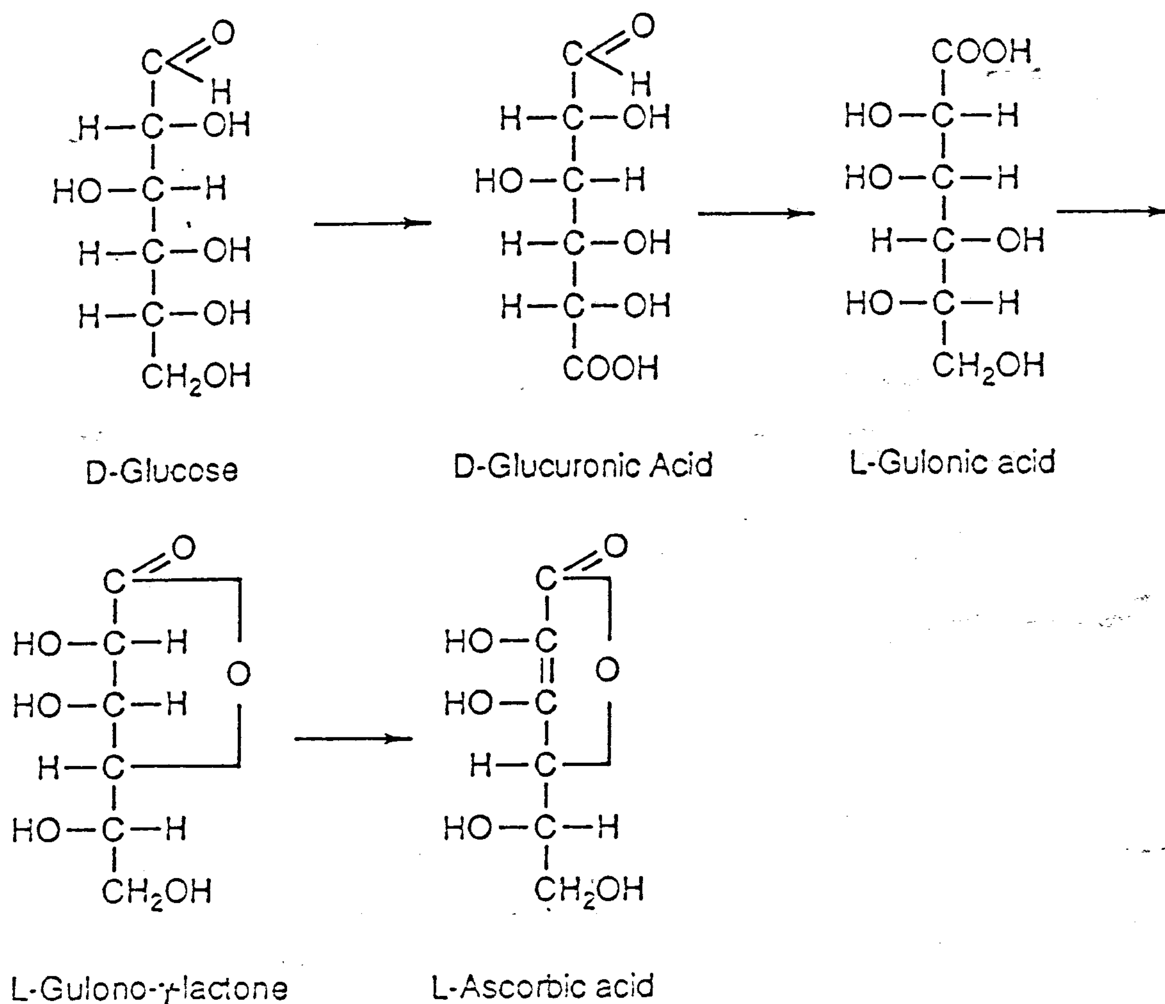


Figure 2 : The metabolic pathway of L-ascorbic acid biosynthesis in animals (28).

1.2.2.1 Metabolism

The recommended daily dietary allowances (RDAs) over the past few decades have varied from as little as 10mg to a thousand times as much. The recommended RDA for an adult is 60mg, while the RDA for the aged is 150mg (33). Milk is the only animal produce that provides a significant amount of the vitamin (1-5mg per 100g), although there is some in liver. The best sources are fresh fruits

(particularly citrus, tomatoes and green peppers), baked potato (17mg per 100g) and leafy vegetables.

Compounds which are incapable of being synthesized within the cell need to be transported in two ways: across cell membranes and through biological fluids. Transportation through biological fluids can involve simple diffusion, hydrodynamic flow and protein binding (34).

It is believed that protein binding is of little significance in the transport of vitamin C in biological fluids and its retention in cells (34,35). Vitamin C can be filtered by the kidney and ultra filtered from plasma suggesting that protein binding in the blood is minimal (34,35). In addition, vitamin C is rapidly lost from energy-depleted cells, suggesting that binding within the cell is also probably limited. On the other hand, studies have shown binding of Asc to serum albumin and that this plasma protein can increase the stability of vitamin C (34). No more can be deduced as the full extent of associations and binding strength remain to be determined (34).

The polarity of Asc, which favours its presence in aqueous fluids, also hampers its passage across the hydrophobic cell membrane. This, and the high concentrations, make it almost certain that the vitamin needs carrier mechanisms to enter the cell (34,35). Unfortunately, understanding of the transport mechanism into the cell remains unclear. Most cells appear to be able to transport dehydroascorbic acid (DHA), the oxidized form of Asc. The process involves facilitated diffusion, *i.e.* with a carrier but not against the an electrochemical gradient or requiring energy. Continuous uptake of dehydroascorbate is due to the cells ability to rapidly reduce it to Asc and hence conserve a low intracellular concentration of the oxidized form of the vitamin. The possibility of co-transport of Asc by this system provides an additional mechanism for moving Asc across cell membranes (34). Once in the cell, transportation down the concentration gradient into the blood via facilitated diffusion occurs.

Asc can act as a reducing or oxidizing agent depending on the reaction and circumstances. One of the principal reasons for the great interest in this aspect of vitamin C function, is that the role of vitamin C in living systems is undoubtedly connected to its oxidation-reduction behaviour. This phenomenon may ultimately be the key in understanding the biological mechanisms of the actions of vitamin C (33,36). L-Asc is oxidized by dioxygen to dehydroascorbic acid (see Figure 3), and both have antiscorbutic and other physiological functions of vitamin C. Both Asc and DHA are ultimately excreted in the urine as oxalic acid (31,37).

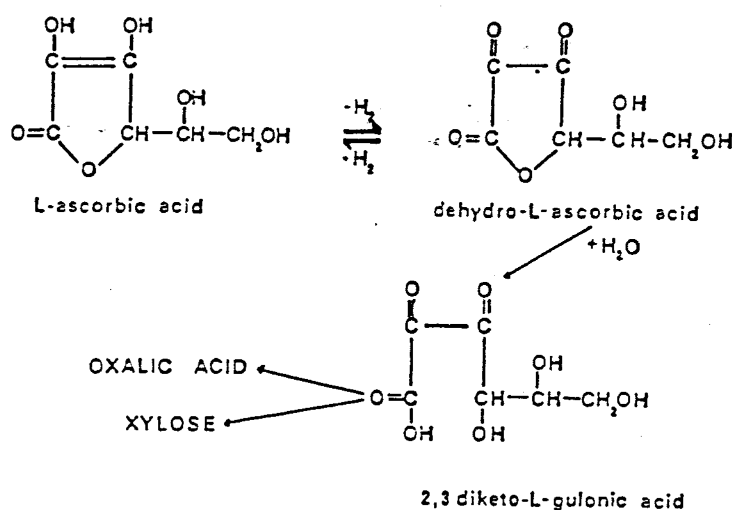


Figure 3 : Vitamin C metabolism in humans (37).

1.2.3 FUNCTIONS OF ASCORBIC ACID

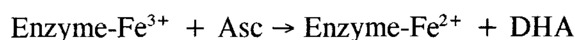
Asc is of enormous biological importance. Functions include involvement in enzyme reactions, synthesis of hormones, neurotransmitters, collagen, carnitine, detoxification of exogenous compounds and cytochrome P450 activity. In addition, it plays a major role as an antioxidant and free radical scavenger, protecting cells against lipid peroxidation. Furthermore, it has been shown to function in reconstituting vitamin E for protection of lipid membranes (38).

Uncertainty exists regarding the actual concentrations of intracellular Asc but it has been estimated to be as high as several millimolar (39). In man the highest concentration of Asc is located in the adrenals, ovaries, brain, pituitary gland, liver, spleen, blood cells and extracellular fluid surrounding the lung and eye (33). In this thesis, discussions of functions or suggested functions will be limited to the areas of relevance to cancer.

1.2.3.1 Ascorbic acid in enzyme reactions

Reactions primarily involving Asc are hyroxylations requiring molecular oxygen (see Figure 4). These reactions usually involve Fe^{2+} or Cu^{2+} as a cofactor (29). Asc is known to stimulate numerous dioxygenases that contain prosthetic Fe^{2+} and monooxygenases with prosthetic Cu^{2+}. Although other reducing agents may replace Asc, at least to some extent, Asc shows the greatest stimulation of these activities.

Asc is believed to play one of two roles: (i) as a source of electrons for reduction of O_2; *i.e.* as a cosubstrate (see Figure 4), or (ii) as a protective agent, in that it provides electrons to keep prosthetic metal ions in the reduced state (29,40). These enzymatic reactions are important to many biochemical processes, for example hydroxylation reactions.



$$\text{Enzyme-Fe}^{3+} + \text{Asc} \rightarrow \text{Enzyme-Fe}^{2+} + \text{DHA}$$

Figure 4: The suggested role of ascorbic acid as a cosubstrate in an enzyme reaction (40).

1.2.3.2. Ascorbic acid in the extracellular and intracellular matrix and collagen biosynthesis

Most cells in the body are firmly embedded in a highly viscous ground substance. The ubiquitous ground material infiltrates every interspace and sequesters every stationary cell from its neighbours. It forms the immediate contact environment of all cells and it must be traversed by molecules entering or leaving the cell. Evidence suggests that the interface between a cell membrane and its immediate extracellular environment is the vital factor in the whole proliferative process (31,41). Variations in the composition of the extracellular environment exert a significant effect on cell behaviour, hence the cells obtain a powerful means of modifying their immediate surroundings. Thus a proliferating cell and its contact environment comprise a balanced scheme in which each component influences the other; this synergism is involved in all forms of cell division and is of particular importance in cancer. Until recently, emphasis on cancer research tended more towards the cell, neglecting the other half of the proliferation equation (31,41).

The ground substance of the intercellular matrix is a complex aqueous gel containing electrolytes, metabolites, dissolved gases, trace elements, vitamins, enzymes, carbohydrates, fats and proteins. The characteristic viscous property is dependent on the abundance of specific long chain acid peptidoglycan polymers, the glycoaminoglycans (GAGS) and the proteoglycans. This network of high-molecular weight polymers form the structurally stable hydrophilic mesh, which in turn is fortified at the microscopic level by a 3-dimensional web of collagen fibres (31,41). The single-stranded long chain polymers comprising GAGs have molecular weights ranging from 10^1 - 10^6. Hyaluronic acid (comprising repeating molecular units of N-acetylglucosamine and glucuronic acid), chondroitin (built up from N-acetylgalactosamine and glucuronic acid), and sulphate esters

(chondroitin sulphate) are the standard forms. Proteoglycans are macromolecules of a more complex multibranched structure. Present evidence (41) suggests a primary GAG core exists, to which secondary protein cores are attached at spaced intervals by link proteins.

A notable property of the intercellular substance is its very high viscosity and cohesiveness, which in turn is dependent upon the chemical integrity of the large molecules. The structural integrity and viscosity can be eliminated by the hydrolyzing action of certain related enzymes known by the generic name hyaluronidase. Most cells in the body are able to synthesize hyaluronidase. The network of the intercellular ground substance is in dynamic equilibrium with GAG and its breakdown products *via* the catalytic action of hyaluronidase and subsequent excretion (41). Claude Bernard (cited in 41) states that it is in this slowly changing environment that all cellular activity takes place. Depolymerization of matrix proteoglycans is initiated by the combined action of the glycosidases and neutral proteases. Both of these are believed to be released simultaneously from lysosomes during cell division. It now seems reasonable to suggest that continuous release of these hydrolytic enzymes from neoplastic cells is responsible for their invasive capability, for the selective routing by increased diffusion of nutrients towards the tumour cell, and perhaps for even sustaining the whole momentum of autonomous neoplastic proliferation (31).

The collagen component of the extracellular matrix is also vulnerable to the same hydrolytic enzyme activity. Collagen synthesis is a complex process of protein synthesis, postranslational modifications, protein secretion and extracellular matrix formation. Collagen is a unique animal protein: glycine comprises up to one third of its amino acid residues with an abundance of proline or 4-hydroxyproline and a few of 3 hydroxyproline and hydroxylysine residues (27,29,31,40). Hydroxylation of prolyl and lysyl residues in collagen is catalyzed by the respective hydroxylases. The enzyme prolyl 4-hydroxylase requires ferrous ion, α-ketoglutarate, oxygen, correct substrate peptide and Asc for maximum activity (27,29,31,40).

The exact role of Asc in collagen synthesis has been the focus of numerous studies. Lack of Asc dramatically reduces hydroxylation of prolyl and lysyl residues into hydroxyproline and hydroxylysine of mature collagen during ribosomal assembly, leading to the instability of the triple helix of collagen. This instability results in increased collagen catabolism which is demonstrated in scurvy and in cancer (27,29,31,42) in a variety of cell types. Addition of Asc has brought about increased transcription, translation, and stability of mRNA for procollagen. Assuming cell proliferation is reliant on the depolymerization of the ground substance by cellular hyaluronidase, it can be seen that there

are two methods of exerting some therapeutic control of cancer and other disease states in which extreme cellular proliferation is a harmful feature. An attempt to strengthen the ground substance is one such way and the other is to directly neutralize the cellular hyaluronidase by limiting or inhibiting its action (41). There is strong evidence (41,43) to suggest that Asc is in some way involved in the synthesis of a physiological hyaluronidase inhibitor (PHI). Thus if the elementary theory of cellular proliferation is correct, hyaluronidase catalyzes the hydrolysis of the GAG in the immediate environment allowing cells freedom to divide and migrate. PHI might then prove to be a valuable therapeutic agent in controlling numerous forms of excessive proliferation, including cancer. It may not be necessary to synthesize PHI, but rather it may be possible that the body, given enough Asc, could synthesize a sufficient quantity of PHI (41). Thus in summary: in cancer, to obtain sufficient inhibition of cell migration, an excess of Asc maybe essential and is one factor open to therapeutic intervention.

1.2.3.3 Ascorbic acid and nitrosamines

N-Nitroso compounds are known to induce a wide variety of tumours in most species of animals. This suggests that N-nitroso compounds are important in human carcinogenesis. Such nitrosamines are thought to come from exogenous and endogenous sources (29,31,44-46).

Asc is a potent inhibitor on N-nitrosamine formation (7,47), the precursors being nitrates and nitrites and secondary or tertiary amines (47). Nitrites are present in water and foods, particularly vegetables, in addition to cured meat products and cheese. Bacteria in food and in saliva convert the nitrates from these sources to nitrite, which is the important nitrosating agent. On the other hand, nitrogen oxides produced in flame ovens, cigarette smoke and other atmospheric pollution can act as nitrosating agents (44). Nitrosamines are formed by reaction of nitrite (NO_2) with amines under conditions of low pH (pH 2-4) as is present in the stomach (29,31,46). A number of the cancer cases in humans may be attributed to the action of these agents.

In cancer cells Asc, has the ability to react with the nitrosating agents, converting them to products which do not nitrosate. The reactions involve nitrous oxide (NO) with either Asc or its anion (DHA) therefore competing with amine-nitrosation reactions (29). Dietary supplementation with Asc was also been carried out to determine its effectiveness in inhibiting cancer by these preformed chemicals. Studies (cited in 46) showed protective effects of vitamin C against skin, respiratory tract, and kidney cancer; either inhibition, enhancement or no effect in the mammary gland and colon cancer, and no

effect of Asc supplementation in the urinary bladder.

1.2.3.4 H_2O_2 formation by and antioxidant role of ascorbic acid

It is suggested in the literature that Asc can generate hydrogen peroxide (H_2O_2) upon oxidation by molecular O_2 in biological systems (36,48). Asc oxidation involves single electron transfer reactions, which result in the formation of H_2O_2 (49).

This highly reactive H_2O_2 is termed a reactive molecule and is capable of causing cellular damage. The sites of formation of this molecule include all cellular constituents in addition to the cytosol. Deleterious effects caused by these molecules involve H_2O_2 reactions with polyunsaturated fatty acid (PUFA) intracellular membranes, nucleotides in DNA and critical sulfhydryl bonds in proteins (50).

Peterkofsky *et al* (51) proposed that the antitumour effect of Asc was mediated by H_2O_2, as the latter was found to be toxic to fibroblast cultures. Benade *et al* (48) found Asc to be toxic to Ehrlich ascites carcinoma cells *in vitro* and proposed that the toxicity is linked to H_2O_2 formation. It has been suggested that the toxicity of Asc to tumour cells may be due to low catalase levels in tumour cells. The catalase enzyme is responsible for the detoxification of H_2O_2 within cells (52,53). Asc inhibits catalase activity, hence supplementation of tumour cells with Asc will result in the decreased detoxification of H_2O_2 by catalase ultimately resulting in an accumulation of H_2O_2 to cause cell death (53).

In contrast, Asc is an excellent water-soluble antioxidant and free radical scavenging nutrient protecting cells from destruction by oxidants (33,40,54-57). Free radicals, including hydroxy, hypochlorite, peroxy, alkoxy, superoxide, hydrogen peroxide, and singlet oxygen are generated by autooxidation, radiation, or from the activities of oxidases, dehydrogenases and peroxidases. Vitamin C is known to act as an antioxidant both *in vitro* and *in vivo* and its function as a free radical scavenger enables vitamin C to become the cells primary defence mechanism against oxidative damage. Increasing evidence implies the involvement of free radicals and oxygen species in numerous pathological events, cancer and ageing (56).

1.2.4 ASCORBIC ACID AND CANCER

As already mentioned, some micronutrients are known to possess anticarcinogenic properties. Vitamin

C has been suggested (5,6,11,16,22,26,29,39,40,43-45,48,49,58-62) and disputed (52,63-69) as an anticancer agent. The antitumour activity of Asc is reported to be due the its chemical properties, and not due to its metabolism (70).

A reduced status of Asc in serum and plasma of cancer patients and tumour-bearing animals have been reported in several studies (43,71). The first large-scale trial of Asc supplementation in human cancer therapy was reported by Cameron and Pauling (72) in which 22% of the Asc-treated patients were alive compared with only 0.4% of the control group after one year of study. Cameron (73) has since published a protocol for the use of vitamin C in the treatment of cancer, emphasing continuous use of vitamin C as opposed to intermittent administration. In contrast, Campbell and Jack (74) cautioned the use of mega Asc therapy in cancer treatment.

Immunological studies have reported that Asc can induce immunity against some cancers in mice. In addition to the immunizing actions are changes in the surface structure of the cancer cells (75). Reversion by Asc of transformed cells to normal morphology has been proposed (76-78). These studies suggest that low concentrations of Asc in C3H/10T½/CL8 cells can be effective in suppressing oncogenic progression only prior to a stage where an initiated cell achieves the capacity to grow in semi-solid medium and to produce tumours in immunosuppressed animals (76,77).

Epidemiological evidence links increased intake of fruits and vegetables with decreased risk of most cancer types (7). Of 170 epidemiological studies (cited in 79) of cancer at all sites, 132 exhibited a statistically significant protective effect associated with the highest intakes of fruits and vegetables. Individuals with low fruit/vegetable intake (approximately 20-30% of the population) have cancer rates at least twice that of individuals with high intakes. This is suggestive of an important role for vitamin C in cancer prevention. In 90 epidemiological studies by Block (38) in which he examined the role of vitamin C or vitamin C rich foods in cancer prevention, the vast majority showed statistically significant protective effects. This evidence is particularly strong for the cancers of the oesophagus, oral cavity, stomach and pancreas, while other studies have suggested protective effects in cancers of the cervix, rectum and breast. Graham *et al* (cited in 80) suggest a protective role for Asc in laryngeal cancer. Although there are encouraging data on the anticarcinogenic effects of Asc, higher than recommended levels have been shown to produce adverse effects under some conditions (80).

1.3 VITAMIN E

1.3.1 HISTORY

Studies by Osborne and Mendel and by Mattil and Conklin in 1922 (cited in 81) demonstrated that laboratory rats fed a semi-purified diet providing an abundance of the vitamins A, B, C and D exhibited good growth and stamina but often failed to support reproduction. At this time Evans and his coworker Bishop (81) undertook studies in which it was reported that reproduction failed in pregnant females fed diets containing all of the then known nutrients. Although oestrus, mating and every recognizable phase of the beginning of a normal pregnancy had resulted, the foetuses soon died and were resorbed. It was against this background that Evans and Bishop (82) demonstrated the evidence of an unknown dietary factor, initially named factor X. Wheat germ, lettuce and dried alfalfa leaves were recognized as sources of this fat soluble factor (81-83). Single daily drops of wheat germ oil completely prevented this gestation resorption, whereas rich sources of vitamins A, D and cod liver oil not only failed to prevent resorption but also increased the severity of the defect. Barnett Sure (cited in 82), in 1924, confirmed Evans's finding in independent studies, concluding that this fat soluble reproductive factor was a new vitamin. In 1925, Evans adopted the letter E as the next serial alphabetical designation. Thus the first five years of vitamin E history was that of its recognition solely as a dietary factor necessary for fertility in the male and female rat.

During the next decade or two fruitless attempts were made to acquire evidence of practical applications of this discovery in terms of reproductive failures in vetinary and clinical medicine. In 1931 Adamstone (83) recorded mortality in vitamin E deficient chick embryos, while Pappenheimer and Goettsch described vitamin E deficient encephalomalacia in the chick and nutritional muscular dystrophy in guinea pigs and rabbits, hence confirming Evans and Burr's idea (1928) that vitamin E was not implicated only in anti-sterility (81,83).

Vitamin E research had been severely impeded by the lack of a more potent source of the vitamin than wheat germ oil, and by dependence upon time-consuming and incompletely reliable bioassays for determining the vitamin E content of plants and animals. Evans *et al* (82-84) at Berkeley isolated an active substance from wheat germ oil and found it to have very high vitamin E activity.Fernholz (cited in 82) in 1938 undertook thermal degradation of the vitamin, demonstrating that it contained a phytyl chain and a hydroquinone moiety, and suggested a structure of the vitamin later proven to be correct. The structure of vitamin E was subsequently determined and given the name tocopherol.

1.3.2 STRUCTURE AND METABOLISM OF VITAMIN E

Vitamin E refers to a family of fat soluble phenolic compounds called tocopherols (85,86). Eight forms of vitamin E are known to exist in nature: α-, β-, γ-, and δ- tocopherols contain saturated phytol side chains, while α-, β-, γ-, and δ- tocotrienols possess three double bonds in the side chain (82,85-87), δ-tocotrienol is the first member of the group to be formed by biosynthetic processes in plants, after which methylation occurs leading to the consecutive formation of λ-, β-, and α-trienols and hydrogenation of these produce the respective tocopherols. The tocopherols possess three asymmetrical carbon atoms located at C-2, C-4' and C-8' (82) (See Figure 5).

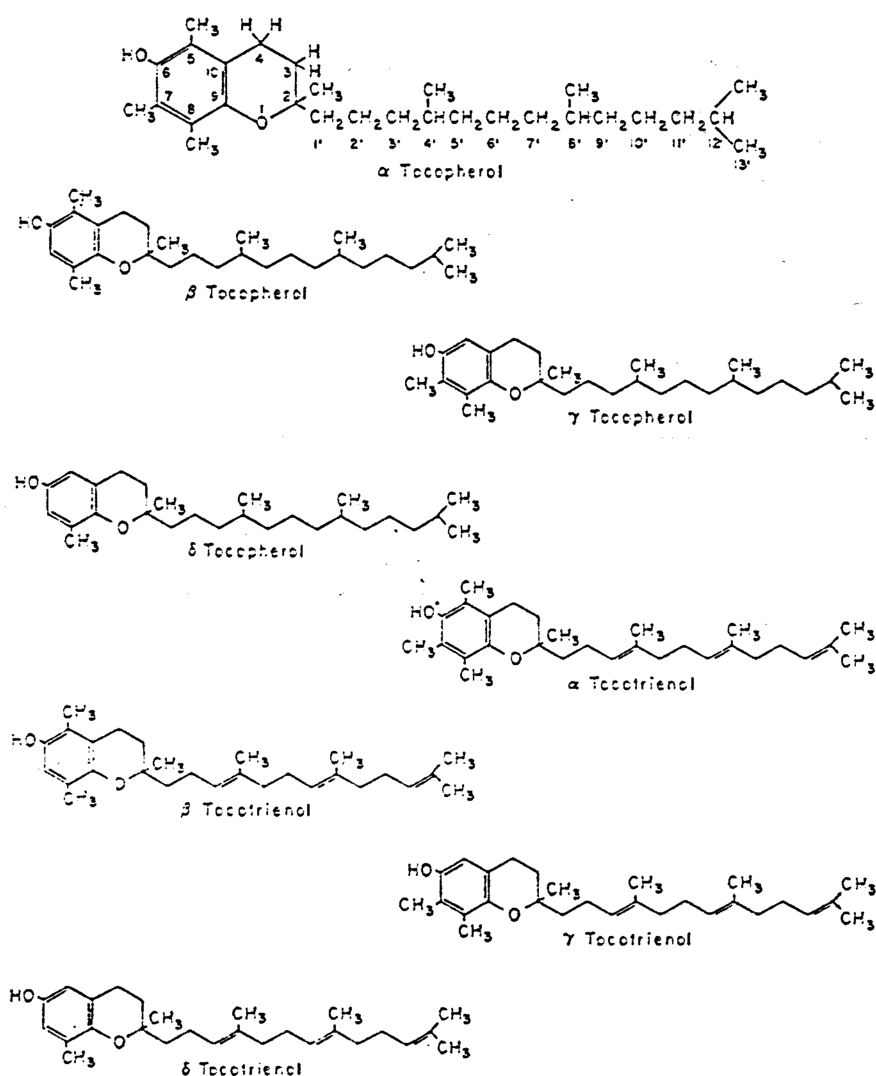


Figure 5: Chemical structures of naturally occurring tocopherols and tocotrienols (82).

The most biologically active form of vitamin E is α-tocopherol (α-TOH) (82,85,86,88-90). α-TOH (see Figure 5) is a 6-hydroxychroman derivative with methyl groups in positions 2,5,7 and 8 and a 16-carbon aliphatic side chain attached at carbon 2. The phytyl side chain at position 2 facilitates the incorporation and retention of the α-TOH molecule in biological membranes, so that the biologically active site of the molecule in the 6-hydroxyl position is optimal (85,86,90).

Two primary sources of vitamin E in commercial use are d-α-tocopherol and dl-α-tocopherol, in addition to the acetate and succinate esters of these compounds. The acetate esters are prepared commercially by reaction of the alcohol forms with acetic acid and they do not exist in nature (82).

Vitamin E is a potent anticancer agent in terms of both cancer prevention and regression. This has been verified in various animal experiments and cell culture studies (49). Of the various forms of vitamin E tested for antiproliferative effects on tumour cells - namely D-α-tocopherol nicotinate, DL-α-tocopherol free alcohol, Aquasol DL-α-tocopherol acetate and D-α-tocopherol succinate, the succinate form of natural D-α-tocopherol (see figure 6) (1), has been shown to be the most effective form *in vitro* (49,91-98). Although the precise reasons for this are unclear, the following possibilities have been suggested: (a) vitamin E succinate may be marginally more soluble and stable in growth medium or solution; (b) it may easily cross the cell membrane, and (c) it may be converted to α-TOH more slowly, so that the intracellular level of α-TOH remains high for a longer period of time (49).

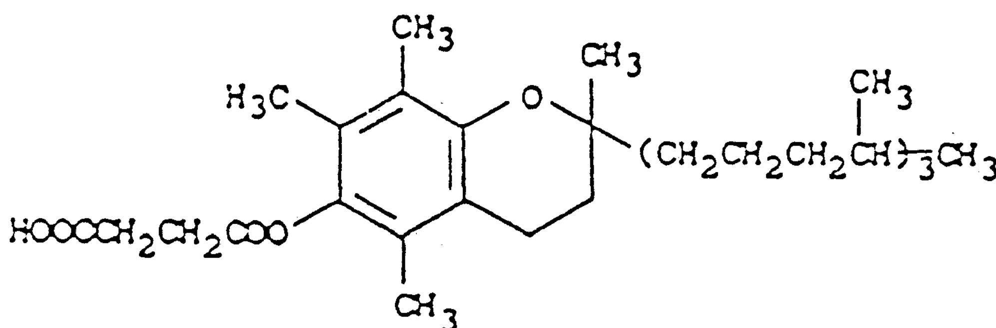


Figure 6: α-Tocopherol acid succinate (1).

Vitamin E is an essential nutrient for man as it cannot be synthesized *de novo* and is therefore obtained from the diet (99). The required dosage for most vitamins is based on the level at which deficiency becomes clinically evident. The minimum nutritional requirement for adults is in the range 5-13mg of d-α-TOH or its equivalent (99-102). The average intake of a balanced adult diet ranges from 7 to 9mg, and the RDA is 8-10mg per day (99,102). Vitamin E is a fat-soluble vitamin found in the diet primarily in vegetable oils, cereal grains, green plants, egg yolk, milk fat, liver, nuts and vegetables (100-102). TOHs occur as free phenols in most food sources, however α-TOH esters are widely used in pharmaceutical formulations and dietary supplements because the esters are much more resilient to oxidation (81,102,103).

The mechanism by which vitamin E is absorbed and transported in plasma is well understood, but its intracellular transport is not well recognized. Due to its hydrophobicity, vitamin E requires a special transport system in aqueous environments of the plasma, extracellular space and cell cytoplasm. Unlike other vitamins, vitamin E does not have a special carrier protein in plasma but is transported in plasma by lipoproteins such as high density lipoprotein (HDL), low density lipoprotein (LDL), and very low density lipoprotein (VLDL) (104,105). Dietary studies have indicated that no discrimination between the homologues in the intestine occurs, as TOH is absorbed from the gut in micelles in which the formation depends on bile salts and pancreatic lipase (86,104-107). Absorption of TOH occurs mainly in the upper and middle thirds of the small intestine of animals and is enhanced by medium chain triglycerides but inhibited by long chain polyunsaturated fatty acids (PUFAs). Micelles containing TOH, free fatty acids, monoglycerides and other fat soluble vitamins passively diffuse through the brush border (82,104). Transfer of micelles from the cell thus requires several stages. In mammals, it must proceed through the lateral or basal plasma membrane of the cell and through the basal lamina and then enters the fluid of the lamina propria. It is from here that the vitamin enters the capillaries of the lymph and is transported in the intestinally-derived chylomicrons which in turn are swiftly catabolized in the circulation by lipoprotein lipase bound to the endothelial wall (107). FAs and TOH are transferred to tissues during lipoprotein lipase-catalyzed hydrolysis of triacylglycerols to free FAs and monoglycerols. Resulting chylomicron components, including apolipoproteinic cholesterol, phospholipids and TOHs are delivered to plasma HDLs, while apolipoprotein E is transferred in the reverse reaction, *i.e.* from HDL to the chylomicrons. The binding of apolipoprotein E to chylomicron remnants leads to a rapid clearance of the chylomicron remnant particles from the plasma by the liver via specific apolipoprotein E receptors (108).

Studies by Behrens and Madère (109) demonstrated the involvement of a low molecular weight (about 32 KDa) TOH-binding protein (TBP) which was extremely specific for the α-form of the vitamin. The cytosolic liver TBP may serve as an intracellular transfer protein (cited in 108) shuttling α-TOH between subcellular components particularly to proximal VLDL. In addition, evidence suggests (cited in 108) that the liver TBP may play a part in maintaining plasma α-TOH levels and controlling excretion of vitamin E via the bile. How vitamin E is returned from the tissues to the circulation for reprocessing and recirculation or removal by liver, still remains to be answered (104,108). Vitamin E is secreted from the liver into the plasma in triacylglycerol-rich VLDLs. Hydrolysis of VLDLs by lipoprotein lipase delivers FAs and vitamin E to tissues and yields LDL. LDL carries the major portion of plasma vitamin E and appears to exchange vitamin E readily with HDL, providing a further means for the delivery of vitamin E to tissues via the LDL receptor-mediated uptake pathway (108).

1.3.3 FUNCTIONS OF VITAMIN E

Functions to be considered are those relevant to cancer. Cancer is believed to be a consequence of external factors combined with a hereditary predisposition for cancer. Increased cancer incidence with the onset of age may be due in part to the increased level of free radical reactions with age, together with the dwindling ability of the immune system to eliminate altered cells. Several functions of vitamin E are relevant in considering its role in cancer (110). In a addition to enhancing the humoural and cellular immune responses (111) vitamin E's reactivity with free radicals is considered to be its major biochemical function (88,110,112,113). Furthermore vitamin E is also known to inhibit the conversion of nitrites to nitrosamines in the body.

1.3.3.1 Vitamin E and nitrosamines

Vitamin E shares with vitamin C the ability to inhibit nitrosamine formation in the stomach (18,25, 45,76,102,110,113,114). Vitamin E also acts in the lipid membrane where nitrosation occurs more readily. α-Tocopherol prevents nitrosamine formation *in vitro* by destroying nitrite anions and *in vivo* by preventing the nitrosative cleavage of the amine to a nitrosamine (25,76,115). Since 80% of nitrosamines are carcinogenic, the prevention of nitrosamine formation has potential significance for the prevention of cancer.

1.3.3.2 Vitamin E and p53

p53 is a gene that has received vast attention as a tumour suppressor gene since its discovery in 1979 (116-120). p53 encodes a 53 KDa protein that arrests the growth of cells containing damaged DNA, thereby helping to conserve genetic stability. It functions as a transcription factor with high affinity for specific DNA target sequences, and in response to DNA damage, p53 up regulates the expression of target genes such as the gene encoding p21, which in turn blocks cell proliferation in G1 of the cell cycle or functions as an essential trigger of apoptosis (117,118). The p53 protein is composed of 393 residues and is divided into three structural domains. These three structural domains are known as the amino-terminal trans-activation domain, the central "core" domain and finally the carboxyl terminus (117). This "wild type " of p53 may be converted by mutation to a "mutant type" of p53 that can act as an oncogene, perhaps alone or together with other oncogenes. Mutant forms not only deprive cells of the "wild types" beneficial effects in cells but can spur abnormal cell growth. The "mutant type" of p53 is expressed in various forms of malignant tumours including carcinomas of breast, lung, colon, stomach, oral mucous membrane and osteogenic sarcoma (116,119). There is evidence (117) to suggest that mutations in the central core domain are linked with cancer. Such cancers are particularly difficult to treat, since mutant p53 confers resistance (117) to radiation and chemotherapy. Tumours containing abnormal forms of the p53 protein are the most aggressive and are particularly prone to spread and cause death quickly. Numerous pharmaceutical companies already have teams working on cancer therapies that target p53.

Since vitamin E has potent activity in cancer inhibition *in vitro* and since the concept of cancer suppressor genes has been established, Schwartz *et al* (116) undertook an experiment using the hamster buccal pouch model to seek the mechanism of vitamin E's anticancer action in the stimulation of a cancer suppressor gene or inhibition of related oncogenes. They found stimulation of "wild type" p53 by vitamin E and decreased "mutant" p53 expression by vitamin E. They propose three suggestions for vitamin E anticancer action: stimulation of a cancer repressor gene; prevention of p53 mutation to oncogenic forms by promoting DNA repair and by preventing mutation of other proto-oncogenes which may function together with "mutant" p53 (116).

1.3.3.3 Vitamin E and membranes

Mammalian cell membranes consist of a lipid bilayer primarily of phospholipids and cholesterol. Proteins having vital cellular functions, such as receptors, transporters and enzymes, are embedded

in the lipid bilayer (121). The oxidation of PUFAs associated with membranes can result in the disruption of numerous cellular functions and cause leakage of intracellular contents. This process, termed lipid peroxidation, is believed to be a major factor in causing numerous toxicities. PUFAs susceptibility to lipid peroxidation is counterbalanced in biological reactions by numerous endogenous antioxidant enzyme systems and antioxidants (122,123).Vitamin E is a potent chain breaking antioxidant which inhibits lipid peroxidation in membranes (84,88,107,108,122,124-128). Since α-TOH is required for protection of mitochondrial and microsomal membranes even when the diet contains enough synthetic antioxidant, it is clear that d-α-TOH appears to have the advantage over other TOHs and organic antioxidants by having the best access and longest retention in the tissues (82). Lucy (125) suggested that vitamin E stabilizes membranes by virtue of specific physicochemical interactions between its phytyl side chain and the fatty acyl chains of polyunsaturated phospholipids particularly derived from arachidonic acid. Lucy and Diplock (127) proposed that the interaction of α-TOH with phospholipids containing arachidonyl residues comprises two reactions. The methyl group at C4' of α-TOH can fit into a pocket provided by the *cis* double bond nearest the carboxyl group. The methyl group at C8' of α-TOH is then aligned and can interact similarly with the third *cis* double bond. Thus in the complex formed, the hydroxyl group of the chromanol ring of α-TOH and the polar groups of the phospholipid all lie at the same end of the complex where they are thought to participate in polar interactions at the membrane surface (82,127). Therefore membranes lacking vitamin E would be expected to be less stable. In addition, according to Lucy and Diplock (125,127), vitamin E in the membrane would reduce membrane permeability and vitamin E deficiency would increase permeability. Scott *et al* (cited in 129) agree with the postulations of Lucy (127) who suggests that the formation of a vitamin E-arachidonate complex in the lipid membranes may have three functional consequences: (i) inhibition of peroxidative destruction of PUFAs in cells and subcellular membranes, (ii) prevention of permeability in biological membranes containing relatively high levels of PUFAs, and (iii) possible prevention of degradation of the membrane phospholipids by membrane-bound phospholipases (82).

1.3.3.4 Antioxidant role of vitamin E

Free radical-mediated lipid peroxidation has been suggested to be critically involved in numerous toxicities, and post ischemic reoxygenation injury, in addition to the degenerative processes associated with ageing (110,130,131). The close relationship between free radical activity and malignancy has also been well documented (132). The hypothesis appears to be that free radical damage of cellular materials would result in triggering or transforming non-malignant cells to malignant ones (133). An

increase in the activated forms of oxygen in the cell due to overproduction or/and the inability to destroy them may lead to severe damage of cell molecules and structures (130). It is well known that vitamin E (α-, β-, γ- and δ tocopherol) acts as an antioxidant in protecting PUFAs and other lipids from peroxidation (134). α-TOH is chemically and biologically the most active lipid-soluble, phenolic antioxidant present in mammalian tissue (135-140). Dam (cited in 141) suggested that the principal role of vitamin E as an antioxidant may be to neutralize free radicals which could initiate a chain reaction, largely in highly unsaturated lipids, bringing about the formation of peroxides and products of their subsequent degradation. Studies have shown α-TOH to be a potent inhibitor of the propagation step of lipid peroxidation (138,142). The antioxidant properties of TOHs have been ascribed to hydrogen transfer from the OH group in the TOH (138). Each TOH molecule can react with two peroxyl radicals (LOO·) (135,136,143), as shown in Figure 7.

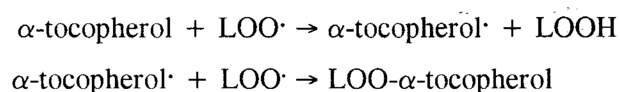


Figure 7 : α-Tocopherol inhibition of phospholipids (135).

The first product is the α-tocopheroxyl radical (α-tocopherol·), which is a resonance-stabilized, oxygen-centred radical. The α-tocopheroxyl radical can react with another peroxyl radical to form a stable adduct (136). From the high affinity of α-TOH for peroxyl radicals it would appear that the structure of the head has been optimized to obtain maximum antioxidant effectiveness, while the phytyl chain gives the compound its lipophilicity and solubility in biomembranes (139).

Though it is a major, if not the only chain-breaking antioxidant in mitochondrial membranes, vitamin E is present in extremely low concentrations, normally less than 0.1nmol per mg of membrane protein, in other words one molecule per 1000 to 2000 membrane phospholipid molecules. On the other hand, lipid peroxyls can be generated in membranes at a rate of 1 to 5nmol per mg of membrane protein per minute. However, destructive oxidation of membranes rarely occurs, nor is vitamin E rapidly depleted (84). An exceptionally efficient mechanism for regenerating or recycling vitamin E must exist in order to sustain such small but effective concentrations. Recycling of vitamin E by enzymatic and non-enzymatic means have been demonstrated, with vitamin C being implicated in the recycling of vitamin E (14,49,84,143,144).

Since reactive oxygen species have been implicated in cancer initiation and promotion, evidence

(144) suggests that vitamin E and other antioxidants alter cancer incidence and growth by functioning as anticarcinogens, quenching free radicals or reacting with their products.

1.3.4 VITAMIN E AND CANCER

Vitamin E has been suggested (145-160) and disputed (58,161-164) as an anticancer agent. The antitumour activity of vitamin E has been reported to be due to its chemical properties and not to its metabolism (135-137).

A reduced status of vitamin E in serum and plasma of cancer patients and tumour-bearing animals has been reported in several studies (113,130,145,146,151,155), suggesting that a deficiency in vitamin E could ultimately lead to diseased states. Immunological studies have reported that vitamin E can induce immunity against infection (20,111,112,158,159). In addition to its immunizing actions, vitamin E can induce morphological alterations and growth inhibition in melanoma cells (94), mouse and human neuroblastoma cells, and rat glioma cells in culture (92). These studies suggest that vitamin E can induce multiple effects during the management of tumours including cell death, differentiation, inhibition of cell division, reduction of the toxic effects of certain chemotherapeutic agents and stimulation of the hosts immune system (94). These effects of vitamin E are dependent upon the type of cancer and the type of therapeutic agent.

Influences of vitamin E in cancer, using epidemiological data, are difficult to assess due to a number of factors:
i. The vitamin is present in a wide variety of food items in low quantities and it becomes difficult to group individuals according to their level of intake. Moreover, not all dietary vitamin E consumed is available for absorption and storage and this again varies among individuals.
ii. The vitamin is relatively unstable during storage and can vary greatly within any individual foodstuff.
iii. No specific and clear-cut vitamin E deficiency has been established.

Hence, epidemiological data on the association of vitamin E and cancer risk are not vast, and those that are available are inconsistent. In 1984 two groups (cited in 160) reported an association between serum vitamin E levels in women and their risk of developing breast cancer. While one reported that low serum vitamin E was related to a significantly high risk of breast cancer, the other failed to note such an association. Menkes et al (155) also reported a significant association between the vitamin

E level and risk of lung cancer, while Norma (161) assessed the serum of 6 800 men and failed to find any correlation between vitamin E and cancers of the colon, lung, stomach, rectum and urinary bladder. Xuan *et al* (cited in 159) suggested after studies done on miners at high risk of lung cancer that vitamin E in combination with other nutrients could be used for chemoprevention. Packer (110) also indicated that adequate intake could provide protection against diseases including cancer caused by pollutants and lifestyle patterns. Knept (113) also suggests that vitamin E may have anticancer properties as a lipid antioxidant and free radical scavenger, thus supporting the hypothesis that dietary vitamin E is associated with decreased cancer risk in man.

Since the combined supplementation of vitamin E succinate and Asc are to be examined in this thesis, it is important to consider their synergistic action *in vivo* and *in vitro*.

1.4 SYNERGISTIC ACTION OF VITAMIN E AND VITAMIN C

In 1941, Goulmbic and Matill (29), demonstrated that although vitamin C alone was not effective in preventing the autooxidation of fat *in vitro*, it could, in the presence of vitamin E, greatly extend the inhibition period produced by vitamin E alone. In 1968, Tappel (cited in 162), suggested that Asc may reduce the tocopheroxyl radical back to the starting TOH. Subsequent *in vitro* (29,163-172) and *in vivo* (173) experiments demonstrated that vitamin C can indeed reduce the α-tocopheroxyl radical (Figure 8).

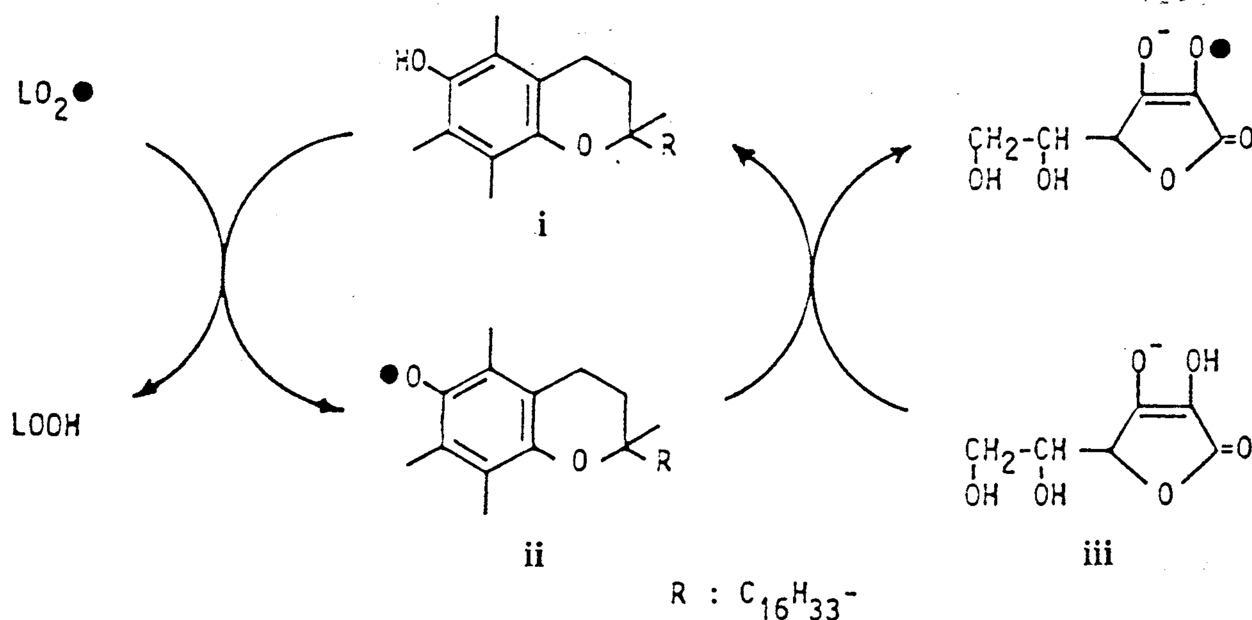


Figure 8 : Regeneration of vitamin E from vitamin E radical by vitamin C (18).

The sparing action of vitamin E by vitamin C is attributed to the reduction of the vitamin E radical by vitamin C to regenerate vitamin E. As depicted in Figure 8, vitamin E (i) scavengers the lipid peroxyl radical ($LO_2\cdot$) to halt the chain propagation and the resulting vitamin E radical (ii) is reduced by Asc (iii) to regenerate vitamin E (18). This suggests that vitamin C represents a pool of antioxidant potential which could be delivered to peroxidized membranes through the regeneration of lipid-soluble vitamin E (103,143,167,168). The delayed consumption of vitamin E is known as the "sparing effect" (exerted by vitamin C) and is defined as the ability of one antioxidant to slow the depletion of another (103). Although vitamin E and C have been proposed to act synergistically, the question that remains is how a lipid-soluble membrane-bound antioxidant such as vitamin E can interact with a water-soluble cytosolic bound antioxidant such as vitamin C. It has been shown (174) that in a membrane system, the phenolic group of the vitamin E is located near the polar heads of the phospholipids that comprise the lipid layer, and the phytyl chain of vitamin E lies parallel to the phospholipid fatty acyl chains. During lipid peroxidation, resulting lipid peroxyl chains create a significant dipole within the membrane that allows the peroxyl radical moiety on the lipid chain to partition to the membrane-water interface. This facilitates repair of the lipid peroxyl radical by vitamin E and results in the production of a stable vitamin E radical at the membrane-water interface. This in turn allows water-soluble vitamin C access to the membrane-bound vitamin E radical for recycling (174).

1.4.1 VITAMIN E AND C AND CANCER

As indicated there is some uncertaintity about the role of vitamin E and C in carcinogenesis and a possible synergistic effect of the two vitamins in regulating tumour cell growth has not been investigated to date. A possible effect of Asc and α-TOH on tumour cell growth maybe mediated through numerous metabolic pathways which influence cell growth. One such pathway is the arachidonic acid cascade pathway (Figure 9) which involves the cleavage of AA from the sn-2 position of the membrane phospholipid via the action of the enzyme phospholipase A_2 (PLA_2). The conversion of AA to prostaglandins (PGs) and the effect of the latter on adenylate cyclase (AC) activity influences the production of cyclic adenosine monophosphate (cAMP), a known regulator of cell growth. This pathway is also influenced, by exogenous essential fatty acids (EFAs), cyclooxygenase (COX) and a calcium (Ca^{2+}) requirement of a number of the enzyme reactions. Since this study involves an investigation of the influence of Asc and α-TOH on the various AA metabolites in the above mentioned pathway and their relationship and possible association with tumour cell growth, a review of the pathway and its metabolites is necessary.

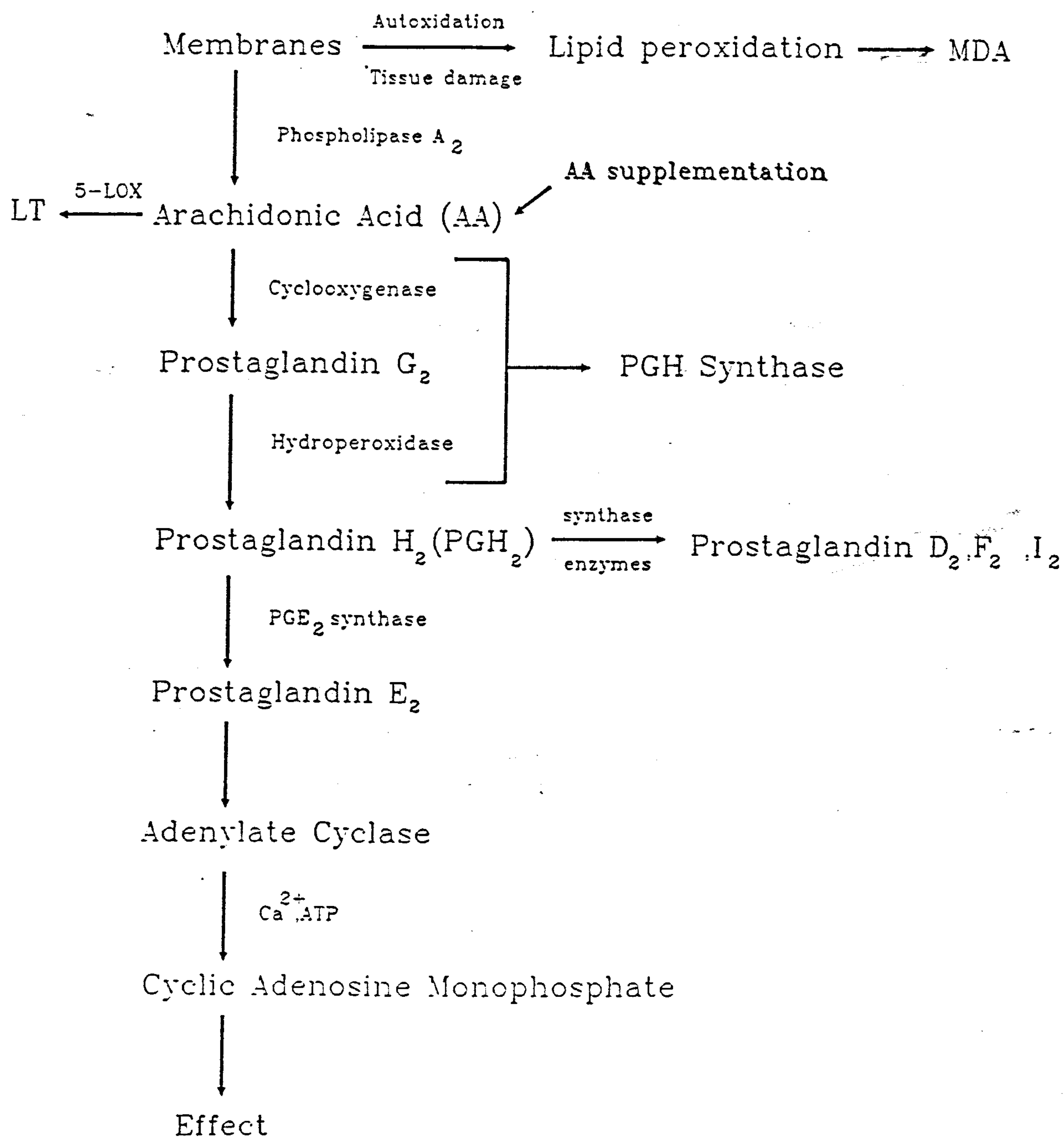


Figure 9 : Schematic representation of the AA cascade pathway investigated in this study.

1.5 CELL MEMBRANES AND FATTY ACIDS

1.5.1 FLUID MOSAIC MODEL OF BIOLOGICAL MEMBRANES

The dynamic state of the lipids in the bilayer of cell membranes was described in 1972 by Singer and Nicolson (121,175-177). They proposed the fluid mosaic model of membrane structure and proposed that this was applicable to most biological membranes.

The accepted model of membrane structure is a dynamic, asymmetric lipid matrix of phospholipds and cholesterol with globular proteins embedded across the membrane to various degrees (178). Most phospholipids are in the bilayer arrangement and may be closely associated with the integral membrane proteins or loosely associated with peripheral proteins, in which the integral proteins are critical to the integrity of membranes (175,178). The peripheral proteins are associated with the membrane surface and hence are easily released from the membrane (179). Several forms of non-covalent and covalent forces are involved in the interaction of membrane protein with membrane lipid (180). Major features of the model include the following:

i. membranes are two dimensional protein solutions in a fluid lipid bilayer phase, in which the "membrane fluidity" refers to the physical state of the central core of fatty acyl chains comprising the bilayer structure. The central core of fatty acyl chains serves the dual purpose of being a solvent for the integral membrane proteins and a permeability barrier (121,175,178,181,182).

ii. a small proportion of membrane lipids interacts specifically with particular membrane proteins in which the association reflects the specific function of the protein (121,175,183-185).

iii. membrane proteins possess unique characteristics that distinguish them from other globular proteins (176). Membrane proteins are free to diffuse in the lipid matrix, however, there appear to be areas of restricted mobility, due to special protein-protein, lipid-protein or lipid-lipid interactions (175,176,180).

Membrane fluidity is influenced by temperature, drugs and nutrition (176,186). Thus biological membranes are changing, dynamic and responsive structures in terms of their constituents. This vast diversity brings about unique differences among similar membrane types of different cells (180).

1.5.2 BIOSYNTHESIS OF FATTY ACIDS

Phospholipids and cholesterol are the main forms of lipid present in cell membranes. Lipids also serve as the metabolic fuel for the cell, with fatty acids being one of the main oxidative substrates (121). Another form of lipid, triacylglycerol, is a storage form of fatty acid within the cell, in which its FA content can be used either for energy or for membrane lipid synthesis (187). Most of these saturated and monosaturated fatty acids are derived from the diet or from *de novo* synthesis by the condensation of acetate units followed by direct oxidative desaturation of the long fatty acids. In contrast, PUFAs can be derived only from dietary lipids since mammalian tissues lack the enzymes capable of synthesizing linoleic acid and α-linoleic acid which are the precursors of all the PUFAs found in animal cells (188).

1.5.2.1 Unsaturated fatty acids

In 1930, Burr and Burr (cited in 189-191) first suggested that some mammalian tissue could not synthesize certain unsaturated FAs and needed to be acquired from the diet. These are termed essential FAs (188,192), in which there are two families of essential FAs, the n-6 (ω 6) and n-3(ω 3) family. The n-6 EFAs have considerably more biological activity, and there is a debate as to whether the n-3 family are in fact essential.

Dietary n-6 EFAs are supplied abundantly in the diet in the form of linoleic acid which in turn is converted to arachidonic acid via desaturase and elongase enzymes (193-196). The available n-6 fatty acids are stored as esters of glycerol and cholesterol and are the *in vivo* precursors for the PGs.

1.5.3 FATTY ACIDS IN THE BIOMEMBRANES

Membrane fluidity now appears to be of general importance for processes ranging from passive permeability and lateral diffusion to cell recognition, differentiation and malignant transformation (181). The FAs esterified to form complex lipids constitute the hydrophobic core of the membrane that provides anchorage for the numerous integral and intrinsic proteins that function as enzymes, transporters, and ion channels. The FA profiles of complex lipids vary with the type of lipid, position within a phospholipid, body organ, organ region, cell type, and in some cases, even to selective domains within the plasma membrane (73). FA components of the membrane lipids could affect membrane function in one of four ways: (i) effects on lipid fluidity (184,186,190,197) (ii); effects

on lipid thickness (184,197); (iii) effects on lipid phase (175,186,197); and (iv) by specific interaction with membrane proteins. Such effects may influence enzymes such as AC which is particularly sensitive to changes in membrane fluidity, hence increased membrane fluidity augments enzyme activity whereas activity is reduced when membranes become more rigid (121,186,198). This is a result of the membrane FAs directly affecting the dynamic properties of a protein through their influences on the bulk physical characteristics of the hydrophobic core of the bilayer (184). Modifications of the phospholipid fatty acyl composition can also effect cellular functions such as PG synthesis and cell growth. Furthermore, the increase in lipid peroxidation that can result from PUFA supplementation may have effects on other cellular processes besides inhibiting cell proliferation as discussed earlier (121).

1.5.4 MEMBRANES AND CANCER

Interest in the physical and chemical properties of cellular membranes in cancer research has been steadily growing (199). Neoplastic transformation leads to dramatic change in the lipid structural organization within tumour membranes (199,200). Tumour plasma membranes generally exhibit decreased fluidity, in that they are structurally changed towards a higher rigidity (199,201). The loss of fluidity is often caused by free radical and lipid peroxidation processes in which the unsaturated bonds are the prime targets (53,202-204). Furthermore, alterations in certain cellular functions including carrier-mediated transport receptor binding, ion channels and eicosanoid production have also been observed (200).

The functioning of the cancer cell is determined in part by peculiarities of its external surface. Cancer cells are unable to bind calcium adequately, and do not adhere to one another tightly, but tend to separate and disseminate to the surrounding tissues. Hence, many of the malignant features of cancer cells may be attributed to the cell surfaces. Studies by Coman and Anderson (205) showed that when comparing the ultrastructure of normal squamous epidermal and Vx2 epidermoid carcinoma cells, vast differences in the ultrastructure were noted. The significance of difference in surface structure to the neoplastic state of the cells remains unknown, but, implies that membranes are credible targets for neoplastic therapy, a possibility that has generally been overlooked (200).

1.6 FREE RADICAL FORMATION AND LIPID PEROXIDATION

Living systems have evolved to survive in the presence of molecular oxygen, and for most biological

systems, life depends on it. Although critical to the maintenance of life, exposure to oxygen is not without risks. It is extremely toxic at concentrations slightly higher than that in air, and this toxicity may be important in several biological processes including cancer. Hence, life in the presence of oxygen involves a delicate balance between the obligatory role of oxygen in respiration and the need to protect biological systems from the detrimental effects of the partially reduced intermediates (206). These intermediates include superoxide, anion, radical, hydrogen peroxide and hydroxyl radical (55,133,136,206-210). Autooxidation is a free radical chain process consisting of chain initiation, propagation, and terminal steps (133,136,211)

A free radical can be defined as a chemical species possessing an unpaired electron, hence it can be referred to as a fragment of a molecule. Free radicals can be formed in three different ways (54,211) (Figure 10).

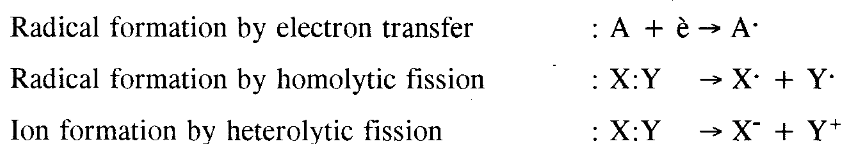


Figure 10 : Three different ways of free radical formation (54).

Electron transfer is by far the most common process in biological systems. Free radicals can be negatively charged, positively charged or electrically neutral (54). Initiation of peroxidation occurs by the attack of a species capable of abstracting hydrogen atoms from fatty acid side chains (212). Species capable of doing this are hydroxyl (OH·), peroxyl (LOO·), alkoxyl (LO·) and alkyl radicals (L·). Hence the first step in the initiation of lipid peroxidation from a PUFA is attack by a free radical species (R·) which is capable of abstracting one of the doubly allylic hydrogen atoms on the carbon atom between two double bonds (See figure 11).

Propagation follows initiation. The product of the radical attack upon a polyunsaturated fatty acid (LH) is a delocalized pentadienyl radical (L·) which in turn reacts rapidly with oxygen to form the peroxyl radical (LOO·). The peroxyl radical can then extract a hydrogen atom from a PUFA to yield yet another free radical (L·) and a peroxide (LOOH) (136). Singlet oxygen which can initiate lipid peroxidation from PUFA, may represent one of the ways that this electronically excited species of oxygen is toxic to biological systems (136,210). One of the most common ways of initiating lipid peroxidation is by means of the metal-catalyzed breakdown of peroxides already present in the system.

Both oxidized and reduced transition metals (iron or copper), can catalyze the decomposition of peroxides to form either alkoxyl, alkyl or hydroxyl radicals. These species can in turn re-initiate the peroxidative process (Figure 11).

Compounds that react with chain-propagating radical species and result in the formation of species no longer capable of hydrogen abstraction are considered chain breaking antioxidants. Termination of chain propagation (Figure 11) involves a variety of compounds which function as chain-breaking antioxidants such as phenols, aromatic amines and conjugated polyenes (136). Apart from these antioxidants, most cell types possess proteins and enzymes that can specifically remove reactive species (210). These proteins include superoxide dismutase (SOD), catalase, peroxidase and the glutathione-dependent systems (136,210,213).

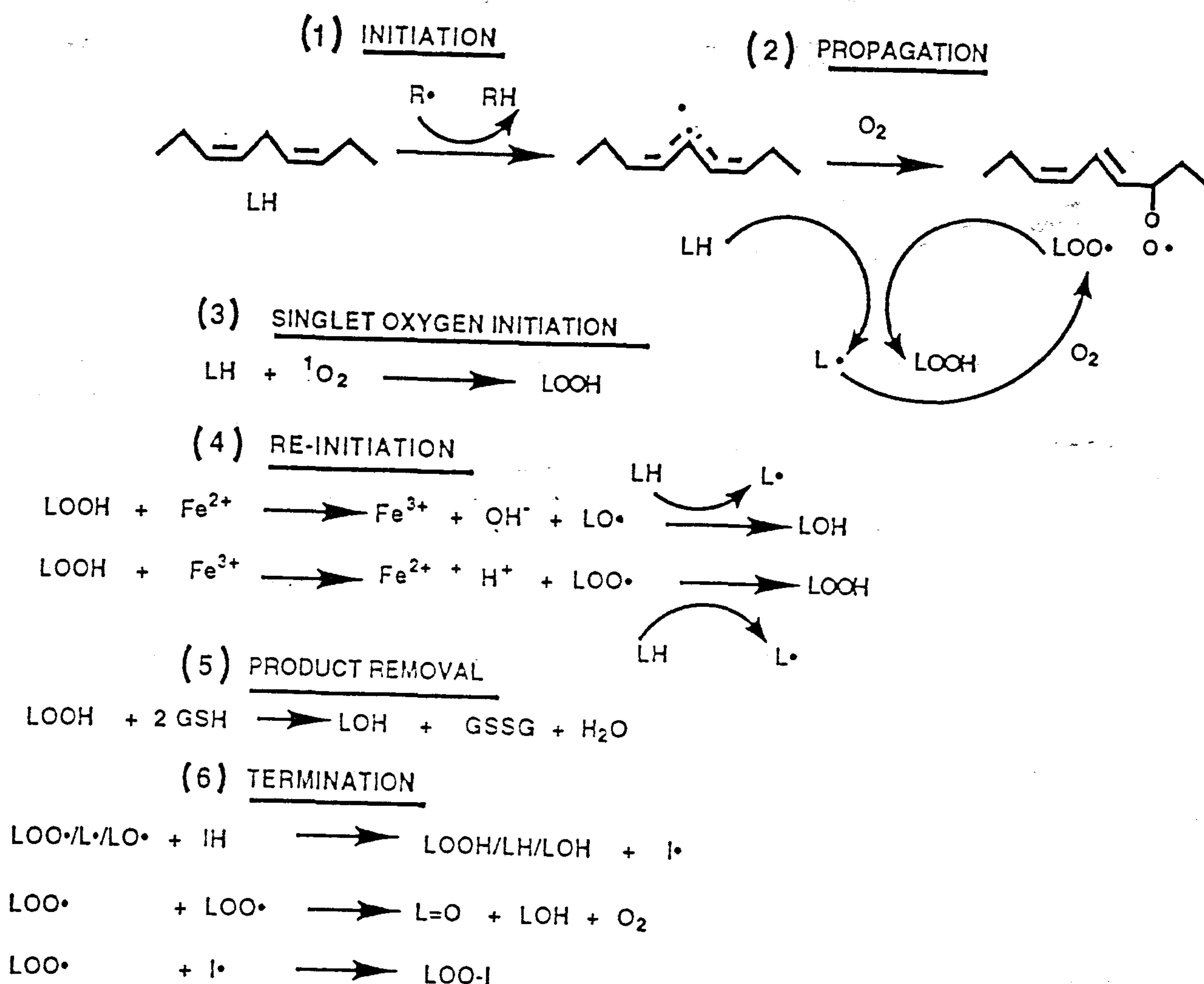


Figure 11: Lipid peroxidation and sites of action of antioxidants in lipid peroxidation (136).

Of special interest to this study is the involvement of lipid peroxidation in cancer. The major effects of the products of lipid peroxidation are the inhibition of DNA synthesis, cell division, and tumour growth (123). Substantial evidence on the other hand has reported that active oxygen species including free radicals play a role in the tumour initiation and promotion processes in multistage carcinogenesis (3,4,8-11,54,133,211,214). However, studies by Salim (215) illustrated that by removing oxygen-derived free radicals, the development of hepatic metastases is impaired and survival is prolonged in rats with 1,2-dimethylhydrazine-induced colonic cancer. In contrast, Das *et al* (216) showed that human breast carcinoma (AR-75-1) exhibited increased superoxide production which may be attributed to SOD and glutathione peroxidase activities. Lipid peroxidation products a consequence of free radical reactions have been suggested to influence the way in which signals for proliferation cross membranes. It is not yet known whether the observed increased resistance to lipid peroxidation is a property of malignant cells or whether it is simply a feature of rapidly dividing cells in proliferating tumours. Goldring *et al* (214) have proposed that lipid peroxide levels are inversely related to cell growth, irrespective of whether the cells are malignant or not. Studies by Cheeseman et al (217) showed reduced rates of lipid peroxidation in Yoshida hepatoma cells while Slater (218) reported that liver tumours undergo lipid peroxidation very slowly and have noted a number of contributory factors *viz*; lower content of PUFAs which are substrates for PUFA-peroxidation; enhanced cholesterol levels; decreased NADPH-cytochrome P450 reductase activity which donates electrons to reducible substrates which serve to initiate peroxidation; and low cytochrome P450 content which is known to act as a source of new radicals for chain propagation.

It has also been observed that enhanced lipid peroxidation *in vitro* induced by the addition of PUFAs to the culture medium resulted in a cytotoxic effect in cancer cells. This led (219) to the proposal that specific PUFAs may be used to treat patients with cancer on the assumption that there is reduced tumour lipid peroxidation in the first instance. Some patients, however, have reduced antioxidant levels and exhibit increased lipid peroxidation-malondialdehyde (MDA) which has mutagenic properties. MDA is generated during peroxidation of PUFAs in the cell membranes by free radicals and the breakdown of prostaglandin H_2 (PGH_2), the immediate precursor of other PGs (219).

1.7 PROSTAGLANDINS

PGs consist of a large group of cyclic derivatives of C_{20} oxygenated unsaturated FAs (196,220) and have been detected in virtually all cells and tissues of animals (220,221). PGs augment a variety of biological effects and appear to play a regulatory role in a number of systems (220). The term

"prostaglandin" is a misnomer: it was coined by von Euler in 1935 in the belief that this active substance emanated from the prostate gland (222,223). Today, PG is a term applied to a series of compounds derived enzymatically and non-enzymatically from C_{20} FAs such as AA (Figure 12) (9).

PGs are not stored in tissues (9,224-227) but are formed upon stimulation of cells. Figure 12 summarizes the three phases of prostanoid formation: (a) mobilization of AA from membrane phospholipids; (b) sequential conversion of released AA to the prostaglandin endoperoxides PGG_2 then PGH_2; and (c) isomerization/reduction of PGH_2 to the various prostaglandins (224,227). To fulfil a function, the PGs have to be synthesized at rates sufficient to overcome inactivation (228).

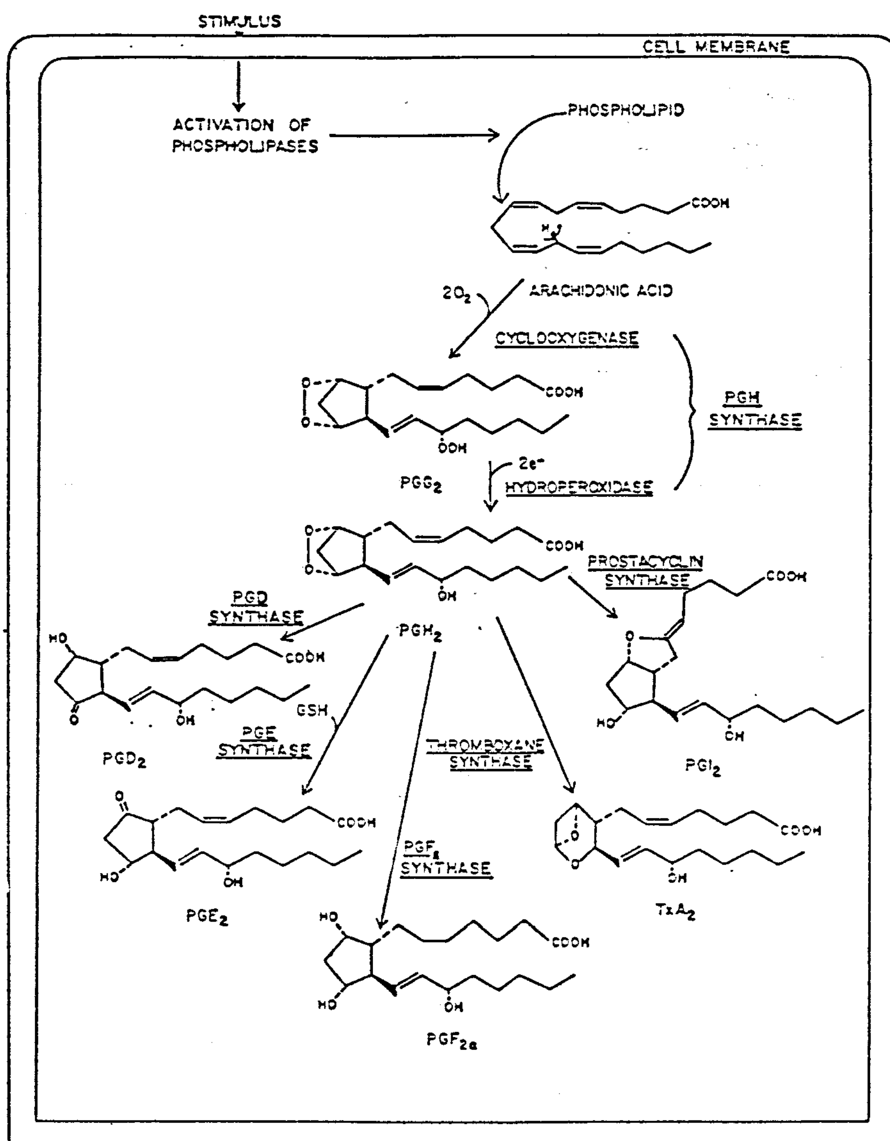


Figure 12: Biosynthetic pathway for prostanoid formation (229).

1.7.1 STRUCTURE AND PROPERTIES OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE

cis-PUFAs in which the conjugation is interrupted by methylene groups, belong to the group of essential FAs which can be converted into PGs with the help of enzyme systems (230). AA is by far the most biologically important EFA of the $\omega 6$ series. Besides normal β-oxidation, the common fate for all FAs in the body, AA can be enzymatically oxygenated by three different enzyme types: (i) by lipoxygenases to form hydroperoxyeicosatetraenoic acids; (ii) by COX to form PG endoperoxides and (iii) by monooxygenases (231). AA can interact with prostaglandin endoperoxide synthetase (PES) (E.C. 1.14.99.1) (also referred to as FA COX and PGH synthase), a membrane-bound multi-enzyme complex (222,232). The ubiquitous PES catalyzes the first committed step in the formation of PGs and TXs from AA.

PES has two activities, a cyclooxygenase which converts AA to PGG_2 and a peroxidase which reduces PGG_2 to form PGH_2 (233-237). PGH_2 is converted to a combination of prostanoids by numerous synthases depending on the cell or tissue type (238,239).

The PES is a glycoprotein with two identical heme-containing subunits (240,241) in which the primary site of action for the enzyme in the endoplasmic reticulum (ER) has an abundance of heme (242-244). The synthase has been purified to homogeneity as a dimer of 70kDa subunits (239,242). The amino acid sequence has been deduced from the nucleotide sequence of the cloned synthase gene (245,246). The 2.7 Kb cDNA encodes a protein of 600 amino acids including a signal sequence of 24 amino acids. The glycosylated enzyme (232,247) has three potential sites for N-glycosylation (248). A hydrophobic region (amino acids 267-282) may be a site of a transmembrane domain (246). Based on experimental observations, a model predicting COX topology in the ER with different putative functional domains has been proposed (246). The amino-terminal and the carboxy-terminal end of the protein are separated by one transmembrane-spanning region (Figure 14). Distinct peroxidase and dioxygenase domains are predicted to be cytoplasmic and in the COOH terminal half of the protein. The NH_2-terminal portion of the protein has three glycosylation sites, in addition it has been speculated that there is an epidermal growth factor-like domain on the NH_2-terminal side of the protein (247). Serine 530 is the "active site serine" on the membrane.

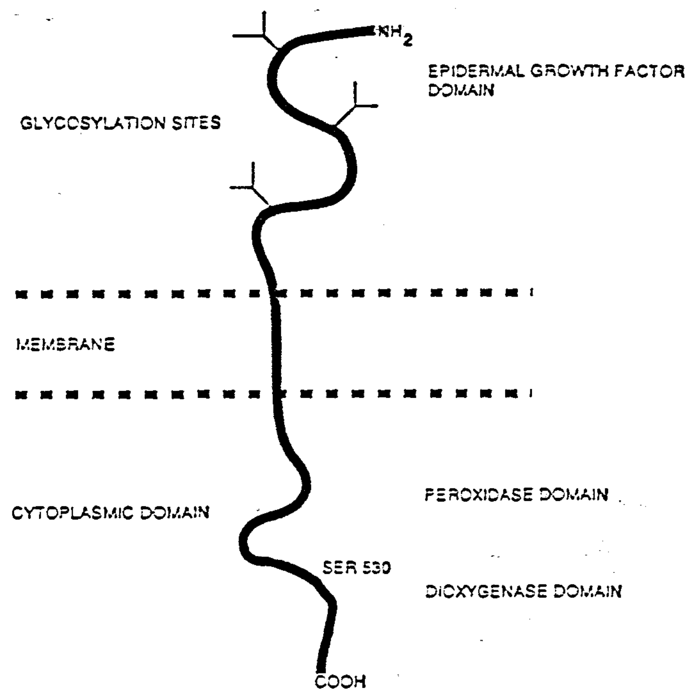


Figure 13 : A predicted model for the cyclooxygenase enzyme. Cyclooxygenase (solid line) is shown traversing the membrane (interrupted lines) once. Carboxy-terminus is cytoplasmic and contains putative dioxygenase domain and peroxidase domain (247).

Two COX enzymes have been described which are almost identical at the protein level but which differ markedly at the gene level (249). The first isoform (COX 1) is constitutively expressed, that is messenger RNA (mRNA) is constantly transcribed from the gene and the protein is synthesized at a steady rate independent of extracellular signals (249,250). The second isoform (COX 2) is an inducible form (248-254) in which the gene is said to be sensitive to extracellular signals and its expression may be increased by activation of cell surface receptors (249).

This finding led to interest in the mechanism of non steroidal anti-inflammatory drugs (NSAIDS). The NSAIDS are known to exert their effects through the inhibition of PG synthesis by interaction with CO_2 (256). In addition to NSAIDS, glutathione peroxidase also suppressed PGH synthase activity (257,258). On the other hand, lipid hydroperoxides serve as essential activators of PES. Peroxides activate COX, leading to several metabolic events linking the regulation of cellular peroxide levels to an indirect regulation of the production of PGs by COX (242,259), while phenol and tryptophan

are known to stimulate the peroxidase of the PES (241,260).

1.7.2 MECHANISM OF ACTION OF PROSTAGLANDIN ENDOPEROXIDE SYNTHASE

Biosynthesis of PGs is initiated by PES, a dioxygenase which incorporates two moles of oxygen into each mole of AA to form PGG_2 (234). This same enzyme then converts the PGG_2 to PGH_2 by reducing the hydroperoxy group into a hydroxyl moiety in a peroxidase-type reaction (235,260,261). (See figure 14).

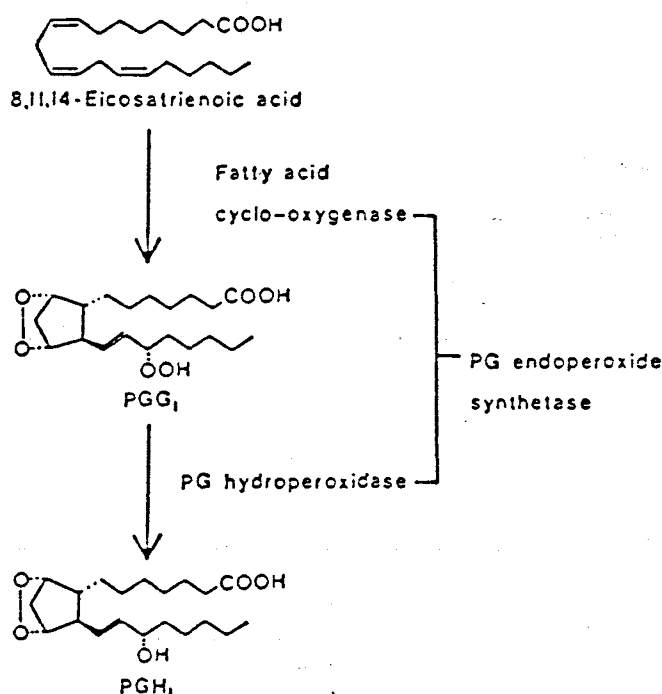


Figure 14: Reactions catalyzed by prostaglandin endoperoxide synthetase (262).

The COX reaction requires the continued presence of hydroperoxides, and much evidence (263) suggests that peroxidase plays an important role in the hydroperoxide-dependent initiation of the COX (263,264). The hydroperoxide and ferriheme interact to form a hydroperoxy radical (probably enzyme bound) in an initiation step for the overall reaction. This radical, with the help of the COX enzyme, propagates the reaction by abstracting the 13-S hydrogen (258). Oxygen then reacts with the alkyl radical at carbon 11 and cyclization occurs (265), hence one molecule is retained as a peroxide linking C9 and C11, while the second molecule of oxygen appears as a hydroperoxide at C18 (258,265,266). Experiments with ^{18}O have shown that both oxygen atoms at 9- and 11- positions originated from the same molecule of oxygen, demonstrating that the incorporation of oxygen involves a dioxygenase

reaction (267). Peroxidation of the 15-hydroperoxy group of PGG_2 to a 15-hydroxy group, producing PGH_2, is catalyzed by the hydroperoxidase activity of the PES (237). In addition PG hydroperoxidase is capable of oxidizing NADH and NADPH in a series of reactions which involves the formation of radicals ·NAD and ·NADP (268). These radicals readily react with oxygen to form the superoxide anion. Thus superoxide generation is a product of a side-chain reaction, dependent on the presence of suitable reducing co-substrates (268).

COX has an obligatory requirement for a hydroperoxide activator. Any modification which reduces hydroperoxide concentrations below about 10nM can inhibit COX activity (229). A peculiar characteristic of the COX is the time course of the enzyme reaction. The COX activity slows down gradually as soon as the reaction commences and ceases after 1-2 minutes. The cessation of the reaction is neither due to exhaustion of substrate nor accumulation of an inhibitor since the reaction can be resumed by the addition of fresh enzyme, but not by the further supply of substrate. Such a phenomenon has been observed by many investigators (258,260,268,269) and is referred to as a self catalyzed destruction or self-deactivation. The enzyme may be inactivated by an active oxygen species which is released during the hydroperoxidase reaction. Reduction of the 15-hydroperoxide group of PGG_2 to a hydroxyl may produce an active oxygen species if the hydroperoxide is not fully reduced to water, resulting in the destruction of the enzyme (237). Vitamin E, like phenol, may provide additional reducing equivalents to generate PGH_2 and vitamin E radicals, without the concomitant release of oxidizing equivalents.

1.7.3 BIOSYNTHESIS OF PROSTAGLANDINS D_2, E_2, $F_{2\alpha}$, and I_2

Biosynthesis of PGs requires the release of the esterified precursor AA from tissue lipids, AA in turn is converted to PGH_2 by PES, the central enzyme in the PG biosynthetic pathway. PGH_2 is at a pivotal point in the divergent pathways leading to the synthesis of various types of PGs and thromboxanes TX (237,270) (see Figure 15).

Although products are derived from a common cyclic endoperoxide intermediate, differences in optimal conditions for the formation of each, and differences in the effects of inhibitors on each, show that separate enzymes are involved in the formation of the different products from the endoperoxide intermediate, PGH_2 (271).

Enzymes utilizing PGH_2 as substrate, are isomerases which attack the 9,11-endoperoxide bridge of

PGH_2. PGH_2 is the substrate for the enzymes PGD, PGE, PGF and PGI synthases (237), and depending on the cell or tissue type, PGH_2 is converted to a combination and varying concentrations of the prostanoids PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 (238).

1.7.3.1 PGD synthase

The conversion of PGH_2 to PGD_2 is an isomerization of the 9,11-endoperoxide moiety to 9α-hydroxyl and 11-keto groups of PGH_2 (Figure 15) (237). The enzyme is found predominantly in the cytosol (237) and can be purified to homogeneity. A survey done on rat tissue indicated that PGD isomerase activity was higher in the brain and spinal cord than in other tissues (232,237). PGD_2 is the principal COX product of rat and human mast cells (232).

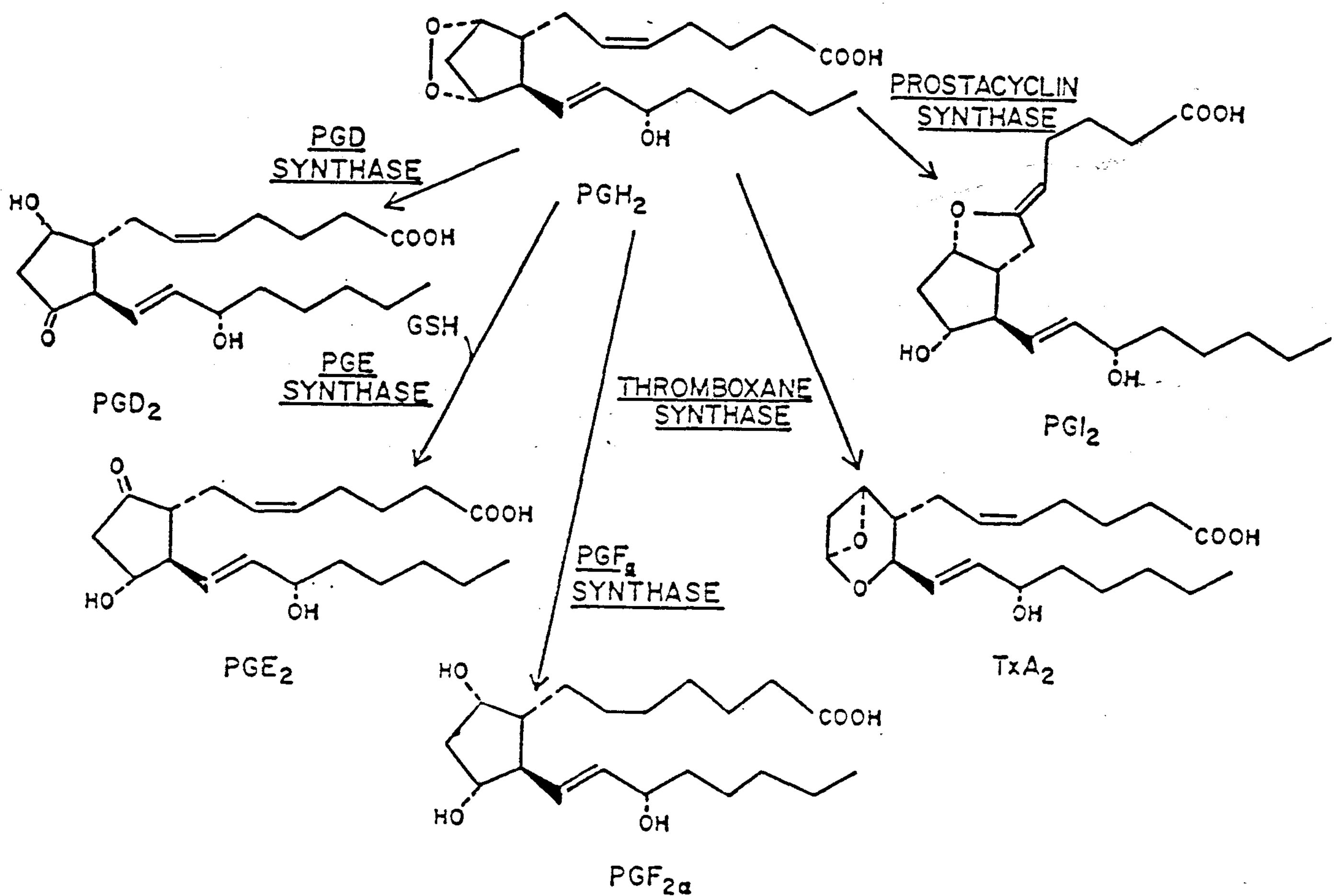


Figure 15: Modified biosynthetic pathway for prostanoid formation (229).

1.7.3.2 PGE synthase

The conversion of PGH_2 to PGE_2 is an isomerization of the 9,11-endoperoxide to 9-keto and 11α-hydroxyl groups (Figure 15). Initially, the enzyme system catalyzing the overall synthesis of PGE_2 from AA was referred to as PG synthase (E.C. 1.14.99.1). Glutathione is required as a specific coenzyme for the reaction, in which glutathione is believed to act as a nucleophile in a 1,2-hydride shift during the isomerization of endoperoxide (272).

1.7.3.3 PGF synthase

There are three possibilities for the synthesis of $PGF_{2\alpha}$. Firstly the conversion of PGH_2 to $PGH_{2\alpha}$ can involve a net two-electron reduction of PGH_2 (229,266). Thus the overall reduction is a reductive cleavage of the 9,11-endoperoxide of PGH_2. The second pathway is the conversion of PGE_2 to $PGF_{2\alpha}$. The 9-keto group of PGE_2 can be reduced to a hydroxyl group. This conversion occurs in various tissues (273,274). The third mechanism of $PGF_{2\alpha}$ synthesis is the reduction of 11-keto of PGD_2 (275,276).

1.7.3.4 PGI synthase

The formation of PGI_2 is an isomerization of the 9,11-endoperoxide of PGH_2 into a 6,9-epoxide and an 11α-hydroxyl group (Figure 15). PGI_2 is unstable, especially at acidic pH, and is readily transformed into 6-keto-$PGF_{2\alpha}$, a stable but inactive product (235). PGI synthase is inhibited by hydroperoxide of various unsaturated FAs (277-279), while Asc is thought to protect PGI synthase by scavenging hydroxyl radicals which are produced from hydroperoxides (280,281).

1.7.4 FUNCTIONS OF PROSTAGLANDINS

PGs have interested investigators ever since their initial discovery in the 1930s, (282), of their remarkable versatility and wide range of effects (223).

PGs are cell-to-cell messengers produced by eukaryotic cells in response to extracellular stimuli (283). Many of the effects of PGs are manifested via changes in the intracellular concentration of cAMP (283). PGE_2, $PGF_{2\alpha}$ (284,285) and PGI_2 (286,287) all enhance the formation of cAMP. PGs can either inhibit or facilitate tumour metastasis (9,288), host immunoregulation (289), tumour promotion

(276), and cell proliferation (226,290), depending on the cell system examined. PG production is related to cell growth, and cells in the quiescent state (Go phase) are the most active PG producers (291).

PGs are transported actively into cells by a specific carrier on cell membranes and the growth inhibitory effects of these PGs is closely related to this uptake (292). Prostanoids leave the cell by facilitated diffusion (239).

1.7.4.1 Functions of prostaglandin D_2

Although the biological function of PGD_2 is not fully understood, PGD_2 has been described as an antithrombotic agent (293,294), and a neuromodulator (295,296).

1.7.4.2 Functions of prostaglandin E_2

The pharmacology of PGE is quite diverse. PGE_2 is known to be a vasodepressor (223,270) and bronchodilator (270). In the kidney, PGE_2 can increase sodium excretion and decrease vasopressin-mediated water reabsorption. Both of these responses appear to involve interactions with AC (280). Furthermore low doses of PGE_2 were shown by Bygdeman (cited in 223) to stimulate contraction of the uterus.

1.7.4.3 Functions of prostaglandin $F_{2\alpha}$

$PGF_{2\alpha}$, a closely related member of the PGE family, also raises blood pressure and $PGF_{2\alpha}$, in addition to PGE_2, $PGF_{2\alpha}$ was shown to stimulate contraction of the uterus (223,297).

1.7.4.4 Functions of prostaglandin I_2

Prostacyclin (PGI_2), produced by the blood vessel wall, is the most potent natural inhibitor of platelet aggregation known (298,299). Furthermore, PGI_2 is a potent pulmonary vasodilator, where it binds to specific platelet receptors and activates AC, raising the cAMP level in the platelets.

1.7.5 PROSTAGLANDINS AND CANCER

Eicosanoids can influence the carcinogenic process in various ways. PG levels can either inhibit or stimulate tumour growth or influence tumour migration and the metastatic potential (200,289).

PGs, especially of the E series, have been shown to be elevated in a large number of tumours (9,220,228,300-304). Uncertainty remains as to whether the increase in the synthesis and release of PGs is the cause or the effect of increased proliferation rates, or whether it merely accompanies this phenomenon (305). Two mechanisms have been proposed for the increased PG production by tumours: tumour tissue could contain elevated amounts of PG synthetic enzymes and/or increased substrate may be available due to altered enzyme activities within the PG biosynthetic pathway (306). Interaction of PGs with growth factors has been reported and may contribute to the ability of the tumour cells to establish themselves at a metastatic site (300,303). In contrast, PGs in a number of situations have been shown to elicit a bell-shaped dose response curve with low concentrations stimulating and higher concentrations inhibiting cell proliferation (307).

With respect to the PGs to be considered in this study, in 1979, studies by Fitzpatrick *et al* (308,309) demonstrated that PGD_2 had an antimetastatic effect on mouse melanoma B16F10 cells. In 1982, Fukushima *et al* (310) presented evidence that PGD_2 had potent cytotoxic activity on several lines of tumour cells such as mouse leukaemia and several human leukaemia cells *in vitro*. In 1983, studies by Simmet and Jaffe (311), Bregman and Meyskens (312) reported that PGD_2 was a potent inhibitor of mouse melanoma growth *in vitro*. The antineoplastic PGD_2 is thought to exert its effects by inhibiting DNA synthesis (313,314) and through its inhibition of platelet aggregation which is not favoured in the metastatic process (309). PGs of the E series are known to inhibit the growth of a number of tumour cell lines (315). Numerous *in vitro* (257,316,317) and *in vivo* studies (318,319) have demonstrated PGE_2 inhibitory effect on cell growth which is thought to be mediated via the cAMP-AC linked system (320). $PGF_{2\alpha}$ production is increased in several cell lines transformed by chemicals or oncogenic viruses (321). $PGF_{2\alpha}$ has been reported to increase proliferation of cultured Swiss 3T3 and Raj Leukaemia cells (cited in 322), while $PGF_{2\alpha}$ had no effect on cell growth in murine mastocytoma cells (323). However, in human gastric carcinoma cell line Kato III, $PGF_{2\alpha}$ significantly and dose-dependently inhibited cell growth. The study by Nakamura *et al* (285) suggested that inhibition of growth was mediated via the stimulation of cAMP. PGI_2 is known to be both a negative and a positive modulator of tumour growth. PGI_2 production inhibited the growth of subcutaneous B16 tumours (324). However, Honn and Meyer (324) suggest that the modulation of

PGI_2/TXA_2 ratios may be an important therapeutic locus in the control of tumour growth. PGI_2 is a powerful antimetastatic agent against B16 melanoma cells. This effect, which may result from the platelet anti-aggregatory action of PGI_2 is potentiated by a PDE inhibitor. Inhibitors of PGI_2 synthesis increase metastasis (325). PGI_2 and agents that may increase endogenous PGI_2 synthesis or prolong its activity are suggested as new antimetastatic agents (325).

1.8 CALCIUM

Ca^{2+} is the most abundant cation in vertebrates (326) and Ca^{2+} is both an essential structural body component and a functional element in living cells. It is a key component in conserving the membrane integrity in addition to being a pivotal regulator for a wide variety of cell functions in its role as a second messenger (327). Of particular interest in this study is the association of Ca^{2+} with PLA_2 and AC activity.

1.8.1 CHARACTERISTIC PROPERTIES OF CALCIUM

Calcium is characterised by its charge, co-ordination number and unhydrated radius (328). The concentration of Ca^{2+} ions in the resting cytoplasm of cells is very low (10^{-7} M or less) (329), while that of the extracellular free Ca^{2+} concentration is in the region of 1mM. Hence a very steep electrochemical gradient of Ca^{2+} exists across the plasma membrane for most animal cell types. Together with the concentration gradient, an electrical driving force for Ca^{2+} entry prevails, since the intracellular space is usually found to be at a potential of about -60mV relative to the outside (326,330). The Ca^{2+} concentration in the cytosol rises to about 1μM following a Ca^{2+} mobilization stimulus. Ca^{2+} is involved in electrical signalling processes as well as transmembrane chemical signalling (330).

Ca^{2+} cannot be synthesized or broken down, the only mechanisms available for causing large-scale changes in Ca^{2+} concentration involve alterations in compartmentation and movements across membranes. Ca^{2+} ions are "sticky", which enables it to bind to various ligands such as proteins and phosphate groups. Ca^{2+} bound to such ligands enables the ion to regulate metabolic processes (330). One such protein is the calcium-dependent regulatory protein (CDR,calmodulin) which is an acidic, low molecular-weight, ubiquitous Ca^{2+} binding protein of mammalian cells. Calmodulin is recognized as the mediator of Ca^{2+} dependent control of an increasingly large number of enzymes (331).

1.8.2 INTRACELLULAR TRANSPORT OF CALCIUM

Given the extreme electrochemical gradient of Ca^{2+} across membranes, two systems exist for the active outward transport of Ca^{2+}. The two well documented systems are the Ca^{2+}-stimulated ATPase and a Na^{+}-Ca^{2+} exchange systems, in which many but not all cell types seem to contain both systems. The level of activity of the Na^{+}-Ca^{2+} exchange system varies considerably from tissue to tissue, being weak or absent in erythrocytes and liver cells and highly active in excitable cells such as those of heart and nerve. A detailed account of these systems is given by Dawson (330).

1.8.3 FUNCTIONS OF CALCIUM

1.8.3.1 Calcium and cell proliferation

Ca^{2+} availability is the key controller of cell proliferation in multicellular organisms and a defect in the Ca^{2+} levels appears to be the common determinant of unrestrained proliferation in cancer cells. Durham and Walton (329) suggest that the proliferation rates of both normal and cancer cells is controlled by the availability of Ca^{2+} ions from the stores inside the cell at a particular time and place (329).

Malignant cells lose the ability to maintain cytoplasm Ca^{2+} concentrations below a critical point. As a result, in contrast to normal cells they can proliferate when extracellular fluid Ca^{2+} ion concentrations are very low (328,332). These findings are substantiated by Swierenga *et al* (cited in 333) who showed that neoplastic cells with high tumourigenicity were able to proliferate in a low Ca^{2+} medium whereas proliferation of non-neoplastic cells in low Ca^{2+} medium was much lower, suggesting that proliferation is controlled by extracellular Ca^{2+} (328). Thus the Ca^{2+} requirement of neoplastic cells is very low in comparison to that of normal cells (329). This is consistent with the observation that hepatoma cells contained at least 100% more Ca^{2+} than normal liver. In addition, the Ca^{2+} content was found to escalate progressively with increasing age of the tumour (334).

Ca^{2+} and /or calmodulin play significant inhibitory roles in DNA synthesis and cell proliferation (335-337). cAMP has the ability to mediate Ca^{2+} levels (188) with cAMP regulating the cell cycle at the various phases (338).

1.8.3.2 Calcium and enzymes

Many key enzymes which are involved in AA esterification or release are dependent on metal ions. Ca^{2+} stimulates acyl hydrolase activity (339), while PLA_2 requires elevated cytosolic Ca^{2+} for the activation and subsequent release of AA from tissue lipids in numerous stimulated cell types (339,340). 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 (PKC) and subsequent phosphorylation of PLA_2 due to prior activation of phospholipase C (PLC) (339,341). Hence, Ca^{2+} becomes directly stimulatory to PLA_2 as exhibited in cell homogenates and isolated membranes. Thirdly, Ca^{2+} is required for the activation of PLA_2 by G proteins (339,342,343). Following release of AA from precursor lipid stores, prostanoids are formed by oxygenation of free AA by prostaglandin endoperoxide synthase (PES).

Ca^{2+} also acts as a second messenger in eliciting a physiological response via Ca^{2+}-modulated proteins. AC forms reversible Ca^{2+}-dependent complex with calmodulin, with calmodulin-Ca^{2+} interacting directly with the catalytic subunit of AC (344). The various hormone-responsive AC systems, including the B16 melanoma (345) are Ca^{2+} dependent. However, comparatively few have been shown to be regulated by calmodulin. 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 a stimulator of AC. However, G proteins are also involved in the action of certain receptors that inhibit Ca^{2+} channels (346). Thus G proteins have direct effects on plasma membrane Ca^{2+} channels (330).

1.8.4 PHOSPHOLIPASE A_2

Studies over the last several years have led to the expansion of knowledge on the diversity of forms and function of mammalian sn-2 acylhydrolases. The release of FAs from phospholipids by these enzymes although proposed over a hundred years ago has only recently been more defined. Of particular interest in this study is AA release.

1.8.5 CLASSIFICATION AND PROPERTIES OF PHOSPHOLIPASE A_2

Phospholipases A_2 (PLA_2; phosphatide sn-2-acylhydrolases; EC 3.1.1.4) catalyze the hydrolysis of the sn-2 fatty acyl chain of various phospholipid (PL) substrates to yield FAs and lysophospholipids (347-349). PLA_2s are a heterogenous family of enzymes that can be classified into two classes based

on their molecular weight (350,351). Secretory PLA_2s ($sPLA_2$) are small proteins (about 14 KDa) which unselectively cleave any FA from the sn-2 position of phospholipids. $sPLA_2$ requires millimolar levels of Ca^{2+} for activity and have a high sulphide content (352-354). The $sPLA_2$ are further divided into three groups based on their amino acid sequence, namely group I, II and III. Pancreatic PLA_2 is in Group I (347,353,355), Group II includes human synovial fluid and platelet PLA_2s (347,353,356) and Group III includes bee venom PLA_2 (347). Group IV, the high molecular mass (80-110kDa) cytosolic PLA_2s ($cPLA_2$) are activated by submicromolar amounts of Ca^{2+} and preferentially hydrolyse phospholipids that have AA at the sn-2 position (347,352,357). This enzyme is present in the cytosol and translocates to membranes in response to physiological concentrations of Ca^{2+} (352-354,357-359), thus mediating transient physiological eicosanoid synthesis. $cPLA_2$ lacks disulphide bonds and exhibits no amino acid sequence homology with Type I and II although it does have a Ca^{2+} binding domain like that of PKC (353). $cPLA_2$ is of particular interest in this thesis due to its AA selectivity, which is the key substrate involved in the regulation of eicosanoid synthesis via the cyclooxygenase pathway.

A number of factors influence PLA_2 activity. Acidic phospholipids are generally hydrolysed faster than neutral ones (360). Studies have shown that the $cPLA_2$ activity is stimulated by anionic phospholipids which act to increase the affinity of PLA_2 for Ca^{2+} (361-364). The activity of this enzyme is influenced by the rate of enzyme attachment and release of AA from the phospholipid monolayer where $cPLA_2$ carries out its hydrolyzing activity (357,362). Most PLA_2s show an absolute requirement for Ca^{2+} but some intracellular enzymes are activated substantially by Sr^{2+}(362,365). The concentration of free AA within resting cells is unknown but is believed to be maintained at low levels. An increase in the release of free AA from phospholipids has been observed in many cell types (366) upon stimulation. PLA_2, is competitively inhibited by unsaturated FAs, particularly AA. Hence FA inhibition may represent one mechanism of regulation for PLA_2 and thereby eicosanoid production (367-370). Subsequently to these findings, researchers have reported that AA metabolites are potent mediators of cell proliferation, glucocorticoids and allergic and inflammatory reactions, PLA_2 inhibitors are expected to have a variety of therapeutic effects on cell proliferation, glucocorticoids, allergic and inflammatory reactions (357,371,372).

1.8.6 FUNCTIONS OF PHOSPHOLIPASE A_2

The presence of PLA_2 in biological membranes is a prerequisite for proper membrane turnover in cellular metabolism and is considered to be involved in membrane remodelling (349,373), exocytosis

(374) and the repair of oxidative damage (349). Membrane-associated PLA_2 prevents membrane oxidative damage by providing a membrane sparing effect by the elimination of lipid peroxidation products (375). In addition, PLA_2 activity leads to a rapid release of large amounts of PGs (376), which in turn stimulate second messengers.

1.8.7 G PROTEINS AND PLA_2 MECHANISMS

Two major routes of receptor-mediated regulation have been implicated in the control of AA mobilization and release. One route is activation of PLA_2 via a receptor linked G-protein not requiring PLC (342,347,377-379), and the other is indirect activation through a PLC-mediated pathway increasing Ca^{2+} and activating PKC (347,353,380-382). In the first route, PLA_2 is directly linked to a G-protein and hence dependent on GTP (343,346,367,376-378). This leads to a direct release of AA, without prior activation of other phospholipases.

Termination of a receptor-mediated signal transduction is a vital requirement. The system in which PLA_2 activity is linked to G proteins, reassociation of the inactive heterotrimer following activation of the GTPase associated with the α-subunit of the G-protein occurs (367). In addition to shutting off the signal at the G protein level other mechanisms exist which could turn off the systems at the receptor level of the G-protein(s). These include reduction of cytosolic Ca^{2+} levels, activation of protein kinases, feedback inhibition as well as the second messenger, cAMP (366,367).

1.9 ADENYLATE CYCLASE AND CYCLIC ADENOSINE MONOPHOSPHATE

AC was discovered by Sutherland and Rall in 1957 (383). AC (ATP pyrophosphate-lyase[cycling] EC 4.6.1.1), the catalytic protein converts ATP to adenosine 3',5'-cyclic monophosphate (cAMP) and pyrophosphate (331,384,385). cAMP acts as a second messenger for many systemic and local hormones and consequently controls many physiological processes at the cellular level (198,386).

1.9.1 STRUCTURE AND PROPERTIES OF ADENYLATE CYCLASE

AC occurs in both prokaryotic and eukaryotic organisms and most probably in plants (331,383). AC, a glycoprotein, is a single subunit enzyme of either 115kDa or 150kDa (346). However, this polypeptide exists with the AC system which is ubiquitous, multi-component and membrane-bound (198,331,383). The AC system is composed exclusively of intrinsic membrane proteins and depends

on their proper integration in a membrane for hormonal regulation and hence adequate functioning (331).

Initially, the hormone response AC system in the cell membrane exhibited a three component model comprising a hormone receptor, a catalytic unit, and a guanine nucleotide-binding regulatory protein (198,331,387-390). However, research has shown that the receptor and the GTP-binding regulatory protein components are more complex. The hormonally regulated AC system is now shown to comprise of five main components: the stimulatory receptor Rs, the stimulatory guanine-nucleotide binding and hydrolyzing regulatory protein Gs, which itself is composed of three subunits (α,β,γ), the catalytic moiety of the AC, the inhibitory receptor Ri and the inhibitory GTP-regulatory protein Gi, which also consists of three subunits (346,388,391,392). AC stimulation via hormones appears to be a transmembrane phenomenon (393), as the various components are asymmetrically arranged in the membrane (198). The catalytic site on the enzyme, which is responsible for the formation of cAMP from ATP, is thought to face the inside of the intact cell, while the receptor site, which interacts with specific circulating hormones, faces the external medium (393). AC, like many integral membrane-bound enzymes, is regulated by the nature of its membrane lipid environment (121,198).

1.9.2 PROPERTIES OF THE ADENYLATE CYCLASE COMPONENTS

1.9.2.1 The receptor component

Various hormones have their own specific receptor sites on the receptor component, of which glucagon and epinephrine are the best documented (178,394). Some receptors, however, are known to recognize more than one hormone (198). The receptor-hormone complex acts by facilitating the binding of a guanine (or related purine) nucleotide to the regulatory site (198). Hormonal stimulation of AC is observed only in intact membranes.

1.9.2.2. The regulatory component

Nucleotides, particularly guanine nucleotides, play a vital role in regulating the function of hormone-sensitive AC systems (395). Two major subunits suggest two distinctly separate guanine regulatory sites for AC. One site appears to mediate inhibition of AC, and is denoted the Gi or Ni site (388,396,397), while occupancy of the Gs leads to AC stimulation (387,388,396,397).

The regulatory protein is essential to functionally couple agonist occupancy of the receptors with activation of the catalytic moiety. Hence guanine nucleotides not only modulate the catalytic activity of AC but also exert specific regulatory effects on agonist binding to receptors (389).

1.9.2.3 The catalytic component

Little is known about the physical properties of the catalytic component of AC, however it is known that this component has a relatively hydrophobic surface area (198,331). In addition, the catalytic component must have a substrate site for ATP as well as a binding site for the cation. Hence, this component presumably binds to a divalent cation-ATP complex (198,398).

1.9.3 MECHANISM OF ADENYLATE CYCLASE ACTIVITY

In 1971 it was reported that the receptor-sensitive signal transduction was not only regulated by hormones but also guanosine triphosphate (GTP) as originally suggested in 1956 by Sutherland (cited in 346). The progress that led to the discovery of hormone signalling is reviewed in Pertseva (399). The transmembrane signalling systems usually consist of two major components: the specific binding site (receptor) and the effector components-namely the enzymes or channel proteins (391). GTP is an essential cofactor for hormonal stimulation of AC. The actions of GTP require interaction of the nucleotide with specific guanine nucleotide-binding proteins, which in turn serve as regulators of the appropriate catalytic entity (400,401). Hence regulatory proteins function as transducers of information between receptors for hormones and the ultimate effector (402). AC has a type II signalling mechanism, generating second messengers (399). The binding of an agonist to the receptor is followed by an interaction of the Rs complex with the stimulatory G-protein (Gs) (391) (See figure 16).

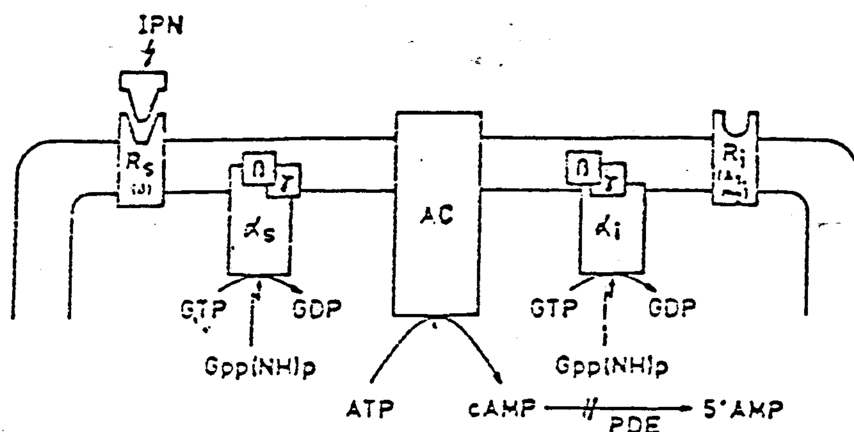


Figure 16 : Mechanisms involved in the regulation of the adenylate cyclase activity by stimulatory (s) and inhibitory (i) processes (391).

Gs binds to GTP and cations in addition to linking the receptor Rs to the catalytic unit (198,387,388,403). AC is essentially inactive in the absence of the divalent cation Mg^{2+} (339,404). The activated GTP-bound Gs induces an active conformation in the catalytic unit of AC, resulting in the formation of cAMP (391). The presence of high levels of GTP ($\geq$ 1μM), activates the catalytic unit in the conversion of ATP to cAMP (198,391). The activation of intracellular cAMP-dependent protein kinases results in the phosphorylation of specific protein substrates. Stimulation of the inhibitory receptor Ri induces dissociation of the β- and γ- subunits from αs and the subsequent tight binding to αi, brings about the inactivation of the catalytic unit (391) due to the binding of GDP (346).

1.9.4 FACTORS INFLUENCING ADENYLATE CYCLASE ACTIVITY

1.9.4.1 Membrane lipid composition

AC is sensitive to changes in membrane fluidity. Increased membrane fluidity enhances enzyme activity whereas activity is reduced when membranes become more rigid (178,198). AC activity is affected by changes in the composition of the phospholipid fatty acyl chains and polar head groups (121), hence modification of AC activity is influenced by the nature of the dietary fat (405). To explain this phenomenon, it has been suggested that AC can exist in numerous different conformations exhibiting varying activities, and that changes in membrane lipid composition cause the enzyme to

shift from one conformation to another (121).

AC activity is also affected by changes of membrane lipid environment (406). Lipid peroxidation in membranes is known to decrease membrane fluidity, exerting a negative effect on the enzyme activity and in doing so inhibits AC activity (407,408). In contrast, Canuto et al (409) found that in AH-130 Ascites Hepatoma, enhanced AC activity was not only related to membrane fluidity but also to the increase of lipid peroxidation.

1.9.4.2 Prostaglandins

Regulation of the AC system appears to involve PGs (410), which have been shown to be associated with activation of AC and an increase in cellular cAMP concentrations (411). Stimulation of AC activity by PGs is initiated through the binding of these agonists to their specific receptors on the outer surface of the membrane bilayer (341,412-415). PGE_2 (416-421), PGD_2 (286,422) and PGI_2 (286,287,422) are reported to have both stimulatory and inhibitory effects on AC activity, hence altering cAMP production accordingly, while $PGF_{2\alpha}$ (415,423,424) has been reported to have only a stimulatory effect on AC activity. Separate receptors are present for each PG suggesting the effects brought about by PGs are independent of each other (415).

1.9.4.3 Calcium-dependent regulatory protein

The calcium-dependent regulatory protein (calmodulin) is recognized as the mediator of Ca^{2+}-dependent control of an increasingly large number of enzymes (331). AC has demonstrated a need for CDR for the stimulatory effects of Ca^{2+} on AC activity (426). CDR-Ca^{2+} is however found to activate some ACs but not all of the AC types (331,345,426).

1.9.5 CYCLIC ADENOSINE MONOPHOSPHATE

cAMP is a ubiquitous molecule in which numerous activities are facilitated by hormonal actions which utilize cAMP as a second messenger or mediator (427). The magnitude of the effects of cAMP depends on its concentration (428). Since the discovery of cAMP by Earl Sutherland (cited in 392,429), cAMP has attracted much attention for the explanation of a variety of cellular phenomena. The conversion of ATP to cAMP by AC takes place in the presence of a divalent cation, such as Ca^{2+}, Mg^{2+} or Mn^{2+} (404,430,431). The enzyme phosphodiesterase (PDE) is instrumental in

controlling the concentration of cAMP within cells, and in doing so altering the biological activity of cAMP (432). cAMP is converted to 5' adenosine monophosphate (AMP) by PDE in the presence of Mg^{2+} (433-435). Numerous forms of PDEs are known to exist (436-439).

1.9.5.1 Biological functions of cyclic adenosine monophoshate

An important property of cAMP is that it affects the activity of different cell types in various ways (431). cAMP not only mediates the actions of most biogenic amines and polypeptide hormones but also influences such fundamental processes as cell division, cell motility, morphology, contact inhibition and the synthesis of proteoglycans (431-433,440,441). Furthermore, in cancer, cAMP may be a modulator of the activity of several oncogenes (429).

S
↓ [cAMP]
[cAMP] ↑
↑ [cAMP]
G1
G2
[cAMP] ↑
D.
[cAMP] ↓

G1 - preparation for chromosome replication
S - DNA synthesis
G2- preparation for cytokinesis
D - cytokinesis or cell division

Figure 17: cAMP regulation of the cell cycle (442).

Three observations provide evidence for the role of cAMP in the control of cellular growth: (i) cAMP levels are lower in transformed cells than normal cells (443), (ii) agents which increase cellular levels suppress growth and (iii) agents which decrease cellular cAMP levels stimulate growth (444,445). cAMP concentrations vary within the cell during the different phases of the cell cycle. These fluctuating levels of cAMP in turn play a significant role in controlling the cell cycle (394,442).The life cycle can be divided into four phases (See Figure 17).

The cell cycle begins at cytokinesis or cell division. As the cell matures it goes through a G1 (Gap 1) phase, during which it prepares to replicate its chromosomes; it then progresses through to an S (for DNA synthesis) phase, during which it replicates its chromosomes. Thereafter it pauses in a

second gap, or G2 phase, during which it prepares for mitosis and cytokinesis; finally it ends its life as an individual upon dividing into two new individuals during the D phase (328,394,446). The length of these periods depends on the cell type and conditions of growth. Mitosis (D phase) is usually the shortest phase (394).

The G1 phase is the most susceptible to inhibition by shortages of essential nutrients and is the most responsive to growth factors and hormones. This is due to the fact that the conditions must be optimal before the cell will risk starting the chromosome subcycle. In the G1 phase there is a prolonged cAMP surge in a variety of cells (394,423,446). A further prolonged surge occurs in the late S phase or G2 phase of some cells (431,445-447). These elevated levels of cAMP keep the cell in the quiescent state and it is only once the cAMP levels drop, that the cell is then stimulated to resume cycling. Hence, elevated levels of cAMP in the S phase, prevent DNA synthesis (442,446). Therefore, cAMP functions as a negative regulator of DNA synthesis (328,446).

1.9.5.2 Cyclic adenosine monophosphate and prostaglandins

Various effects of PGs on the regulation of growth, morphology and functional differentiation on several cell lines in culture appear to be mediated through the cellular concentrations of cAMP (447) as they can alter the intracellular levels of cAMP and/or Ca^{2+}(448).

PGE_2 (420,421,449), PGD_2 (386,422,447), PGI_2 (387,405,422) and $PGF_{2\alpha}$ (415,423,449) are known to increase cAMP accumulation in various cell lines. In mastocytoma cells (447) PGI_2 was the most potent PG in elevating cAMP within the cells, with $PGF_{2\alpha}$ being the least potent, whereas in dog thyroid, $PGF_{2\alpha}$ was the most potent PG (423). Cell proliferation shows an inverse relationship with these PG-induced changes in cAMP levels (450).

PGE_2 can activate cAMP formation in normal cells (451), hence elevated PGE_2 levels resulted in increased cAMP formation which suppresses DNA synthesis (416). Furthermore cAMP acts as the second messenger for PGE_2-mediated modulation of biological functions in various cells (452,453). PGE_2 at concentrations greater than 10^{-6}M stimulated platelet AC and raised cAMP synthesis (454), whereas concentrations as low as 10^{-6}M inhibited AC and cAMP, reducing the platelet content below controls (449). In general, it appears that PGE_2 (449,455), PGD_2 (447), PGI_2 (287,447) and $PGF_{2\alpha}$ (415,449) stimulate cAMP formation directly via association with their respective receptors resulting in cAMPs regulation of various biological functions.

1.9.6 ADENYLATE CYCLASE ACTIVITY, CYCLIC ADENOSINE MONOPHOSPHATE AND CANCER

In general, tumour and transformed cells have levels of cAMP significantly lower than those of normal cells (427,456-461). Low intracellular levels of cAMP in transformed and malignant cells may be due to alterations in the activity of AC and/or PDE (386,462), hence altering the pattern of cAMP metabolism (458). AC has two binding sites for Mg^{2+}. One is the catalytic site and the other is the Gi site. In transformed cells, the second regulatory site is altered such that high concentrations of Mg^{2+} are unable to enhance catalytic activity (398). Furthermore, cAMP itself is an inducer of PDE (386). Moreover, lower cAMP content in transformed cells may also be attributed to some modification of the plasma membrane (398).

In transformed cells, various modifications are known to occur in the plasma membrane. Since AC is associated with the plasma membrane, altering its AC receptor sites results in decreased activity and hence decreased cAMP levels (453,462). Furthermore, lipid peroxidation decreases membrane fluidity inhibiting AC activity (415). It has been reported (386) that during oncogenesis AC activity is always elevated, resulting in enhanced cAMP levels in these cells. This alteration in cAMP metabolism during tumour development could have a functional role in oncogenesis or could just be an accompaniment to the cellular changes (386,429). However, cAMP does not appear to function as a second messenger for oncogenes (386).

The concentration of cAMP appears to control the growth rate of cells. Numerous *in vitro* studies have shown an inverse correlation between the growth rate and intracellular cAMP levels (442,443,459,462,463). Hence, it has been suggested that the abnormal properties of transformed cells, *e.g.* growth rate, morphology and loss of contact inhibition, can be reverted towards those of normal cells by treatment with agents that raise cAMP levels (435,464,465). Such agents could be PDE inhibitors, such as theophylline, caffeine (466,467) and Asc (464) which increase the endogenous intracellular cAMP pool size. This implies that many of the abnormal properties of transformed cells may be due to decreased cAMP levels (462), as a result of decreased AC activity. However, numerous *in vivo* studies with animal tumours have also shown increased cAMP levels in the cells (447,468). Hence there is some uncertanty regarding the exact relationship between cAMP levels and tumour cell growth.

1.10 OBJECTIVES

As indicated earlier there is some uncertaintity about the role of vitamin E and C in anticarcinogenesis, although it has been suggested that these effects maybe due to their synergistic antioxidant properties and/or their combined effect on various metabolic pathways such as the AA cascade pathway shown in Figure 9 (repeated on this page).

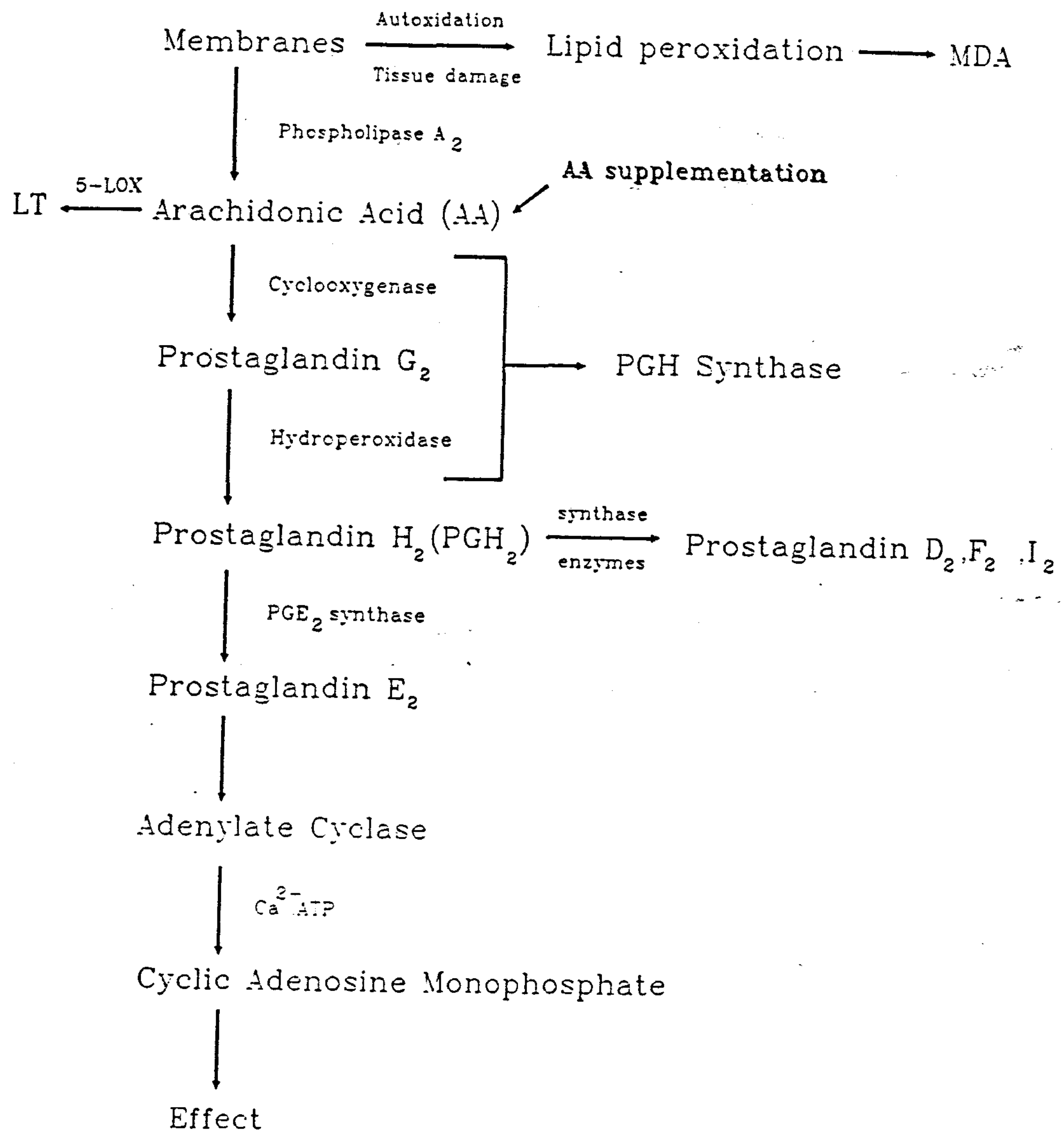


Figure 9: Schematic representation of the AA cascade pathway investigated in this study.

The principal objective of this dissertation was to investigate the effect and role of combined vitamin E succinate and Asc supplementation over a nutritional range on the growth and cellular functions of *in vitro* cultured normal and malignant cell lines.

The effect of vitamin E succinate and Asc on malignant BL6 and non malignant LLCMK cell growth could possibly be mediated through metabolic pathways which influence this cell growth. The pathway that was studied in this regard was that of the AA cascade pathway (Figure 9, repeated on page 55), which involves the cleavage of AA from esterified phospholipid stores via the action of PLA_2. The conversion of AA to PGs and the effect of the latter on AC activity and cAMP (a known regulator of cell growth) was also determined. The pathway is also influenced by exogenous EFAs, 5-LOX, COX and Ca^{2+} levels. In addition to the role of 5-LOX and COX activity in AA metabolism, their role in free radical formation and lipid peroxidation was also examined.

Hence, the effect of combined vitamin E succinate and Asc supplementation on the activity and the metabolism of this pathway, in addition to AA metabolites which influence this pathway, was investigated.

CHAPTER 2

COMBINED NUTRIENT SUPPLEMENTATION AND CELL GROWTH

2.1 INTRODUCTION

The technique of *in vitro* culture of animal cells has expanded considerably over the past few years, and is now widely used in many disciplines (469). The properties of individual cell lines makes them useful for a wide variety of purposes in biological research, diagnosis and commercial applications (470). The development of continuous cell lines has attained great importance in research and has numerous advantages over a finite cell line (469). Finite and continuous cell culture techniques allow for the supplementation of the cultures with various nutritional factors and the monitoring of the effects of these factors on cell growth and cell metabolism.

In attempts to find a treatment for cancer, cultured malignant cell lines have proved invaluable. These cell lines have been supplemented with various anticarcinogenic chemical compounds, in addition to various natural biological substances as a means of halting or delaying tumour cell growth (80). The potential of these natural substances as a preventative measure against neoplasia development is of paramount importance as numerous sources clearly indicate that natural foods contain factors that protect against cancer (7,80). Thus a relationship between diet and cancer incidence is clearly indicated in human populations (7,80) and the role of vitamins in the prevention and management of neoplasms is becoming increasingly evident.

For example the beneficial effects of vitamin C in the prevention (31) and management (31,52) of neoplasms has been extensively reported. Supplementation of a mixture of vitamin C with B_{12} increased the survival rate of mice bearing P388 leukaemia and Ehrlich carcinoma (471). Sodium Asc, in combination with some tumour therapeutic agents, namely 5-fluoro uracil, bleomycin sulphate, sodium butyrate, cAMP-stimulating agents and X-radiation, has been found to potentiate the growth inhibitory effect on neuroblastoma cells in culture (53). Furthermore, vitamin C supplemented to CHO clone A cells *in vitro* inhibited cell growth (472). Results of administration of Asc as a therapeutic agent to cancer patients suggests that it could assist in overcoming Asc deficiency and assist in stabilizing malignant cell development, hence halting further metastasis (31,68,473).

Vitamin E succinate has also been shown to induce differentiation and growth inhibition in certain animal and human tumour cells in culture (49,93). Numerous studies have been conducted to evaluate the role of vitamin E in experimental carcinogenesis (5), although the studies that have been carried out (cited in 80) have reported decreased tumour growth with α-TOH supplementation. Supplementation of vitamin E succinate reduced the growth of transplanted mouse melanoma and neuroblastoma cells *in vivo* (49). Furthermore, supplementation of vitamin E succinate to melanoma cells *in vitro* induced morphological changes and growth inhibition (94), while vitamin E succinate has been reported to inhibit cell growth of glioma and neuroblastoma cells in culture (92). Treatment of vitamin E succinate and PGs such as PGE_2 have been shown to act in an additive manner to inhibit the proliferation of human oral squamous carcinoma cells (SCC-25) (91), while vitamin E succinate enhances the growth inhibitory effect of vincristine in a synergistic fashion (474) in neuroblastoma and glioma cells in culture.

These findings suggest that the inability of tumour cells to maintain biochemical processes may be partly due to the deficiency or increased requirement of essential nutrients such as Asc and vitamin E. Emphasizing this possibilty, tumour cells lose their ability to maintain the complex intracellular and extracellular matrix (31) as well as cell membrane integrity (123,199). Furthermore, administration of α-TOH to cancer patients could aid in overcoming the cellular damage caused by active oxygen, preventing the promotional phase of carcinogenesis and stabilizing cell membranes (5,475). The suggestion that vitamin E and C can modify the effect of pharmacological agents on tumour cells in culture is somewhat unique (52,49). The extent of modification depends upon the type of tumour, vitamin and the pharmacological agents. As a result of the growing evidence for vitamins A, C and E as nutritional adjuncts in the treatment of cancer in one study, patients with disseminated malignancies were placed on large doses of A, C and E. Results from this study suggest that doses of A, C and E are of great value in the treatment of these cancer patients (476).

While the inhibitory effects of Asc and vitamin E succinate on the growth of numerous cell lines *in vitro* have been well documented, the addition of combined Asc and vitamin E to cell lines and the subsequent growth effects has not. A synergistic action of these two vitamins has, however been reported (103,143,167,168).

In addition to vitamin supplementation of tumour cell lines, other nutrients which can induce changes in fluidity and stability of all membranes are the essential FAs (121,184,186). In tumour cells, there may be substantially reduced quantities of n-6 EFAs (193). Supplementation with EFAs has been

shown to inhibit the growth of malignant tumours *in vitro*, enhancing EFA levels resulting in increased PG production (194). Eicosanoid production, in turn, is essential in the generation of secondary metabolites, *eg* cAMP (see Chapter 1), which control metabolic pathways including those associated with the control of cell growth.

In this study, cells were supplemented with combined vitamin E succinate and Asc and the influence of the combination of these compounds on the growth of non malignant LLCMK (monkey kidney) and malignant BL6 (murine melanoma) cell lines was monitored. In addition, the effects on cell growth combinations of vitamin E succinate, Asc and ^{3}H vitamin E succinate, ^{14}C ascorbic acid and AA, respectively were also determined since supplementation of the two cell lines with these combinations of nutrients and their derivatives was the subject of subsequent studies described in this thesis.

2.2 MATERIALS AND METHODS

MATERIALS

Basal Minimum Essential Medium (MEM) , L-serine, L-ascorbic acid (reduced form-99%),(+)-α-tocopherol acid succinate and succinic acid disodium salt were purchased from Sigma Chemical Co., USA. LLCMK (monkey kidney) and BL6 (murine melanoma) cells were obtained from Stellenbosch University, South Africa. Foetal Calf Serum (FCS) and sterile tissue culture flasks were purchased from Elabtec, Port Elizabeth, South Africa. Novo streptomycin (1g/3ml) and novo penicillin (2 million U) were purchased from Novo Industries (Pharmaceuticals Ltd), South Africa. Unilab, SAARCHEM, South Africa, supplied the $NHCO_3$ and dimethyl sulphoxide (DMSO). Glycine, D-glucose, phenol red, KH_2PO_4 and Na_2HPO_4. $2H_2O$ were purchased from BDH Chemicals Ltd, England. Trypsin was purchased from Boehringer Mannheim, Germany, while ethylenediamine-tetraacetic acid (EDTA) was purchased from Holpro Chemical Co., South Africa. L-[carboxyl-^{14}C] Ascorbic acid, [U-^{3}H] vitamin E acid succinate and 15-[^{3}H] arachidonic acid were purchased from Amersham International, England. A haemocytometer was purchased from Neubauer, Germany.

METHODS

2.2.1 PREPARATON 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 $NaHCO_3$:

0.01g/l Serine	1.68g/l $NaHCO_3$
0.006g/l Glycine	5ml/l Novo-Strep and Novopen Mixture

(1 Vial of sodium benzylpenicillin (10^6U) and one vial streptomycin sulphate (10^6 μg) were combined and then made up to 100ml with milli Q water). Ten litres of media were prepared at a time.

2.2.1.2 Filtration of the medium

The medium was filtered through a millipore filtration unit (Millipore Corporation, USA), using the following three filters: a prefilter, type AW 03 "Membr filter" 50k (size 130); a 0.45μm type HA filter (HAWP 14250); and a 0.22μm type GS filter (GSWP 14250). Initially 500ml of milli Q water was pumped through followed by 200ml of medium, both of which were discarded, while the remaining medium was sterile-filtered into autoclaved Schott bottles. The bottles of medium were incubated at 37°C for 7 days, 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. Thereafter, all medium was incubated at 37°C for 48 hours to test for contamination. 20% (v/v) FCS medium was prepared for use in the first 24 hour growth period for 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 0.33% solution. The trypsin solution contained the following:

8.0g/l	NaCl	0.2g/l	EDTA
0.4g/l	KCl	33.33mg/l	Trypsin
1.0g	D-glucose	0.02g/l	Phenol Red
0 .58g/l	$NaHCO_3$		

10ml Novo-Strep and Novopen solution (prepared as in 2.2.1.1)

The trypsin was filtered directly into the culture flask, through a 0.45μm Millipore filter using a Swinnex-25 holder. The trypsin solution was stored at -20°C until required.

2.2.1.5 Preparation of media containing vitamin E succinate and ascorbic acid

Stock solutions of vitamin E succinate, 5-20mg/ml were freshly prepared in absolute ethanol and diluted 1:1000 in media containing 10% FCS, to give final concentrations of 5, 10, 20 μg/ml vitamin E succinate respectively, in 0.1% final concentration of ethanol. Stock solutions of Asc, 25 and 50mg/ml were freshly prepared and diluted 1:1000 in media containing 10% FCS to give a final concentration of 25 and 50μg/ml, respectively.

Thus four different batches of medium were obtained containing the following combined vitamin E succinate: Asc concentrations: 5:25; 10:50; 10:25 and 20:25μg/ml. The concentrations were chosen following preliminary range-finding experiments to determine the most effective cell growth inhibitory concentrations of combined vitamin E succinate and Asc.

Vehicle controls were prepared with equivalents amounts of succinate (succinic acid) and absolute ethanol as follows: Stock solutions of succinic acid 3.78mg/ml were prepared in milli Q water and diluted 1:1000 in media containing 10% FCS and 0.1% absolute ethanol to give a final volume of 3.78μg/ml. Other concentrations of succinic acid (1.89 and 0.98μg/ml) were prepared by serial dilution from the 3.78mg/ml stock solution.

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 :

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

2.2.2 CELL CULTURE

2.2.2.1 Routine cell culture procedures

All procedures were carried out on a laminar flow bench which had previously been sterilized by exposure to ultra-violet light and all the equipment used was either purchased sterile or autoclaved. Sterile techniques employed included regular swabbing of the hands and bench surface with 70% alcohol, while all equipment was swabbed prior to use. Non-malignant LLCMK (monkey kidney) and malignant BL6 (murine melanoma) cells were maintained at 37°C in 75 cm^2 flasks containing 30ml 10% (v/v) growth media, when they were not required for experimental purposes. The medium was changed approximately once a day.

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

2.2.2.2 Freezing of cells

The storage of either cell line involved the cells being harvested with 10ml sterile trypsin and then centrifuged, and the cell pellet reconstituted with 2ml freezing medium in a cryogenic vial. The cells were stored in liquid nitrogen until required.

2.2.3 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON CELL GROWTH

2.2.3.1 Experimental cell culture procedure

Large flasks of near confluent cultures of either LLCMK or BL6 cells were trypsinised with 10ml sterile trypsin. The flasks were incubated at 37°C until the cells had detached from the flask surfaces. Once the cells had lifted off, they were poured into sterile tubes with caps and centrifuged at 3 000g (Eppendorf Centrifuge 5403, Germany) for 10 minutes. Thereafter, the trypsin solution was poured off and the pellet resuspended in 2ml 10% (v/v) FCS medium. The cells were counted using a haemocytometer. The cell counts allowed 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. In experiments relevant to 3.2.2 and 3.2.3, for which further analytical procedures and data are described in Chapter 3, (the numbers denoting the relevant experiments), 3x10^5 LLCMK or BL6 cells were seeded into 6 sets of 5 25cm^2 flasks. To each of 4 of the 6 sets of flasks was added 10ml medium, containing 10% (v/v) FCS and the varying levels of combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml). The fifth set of flasks, referred to as the control cultures (O) contained only 10ml, 10% FCS medium, while that of the sixth set received 10ml 10% FCS containing 0.1% final volume of ethanol and is referred to as the ethanol control cultures (OE). In experiments relevant to 3.5#, 4.1#, 4.2#, 4.3#, 4.4#, 4.6#, 4.7.2# and 4.7.3# for which further analytical procedures and data are described in Chapter 3 and Chapter 4 respectively (the numbers denoting the relevant experiments), 5x10^5 LLCMK or BL6 cells were seeded into 6 sets of 3 75cm^2 flasks. Exactly the same procedure was carried out as that for the 25cm^2 flasks except that 30ml medium, containing 10% (v/v) FCS was added.

2.2.3.2 Harvesting of the experimental cell cultures

When the first flask reached confluency ($\pm$ 5 days) (determined under a microscope), the cells in all the flasks were harvested under non-sterile conditions using 10ml or 5ml trypsin depending on flask size. The cell suspensions were centrifuged for 10 minutes at 3 000g. The pellet was resuspended in 2ml PBS buffer, unless otherwise stated, and enumerated using a haemocytometer. The cell counts were used as a measure of cell growth, enabling the effect of combined vitamin E succinate and Asc on cell growth to be determined. The counted cells were then used for further experimentation and analysis.

2.2.4 THE EFFECT OF COMBINED VITAMIN E SUCCINATE, ASCORBIC ACID AND ^{3}H VITAMIN E SUCCINATE SUPPLEMENTATION ON CELL GROWTH

2.2.4.1 Experimental cell culture procedure

The experimental procedure described below is relevant to experiment 3.3 in Chapter 3.

Non-malignant LLCMK or malignant BL6 murine melanoma cells were seeded into 6 sets of 3 75 cm^2 flasks, at $5x10^5$ cells/flask. Basal MEM, 30ml containing 10% FCS, and the varying combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) concentrations respectively were added to four sets of flasks. The fifth set of flasks received 30ml 10% (v/v) FCS medium containing 0.1% final concentration of ethanol, and was referred to as control cultures (0E) The sixth set of flasks contained 30ml 10% (v/v) FCS medium and was referred to as control cultures (0). The six sets of flasks were incubated at 37°C, with one media change. During the media change, 0.1μCi of ^{3}H vitamin E succinate was added to all flasks containing the varying combined vitamin concentrations and the control culture set (0E), while control cultures (0) received ^{3}H vitamin E succinate.

The flasks were once again incubated at 37°C and harvested as described in 2.2.3.2.

2.2.5 THE EFFECTS OF COMBINED VITAMIN E SUCCINATE , ASCORBIC ACID AND L-[CARBOXYL-^{14}C] ASCORBIC ACID SUPPLEMENTATION ON CELL GROWTH

2.2.5.1 Experimental cell culture procedure

The experimental procedure described below is relevant to experiment 3.4 in Chapter 3.

The method outlined in 2.2.4.1 was repeated for ^{14}C ascorbic acid supplementation of the cells with the following changes. During media change, 0.15μCi ^{14}C Asc was added to all flasks containing the varying levels of combined vitamin E succinate and Asc concentrations and the control cultures (0E). The control culture (0) was again the sixth set of flasks, with no combined vitamin concentrations or ^{14}C Asc supplementation.

The cells were harvested as described in 2.2.3.2.

2.2.6 THE EFFECT OF COMBINED VITAMIN E SUCCINATE, ASCORBIC ACID AND ^{3}H ARACHIDONIC ACID SUPPLEMENTATION ON CELL GROWTH

2.2.6.1 Experimental cell culture procedure

The above experimental procedure is relevant to experiment 4.3 in Chapter 4.

Non-malignant LLCMK or malignant BL6 murine melanoma cells were seeded into six sets of 3 75cm^2 flasks, at 5x10^5 cells/flask. Basal MEM, 30ml containing 10% FCS and the varying levels of combined vitamin E succinate and Asc concentrations (5:25; 10:50; 10:25 and 20:25μg/ml) respectively were added to four sets of flasks. The fifth set of flasks received 30ml of 10% FCS medium containing 0.1% final concentration of ethanol and was referred to as control cultures (0E), while the sixth set of flasks contained 30ml 10% (v/v) FCS medium and was referred to as control cultures (O). The sixth set of flasks were incubated at 37°C, with one media change. During the media change, 5μCi 15-^{3}H AA was added to all flasks containing the varying combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) concentrations respectively, and the control cultures (0E). The control set (O) was again the sixth set, to which no combined vitamins or 15-^{3}H AA was supplemented.

The above methods were repeated for AA supplementation, with the following changes. During the media change, 2.5μM AA was added to all flasks containing the varying combined vitamin E succinate and Asc concentrations, and the control set of cultures (0E). The control set (0) was once again the sixth set of flasks, with no combined vitamin or AA supplementation.

The cells were harvested as described in 2.2.3.2.

2.2.7 STATISTICAL ANALYSIS

The results obtained were analysed 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 COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON LLCMK AND BL6 CELL GROWTH

Cell growth of LLCMK and BL6 cells found in the relevant experiments in which combined 5:25; 10:50; 10:25 and 20:25μg/ml vitamin E succinate and Asc supplementation, respectively, was performed are presented in Table 2a and Table 2b. Relevant to the discussion in this chapter, for both cell types, is the mean percentage of growth inhibition relative to untreated controls that was obtained in all the experiments. Growth inhibition for each individual experiment will be discussed in Chapters 3 and 4 with respect to the relevant data obtained on cell metabolism in these experiments.

The overall mean growth inhibition, recorded in Table 2a, indicates that combined vitamin E succinate and Asc did not have a significant inhibitory or stimulatory effect on LLCMK cell growth. Combined vitamin E succinate and Asc supplementation at 5:25 and 10:25μg/ml resulted in slight increases in cell growth relative to that of the control cultures (0E), however not significantly so. At 10:50 and 20:25μg/ml combined vitamin E succinate and Asc supplementation, cell growth decreased compared with control cultures (0E), although again no significance was found.

Supplementation of BL6 cells with combined vitamin E succinate and Asc resulted in an overall significant ($p \leq 0.001$) decrease in cell growth when compared to control cultures (0E) (Table 2b). When comparing 20:25μg/ml combined vitamin E succinate and Asc to 5:25μg/ml ($p \leq 0.001$), 10:50μg/ml ($p \leq 0.01$) and 10:25μg/ml ($p \leq 0.001$) a significant decrease in cell growth was again found. Thus, the most significant decrease in cell proliferation (Table 2b) was observed at combined 20:25μg/ml vitamin E succinate and Asc supplementation relative to control cultures (0E) and the other combined vitamin concentration groups.

TABLE 2a : The effect of combined vitamin E succinate and ascorbic acid supplementation on LLCMK cell growth. Results are the mean of five* or three cultures ± SEM.

[vitamin E succinate : ascorbate] μg/ml	LLCMK CELLS									
	Growth inhibition [% of untreated controls]									
	Exp 3.2.2*	Exp 3.2.3*	Exp 3.5	Exp 4.1	Exp 4.2	Exp 4.4	Exp 4.5	Exp 4.6	Exp 4.8	Mean
0E	100 ±4.81	100 ±3.7	100 ±12.2	100 ±6.5	100 ±6.9	100 ±22.1	100 ±3.5	100 ±1.7	100 ±2.7	100 ±7.12
5:25	100.8 ±2.0	99.9 ±1.2	73.5 ±7.6	95.2 ±7.7	274.3[b] ±10.6	201.5[a] ±10.6	77.0[a] ±0.5	121.8 ±5.6	65.1[a] ±5.3	123.2 ±23.2
10:50	87.8 ±14.1	87.5 ±11.2	70.0 ±13.0	104.5 ±7.5	77.9 ±12.4	82.7 ±2.7	31.7[a] ±1.2	121.8 ±4.3	34.4[a] ±1.4	77.6 ±9.8
10:25	97.7 ±1.2	93.5 ±2.2	68.9 ±6.4	107.4 ±4.9	235.2[b] ±9.2	146.8[c] ±4.9	54.5[a] ±3.6	141.2[a] ±7.0	43.9[a] ±5.8	109.9 ±19.6
20:25	86.8 ±3.3	85.4 ±2.7	78.5 ±7.3	98.49 ±8.1	90.2 ±14.0	94.7 ±1.3	33.6[a] ±2.9	87.33 ±16.6	25.1[a] ±4.1	75.6 ±9.0

a - $p \leq 0.001$ relative to control cultures (0E)
b - $p \leq 0.01$ relative to control cultures (0E)
c - $p \leq 0.05$ relative to control cultures (0E)

TABLE 2b : The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth. Results are the mean of five* or three cultures ± SEM.

[vitamin E succinate : ascorbate] µg/ml	BL6 CELLS											
	Growth inhibition [% of untreated controls]											
	Exp 3.2.2*	Exp 3.2.3*	Exp 3.5	Exp 4.1	Exp 4.2	Exp 4.4	Exp 4.5	Exp 4.6	Exp 4.7.2	Exp 4.7.3	Exp 4.8	Mean
0E	100 ±2.2	100 ±1.2	100 ±10.5	100 ±7.9	100 ±0.8	100 ±1.7	100 ±5.8	100 ±3.9	100 ±5.6	100 ±4.2	100 ±3.4	100 4.5
5:25	85.1[b] ±3.3	88.0[b] ±3.2	87.1[b] ±3.2	105.6 ±5.1	49.5[a] ±3.8	25.8[a] ±1.3	76.4[b] ±2.6	81.3[b] ±1.7	85.6[c] ±4.1	107.9 ±5.2	77.0[b] ±4.8	79.0[b] ±7.1
10:50	64.6[a] ±5.5	66.0[a] ±4.8	60.8[a] ±2.6	70.7[b] ±3.1	12.1[a] ±1.5	17.5[b] ±0.3	48.3[a] ±3.0	74.6[b] ±3.6	81.7[b] ±3.2	87.7 ±1.4	52.5[a] ±6.4	57.5[a] ±7.3
10:25	67.2[a] ±0.5	69.8[a] ±1.2	68.0[a] ±2.5	81.4[c] ±5.4	40.7[a] ±2.2	26.7[a] ±1.9	45.2[a] ±6.4	130.6[a] ±1.8	76.8[b] ±3.1	90.3 ±4.5	51.2[a] ±4.0	68.0[b] ±8.5
20:25	13.9[a] ±0.8	15.9[a] ±1.1	30.2[a] ±1.7	63.7[a] ±6.1	3.6[a] ±0.2	1.6[a] ±6.1	24.49[a] ±3.0	15.13[a] ±0.4	57.1[a] ±2.6	69.6[a] ±1.6	20.5[a] ±2.2	28.7[a] ±7.2

a - $p \leq 0.001$ relative to control cultures (0E)
b - $p \leq 0.01$ relative to control cultures (0E)
c - $p \leq 0.05$ relative to control cultures (0E)

TABLE 3a : The effect of vehicle treatments on LLCMK cell growth. Results are the mean of five* or three cultures ± SEM.

Treatments[a]	LLCMK CELLS									
	Growth inhibition [% of untreated controls]									
	Exp 3.2.2*	Exp 3.2.3*	Exp 3.5	Exp 4.1	Exp 4.2	Exp 4.4	Exp 4.5	Exp 4.6	Exp 4.8	Mean
0	100 ±5.1	100 ±0.2	100 ±2.1	100 ±3.7	100 ±3.7	100 ±8.2	100 ±25.2	100 ±2.2	100 ±1.3	100 ±5.7
0E	99.0 ±9.6	105.9 ±8.6	99.6 ±3.2	102.9 ±2.2	90.8 ±6.7	101.9 ±3.2	96.3 ±6.9	102.9 ±2.2	100.6 ±15.2	100.5 ±1.4

a - cells cultured in the presence (0E) or absence (0) of ethanol

TABLE 3b : The effect vehicle treatments on BL6 cell growth. Results are the mean of five* or three cultures ± SEM.

Treatments[a]	BL6 CELLS											
	Growth inhibition [% of untreated controls]											
	Exp 3.2.2*	Exp 3.2.3*	Exp 3.5	Exp 4.1	Exp 4.2	Exp 4.4	Exp 4.5	Exp 4.6	Exp 4.7.2	Exp 4.7.3	Exp 4.8	Mean
0	100 ±7.0	100 ±23.0	100 ±7.3	100 ±6.3	100 ±15.1	100 ±11.0	100 ±8.3	100 ±17.1	100 ±5.6	100 ±3.7	100 ±3.4	100 ±9.8
0E	103.3 ±7.9	88.7 ±22.7	98.4 ±4.7	92.6 ±7.2	110.4 ±7.6	101.5 ±36.4	100 ±2.7	101.7 ±1.9	96.3 ±3.6	137.4 ±2.7	99.1 ±3.9	102.7 ±3.9

a - cells cultured in presence (0E) or absence (0) of ethanol

Tables 3a, 3b and 4 represent the control experiments using the solvent ethanol and succinic acid.

A control experiment using the solvent of vitamin E succinate (absolute ethanol) was done to determine the effect of ethanol on the parameters measured in this study. In both the LLCMK (Table 3a) and BL6 cells (Table 3b), no trend of increased or decreased cell growth was observed when compared with control cultures (0).

A further control experiment (Table 4) was set up with the equivalent concentrations of succinic acid as those present in the vitamin E succinate supplemented cultures and an equivalent amount of ethanol. Supplementation of equivalent amounts of succinic acid and ethanol as present in the vitamin E succinate supplemented cultures did not have a significant inhibitory or stimulatory effect on LLCMK cell growth. A general trend of decreased LLCMK cell growth is however apparent. In BL6 cells, supplementation of succinic acid and ethanol equivalents resulted in no significant decreasing or increasing trend of cell growth.

TABLE 4: The effect of succinic acid and vehicle treatments on LLCMK and BL6 cells, respectively. Results are the mean of five cultures ± SEM.

[Succinic acid] μg/ml	[Ethanol] %	Growth Inhibition [% of untreated controls]	
		LLCMK CELLS	BL6 CELLS
0[a]	0.1	100±7.80	100±9.79
0.89	0.1	100.62±3.13	86.19±3.66
1.89	0.1	85.24±5.29	87.26±5.20
3.78	0.1	86.29±4.30	105.76±6.36

a - cells cultured in the presence of vehicle control

2.3.2 THE EFFECT OF COMBINED VITAMIN E SUCCINATE, ASCORBIC ACID AND ^{3}H VITAMIN E SUCCINATE SUPPLEMENTATION ON CELL GROWTH

A slight decrease (non significant) in cell growth was found in control cultures (0E) of both the LLCMK and BL6 cells, when compared with that of control cultures (0) (Table 5), although not significantly so.

TABLE 5: The effect of vehicle treatment on LLCMK and BL6 cell growth. Results are the mean of three cultures ± SEM.

Treatment	Growth Inhibition [% of untreated controls]	
	LLCMK CELLS	BL6 CELLS
0	100±7.12	100±3.75
0E*	96.35±14.05	94.43±6.31

* - control cultures containing 0.1% ethanol and ^{3}H vitamin E succinate

TABLE 6: The effect of combined vitamin E succinate, ascorbic acid and ^{3}H vitamin E succinate supplementation on cell growth. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	[Ethanol] %	[^{3}H] Vitamin E succinate μCi	Growth Inhibition [% of untreated controls]	
			LLCMK	BL6
0	0.1	0.1	100 ±5.9	100 ±2.5
5:25	0.1	0.1	50.0[a] ±7.3	15.5[a] ±0.3
10:50	0.1	0.1	64.5[b] ±9.8	11.7[a] ±1.2
10:25	0.1	0.1	60.1[b] ±7.3	24.2[a] ±2.1
20:25	0.1	0.1	49.4[a] ±5.2	18.2[a] ±0.7

a - $p \leq 0.001$ relative to respective control cultures (0E)
b - $p \leq 0.01$ relative to respective control cultures (0E)

Supplementation of combined vitamin E succinate and Asc (5:25; 10:50; 10:25 and 20:25μg/ml), and 0.1μCi ^{3}H vitamin E succinate (Table 6) resulted in a trend of decreased cell growth for both cell types. The decrease in LLCMK cell growth recorded was significant at 5:25 ($p \leq 0.001$); 10:50 ($p \leq 0.01$); 10:25 ($p \leq 0.01$) and 20:25μg/ml ($p \leq 0.001$) when compared with control cultures (0E). A similar but much more marked inhibitory effect was observed in the BL6 cells. The decreased cell growth recorded was significant ($p \leq 0.001$) for each combined vitamin concentration group.

2.3.3 THE EFFECT OF COMBINED VITAMIN E SUCCINATE, ASCORBIC ACID AND ^{14}C- ASCORBIC ACID SUPPLEMENTATION ON CELL GROWTH

Cell growth in control cultures (0E) of both LLCMK and BL6 (Table 7), was slightly increased in comparison to control cultures (0), although no significance was recorded.

TABLE 7: The effect of vehicle treatment on LLCMK and BL6 cell growth. Results are the mean of three cultures ± SEM.

Treatment	Growth Inhibition [% of untreated controls]	
	LLCMK	BL6
0	100±6.78	100±2.31
0E*	107.98±7.13	101.25±5.67

* - control cultures containing 0.1% ethanol and ^{14}C ascorbic acid

Supplementation of combined vitamin E succinate and Asc (5:25; 10:50; 10:25 and 20:25μg/ml) together with 0.15μCi ^{14}C Asc resulted a general trend of decreased LLCMK cell growth with a significant ($p \leq 0.01$) decrease at 5:25μg/ml vitamin E succinate and Asc (Table 8). On the other hand, BL6 cells (Table 8) supplemented with varying levels of combined vitamin E succinate and Asc, together with 0.15μCi Asc, showed a marked decrease in cell growth at each combined vitamin concentration with significant ($p \leq 0.001$) decreases at 10:50; 10:25 and 20:25μg/ml combined vitamin E succinate and Asc respectively.

TABLE 8: The effect of combined vitamin E succinate, ascorbic acid and ^{14}C ascorbic acid supplementation on cell growth. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] $\mu g/ml$	[Ethanol] %	^{14}C ascorbic acid μCi	Growth Inhibition [% of untreated controls]	
			LLCMK	BL6
0	0.1	0.15	100 ±4.2	100 ±3.0
5:25	0.1	0.15	71.4[a] ±7.1	75.0 ±5.8
10:50	0.1	0.15	80.6 ±5.2	53.3[b] ±1.2
10:25	0.1	0.15	77.0 ±5.2	58.2[b] ±4.0
20:25	0.1	0.15	81.1 ±6.1	28.2[b] ±0.3

a - $p \leq 0.01$ relative to respective control cultures (0E)
b - $p \leq 0.001$ relative to respective control cultures (0E)

2.3.4 THE EFFECT OF COMBINED VITAMIN E SUCCINATE, ASCORBIC ACID AND ^{3}H-ARACHIDONIC ACID SUPPLEMENTATION ON CELL GROWTH

Combined vitamin E succinate and Asc supplementation together with 5μCi ^{3}H AA resulted in an overall increase in cell growth of LLCMK cells, (Table 9). This increase in cell growth was significant at 5:25 ($p \leq 0.001$); 10:50 ($p \leq 0.001$); 10:25 ($p \leq 0.01$) and 20:25μg/ml ($p \leq 0.05$) respectively. On the other hand, combined vitamin E succinate and Asc together with 2.5μCi AA supplementation resulted in an overall decrease in cell growth of LLCMK cells (Table 10), although not significantly so. Combined vitamin E succinate and Asc together with AA (radioactive and non-radioactive) resulted in an overall significant ($p \leq 0.001$) decrease in cell growth of BL6 cells (Tables 9 and 10). Supplementation of AA (radioactive and non-radioactive) to control cultures did not have any effect on cell growth (Tables 11 and 12).

TABLE 9: The effect of combined vitamin E succinate, ascorbic acid and ^{3}H arachidonic acid supplementation on cell growth. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	[Ethanol] %	15-^{3}H AA μCi	Growth Inhibition [% of untreated controls]	
			LLCMK	BL6
0	0.1	5	100 ±5.4	100 ±8.2
5:25	0.1	5	154.1[a] ±8.7	73.0[c] ±12.7
10:50	0.1	5	140.4[a] ±5.5	33.8[a] ±2.2
10:25	0.1	5	131.6[b] ±5.5	34.6[a] ±3.1
20:25	0.1	5	122.8[c] ±5.6	28.1[a] ±3.1

a - $p \leq 0.001$ relative to respective control cultures (0E)
b - $p \leq 0.01$ relative to respective control cultures (0E)
c - $p \leq 0.05$ relative to respective control cultures (0E)

TABLE 10: The effect of combined vitamin E succinate, ascorbic acid and arachidonic acid supplementation on cell growth. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	[Ethanol] %	[AA] μM	Growth Inhibition [% of untreated controls]	
			LLCMK	BL6
0	0.1	2.5	100 ±4.3	100 ±0.8
5:25	0.1	2.5	87.5 ±4.5	49.3[a] ±2.2
10:50	0.1	2.5	85.4 ±1.9	44.0[a] ±0.2
10:25	0.1	2.5	86.3 ±3.7	28.1[a] ±0.3
20:25	0.1	2.5	85.28[c] ±3.3	24.1[a] ±1.5

a - $p \leq 0.001$ relative to respective control cultures (0E)

TABLE 11: The effect of ^{3}H arachidonic acid and vehicle treatment on LLCMK and BL6 cell growth. Results are the mean of three cultures ± SEM.

Treatment	Growth Inhibition [% of untreated controls]	
	LLCMK CELLS	BL6 CELLS
0	103.7±6.0	105.7±4.0
0E°	106.3±2.5	104.6±3.8
0E*	100±5.4	100±8.2

o - cultures containing 0.1% ethanol
* - control cultures containing 0.1% ethanol and 5μCi ^{3}H- AA

TABLE 12: The effect of arachidonic acid and vehicle treatment on LLCMK and BL6 cell growth. Results are the mean of three cultures ± SEM.

Treatment	Growth Inhibition [% of untreated controls]	
	LLCMK CELLS	BL6 CELLS
0	103.7±2.2	101.7±3.2
0E°	105.7±2.1	104.0±1.0
0E*	100±4.3	100±0.8

o - cultures containing 0.1% ethanol
* - control cultures containing 0.1% ethanol and 2.5μCi AA

2.4 DISCUSSION

Vitamin E supplementation has been shown to have an inhibitory effect on the growth of numerous cell lines *in vitro* (49,91,92,94,96,97,477), while vitamin C has been reported to have both an inhibitory and a stimulatory effect on the growth of numerous cell lines in culture (64,69,76,478-480).

Vitamin E succinate is the most potent form of vitamin E *in vitro* and is believed by Prasad and Rama (49) to be effective in a very narrow concentration range. A concentration of 6μg/ml was found to produce a more marked decrease in cell growth than that produced by a concentration of 5μg/ml in melanoma cells (49). A concentration of 10μg/ml vitamin E succinate supplemented to melanoma cells was lethal. These findings were substantiated by studies by Prasad and Edwards-Prasad (94), Rama and Prasad (92) and Kline *et al* (97). The growth inhibitory effect of vitamin E succinate on mouse melanoma in culture was found to be primarily irreversible (94). In summary, from the above studies, 10μg/ml vitamin E succinate was reported as being the most cytotoxic concentration when supplemented to various cell lines in culture. Furthermore, vitamin E succinate is known to induce

morphological changes in melanoma cells. At high concentrations (10μg/ml) of vitamin E succinate, cells become round and granulated, which is attributed to the toxicity of the vitamin (92).

The effect of Asc supplementation on cell growth appears to depend on the cell type and the level of Asc supplementation. Since melanoma cells have been found to preferentially incorporate Asc, *in vitro* studies have shown that Asc is more toxic to melanotic cells than any others studied (59). The growth of BL6 cell melanoma *in vitro* (481,482) and leukaemic (61) cells was shown to be inhibited at Asc concentrations ranging from 25-200μg/ml, with marked decreases occurring at 50-200μg/ml. On the other hand, it has been reported that non-malignant, osteoblast-like MC 3T3-E1 cells resulted in enhanced proliferation following Asc supplementation (42), while that of Chinese hamster ovary cell proliferation (472) was inhibited by Asc. Interestingly, Asc supplementation of up to 200μg/ml is considered not to cause any morphological changes in mouse melanoma (B-16), mouse neuroblastoma (NBP_2), rat glioma (C-6) and mouse fibroblasts (L cells) in culture (49). However, at higher concentrations (500-1000μg/ml) supplementation of Asc was lethal.

Since the effect of combined vitamin E succinate and Asc supplementation of malignant BL6 murine melanoma on cell growth has not been reported, it was important to determine effective inhibitory concentrations of combined vitamin E succinate and Asc supplementation on BL6 cell growth. With reference to these inhibitory concentrations of vitamin E succinate and Asc, preliminary range finding experiments were set up. Results from these studies showed that the most effective cell growth inhibitory levels of combined vitamin E succinate and Asc were as follows: 5:25; 10:50; 10:25 and 20:25μg/ml respectively.

Since studies by Ottino and Duncan (483) on BL6 cell growth following vitamin E succinate supplementation resulted in marked decreases in cell growth, it may be expected that a more pronounced inhibitory effect on cell growth of these cells following combined vitamin E succinate and Asc since an Asc regenerating capacity of vitamin E (see Section 1.4) could be expected.

Results from these studies showed that supplementation of combined vitamin E succinate and Asc in LLCMK cells resulted in no increasing or decreasing trend in cell growth. However, supplementation of combined vitamin E succinate and Asc of BL6 cells resulted in a decreasing trend in cell growth. The decrease in cell growth of BL6 cells was significant ($p \leq 0.001$) at all combined concentrations (5:25; 10:50; 10:25 and 20:25μg/ml). Furthermore, as expected, it was found that the concentration of the vitamin E succinate relative to Asc was important for maximum inhibition of BL6 cell growth.

When the concentration of vitamin E succinate relative to Asc was increased, a significant inhibitory effect on cell growth was observed. This suggests that the ratio of vitamin E succinate to Asc is a major determining factor for the effectiveness of these vitamins in decreasing cell growth. The reason for this is that vitamin E is known to be inhibitory (49,91,92,94,96,477) to growth of various cell lines, and the regenerative capacity of vitamin C could in turn enhance the antiproliferative effect of vitamin E.

Supplementation of ^{3}H vitamin E succinate and ^{14}C ascorbic acid respectively, to combined vitamin E succinate and Asc supplemented LLCMK and BL6 cells resulted in a significant decreasing trend in cell growth for both cell types. Supplementation of BL6 cultures with combined vitamin E succinate and Asc together with AA (radioactive and non-radioactive), again resulted in significant decreases in cell growth. As the supplemented levels of AA remained constant, it is reasonable to conclude that this inhibitory effect on cell growth was due to the combined vitamin E succinate and Asc supplementation.

Control experiments (Tables 3a, 3b and 4) of equivalent amounts of succinic acid and ethanol were set up to determine the effect of the vehicle, ethanol, and succinic acid on the parameters measured in this study. None were found to have any effect on the growth of BL6 cells. These findings suggest that the growth inhibitory effects observed in BL6 cell are in fact due to combined vitamin E succinate and Asc and not the vehicle or succinic acid. The findings in this study on these controls were supported by studies of Prasad and Edwards-Prasad (94) and Kline *et al* (97).

CHAPTER 3

THE EFFECT OF COMBINED NUTRIENT SUPPLEMENTATION ON THE UPTAKE AND METABOLISM OF THE NUTRIENTS AND ON THE OXIDATION STATES IN CELLS

3.1. INTRODUCTION

Oxygen, a vital element for the life of a cell, is also the source of active species of oxygen which can disrupt cell structure and alter cell function (3). Substantial evidence indicates that the active oxygen species formed, particularly from molecular oxygen, may play a role in the tumour promotion process of multistage carcinogenesis (3,10,11).

Reactive oxygen species (ROS), which include superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, organic peroxide radicals, and singlet molecular oxygen are constantly being generated intracellularly in organisms (484). The aerobic organisms are protected against this oxidative damage by an array of defence mechanisms, which under normal conditions appear to provide adequate protection for the cell membrane. Cellular defences against these transient but damaging species can be grouped under enzymic and non-enzymic mechanisms. These are represented by the protective enzymes SOD, glutathione peroxidase and catalase, and non-enzymic antioxidants such as Asc, β-carotene and α-TOH (17,210). However, the possibility exists that in certain circumstances these defences become defective or even completely overwhelmed, resulting in free radicals and active oxygens attacking lipids, proteins, sugars, CHO, and in DNA inducing oxidation, cleavage, cross-linking and modifications which eventually leads to extensive damage (52-54,210).

Tumour promoters are known to modulate cellular antioxidant defense mechanisms (10), resulting in tumour cells exhibiting decreased enzymatic antioxidant activity (123,485-487). This phenomenon leads to tumour cells accumulating free radicals and lipid peroxides. Furthermore, some human patients have exhibited reduced antioxidant levels and corresponding increased lipid peroxidation-MDA levels which are known to have mutagenic properties (188).

Numerous studies on the relationship between serum vitamin E and cancer incidence have suggested an inverse relationship between the levels of vitamin E and risk of cancer development

(110,146,155,488). A similar relationship has been suggested for that of Asc (43,71). As a consequence, one would expect tumour cells to have reduced free radical and lipid peroxide levels (123,130). Results from studies in Section 2.3.1 have shown that combined vitamin E succinate and Asc supplementation significantly reduced cell growth in BL6 cells. The actual mechanism by which vitamin E succinate inhibits growth is at present unknown (97), although studies by Prasad and Rama (92) have suggested that the inhibitory effects of vitamin E succinate on growth are mediated in part by its antioxidant properties. Vitamin C is reported to regenerate TOH from the tocopheroxyl radical thus restoring the antioxidant ability of the fat-soluble vitamin E (36). This would suggest an enhanced inhibitory effect on BL6 cell growth following combined vitamin E succinate and Asc. In nature, the antioxidant α-TOH is found as free (unesterified) α-TOH (489), while vitamin E esters are non-physiological antioxidants which require esterase activity in order to liberate the free alcohol, α-TOH, with antioxidant properties (93,97,490,491). As yet, no species specific hydrolysis of vitamin E succinate has been demonstrated, however it has been suggested that numerous other ester forms encounter esterases in association with other hydrophobic membranes and other membranous structures present in most cells (490). Uptake mechanisms of α-TOH across plasma membranes are presently undefined (107), while the tocopheryl esters, are absorbed into the cell, also by unknown transfer mechanisms (490,492). Concerning the uptake of Asc by cells, it appears that more than one transport mechanism is operative, although the hydrophilic nature of vitamin C suggests that a carrier mechanism is necessary for Asc entry into the cell (34).

To determine whether the inhibition of BL6 cell growth is the result of a synergistic antioxidant effect of combined vitamin E succinate and Asc, experiments were carried out to examine the effect of combined vitamin supplementation on free radical and lipid peroxidation levels in LLCMK and BL6 cells. In addition, relative uptake of the vitamin E succinate and Asc, as well as the metabolism of vitamin E succinate was determined.

3.2 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON FREE RADICAL AND LIPID PEROXIDATION FORMATION

3.2.1 MATERIALS

2-Thiobarbituric acid (98%) (TBA), Nitro Blue Diformazan (NBD), Nitro Blue Tetrazolium (NBT) and Butylated Hydroxytoluene (BHT) were purchased from Sigma Chemical Co., USA. 1,1,3,3-Tetramethoxypropane (98%)[malondialdehyde-tetramethyl acetal] was obtained from Fluka,

Switzerland. Trichloroacetic acid (98%) (TCA), butanol and glacial acetic acid (99.5%) were purchased from Holpro Chemical Co., South Africa.

3.2.2 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON FREE RADICAL FORMATION

METHODS

3.2.2.1 Cell culture procedures

Refer to 2.2.3.1 and 2.2.3.2. The cell suspensions were centrifuged at 3 000g for 10 minutes. The cellular pellets were resuspended in 1.0ml of PBS, pH 7.4, for the free radical determination.

3.2.2.2 Free radical formation

The principle of this assay is based on the reduction of NBT by the superoxide anion and other free radical species to insoluble diformazan. Free radical formation was determined using a modified NBT-assay as described by Sagar *et al* (493) and Das *et al* (494).

To 1ml cell suspensions, 0.4ml of a 0.1% NBT solution in PBS, pH 7.4 was added and allowed to incubate for 2 hours at 37°C. The reaction was then terminated by the addition of 0.6ml glacial acetic acid, and the relative absorbance values measured at 560nm. The amount of NBD formed was determined from a standard curve generated from NBD as shown in Appendix 1. Final results were expressed as nmoles diformazan/10^4 cells.

3.2.3 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON LIPID PEROXIDATION FORMATION

METHODS

3.2.3.1 Cell culture procedures for lipid peroxidation experiment

Refer to 2.2.3.1 and 2.2.3.2. The cell suspensions were centrifuged at 300g for 10 minutes. The cellular pellets were resuspended in 1.0ml of 0.9% NaCl for lipid peroxidation determination.

3.2.3.2 Lipid peroxidation determination

Lipid peroxidation was determined using the thiobarbituric acid (TBA) assay. The principle of this assay involves the reaction of MDA equivalents with TBA to yield a pink complex. A modified method of Sagar *et al* (493) and Drapper *et al* (495) was used in this assay.

To 1ml cell suspensions, a 0.5ml aliquot of BHT (0.5g/l) was added followed by the addition of 1ml of 25% trichloroacetic acid (TCA). The samples were centrifuged at 2 000g for 20 minutes at 4°C, to remove insoluble proteins. Following centrifugation, 2ml of protein-free supernate was removed from each tube and a 0.5ml aliquot of 0.33% TBA was added to this fraction. All tubes were boiled for 1 hour at 95°C in a water bath. After cooling the TBA-MDA complexes were extracted with 2ml butanol. The absorbance was read at 532nm and MDA levels determined from a standard curve generated from 1,1,3,3-Tetramethoxypropane as shown in Appendix 2. Final results were represented as nmoles MDA/10^4 cells.

3.2.4 STATISTICAL ANALYSIS

The results were analysed 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 analysed.

3.2.5 RESULTS

The effect of combined vitamin E succinate and Asc supplementation on the formation of free radicals

and lipid peroxidation is shown in Table 13.

Supplementation of combined vitamin E succinate and Asc to LLCMK cells resulted in a non significant decrease in free radical levels for the combined vitamin concentrations tested (Table 13). In BL6 cells, however, supplementation of varying levels of combined vitamin E succinate and Asc respectively, resulted in a general increase in free radical levels with a significant ($p \leq 0.05$) increase occurring at 20:25μg/ml. Comparing the levels of free radicals in LLCMK and BL6 control cultures (0E), a lower free radical level was observed in BL6 cells, however not significantly so.

TABLE 13: The effect of combined vitamin E succinate and ascorbic acid supplementation on free radical and lipid peroxidation levels in LLCMK and BL6 cells respectively. Results are the mean of five cultures ± SEM.

[vitamin E [succinate:ascorbate] μg/ml	LLCMK CELLS		BL6 CELLS	
	Free radical formation	**Lipid peroxidation**	**Free radical formation**	**Lipid peroxidation**
	nmoles Diformazan/ 10^4 cells	**nmoles MDA/ 10^4 cells**	**nmoles Diformazan/ 10^4 cells**	**nmoles MDA/ 10^4 cells**
0E	0.058 ±0.005	0.820[e] ±0.031	0.032 ±0.002	0.423 ±0.132
5:25	0.044 ±0.002	0.581 ±0.011	0.047 ±0.004	0.412 ±0.025
10:50	0.053 ±0.008	5.643[a] ±0.191	0.043 ±0.003	3.662[c] ±0.537
10:25	0.045 ±0.002	0.646 ±0.045	0.043 ±0.003	5.328[c] ±0.341
20:25	0.041 ±0.002	6.763[a] ±0.322	0.051[b] ±0.002	29.063[d] ±2.814

a - $p \leq 0.001$ relative to LLCMK lipid peroxidation control cultures (0E)
b - $p \leq 0.05$ relative to BL6 free radical control cultures (0E)
c - $p \leq 0.05$ relative to BL6 lipid peroxidation control cultures (0E)
d - $p \leq 0.001$ relative to BL6 lipid peroxidation control cultures (0E)
e - $p \leq 0.05$ relative to control BL6 MDA levels (0E)

Supplementation of LLCMK cells (Table 13) with combined vitamin E succinate and Asc resulted in no general increasing or decreasing trend in lipid peroxidation levels. Significant increases were,

however, detected at 10:50μg/ml ($p \leq 0.001$) and 20:25μg/ml ($p \leq 0.001$) combined vitamin E succinate and Asc, respectively. In BL6 cells, combined vitamin E succinate and Asc supplementation resulted in an overall increasing trend in lipid peroxidation levels with significant increases occurring at 10:25μg/ml ($p \leq 0.05$) and 20:25μg/ml ($p \leq 0.001$) respectively, when compared to control cultures (0E). From Table 13 it is evident that basal MDA levels in LLCMK cells were significantly ($p \leq 0.05$) higher in control cultures (0E) than in BL6 control cultures (0E).

In general, when comparing free radical formation and lipid peroxidation levels in LLCMK and BL6 cells, it was evident that lipid peroxidation levels were higher in BL6 than in LLCMK cells overall following combined vitamin supplementation at higher levels of the two vitamins, while when comparing free radical levels, no substantial difference in the levels was detected.

3.3 EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON [U ^{3}H] VITAMIN E SUCCINATE UPTAKE

3.3.1 MATERIALS AND METHODS

MATERIALS

[U ^{3}H] Vitamin E succinate [1mCi/m] was obtained from Amersham, Int., England. Emulsifier scintillator plus™ scintillation cocktail was purchased from Packard Company, USA.

METHODS

3.3.1.1 Cell culture procedures

Refer to sections 2.2.3.1 and 2.2.3.2.

3.3.1.2 Homogenization of cells and separation of cellular components

The cell suspensions were poured into a Dounce homogenizer and homogenized 30 times with the tight plunger. The homogenizer was rinsed with 1ml PBS solution, pH 7.4. The various homogenates were then separated into membrane and stroma fractions by differential centrifugation. Cell homogenates were centrifuged (Beckman Model J2-21 Centrifuge) at 480g for 20 minutes, to remove

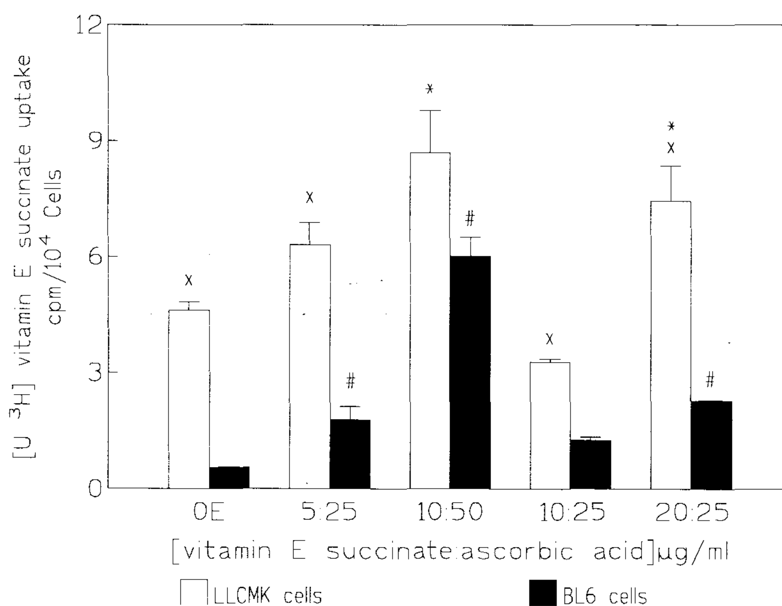
nuclei and non-disrupted cells. The supernatant was retained and centrifuged at 4 000g for 20 minutes to remove the mitochondrial fraction. Once again, the supernatant was retained and centrifuged at 20 000g for 30 minutes, in order to obtain the respective membrane (pellet) and stroma (supernatant) fractions of the homogenized cells. The pellet was resuspended in 2ml PBS solution.

3.3.1.3 Determination of radioactivity in membrane and stroma fractions

Duplicate 1ml aliquots of membrane and stroma fractions were removed and added to scintillation vials containing 10ml of scintillation cocktail. The radioactivity in each membrane and stroma fraction was determined by counting in a Beckman (LS 2800) scintillation counter.

3.3.2 RESULTS

The total uptake of ^{3}H vitamin E succinate by LLCMK and BL6 cells is presented in Figure 18.



x - ($p \leq 0.001$ - $p \leq 0.01$) relative to corresponding BL6 cells
* - ($p \leq 0.01$ - $p \leq 0.05$) relative to LLCMK control cultures (0E)
# - ($p \leq 0.001$ - $p \leq 0.05$) relative to BL6 control cultures (0E)

Figure 18: The effect of combined vitamin E succinate and ascorbic acid supplementation on the uptake of ^{3}H vitamin E succinate by both LLCMK and BL6 cells. The bars are the mean of three cultures $\pm$ SEM.

^3H vitamin E succinate uptake was significantly ($p \leq 0.001$) higher in the control cultures (0E) of LLCMK relative to the uptake of ^3H vitamin E succinate in the control cultures (0E) of BL6 cells. Furthermore, ^3H Depvitamin E succinate uptake was significantly higher in the LLCMK cells supplemented with combined vitamin E succinate and Asc 5:25 ($p \leq 0.001$); 10:25 ($p \leq 0.01$) and 20:25μg/ml ($p \leq 0.001$) relative to the corresponding combined vitamin concentrations in BL6 cells.

Supplementation of LLCMK cells with combined vitamin E succinate and Asc together with ^3H vitamin E succinate resulted in decreased although non significant uptake of ^3H vitamin E succinate at 10:25μg/ml, with a slight increase in uptake at 5:25μg/ml, compared to the relevant (0E) control cultures. However, a significantly increased uptake of ^3H vitamin E succinate at 10:50 ($p \leq 0.01$) and 20:25μg/ml ($p \leq 0.05$) was observed following combined vitamin supplementation relative to control cultures (0E).

Supplementation of BL6 cells (Figure 18) with combined vitamin E succinate and Asc together with [^3H] vitamin E succinate resulted in a general increase in ^3H vitamin E succinate uptake which was significant at 5:25 ($p \leq 0.05$); 10:50 ($p \leq 0.001$) and 20:25μg/ml ($p \leq 0.01$) respectively, relative to control cultures (0E). Studies in our laboratory (personal communications) showed that ^3H vitamin E succinate uptake increased with increasing concentrations of vitamin E succinate supplementation on its own, however no difference in ^3H vitamin E succinate uptake beyond 5-10μg/ml vitamin E succinate is observed. As expected ^3H vitamin E succinate uptake in BL6 cells in this study increased with increasing concentrations of vitamin E succinate, however at 10:25 and 20:25μg/ml combined vitamin E succinate and Asc supplementation a decrease in ^3H vitamin E succinate uptake was observed compared to 10:50μg/ml. This suggests that beyond 10μg/ml vitamin E succinate, uptake of ^3H vitamin E succinate is inconsistent although the decrease in Asc concentration may have influenced the uptake of ^3H vitamin E succinate in these two combined vitamin concentration sets.

Table 14 shows the relative uptake of ^3H vitamin E succinate in membrane and stroma fractions of LLCMK and BL6 cells respectively. Supplementation of LLCMK cells with combined vitamin E succinate and Asc resulted in a general increase in ^3H vitamin E succinate uptake in both membrane and stroma fractions when compared to the control cultures (0E) respectively, although no statistical significance between the values was recorded.

Comparison of membrane and stroma fractions in control cultures (0E) and combined vitamin E succinate and Asc supplemented LLCMK cells showed a general increase in ^3H vitamin E succinate

uptake in the stroma fraction.

Supplementation of BL6 melanoma cells with combined vitamin E succinate and Asc resulted in an increased uptake of ^{3}H vitamin E succinate in membrane and stroma fractions relative to respective control cultures (0E)(Table 14). In the membrane fraction significant uptake of labelled vitamin E succinate was observed at 10:50($p \leq 0.001$); 10:25($p \leq 0.01$) and 20:25(≤ 0.001) respectively, while the uptake in the stroma fraction was only significant ($p \leq 0.001$) at 20:25μg/ml compared to their respective control cultures (0E). Comparison of ^{3}H vitamin E succinate uptake in control cultures (0E) and combined vitamin supplemented membrane and stroma fractions of BL6 cells (Table 14) only showed an increase in uptake of ^{3}H vitamin E succinate in the stroma fraction of the 5:25 and 10:50μg/ml groups.

TABLE 14: The effect of combined vitamin E succinate and ascorbic acid supplementation on the uptake of ^{3}H vitamin E succinate by the membrane and stroma fractions of LLCMK and BL6 cells, respectively. Results are the mean of three cultures ± SEM.

[vitamin E succinate: ascorbate] μg\ml	[U ^{3}H] vitamin E succinate	^{3}H vitamin E succinate uptake in membrane fraction cpm/10^4 cells)		^{3}H vitamin E succinate uptake in stroma fraction (cpm/10^4 cells)	
		LLCMK	BL6	LLCMK	BL6
0E	0.1	1.76 ±0.34	0.21 ±0.02	3.08 ±0.12	0.34 ±0.01
5:25	0.1	2.47 ±0.45	0.41 ±0.15	7.03 ±2.08	1.38 ±0.24
10:50	0.1	2.00 ±0.31	0.99[a] ±0.08	7.92 ±2.08	5.02 ±0.51
10:25	0.1	1.08 ±0.13	0.67[b] ±0.06	1.96 ±0.01	0.598 0.621
20:25	0.1	2.23 ±0.27	1.07[a] ±0.02	4.87 ±1.00	1.21[c] ±0.03

a - $p \leq 0.001$ relative to BL6 membrane control cultures (0E)
b - $p \leq 0.01$ relative to BL6 membrane control cultures (0E)
c - $p \leq 0.001$ relative to BL6 stroma control cultures (0E)

3.4 EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON [CARBOXYL -^{14}C] ASCORBIC ACID UPTAKE

3.4.1 MATERIALS AND METHODS

L-[Carboxyl -^{14}C] ascorbic acid was obtained from Amersham, Int., England.

METHODS

3.4.1.1 Cell culture procedures

Refer to sections 2.2.3.1 and 2.2.3.2.

3.4.1.2 Homogenization of the cells and separation into cellular components

Refer to section 3.2.1.2.

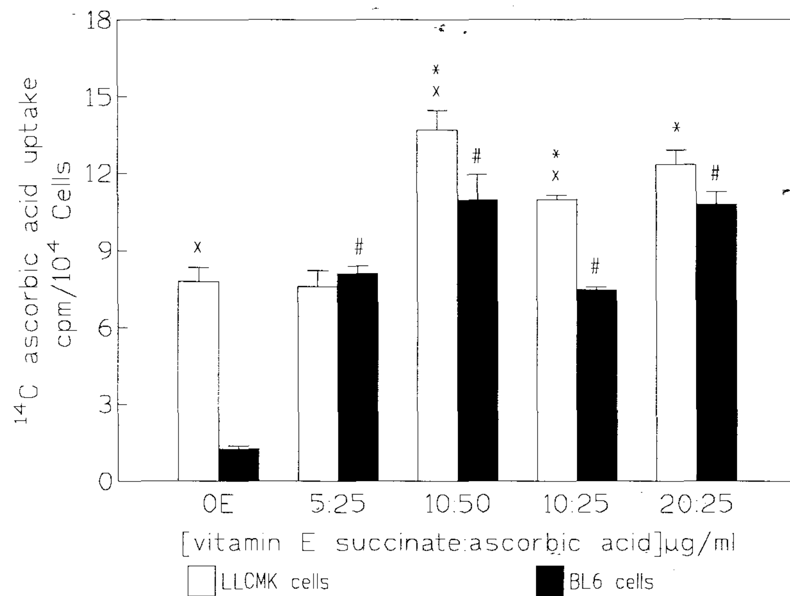
3.4.1.3 Determination of radioactivity in membrane and stroma fractions

Refer to section 3.2.1.3.

3.4.2 RESULTS

The uptake of ^{14}C Asc by LLCMK and BL6 cells (combined membrane and stroma fractions) is shown in Figure 19.

^{14}C Asc uptake was significantly ($p \leq 0.001$) increased in the control cultures (0E) of LLCMK cells relative to the uptake of ^{14}C in control cultures (0E) of BL6 cells (Figure 19) as was the uptake at 10:50 ($p \leq 0.05$) and 10:25 ($p \leq 0.01$) in the LLCMK cells relative to the corresponding BL6 cells. In LLCMK cells (Figure 19) supplemented with combined vitamin E succinate and Asc together with ^{14}C Asc a significant increase in uptake of ^{14}C Asc was found at 10:50 ($p \leq 0.001$); 10:25 ($p \leq 0.01$) and 20:25μg/ml ($p \leq 0.01$) compared with control cultures (0E).



x - (p≤0.001 - p≤0.05) relative to corresponding BL6 cells
* - (p≤0.001 - p≤0.01) relative to LLCMK control cultures (0E)
# - (p≤0.001) relative to BL6 control cultures (0E)

Figure 19: The effect of combined vitamin E succinate and ascorbic acid supplementation on the uptake of ^{14}C ascorbic acid by both LLCMK and BL6 cells. The bars are the mean of three cultures ± SEM.

Supplementation of combined vitamin E succinate and Asc together with ^{14}C Asc in BL6 cells (Figure 19) resulted in a significant ($p \leq 0.001$) increase in ^{14}C Asc uptake at each combined vitamin concentration group relative to control cultures (0E). As expected, an increase in ^{14}C Asc uptake was observed with increasing concentrations of cold Asc with the highest magnitude of ^{14}C Asc uptake measured at 50μg/ml Asc.

Comparison of ^{14}C Asc uptake in control cultures (0E) and combined vitamin E succinate and Asc supplemented membrane fractions with corresponding stroma fractions showed an increase ($p \leq 0.001$) in ^{14}C Asc uptake in the stroma fractions of both cell types (Table 15).

TABLE 15: The effect of combined vitamin E succinate and ascorbic acid supplementation on the uptake of ^{14}C ascorbic acid by the membrane and stroma fractions of LLCMK and BL6 cells, respectively. Results are the mean of three cultures ± SEM.

[vitamin E succinate: ascorbate] μg\ml	^{14}C ascorbic acid μCi	^{14}C ascorbic acid uptake in membrane fraction (cpm/10^4 cells)		^{14}C ascorbic acid uptake in stroma fraction (cpm/10^4 cells)	
		LLCMK	BL6	LLCMK	BL6
0E	0.15	0.97 ±0.02	0.12 ±0.01	6.66[a] ±0.62	1.13[b] ±0.11
5:25	0.15	1.43 ±0.13	0.39[d] ±0.15	9.99[a] ±1.02	6.61[bc] ±1.11
10:50	0.15	1.19 ±0.18	0.45[d] ±0.08	9.07[a] ±1.31	10.47[bc] ±0.51
10:25	0.15	1.49 ±0.14	0.31[d] ±0.06	10.87[a] ±0.31	7.14[bc] 0.621
20:25	0.15	1.31 ±0.17	0.51[d] ±0.02	10.45[a] ±1.80	10.35[bc] ±0.54

a - $p \leq 0.001$ relative to corresponding LLCMK membrane fractions
b - $p \leq 0.001$ relative to corresponding BL6 membrane fractions
c - $p \leq 0.001$ relative to BL6 membrane control cultures (0E)
d - $p \leq 0.05$ relative to BL6 stroma control cultures (0E)

Supplementation of LLCMK cells with combined vitamin E succinate and Asc, resulted in a slight but non significant increase in ^{14}C Asc uptake in both membrane and stroma fractions when compared to respective control cultures (0E). ^{14}C Asc uptake in the BL6 cells (Table 15), increased significantly ($p \leq 0.001$) in the stroma fractions of supplemented cells when compared to control cultures (0E), while a similar significant ($p \leq 0.05$) increasing trend in ^{14}C Asc uptake in the membrane fraction of supplemented cells was observed.

3.5 EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON VITAMIN E SUCCINATE METABOLISM

3.5.1 MATERIALS AND METHODS

MATERIALS

A reverse phase 10μBondaclone ID C^{18} HPLC column (300x390mm) was purchased from

Phenomenex, USA, while the Guard-Pak precolumns [5μM, ODS] were obtained from Metachemicals, USA. Neostigmine and iminodiacetic acid were purchased from Sigma Chemical Co., USA. Nitric acid and Potassium Chloride (KCl) were purchased from Holpro Chemical Co., South Africa. High Performance Liquid Chromatography (HPLC) grade methanol, acetonitrile, hexane and ethanol were supplied by BDH Chemicals, LTD, England. Sodium dihydrogen orthophosphate was obtained from Unilab, SAARCHEM, South Africa.

METHODS

3.5.1.1 Glassware Preparation

Prior to use, all glassware was treated by the modified method of Buettner (cited in 496). This treatment involved the soaking of clean glassware in 25% nitric acid for 48 hours, followed by another 48 hours in milli Q water containing chelating resin (iminodiacetic acid) (5g/l).

3.5.1.2 Preparation of Ca^{2+} and Mg^{2+} free phosphate-buffered saline solution

To minimize oxidation of α-TOH to the tocopherylquinone, trace amounts of catalytic transition metals such as Ca^{2+} and Mg^{2+} were omitted from the assay buffer PBS. This buffer was prepared as described by Kelley *et al* (496) and contained 2.68mM KCl, 1.47mM K_2HPO_2, 136.8mM NaCl, 8.06mM NaH_2PO_2, 10nM neostigmine and 0.5mg/ml chelating resin, pH 7.4

3.5.1.3 Cell culture procedure

Refer to section 2.2.3.1 and 2.2.3.2, with the following changes. The cell pellets were resuspended in 1.0ml ice cold Ca^{2+}/Mg^{2+} free PBS solution, pH 7.4. To the cell suspension, 20μl of 200mM Asc was added.

3.5.1.4 Extraction of vitamin E and vitamin E succinate

The extraction of vitamin E and vitamin E succinate was carried out using a modified method of Kelley *et al* (496). Following enumeration using a haemocytometer, cell suspensions were centrifuged (Eppendorf 5403 centrifuge) at 750g for 10minutes at 4°C. The supernate was discarded and the pellet wash repeatedly with the addition of the same amount of Asc. To the final pellet wash 5μl of 200mM

Asc was added. The pellet was finger-vortexed and placed on ice for 2minutes and then transferred in a glass Pasteur pipette to a cold Dounce homogenizer. To the cell suspension was added 500μl of 95% ethanol. The samples were vortexed and homogenized 30 times with a tight plunger; followed by the addition of 500μl of ice cold hexane. The cell suspensions were transferred to precooled glass centrifuge tubes and centrifuged at 300g for 3 minutes to separate the phases. An aliquot of the top (hexane) phase was placed in a glass storage vial. The head space air was replaced with nitrogen (N_2) and the vials stored at -70°C until required.

3.5.1.5 Vitamin E and vitamin E succinate determination

The extracted samples were analysed by reverse phase HPLC, using a modified method of Scalia *et al* (497). The hexane extracts were dried under a stream of N_2 and reconstituted in 100μl of mobile phase containing methanol:acetonitrile (60:40, v/v). Separation of vitamin E and vitamin E succinate was performed on a μBondclone C^{18} column (10μm particle diameter, 30cmx3.9mm) fitted with a Guard-Pak precolumn (5μm particle size, ODS) and eluted isocratically with methanol:acetonitrile (60:40, v/v) at a flow rate of 1.5ml.min^{-1}. The reconstituted samples were injected (50μl injection volume) onto the column. Vitamin E succinate and vitamin E were detected by a UV-visible spectrophotometer (Beckman, System Gold, Programmable detector module, Mode 166, USA). The identities of the separated compounds were established by co-chromatography with authentic standards, (+) vitamin E and vitamin E succinate, and the quantities of these compounds in the extract determined using peak areas and the standard curve recorded in Appendix 3.

3.5.2 RESULTS

The effect of combined vitamin E succinate and asc supplementation on cellular vitamin E and vitamin E succinate content in LLCMK and BL6 cells is represented in Table 16.

Supplementation of LLCMK cells with 10:25μg/ml of combined vitamin E succinate and Asc resulted in a marked increase in vitamin E succinate levels being detected, with a significant ($p \leq 0.001$) increase occurring at 20:25μg/ml when compared to 5:25μg/ml combined vitamin supplemented cultures. In contrast, vitamin E levels in LLCMK were not detected, suggesting no esterase activity in LLCMK cells.

TABLE 16: The effect of combined vitamin E succinate and ascorbic acid supplementation on vitamin E succinate and vitamin E levels in LLCMK and BL6 cells. Results are the mean of three cultures ± SEM.

[vitamin E succinate: ascorbate] μg/ml	vitamin E succinate (nmoles/10^4 cells)		vitamin E (nmoles/10^4 cells)	
	LLCMK	BL6	LLCMK	BL6
0E	ND	ND	ND	ND
5:25	0.026 ±0.003	0.012 ±0.007	ND	0.002 ±0.002
10:50	0.009 ±0.004	0.012 ±0.006	ND	0.003 ±0.001
10:25	0.0501 ±0.017	0.007 ±0.001	ND	0.004 ±0.008
20:25	0.249[a] ±0.048	0.012 ±0.001	ND	0.006 ±0.008

ND - not detectable
a - $p \leq 0.001$ relative to 5:25μg/ml LLCMK cells (vitamin E succinate)

Supplementation of BL6 cells with varying levels of combined vitamin E succinate and Asc resulted in no increasing or decreasing trend in vitamin E succinate or vitamin E levels. The levels of vitamin E detected in supplemented BL6 cells with combined vitamin E succinate and Asc increased when compared to 5:25μg/ml, although not significantly.

3.6 DISCUSSION

Ester derivatives of TOH are commonly used for vitamin E supplementation for both experimental and therapeutic purposes (95). Studies by Fariss using vitamin E succinate showed complete protection from the toxic effects of oxygen, including lipid peroxidation induction (95) and Cd-induced (498) toxicity in isolated hepatocytes. Furthermore studies by Pascoe and Reed (492) demonstrated protection by vitamin E succinate against the chemical toxicity of the free radical-producing anticancer drug adriamycin. The mechanism by which vitamin E succinate acts as an antioxidant protecting against toxic effects of free radicals and lipid peroxidation is still unclear, however it has been suggested that vitamin E succinate may act as a carrier for the vitamin E enabling the release and accumulation of TOH at a particular and critical cellular site within the cell (95,498,492,498). This suggests the accumulation of TOH may provide the cells first line of defence against free radical

attack on membrane phospholipids, and may in turn allow the water-soluble vitamin C access to the membrane bound vitamin E radical for recycling of this moiety. This synergistic action would result in the delayed consumption of vitamin E, enhancing the antioxidant potential of the two vitamins (103,143,167,168).

Supplementation of non malignant LLCMK cells with combined vitamin concentrations in this study resulted in a non significant decrease in free radical formation, while lipid peroxidation states exhibited no increasing or decreasing trend. In malignant BL6 cells, however, supplementation of combined vitamin E succinate and Asc resulted in a marked increase in both free radical and lipid peroxidation levels. One possible explanation for combined vitamin E succinate and Asc ineffectiveness in acting as an antioxidant preventing free radical formation and lipid peroxidation levels is that the various forms of vitamin E demonstrate different intracellular compartmental distribution patterns (95,97), which may vary in different cell types. In LLCMK and BL6 cells, studies using radiolabelled vitamin E succinate clearly showed uptake in both membrane and stroma fraction. However, supplementation of radiolabelled vitamin E succinate with varying levels of unlabelled vitamin E succinate affected its distribution in that the majority of vitamin E succinate was located in the stroma. This cytosolic accumulation would increase the lipid membrane susceptibility to lipid peroxidation. Studies by Stich *et al* (cited in 58) showed that melanoma cells preferentially incorporate vitamin C, supporting the data obtained in our studies in which BL6 melanoma cells were shown to incorporate ^{14}C Asc. In BL6 cells it was evident that supplementation of radiolabelled Asc resulted in a significant increase in Asc uptake in the stroma fraction when compared to the membrane fraction.

As previously mentioned, vitamin E succinate in this study does not exhibit any antioxidant properties unless the succinate group is cleaved by esterase activity, liberating the free alcohol, vitamin E, which is an active antioxidant (97). Fariss (498) proposed that the vitamin E succinate cytoprotection may be due to vitamin E succinate acting as a carrier for tocopherol enabling the release and accumulation of TOH at a unique and critical cellular site. In LLCMK cells, supplementation of increasing concentrations of vitamin E succinate resulted in a marked increase in the cellular accumulation of the intact vitamin E succinate molecule, however no free vitamin E was detected in LLCMK cells, demonstrating the absence of esterase activity. In BL6 cells, supplementation with increasing concentrations of vitamin E succinate resulted in an overall increased accumulation of cellular vitamin E succinate. Although vitamin E succinate levels in BL6 cells neither increased nor decreased with increasing concentrations of vitamin E succinate, increased free vitamin E accumulation was detected.

Although these vitamin E levels in BL6 cells may appear very low, vitamin E is present in extremely low concentrations in membranes, normally less than 0.1nmol per mg of membrane protein, in other words one molecule per 1000 to 2000 membrane phospholipid molecules (84). Results obtained for LLCMK cells suggest the absence of specific esterase activities in liberating vitamin E succinate, although accumulation of vitamin E succinate was evident. However, results from BL6 cells suggest the presence of various esterase enzymes capable of removing succinate from vitamin E succinate to liberate vitamin E. A possible reason for the enhanced hydrolysis of vitamin E succinate as suggested by Pascoe and Reed (499) is that the Ca^{2+} content affects the intracellular metabolism of vitamin E and its esters within the cells, which may subsequently govern the outcome of a toxic challenge.

In summary, the results obtained confirm the uptake of vitamin E succinate and Asc in BL6 cells. Furthermore, cleavage of the succinate does in fact occur, thus we propose that vitamin E and Asc may act synergistically resulting in the "sparing effect" of vitamin E (see Section 1.4). The inability of combined vitamin E and Asc supplementation to reduce free radical formation and lipid peroxidation levels within the BL6 cells may not be due to their ineffectiveness as antioxidants but rather the presence of other contributing factors which influence the free radical and lipid peroxidation tone within the BL6 cells. Another possible source of free radical and lipid peroxidation within the cells may be increased eicosanoid metabolism in which COX activity in the pathway is believed to be a prime source of intermediatory free radical species (237,242,268). Furthermore researchers suggest that reduced levels of antioxidant enzymes may occur in tumour cells which in turn leads to elevated free radical and lipid peroxidation levels (185,187). Vitamin E has numerous other roles, once inside the cell, besides its role as an antioxidant. Vitamin E might alter membrane stability or function by interacting with the unsaturated EFA portions of phospholipids (lipophilic TOH moiety) (95,498). Several studies indicate that administration of vitamin E stabilizes membranes (500) and alters membrane enzymatic activities (501,502). In order to explain the lack of antioxidant affect of vitamin E succinate and Asc found in the cells used in this study alternative metabolic pathways were investigated. Since vitamin E has been suggested to modulate the regulatory proteins in signal transduction pathways as well as the modulation of key regulatory enzyme activities in the eicosanoid pathway (231,503), the effect of combined vitamin E succinate and Asc supplementation on the eicosanoid pathway and its metabolites associated with cell growth were subsequently investigated in LLCMK and BL6 cells.

CHAPTER 4

COMBINED NUTRIENT SUPPLEMENTATION AND SECOND MESSENGERS

As mentioned earlier the mechanism(s) by which combined vitamin E succinate and Asc exert anticarcinogenic effects *in vitro* are unclear, although it has been suggested that these effects may be due to their synergistic antioxidant properties. Studies in Chapter 3 suggested that the inhibition of BL6 cell growth observed in this study was not mediated in part by the synergistic antioxidant action of combined vitamin E succinate and Asc but rather mediated through metabolic pathways which influence cell growth. This suggestion derives from the fact that free radical formation and lipid peroxidation levels increased after combined vitamin supplementation. Since vitamin E has been suggested to modulate the biosynthetic pathway involving AA metabolism at various points, the AA cascade pathway (refer to Figure 9, page 55), which involves the cleavage of AA via the action of PLA_2, was investigated. The conversion of AA to PGs and the effect of the latter on AC activity and cAMP (a known regulator of cell growth) was also determined. The pathway is also influenced by exogenous EFAs, 5-LOX, COX and Ca^{2+} levels. In addition to this, the role of COX and 5-LOX activity in AA metabolism as well as their role in free radical formation and induction of lipid peroxidation was examined. Hence we investigated the effect of combined vitamin E succinate and Asc supplementation on the activity of the pathway, and of AA metabolites which influence it.

4.1 COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION AND CALCIUM LEVELS

4.1.1 INTRODUCTION

Ca^{2+} is both an essential structural body component and a critical functional element in living cells (327). Ca^{2+} binds to various ligands such as proteins and phosphate groups (504) which in excess, can lead to a decrease in membrane fluidity if such groups form part of membranes (390). The function of this Ca^{2+}-binding is to enable Ca^{2+} to act as a pivotal regulator in metabolic processes (327,329,504) both as a major second messenger, and as free intracellular Ca^{2+} in cells (504). Signal mediation by cytosolic Ca^{2+} ions within cells plays a vital role in many cellular events (505). For example, many of the key enzymes involved in AA esterification or release are dependent on Ca^{2+}. PLA_2 requires elevated cytosolic Ca^{2+} for activation and subsequent release of

AA from numerous tissue lipids in various stimulated cell types (339,340) as the concentration of AA within resting cells is believed to be maintained at low levels (339,366). The regulation of Ca^{2+} levels in cells is mediated by several actions and is very complex. Intracellular Ca^{2+} concentration in a pituitary cell line has been shown to be controlled by second messenger AA and its metabolites. AA was shown not only to augment intracellular Ca^{2+} mobilization within the cells but to stimulate the entry of Ca^{2+} into these cells (506). AA is central to the process of second messenger metabolism as AA may serve as a positive or negative feedback regulator of the effects of intracellular Ca^{2+}.

The availabilty of Ca^{2+} within a cell is believed to be a key regulator of cell proliferation in multicellular organisms. Defects in Ca^{2+} control processes are a common determinant of unrestrained cell proliferation (329,335,356). Calcium concentrations within tumour cells vary in different cell types. Some malignancies are frequently associated with hypercalcaemia rather than hypocalcaemia. This phenomenon has been useful in the detection of certain tumour cell types (507-509).

Since Ca^{2+} levels play an important role in modulating cell proliferation, and earlier studies in Chapter 2 showed significant growth inhibition in BL6 cells following combined vitamin E succinate and Asc supplementation, it was necessary to determine the levels of intracellular Ca^{2+} in non-malignant LLCMK and malignant BL6 cells, as well as the effect of combined vitamin E succinate and Asc supplementation on intracellular Ca^{2+} levels.

4.1.2 MATERIALS AND METHODS

MATERIALS

A GBC 909 Atomic Absorption Spectrophotometer was used for Ca^{2+} detection. $CaCl_2$ was purchased from Merck, Darmstadt, Germany, while nitric acid (HNO_3) was obtained from Unilab, SAARCHEM, South Africa.

METHODS

4.1.2.1 Glassware preparation

All glassware was acid washed prior to use with 25% HNO_3 solution at 60°C overnight. Thereafter it was rinsed with milli Q water.

4.1.2.2 Cell culture procedures

The methods described in 2.2.3.1 and 2.2.3.2 were 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 Ca^{2+} concentrations in separate cell fractions were below detection limits.

4.1.2.3 Acid digestion of the cells and calcium detection

On completion of cell enumeration, the suspensions were centrifuged at 3 000g for 10 minutes and the supernatant liquid discarded. The cells were washed with 1ml Tris HCl buffer and again centrifuged at 3 000g for 10 minutes. The supernatant material was again discarded and the centrifuge tubes drained. The remaining pellet was resuspended in 0.2ml concentrated HCl and boiled at 100°C for two hours. After 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 10minutes to remove the resultant debris. The supernate was assayed for Ca^{2+} content by flame atomic absorption spectroscopy. The conditions used for detection were as follows; fuel source 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 also assayed using flame atomic absorption spectroscopy. The Ca^{2+} standard curve is recorded in Appendix 4.

4.1.2.4 Statistical analysis

The results obtained were analysed 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 analysed.

4.1.3 RESULTS

Ca^{2+} levels in LLCMK cells of control cultures (0E) and 10:50; 10:25 and 20:25μg/ml combined vitamin E succinate and Asc supplemented cells were significantly ($p \leq 0.001$) higher than the Ca^{2+} levels detected in corresponding BL6 cultures (Table 17).

Combined vitamin E succinate and Asc supplementation of the LLCMK cells (Table 17) resulted in a very erratic effect on Ca^{2+} levels within the cells relative to the control cultures (0E). At 10:25μg/ml combined vitamin E succinate and Asc supplementation there was a significant ($p \leq 0.05$) increase in Ca^{2+} levels was observed while, at 5:25μg/ml a significant ($p \leq 0.05$) decrease in Ca^{2+} levels relative to control cultures (0E) was observed. At 10:50μg/ml and 20:25μg/ml combined vitamin E succinate and Asc supplementation of LLCMK cells resulted in no observed effect on Ca^{2+} levels relative to control cultures (0E).

Combined vitamin E succinate and Asc supplementation of the BL6 cells markedly increased the Ca^{2+} levels in these cells relative to control cultures (0E) with significant ($p \leq 0.001$) increases in Ca^{2+} levels at 10:50; 10:25 and 20:25μg/ml relative to control cultures (0E).

TABLE 17: The effect of combined vitamin E succinate and ascorbic acid supplementation on calcium levels in LLCMK and BL6 cells respectively. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	Calcium concentration (nM/10^4 cells)	
	LLCMK	**BL6**
0E	5.02^{a} ±0.46	0.49 ±0.04
5:25	1.85^{b} ±0.67	0.46 ±0.11
10:50	4.33^{a} ±0.68	1.15^{c} ±0.05
10:25	7.15^{ab} 0.61	1.63^{c} ±0.04
20:25	4.34^{a} ±0.61	1.95^{c} ±0.02

a - $p \leq 0.001$ relative to corresponding BL6 cells
b - $p \leq 0.05$ relative to LLCMK control cultures (0E)
c - $p \leq 0.001$ relative to BL6 control cultures (0E)

4.1.4 DISCUSSION

The level of free Ca^{2+} in pancreatic acinar carcinoma cells has been reported to be lower than in normal acinar cells (510). Results from this study showed that Ca^{2+} levels in BL6 cells were lower than the Ca^{2+} levels found in the LLCMK cells, which supports the above finding. Ca^{2+} levels in

different tumour cells vary relative to one another. BL6 melanoma cells have been reported to have considerably higher Ca^{2+} content than normal liver and kidney cells (504). However, studies by Hill (510) showed that all B16 melanoma cell lines had lower intracellular free Ca^{2+} levels than a transformed murine melanocyte cell line, Melan A. Thus it would seem that BL6 melanoma cells are characterised by lower Ca^{2+} content. Despite this, studies (511) on 9 human melanoma cell lines suggested that growth was not affected in Ca^{2+} depleted medium, whereas normal cells ceased to proliferate. The mechanism for continued growth of neoplastic cells at low Ca^{2+} remains unclear (511), but the situation is consistent with the suggestion that intracellular Ca^{2+} in these cells is high and requires no extra Ca^{2+}, or that these cells have adapted to a low extracellular Ca^{2+} environment. Thus a change in intracellular free Ca^{2+} could be relevant to the development of neoplasia (510).

The effect of combined vitamin E succinate and Asc on Ca^{2+} levels in LLCMK and BL6 cells has not previously been reported. High Ca^{2+} content was detected in LLCMK cells supplemented with combined vitamin E succinate and Asc with no increase or decrease trend in Ca^{2+} levels being dectected. As regards the BL6 cells, combined vitamin E succinate and Asc significantly increased Ca^{2+} levels within these cells compared with the control cultures (0E), except for the 5:25μg/ml group. The effect of combined vitamin E succinate and Asc supplementation on Ca^{2+} levels within these cells could be due to the ability of vitamin E to modulate membrane fluidity, which could in turn account for the observed changes in Ca^{2+} levels. The only mechanism available for causing large-scale changes in Ca^{2+} concentration involves movement either across the plasma membrane or across intracellular membranes (330).

As Ca^{2+} levels play an important role in modulating PLA_2 activity (339,340) and since marked changes in intracellular Ca^{2+} have been shown to occur in LLCMK and BL6 cells supplemented with combined vitamin E succinate and Asc, determination of the effect of combined vitamin supplementation on PLA_2 activity in LLCMK and BL6 cells required investigation.

4.2 COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION AND PHOSPHOLIPASE A_2 ACTIVITY

4.2.1 INTRODUCTION

Phospholipids are the major structural components of cell membranes (121) and are involved in numerous biological responses including signal-transduction, second messenger generation, cell

membrane integrity and the activity of membrane bound enzymes (190). It is the fatty acids present at the 1 and 2 position on the phospholipid molecule which determine and maintain membrane fluidity (190). The presence of PLA_2 in biological membranes is a prerequisite for proper membrane turnover (changes in chemical and physical properties of the membrane) in cellular metabolism for signal transduction and in membrane remodelling (349,373).

PLA_2 removes FAs, mostly AA, from the sn-2 position on the phospholipid glycerol backbone (282). The concentration of free AA within resting cells is believed to be maintained at low levels. The changes in AA which lead to an increase in the synthesis of the eicosanoids results from receptor-stimulated lipid hydrolysis as a result of PLA_2 activation (366,512). Four types of PLA_2 exist, with the membrane-bound 85 kDa PLA_2 (347,349,352,364) being important in this study, as this particular PLA_2 cleaves AA from the sn-2 position which is an important substrate in the pathway studied. The high molecular mass $cPLA_2$ translocates to membranes in response to submicromolar changes in Ca^{2+} (513). This observation is of particular significance because many of the ligands that cause rapid production of eicosanoids are Ca^{2+}-mobilizing agents.

In diseased states such as neoplastic transformation, it has been reported that lipid structures are profoundly altered and hence the membrane fluidity of a number of tissues is modified (123,200). This change can affect the activity of membrane-bound enzymes resulting in the possible impairment of PLA_2 activity in malignant cells. The aim of this part of the study was to determine PLA_2 activity in LLCMK and BL6 cells, as well as the effect of combined vitamin E succinate and Asc supplementation on PLA_2 activity.

4.2.2 MATERIALS AND METHODS

MATERIALS

1-Palmitoyl-2-[1-^{14}C] arachidonyl-sn-glycerol-3-phosphatidyl choline [1-^{14}C-PC] was supplied by New England Nuclear Products, Boston, USA. Taurocholic acid, fatty acid-free bovine serum albumin (BSA) and phosphatidylcholine (PC) were purchased from Sigma Chemical Co., USA. Silica gel 60 Aluminium TLC plates were supplied by Merck, Darmstadt, Germany. Chloroform was obtained from BDH Chemicals, LTD, England, while Folin-Ciocalteu reagent was purchased from Unilab, SAARCHEM, South Africa.

METHODS

4.2.2.1 Cell culture procedures

Methods described in 2.2.3.1 and 2.2.3.2 were repeated except that cell pellets were resuspended in 1ml of 0.1M Tris HCl buffer, pH 7.4.

4.2.2.2 Homogenization of cells and enzyme preparation

The method outlined in 4.2.1.2 was repeated, except that the pellet was resuspended in 2ml of 0.1M Tris HCL buffer, pH 7.4.

4.2.2.3 Protein Determination

Protein determinations were performed using a modified method of Lowry *et al* (cited in 514). The protein standard curve is recorded in Appendix 5.

4.2.2.4 Determination of phospholipase A_2 activity

Membrane PLA_2 activity was assayed using a modification of the methods of Ballou and Cheung (369,370), and the method of Krumhardt and Dupont (515). 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=52mCi/mmol).

A 200μl aliquot of membrane suspension was added to 50μl of substrate and incubated at 57°C for 10 minutes. The substrate contained Tris-HCl buffer, 50mM taurocholic acid, 20mM calcium chloride, 0.05g% fatty acid-free bovine serum albumin and 0.03μCi of radioactive labelled substrate. On addition of membrane suspension and after a 10 minute incubation period, 100μl aliquots of reaction mixture were removed and added to 100μl of 100% ethanol containing 2% glacial acetic acid and 330μM AA, to terminate the reaction. This solution was vortex mixed and 100μl applied to silica gel 60 Aluminium plates, which separated the [1-^{14}C]AA released from the labelled substrate in a mobile phase of chloroform:methanol: H_20 (65:25:4, v/v/v). AA and PC were used as markers. After the development of the TLC plates, bands of the silica gel were scraped into scintillation vials containing 10ml of a scintillation cocktail. Radioactivity was determined by liquid scintillation

counting. PLA_2 activity is determined as the net release of ^{13}C AA from labelled substrate and is expressed as pmol of AA released per minute per mg membrane protein.

4.2.3 RESULTS

Shown in Table 18 is the effect of combined vitamin E succinate and Asc supplementation on PLA_2 activity of both LLCMK and BL6 cells.

TABLE 18: The effect of combined vitamin E succinate and ascorbic acid supplementation on phospholipase A_2 activity detected in the respective LLCMK and BL6 cells. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	**PLA_2 activity (pmol AA/min x mg protein)**	
	LLCMK CELLS	**BL6 CELLS**
0E	12.78 ±0.48	11.20 ±0.12
5:25	5.67^{b} ±0.17	55.56^{ac} ±5.32
10:50	4.91^{b} ±0.21	134.89^{ac} ±3.63
10:25	8.34^{b} ±0.31	139.92^{ac} ±2.37
20:25	4.44^{b} ±0.32	163.19^{ac} ±6.13

a - $p \leq 0.001$ relative to corresponding supplemented LLCMK cells
b - $p \leq 0.001$ relative to LLCMK control cultures (0E)
c - $p \leq 0.001$ relative to BL6 control cultures (0E)

A comparison of the PLA_2 activity in the two cell types revealed that the PLA_2 levels in the BL6 control cultures were slightly lower than that of the corresponding LLCMK control cultures (0E). PLA_2 activity in combined vitamin E succinate and Asc supplemented BL6 cells is, however, significantly ($p \leq 0.001$) increased compared with the corresponding LLCMK cells (Table 18). PLA_2 activity in LLCMK cells supplemented with varying vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) levels resulted in a significant ($p \leq 0.001$) decrease in activity relative to control cultures (0E). However, in combined vitamin E succinate and Asc supplemented BL6 cells, a significant ($p \leq 0.001$) increase in PLA_2 activity was detected relative to control cultures (0E).

4.2.4 DISCUSSION

Studies by Buckley *et al* (377) demonstrated that both Ca^{2+}-dependent and Ca^{2+}-independent pathways are involved in PLA_2 activation. The 85kDa $cPLA_2$ of interest in this study shows an absolute requirement for Ca^{2+} (360) in the enzyme's translocation from the cytosol to particulate cell fractions (347,357,358) and has a substrate preference for sn 2-arachidonyl-containing phospholipids (347). In the present study 1-palmitoyl-2-[1-^{14}C] arachidonyl-sn-glycerol-3-phospatidylcholine was utilized as substrate during the PLA_2 assay performed.

A difference exists between soluble PLA_2 and membrane-bound PLA_2 activity in terms of substrate specificity and the rate of catalysis (373). Work by Hendrickse *et al* (381) demonstrated that PLA_2, the key enzyme of AA release, was increased in colorectal cancer tissue as compared with that of normal tissue. In this study, PLA_2 activity in BL6 supplemented cultures was significantly higher than the PLA_2 activity in the LLCMK cells, which supports the above finding. Comparison of PLA_2 activity found in the control cultures (0E) of LLCMK and BL6 cells, revealed lower PLA_2 activity in the malignant BL6 cells. Tumour cells are reported to have altered EFA composition in their membranes. This in turn modulates the activity of membrane-associated enzymes, such as PLA_2 and AC (190). Hence, the lower PLA_2 in the BL6 cells could be due to changes in the EFA content of the BL6 cell membrane. Thus it would seem the PLA_2 activity is regulated in part by the EFA composition of the phospholipid bilayer in which they reside, and not by substrate availability (515).

In LLCMK cells, PLA_2 activity decreased significantly compared with control cultures (0E), whereas in the BL6 cells, PLA_2 activity increased significantly with varying levels of combined vitamin E succinate and Asc supplementation. The mechanism by which combined vitamin E succinate and Asc supplementation increases PLA_2 activity in BL6 cells is not clear, although certain factors should be considered. Studies by Stoll and Duncan (481) reported decreased PLA_2 activity upon Asc supplementation in BL6 cells. Since it is believed that tumour cells have altered membrane fluidities (123,190), and vitamin E is known to stabilize membranes by virtue of specific physicochemical interactions (95,125,127) the significant increase in PLA_2 activity observed following combined vitamin E and Asc supplementation may be due to the ability of vitamin E to modulate membrane fluidity through specific physicochemical interactions in membrane stabilisation. Asc in turn could regenerate vitamin E which could also account for the increase in Ca^{2+} levels observed (Section 4.1) and in turn would result in increased PLA_2 activity.

The reason for the inhibition of PLA_2 activity in LLCMK cells following vitamin E succinate and Asc supplementation is unclear. The effect of vitamin E succinate may be due to indirect inhibition of PLA_2 through the regulation of intracellular Ca^{2+}, as submicromolar changes in Ca^{2+} levels are known to regulate PLA_2 activity.

In summary, these experiments revealed that when compared with the non malignant LLCMK cells, PLA_2 activity was significantly higher in supplemented malignant BL6 cells. It was also found that combined vitamin E succinate and Asc supplementation of BL6 cells resulted in increased PLA_2 with increased vitamin E succinate concentrations, which may in turn affect AA levels within the cells. A further factor to consider in terms of cellular AA content is the possible effect of combined vitamin E succinate and Asc supplementation on AA uptake into the cells, and the influence that exogenous AA in the medium may exert on the cellular levels of AA.

4.3 ARACHIDONIC ACID LEVELS WITH COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID AND ARACHIDONIC ACID SUPPLEMENTATION

4.3.1 INTRODUCTION

AA is esterified to specific phospholipids, most notably phosphatidylcholine and phosphatidylethanolamine, hence cells contain only small amounts of free AA (339,366). FA supplementation of cells *in vitro* or changes in dietary fat intake can lead to marked alterations in the FA composition of membrane phospholipids (183,184,516) without disrupting basic membrane or cellular integrity (200). The activities of membrane-bound enzymes are dependent on the lipid *milieu* surrounding the protein and enzymes, hence alteration in lipid composition may induce changes in the activities of membrane-bound enzymes (184,198), membrane fluidity and cellular functions such as cell growth (517). Membranes isolated from tumour cells are reported to have profoundly alterated the EFA composition (123). Neuroblastoma cells in culture are able to rapidly incorporate exogenous FAs from the medium (518). The exogenous FAs are incorporated into membrane phospholipids by the action of acyltransferases (519).

AA is a 20 carbon PUFA which is released from the phospholipids after stimulation of PLA_2 and is converted to PGs via the COX pathway (519). AA and its metabolites (PGs) act in various ways as intercellular and intracellular messengers (233,366). It is generally believed that changes in free AA levels would lead to alterations in eicosanoid synthesis (366).

This study examined the ability of the LLCMK and BL6 cells to take up AA from the culture medium, together with the effect of combined vitamin E succinate and Asc supplementation on this uptake. The effect of combined vitamin E succinate and Asc supplementation on AA levels in the membrane and stroma fractions of these cells was also investigated.

4.3.2 MATERIALS AND METHODS

MATERIALS

Refer to section 2.2. A SP 2330 gas-liquid chromatography (GLC) column manufactured by Supelco was supplied by Anatech Instruments, South Africa. Methylated arachidonic acid was purchased from Sigma Chemical Co., USA. BF_3 methanol reagent (14%) was purchased from Merck, Darmstadt, Germany. Potassium Hydroxide (KOH) was purchased from Unilab, SAARCHEM, South Africa.

METHODS

4.3.2.1 Preparation of methanolic KOH (10%)

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

4.3.2.2 Cell culture procedures

The relevant methods are referred to in 2.2.6.1 and 2.2.3.2.

4.3.2.3 Homogenization of cells and separation of cellular components

The method was repeated as described in 3.2.1.2, except that the pellet was resuspended in 2ml PBS solution.

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

The membrane and stroma fractions, respectively, of the two cell types, supplemented with 15-^{3}H AA, were placed into scintillation vials containing 10ml scintillation cocktail. The amount of ^{3}H AA

in each fraction was determined by scintillation counting.

4.3.2.5 Saponification, esterification and extraction of fatty acids

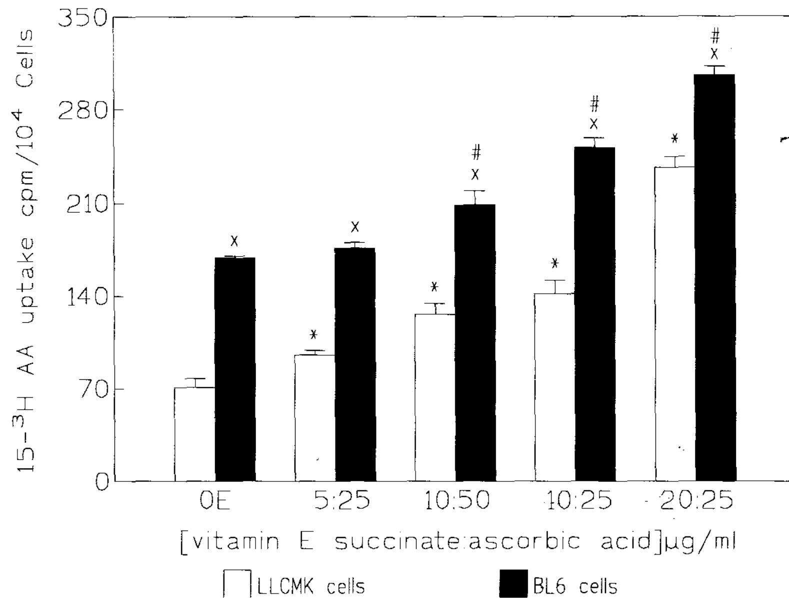
The method used was that of Stoll (520). The various cell fractions were transferred into round bottomed flasks and 2ml of 10% methanolic KOH was added to each. The lipids were saponified by heating with reflux under N_2 for 45 minutes at 85°C. On completion of saponification, the free FAs were acidified by adding 1ml of 7N HCl. The acidified FAs were extracted twice with 3ml of distilled petroleum ether and vortex mixed for 2 minutes each time. The distilled petroleum ether extracts were pooled and evaporated to dryness under N_2 at 60°C. The residual FAs were methylated by heating with 0.3ml BF_3-methanol reagent, with reflux and under N_2 for 5 minutes at 100°C. The FA esters were again extracted twice using 1ml petroleum ether and shaken for 2 minutes each time. The pooled extracts were evaporated to dryness under N_2 and stored in darkness at -20°C until required.

4.3.2.6 Free fatty acid analysis by gas-liquid chromatography

Separation of the extracted free FAs, which were reconstituted in 20μl distilled petroleum ether, was achieved by GLC, with a SP 2330 fused capillary column in the Hewlett-Packard 5890 Gas Liquid Chromatograph. The temperature programme involved an initial heating of the column at 130°C for 15 minutes, followed by a rise in temperature of 4°C/minute to a final oven temperature of 220°C. The column temperature was held at 220°C for the final 7.5 minutes of the run. A 1μl aliquot of the reconstituted FAs was loaded onto the SP 2330 column. The concentration of AA (20:4), which forms part of the n-6 linoleic acid series, was measured. Results are expressed as percentage composition of AA relative to the total amount of FA in that sample.

4.3.3 RESULTS

^{3}H AA levels in the BL6 control cultures (0E) were significantly ($p \leq 0.001$) higher than the ^{3}H AA levels of the LLCMK control cultures (0E) (Figure 20), as was the uptake of ^{3}H AA by BL6 compared with the corresponding LLCMK cells. Combined vitamin E succinate and Asc supplementation in LLCMK cells resulted in a significant ($p \leq 0.001$) increasing trend in the uptake of total ^{3}H AA by the cells. In the BL6 cells, combined vitamin E succinate and Asc supplementation (with the exception of 5:25μg/ml) also resulted in a significant ($p \leq 0.001$) increase in total ^{3}H AA uptake.



x - $p \leq 0.001$ relative to corresponding LLCMK cells
* - $p \leq 0.001$ relative to LLCMK control cultures (0E)
# - $p \leq 0.001$ relative to BL6 control cultures (0E)

Figure 20: The effect of combined vitamin E succinate and ascorbic acid supplementation on total ^3H arachidonic acid uptake by LLCMK and BL6 cells. Each bar represents the mean of three cultures ± SEM.

Uptake of ^3H AA in the membrane fractions of LLCMK cells was generally higher than that in stroma fractions except in the control cultures (0E). A similar higher ^3H AA uptake by membrane fractions of BL6 cells than stroma fractions in all cultures was also found.

Uptake of ^3H AA in the membrane and stroma fractions of LLCMK cells was increased in the vitamin E succinate and Asc supplemented cells, and this increase was significant in most concentrations as can be seen from Table 19. In BL6 cells there was also an increase in ^3H AA uptake in both fractions as a result of supplementation, but in these cells this was only significant at the 20:25μg/ml level of supplementation in the stroma fraction.

TABLE 19: The effect of combined vitamin E succinate and Asc supplementation on the uptake of 15-^{3}H arachidonic acid by membrane and stroma fractions of LLCMK and BL6 cells respectively. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	15-^{3} H AA μCi	15-^{3}H AA in membrane cpm/10^4 cells		15-^{3}H AA in stroma cpm/10^4 cells	
		LLCMK	BL6	LLCMK	BL6
0E	5	34.97 ±3.72	96.19 ±11.92	36.02 ±2.86	72.73 ±12.29
5:25	5	54.45[a] ±4.26	113.14 ±6.62	41.29 ±1.02	63.13 ±4.55
10:50	5	71.71[b] 2.09	116.42 ±17.50	54.82 ±5.95	92.40 ±6.65
10:25	5	71.04[b] ±2.04	147.91 ±11.99	70.93[c] ±11.74	103.58 ±7.29
20:25	5	125.08[b] ±6.93	183.08 ±14.57	103.63[d] ±7.37	122.63[e] ±13.51

a - $p \leq 0.01$ relative to control cultures (0E) in LLCMK membrane fractions
b - $p \leq 0.001$ relative to control cultures (0E) in LLCMK membrane fractions
c - $p \leq 0.05$ relative to control cultures (0E) in LLCMK stroma fractions
d - $p \leq 0.001$relative to control cultures (0E) in LLCMK stroma fractions
e - $p \leq 0.01$ relative to control cultures (0E) in BL6 stroma fractions

In terms of percentage AA composition the addition of 2.5μM of AA to the medium had no effect on the amount of AA found in the membrane or stroma fractions of both cell types (Table 19). There was also no significant difference in percentage AA found in the membrane *versus* stroma fractions in the control and supplemented LLCMK cells, while in BL6 there was a significantly ($p \leq 0.001$) higher AA composition in the membrane fraction relative to the stroma fractions.

Vitamin E succinate and Asc supplementation to LLCMK cells also had no effect on AA composition in the menbrane fractions although there was a noticeable increase in the 20:25μg/ml group. In the stroma fractions of the cells, percentage AA composition decreased relative to controls, although not significantly so. In BL6 cells the supplementation of combined vitamin E succinate and Asc resulted in a significantly (see Table 20) higher percentage AA in all groups, relative to controls, with the relative increase being more pronounced in the stroma fractions.

TABLE 20: The effect of combined vitamin E succinate and ascorbic acid and arachidonic acid supplementation on the percentage arachidonic acid composition in the respective membrane and stroma fractions of the LLCMK and BL6 cells. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	[AA] μM	% AA in membrane		% AA in stroma	
		LLCMK	BL6	LLCMK	BL6
0E	0	4.25 ±.3	3.10[a] ±0.20	4.96 ±2.10	0.10 ±0.02
0E*	2.5	4.1 ±1.31	2.17[a] ±0.03	5.20 ±1.71	0.08 ±0.01
5:25	2.5	3.84 ±1.52	4.07[ab] ±0.32	4.68 ±0.91	3.82[c] ±0.15
10:50	2.5	4.25 ±1.01	4.52[ab] ±0.03	2.94 ±1.04	3.98[c] ±0.08
10:25	2.5	4.75 ±.93	9.12[ab] ±0.42	2.80 ±0.05	4.89[c] ±1.10
20:25	2.5	6.75 ±0.04	4.64[ab] ±0.36	3.93 ±.09	4.05[c] ±1.51

a - $p \leq 0.001$ relative to corresponding BL6 stroma fractions
b - $p \leq 0.001$ relative to membrane BL6 control cultures (0E*)
c - $p \leq 0.001$ relative to stroma BL6 control cultures (0E*)

4.3.4 DISCUSSION

Studies by Denziot *et al* (519) demonstrated that a human gastric cancer cell line supplemented with AA could accumulate exogenous AA in its cellular membrane lipids. This AA is preferentially incorporated into phosphatidylcholine, followed by a transfer of AA to phosphatidylethanolamine (519). The uptake of ^{3}H AA by the LLCMK and BL6 cells in this study indicates the ability of these cells to accumulate exogenous AA, however, the mechanism of permeation into the cells remains unclear. Vitamin E is believed to have no effect on the uptake and distribution of AA in the major cellular lipid fractions of medial cells in culture (501), although vitamin E is known to have profound effects on AA metabolism. These effects are exerted on biosynthetic pathways involving AA. Hence alternative mechanisms must be involved in the uptake and distribution of AA (503). The AA entry step appears to be insensitive to the intracellular metabolism of the substance and hence not driven by transmembrane concentration gradients (521,522). It is believed that EFA and AA uptake, at least in part, could be carrier-mediated by a membrane fatty acid-binding protein (523,524). In addition, because EFAs, at physiological pH, enter cells as anions, their influx should occur coupled to

movement of a positive charge (521,52). The potential difference may directly affect the permeability of the plasma membrane toward AA in several ways: it may influence AA-lipid partitioning or it may supply the energy required (522). However, a consistent characteristic of AA transport is that of a unidirectional influx exhibiting features of a saturable process (521).

The BL6 cells supplemented with only ^{3}H AA in this study incorporated a significantly greater amount of AA than the corresponding LLCMK cells. Due to altered PUFA content in tumour cells (121), it seems possible that these BL6 cells would incorporate more AA as a result of changes in membrane composition. This observation is further substantiated when, on examing percentage AA composition in BL6 cells, it was found to be greater than in the corresponding LLCMK cells.

In both LLCMK and BL6 cells there was a generally higher uptake of ^{3}H AA found in the membrane than stroma fractions of control and supplemented cells. In both cell types an increase in ^{3}H AA uptake in both membrane and stroma fractions, and in total ^{3}H AA, was found as a result of vitamin E succinate and Asc supplementation. As regards to AA composition in the cells, combined vitamin supplementation resulted in increased levels of AA in both membrane and stroma fractions of BL6 cells. In LLCMK cells, however, an increase (although non-significant) in percentage AA as a result of vitamin supplementation was found only at the 20:25μg/ml concentration in the membrane fraction, while in the stroma fractions (of the supplemented cells) a decrease in percentage AA was in fact found.

The increase in AA levels in BL6 cells as a result of vitamin E succinate and Asc supplementation could, partly at least, be accounted for by the increased uptake of the EFA. The fact that the increased levels of AA as a result of vitamin supplementation was more noticeable in the stroma fraction could be explained by increased AA uptake as well as increased AA release from phospholipid by PLA_2, since the magnitude of increase in percentage AA composition in the stroma fraction of these cells far exceeded the relative increase in AA uptake found as a result of this supplementation. In Section 4.2 supplementation of BL6 cells with combined vitamin E succinate and Asc was in fact shown to result in elevated PLA_2 activity.

The mechanism by which vitamin E succinate and Asc influences the activity of PLA_2 and subsequent release of AA is unclear, however, PLA_2 activity may not only be regulated by vitamin E',s role in membrane stabilization and Asc regenerative capacity of vitamin E but may also be influenced by intracellular Ca^{2+} as elevated levels of Ca^{2+} are required for PLA_2 activation and subsequent release

of AA (339,340,347).

The dependence of 5-LOX activity on Ca^{2+} and AA levels within a cell, as well as the dependence of COX activity on AA levels, led to the further investigation of both 5-LOX and COX activities in BL6 and LLCMK cells.

4.4 COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION AND 5-LIPOXYGENASE ACTIVITY

4.4.1 INTRODUCTION

AA is the key precursor of a large family of potent physiological effectors. It is the branch point leading to two important pathways: one involves the enzyme COX and leads to the synthesis of PGs and TXs, whereas the other involves the lipoxygenase (LOX) enzymes and leads to the synthesis of LTs and lipoxins-compounds that regulate cellular responses in inflammation and immunity (525). Lipoxygenases are dioxygenases which recognise the 1,4-pentadiene structure of PUFAs and incorporate single molecules of oxygen at specific carbon atoms of substrate FAs, resulting in hydroperoxy acids containing dienes as reaction products (525). To date three animal lipoxygenases have been identified, *viz* 5-, 12-, and 15-lipoxygenases, named after the positions where oxygen is inserted into the AA moiety. 5-Lipoxygenase metabolises AA to 5-hydroperoxyeicosatetraenoic acid (5- HPETE), which is subsequently transformed to leukotriene A_4 (LTA_4) (526,237). Lipoxygenases are nonheme, non sulphur-iron dioxygenases which act on fatty acids containing one or more 1,4,2,2-pentadiene moieties to form 2, E-conjugated hydroperoxides. These enzymes play several physiological roles (526), in particular the product hydroperoxides are precursors of specific regulatory molecules such as LTs and lipoxins in animals (527). LTs comprise a group of biologically highly potent lipid mediators synthesized by Ca^{2+}-dependent 5-LOX from 20-carbon PUFAs, predominantly AA (526,528).

Since investigations on the metabolism of AA by LOX have revealed that intermediate peroxy compounds and hydroxyl radicals are produced in the reactions (237,242,268,529) adding to the overall peroxidation state within the cell, the effect of combined vitamin E succinate and Asc supplementation on 5-LOX activity in LLCMK and BL6 cells was determined.

4.4.2 MATERIALS AND METHODS

MATERIALS

Adenosine-5'-triphosphate (ATP) and (±) 5-Hydroperoxyeicosatetraenoic acid [(±)-5-HPETE] were obtained from Sigma Chemical Co., USA. Calcium Chloride ($CaCl_2$) was purchased from Merck, Darmstadt, Germany, while ethyldiaminetetraacetic acid (EDTA) was purchased from Holpro Chemical Co., South Africa. Sucrose was obtained from Riedel-deHaën, Germany.

METHODS

4.4.2.1 Cell culture procedures

The same procedures were used as in 2.2.3.1 and 2.2.3.2, except that the pellets were resuspended in 1ml of 10mM Tris HCl buffer, pH 8.0 containing 1mM EDTA and 0.25M sucrose.

4.4.2.2 Homogenization of cells and enzyme preparation

The enzyme was prepared using a modified method of Soberman (530). Cell suspensions were poured into a Dounce homogenizer and homogenized 30 times with the tight plunger. The homogenizer was rinsed with 1ml of 10mM Tris HCl buffer, pH 8.0 containing 1mM EDTA and 0.25M sucrose. Resulting cell homogenates were transferred to precooled centrifugation tubes and centrifuged (Beckman Model J2 21) at 10 000g for 10 minutes at 4°C. The resulting supernate was stored at -70°C until required.

4.4.2.3 5-Lipoxygenase assay

A modified method of Soberman (530) and Furukawa *et al* (531) was used. The principle of this assay is based on the addition of ^{3}H AA to the enzyme preparation, followed by separation of the product 5 HPETE from substrate ^{3}H AA by TLC and quantitation and determination of the radioactive product formed by scraping off relevant areas of TLC plates. A reaction mixture was prepared containing 10mM Tris HCl pH 8.0, 4mM ATP, 2mM $CaCl_2$, 20μM 15-^{3}H AA and 75μl enzyme preparation in a final volume of 200μl. This was allowed to react for 10 minutes at 24°C. Following incubation the reaction was terminated by the addition of a 500μl aliquot of ethyl ether:methanol:2N citric acid

(30:4:1, v/v/v). Terminated samples were centrifuged at 3 000g for 5 minutes at 4°C. An aliquot (100μl) of the upper organic phase was removed using a graduated capillary tube and spotted onto precoated silica gel 60F_{254} TLC plates, and dried under a stream of N_2. AA and 5-HPETE standards were used as markers. The plates were developed in ethyl ether:petroleum ether:acetic acid (50:50:1 v/v/v) at 4°C. The silica gel regions corresponding to AA and 5-HPETE of samples were scraped off and transferred to scintillation vials containing 10mls of emulsifier scintillator plus™ scintillation cocktail and counted in a Beckman Scintillation counter (LS 2 800). Final results were expressed as pmoles 5-HPETE/10^4 cells.

4.4.3 RESULTS

LLCMK cells (Table 21) supplemented with combined vitamin E succinate and Asc resulted in no increasing or decreasing trend in 5-LOX activity, however, a significant decrease in 5-LOX activity was observed at 20:25μg/ml($p \leq 0.001$) vitamin E succinate and Asc respectively.

TABLE 21: The effect of combined vitamin E succinate and ascorbic acid on 5-lipoxygenase activity in both LLCMK and BL6 cells. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	5-Lipoxygenase activity pmoles 5-HPETE formed/10^6 cells	
	LLCMK CELLS	**BL6 CELLS**
0E	0.691 ±0.017	0.013[c] ±0.001
5:25	0.571 ±0.003	0.037[b] ±0.008
10:50	0.613 ±.004	0.055[a] 0.003
10:25	0.697 ±0.041	0.055[a] ±0.003
20:25	0.241[a] ±0.021	0.173[a] ±0.007

a - $p \leq 0.001$ relative to respective control cultures (0E)
b - $p \leq 0.01$ relative to respective control cultures (0E).
c - $p \leq 0.001$ relative to LLCMK control cultures (0E)

Supplementation of BL6 cells with varying levels of vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) resulted in an increasing trend in 5-LOX activity with increasing concentrations of vitamin

E succinate (Table 21). The increase in 5-LOX activity was significant at each combined vitamin concentration, 5:25 ($p \leq 0.01$); 10:50; 10:25 and 20:25μg/ml ($p \leq 0.001$) respectively, when compared with control cultures (0E). Comparing basal 5-LOX levels in control cultures (0E), (Table 21), revealed that 5-LOX activity in BL6 cells was significantly ($p \leq 0.001$) lower than in LLCMK cells.

4.4.4 DISCUSSION

5-LOX (EC 1:13:11:14) catalyzes the first two steps in the formation of LTs (532-535). LTs act at nanomolar concentrations in intercellular communication, signal transduction, and on host defence (528). 5-LOX, like COX, requires Ca^{2+} (532,535) and hydroperoxides (534,536) for the activation of the enzyme. Furthermore, LOX undergoes self-catalyzed inactivation during oxidation of fatty acid substrates, however, the chemical nature of this self-destruction phenomenon is not yet clear (536). Vitamin E is known to modulate the 5-LOX enzyme in the biosynthetic pathway of AA metabolism, however the mechanism is unknown (503,529). Numerous studies investigating the effects of vitamin E supplementation on LOX activity are contradictory. Vitamin E added at high concentrations has been shown to inhibit platelet LOX (503,528,537), whereas other studies have found no effect. The LOX activity of human neutrophils has been reported to be enhanced by physiological concentrations of plasma vitamin E, whereas higher concentrations are suppressive (538).

Results obtained in this study with supplementation of combined vitamin E succinate and Asc to LLCMK cells showed no increasing or decreasing trend in 5-LOX, however at 20:25μg/ml combined vitamin E succinate and Asc respectively, a significant decrease in 5-LOX was observed relative to control cultures (0E). In BL6 cells however, a significant increase in 5-LOX activity relative to control cultures (0E) was observed following supplementation of combined vitamin E succinate and Asc. The observed increase in 5-LOX activity was more prominent with the increase in vitamin E succinate supplementation in a dose dependent manner.

The mechanism by which vitamin E succinate and Asc influences the activity of 5-LOX in BL6 cells may be similar to that of the PLA_2 enzyme. 5-LOX activity may not only be regulated by vitamin E's role in membrane stabilization and Asc regenerative capacity of vitamin E, but is also known to be influenced by intracellular Ca^{2+} levels (533,535).

Since investigations on the metabolism of AA by LOX have revealed that intermediate peroxy compounds and hydroxyl radicals are produced in the reactions (237,242,529) adding to the overall peroxidation state, and the fact that these compounds/radicals stimulate COX activity (259), the effect of combined vitamin E succinate and Asc supplementation on COX activity in LLCMK and BL6 cells was determined.

4.5 COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION AND CYCLOOXYGENASE ACTIVITY

4.5.1 INTRODUCTION

Prostanoids are synthesized from free fatty acid by a series of enzymatic reactions beginning with the formation of endoperoxides, in which the cyclooxygenase enzyme is important in the initial step of PG synthesis (507). COX is a membrane-bound multi-enzyme complex (222,232,233) contained in endoplasmic reticulum and nuclear membranes (232,244). The enzyme COX catalyzes oxygen insertion and rapid cyclization of AA into the hydroperoxy derivative prostaglandin PGG_2. This transient hydroperoxy endoperoxide is transformed by the peroxidase component of COX into the endoperoxide PGH_2 (235-238,503). The metabolism of AA by COX enzymes is found both in neoplastic and normal tissues (539). Vitamin E and other lipid antioxidants have been suggested to modulate the biosynthetic pathways in AA metabolism at a number of different points, including the COX enzyme (503,539). COX activity can be enhanced or inhibited by lipid antioxidants, depending on their type and concentration (540). Furthermore, the PG-hydroperoxidase reaction associated with COX activity is believed to generate a variety of peroxy compounds and hydroxyl radicals when catalyzing the conversion of PGG_2 to PGH_2 (234,235,240,242,268) adding to the overall lipid peroxidation tone. It is reported by Panganamala *et al* (541) that vitamin E is not a good inhibitor *in vitro* of COX but has profound effects *in vivo* on PG metabolism.

The present study was undertaken to determine the effect of combined vitamin E succinate and Asc supplementation on COX activity in LLCMK and BL6 cells.

4.5.2 MATERIALS AND METHODS

MATERIALS

Manganese protoporphyrin IX, Arachidonic acid (AA) and Prostaglandin B_2 (PGB_2) were purchased from Sigma Chemical Co., USA, while citric acid was obtained from H.W.O. Chemical Co., USA. Precoated silica gel $60F_{254}$ thin layer chromatography (TLC) plates were obtained from Merck, Darmstadt, Germany. Petroleum ether, ethyl ether and anhydrous sodium sulphate (Na_2SO_2) were purchased from Unilab, SAARCHEM, South Africa. 15-^{3}H Arachidonic acid was purchased from Amersham, Int., England, while tris (Hydroxymethyl)-aminomethan was obtained from Boehringer Mannheim, Germany.

METHODS

4.5.2.1 Cell culture procedures

The same procedures were used as those in 2.2.3.1 and 2.2.3.2, except that the resulting cell pellets were resuspended in 1ml 0.2M Tris HCl buffer, pH 8.0.

4.5.2.2 Homogenization of cells and separation of cellular components

The same procedure was carried out as used in 3.2.1.2, except that the Dounce homogenizer was rinsed with 1ml of a 0.2M Tris HCl buffer, pH 8.0 and the final resulting pellet was resuspended in 2ml 0.2M Tris HCl buffer.

4.5.2.3 Determination of cyclooxygenase activity

The method used for the determination of COX activity was a modified method of Yamamoto (542). The principle of this assay is based on the addition of radioactive substrate ^{3}H AA to the enzyme preparation and the reaction product (PGG_2) separated by TLC based upon their affinity for a non-polar solvent. A reaction mixture containing 70μl of 0.2M Tris HCl pH 8.0, 10μl of 40μM manganese protoporphyrin IX, 5nmol AA and 5μl ^{3}H AA (0.46pmol) was prepared for each sample. The reaction mixture was vortex mixed prior to the addition of 10μl COX enzyme preparation to disperse the AA. The reaction mixture and enzyme preparation were mixed with shaking at 24°C for

2 minutes. The reaction was terminated by the addition of a 300μl aliquot of a mixture containing ethyl ether:methanol:0.2M citric acid (30:4:1,v/v), precooled to -20°C. The tubes containing the terminated reaction mixture were immediately transferred to an ice bath where 0.5g anhydrous sodium sulphate was added, and samples gently mixed. A 150μl aliquot of the upper organic phase was removed using a graduated capillary tube and spotted on precoated silica gel $60F_{254}$ aluminium TLC plates. AA and PGB_2 were spotted as markers (PGG_2 is located between these markers (542) and is not commercially available). The plates were developed in TLC tanks containing ethyl ether:petroleum ether:acetic acid, (85:15:0.1, v/v/v) at 4°C. The plates were then air dried and AA and PGB_2 markers visualised using iodine vapours, with the product PGG_2 of COX activity located between these two markers. The silica gel regions corresponding to AA and PGG_2 were scraped off into scintillation vials containing 10mls of emulsifier scintillator plus™ scintillation cocktail and counted in a Beckman scintillation counter (LS 2 800). The remaining silica gel for each sample run was also scraped off the plate and radioactivity determined as above. Total counts per minute (cpm) was obtained by adding cpm AA + cpm PGG_2 + cpm remainder of plate. Radioactivity in the AA and PGG_2 portions were determined as a percentage of the total cpm. Percentage values were used to calculate the number of pmoles of radioactive PGG_2 formed from radioactive AA by the COX activity. Final results were expressed as pmoles $PGG_2/10^4$ cells.

4.5.3 RESULTS

LLCMK and BL6 cells supplemented with combined vitamin E succinate and Asc resulted in increased COX activity in both cell lines (see Table 22).

LLCMK cells supplemented with combined vitamin E succinate and Asc resulted in increased COX activity, with significant ($p \leq 0.01$) increases occurring at 10:50; 10:25 and 20:25μg/ml respectively, compared with control cultures (0E). A similar trend was observed for that of the BL6 cells, in which significant ($p \leq 0.001$) increases in COX activity were observed at each combined vitamin concentration set relative to control cultures (0E).

TABLE 22: The effect of combined vitamin E succinate and ascorbic acid supplementation on cyclooxygenase activity in the LLCMK and BL6 cells. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	Cyclooxygenase activity formed pmoles PGG_2 /10^6 cells	
	LLCMK CELLS	BL6 CELLS
0E	0.018 ±0.001	0.019 ±0.001
5:25	0.016 ±0.003	0.117[b] ±0.004
10:50	0.032[a] 0.006	0.143[b] 0.003
10:25	0.027[a] ±0.002	0.145[b] ±0.008
20:25	0.032[a] ±0.004	0.343[b] ±0.009

a - $p \leq 0.01$ relative to LLCMK control cultures (0E)
b - $p \leq 0.001$ relative to BL6 control cultures (0E)

4.5.4 DISCUSSION

Recent investigations on the metabolism of AA by COX have revealed that intermediate peroxy compounds and hydroxyl radicals are produced in the PGH_2- forming reactions (237,242,268,529). This suggests the higher the activity of COX, the higher the levels of lipid peroxidation within a system. Studies of the COX catalytic mechanism showed that a variety of lipid peroxides can trigger the acceleration of COX (242,259,529,543), including PGG_2 - the reaction product of the COX enzyme (237).

In addition to serving as an antioxidant in the protection of PUFAs in cellular membranes, vitamin E is active in a number of aspects of eicosanoid metabolism (196). It has been suggested that vitamin E may be involved in the modulation of the activity of key regulatory enzymes in the eicosanoid pathway (196,503), such as the COX enzyme. The role of vitamin E in COX reactions has however been disputed for some time.

Data from these studies have shown that supplementation of combined vitamin E succinate and Asc to LLCMK and BL6 cells resulted in a significant increase in COX activity. Possible explanations for this increased COX activity in both cell lines may be due to the effect of vitamin E in membrane stabilization (500), by virtue of specific physicochemical interactions between its phytyl side chain and the fatty acyl chains (125), and the subsequent altering of membrane enzymatic activities (501,502) such as COX. Furthermore the plane of orientation of the vitamin E molecule in the membrane enables the chromanol ring to participate in polar interactions at the membrane surface, resulting in Asc gaining access to the vitamin E radical formed at the membrane water interface regenerating vitamin E in turn prolonging its action (82,127,129,174). It is proposed that due to this synergistic action, increased COX activity was observed and with increasing concentration of vitamin E, increased COX activity is observed.

Furthermore, the PG-hydroperoxidase reaction (the second step of PG biosynthesis), associated with COX activity, is believed to produce a variety of peroxy compounds and hydroxyl radicals when catalizing the conversion of PGG_2 to PGH_2 (237,268,529,543). Hence any compound capable of increasing the activity of the COX enzyme would increase PG-hydroperoxide activity and indirectly increase the levels of free radicals within a system. Thus, we propose that supplementation of LLCMK and BL6 cells with increasing concentration of vitamin E resulted in the increased COX activity, in turn activating PG-hydroperoxidase activity. This protection of PG- hydroperoxidase by vitamin E is further enhanced by the regenerating capacity of vitamin C. The increased levels of oxygen-containing radicals may attack membrane PUFAs and increase lipid peroxidation levels. Thus our findings support the "scheme" proposed by Panganamala (503) of vitamin E acting as a highly lipid-soluble agent protecting COX from autooxidation without diminishing the concentrations of oxygen-centred radicals in the aqueous phase. Hence, in the BL6 cell system combined vitamin E and Asc supplementation enhances COX activity, in turn increasing the transient PGH_2 levels from AA and increasing free radical and lipid peroxidation levels as found in Chapter 3 .

In LLCMK and BL6 cells, compared with the basal COX levels in the control cultures (0E), a slightly lower activity was observed in the melanoma, BL6 cells. This supports the finding that in tumour cells, COX is decreased in activity compared with that of normal cells, but at present the reason for this reduction is not known (529).

Since prostaglandin synthesis is regulated by the activation of PLA_2 and the subsequent release of AA, and since the net production is dependent on the COX activity, the effect of combined vitamin E

succinate and Asc supplementation on PG production was determined.

4.6 PROSTAGLANDIN E_2 LEVELS AND COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION

4.6.1 INTRODUCTION

PGs are cell-to-cell messengers produced by eukaryotic cells in response to extracellular stimuli (283). PGs are not stored within the cell, thus any stimulation of their release requires the prior mobilization of substrate precursors (222,225-227,283), followed by oxidation by the PES complex (224,237).

PGs, especially the E series, have been shown to be elevated in a large number of tumours (9,220,228,300-304). Increased PGE_2 levels were found in tumours induced by the Maloney Sarcoma virus in mouse leg tissue, compared with normal leg tissue (226,305,320). Untransformed baby hamster kidney (BHK) and Balbc 3T3 fibroblasts in culture secrete small quantities of PGs into the medium, whereas transformation of these cells by polyoma virus results in a significant increase in PGE_2 levels released into the medium (554). Uncertainty remains as to whether the increase in the synthesis and release of PGs is the cause or the effect of increased proliferation rates, or whether it merely accompanies this phenomenon (300). Increased production and secretion of PGs by tumours has led to the proposal that tumour tissue could contain elevated amounts of PG synthetic enzymes and increased substrate availability due to altered enzyme activities within the PG biosynthetic pathway (306). Furthermore, PGs are suggested to interact with growth factors possibly contributing to the ability of the tumour cells to establish themselves at a metastatic site (300,303,306).

ElAttar and Lin (148) were the first to examine the *in vitro* effects of vitamin C and E on PGE_2 levels. They found that a combination of vitamins E and C at $100\mu M$ levels resulted in significant reduction of PGE_2 levels in both the fibroblast and SCC-25 cell line. Since the rate of synthesis and the net production of PGs is controlled by PLA_2 and COX (229,238), the present study was undertaken to investigate the effect of combined vitamin E succinate and Asc supplementation on PGE_2 levels in the two cell types used.

4.6.2 MATERIALS AND METHODS

MATERIALS

Methyl formate was purchased from Fluka, Switzerland. SEP-PAK C^{18} cartridges were obtained from Waters Associated, Inc., Massachusettes, USA. The PGE_2 [^{125}I] assay kit with Amerlex-M™ magnetic separation was purchased from Amersham International, Amersham, UK.

METHODS

4.6.2.1 Cell culture procedures

The methods recorded in 2.2.3.1 and 2.2.3.2 were repeated.

4.6.2.2 Homogenization of cells and separation of cellular components

The method described in 3.2.1.2 was repeated, except that 2ml milli Q water was used to rinse the homogenizer. Thus, the pellet (membrane fraction) was resuspended in 2ml PBS solution and 2ml milli Q water.

4.6.2.3 Extraction and isolation of prostaglandins

The PGs were extracted according to a modified method of Powell (545,546,cited in 547). The 4ml cellular fractions were added to 15ml cold ethanol (95%) and shaken for 10 minutes. The samples were diluted to 100ml with precooled water and vortex mixed for 1 minute. The pH of the suspension was adjusted to 3.0 using 1N HCl. The samples were then passed through SEP-PAK C^{18} cartridges which had previously been wetted with 20ml of 80% aqueous ethanol followed by 20ml of water to remove excess ethanol. The cartridges were washed with 10ml water and 10ml petroleum ether, before eluting the PGs with 5ml of methyl formate. The eluent was dried under a stream of N_2 at 25°C. The SEP-PAK C^{18} cartridges were regenerated for re-use by washing with 20ml of 80% aqueous ethanol solution followed by 20ml of water.

4.6.2.4 Prostaglandin E_2 [^{125}I] assay

This assay is based upon the competition between unlabelled prostaglandin E_2 (methyl oximate derivative) and a fixed quantity of ^{125}I-labelled prostaglandin E_2 (methyl oximate derivative) for a limited number of binding sites on a prostaglandin E_2 specific antibody which is specific for the methyl oximate derivative (548). Prior to analyzing the extracted PG fractions using the assay system, the PGs were converted to their methyl oximate derivatives as follows: the dried PG fractions were reconstituted with 100μl of assay buffer, pH 7.0. A 100μl aliquot of methyl oximation reagent (supplied with the PGE_2 assay kit) was added to the reconstituted sample and vortex mixed. The resulting solutions were incubated at 60°C for 1 hour to allow methyl oximation of the sample to occur. Samples were then diluted to a final volume of 500μl with PBS (pH 7.0) and assayed using the radioimmunoassay procedure as described in the assay protocol. A 100μl aliquot of sample was pipetted into appropriately labelled tubes. Tracer (100μl aliquots) was added to each tube followed by 100μl of antiserum. All the tubes were vortex mixed and incubated for 2 hours at 25°C in a water bath. After incubation, 250μl Amerlex-M second antibody reagent was added to each tube. The tubes were incubated for a further 15 minutes at room temperature before separating the antibody-bound fraction by centrifuging at 1500g for 10 minutes at 4°C. After centrifugation, the supernatant was poured off and discarded and the radioactivity present in each tube was determined by counting for 1 minute in a Packard auto-gamma scintillation counter. The pgPGE_2/ tube was read directly from the PGE_2 standard curve as represented in Appendix 6.

4.6.3 RESULTS

The PGE_2 concentration in the LLCMK control cultures (0E) and corresponding vitamin-supplemented cultures was significantly ($p \leq 0.001$) higher than the PGE_2 concentration found in the BL6 control cultures and corresponding vitamin supplemented cultures (Table 23).

In LLCMK cells, PGE_2 concentrations increased slightly with combined vitamin supplementation, with a significant ($p \leq 0.001$) increase observed at 10:50μg/ml relative to control cultures (0E). A similar trend was observed in the BL6 supplemented cells (Table 23) except that significant increases in PGE_2 concentrations relative to BL6 control cultures were observed at 10:50μg/ml ($p \leq 0.01$) and 20:25μg/ml ($p \leq 0.001$) combined vitamin E succinate and Asc supplementation.

TABLE 23: The effect of combined vitamin E succinate and ascorbic acid supplementation on prostaglandin E_2 concentration in LLCMK and BL6 cells, respectively. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	[PGE_2] pg/10^4 cells	
	LLCMK CELLS	BL6 CELLS
0E	13.55[a] ±1.78	1.65 ±0.07
5:25	13.59[a] ±1.23	2.33 ±0.11
10:50	38.44[ab] ±0.74	4.74[c] 0.15
10:25	15.89[a] ±0.06	2.49 ±0.08
20:25	15.79[a] ±0.74	12.26[d] ±0.89

a - $p \leq 0.001$ relative to BL6 control cultures (0E) and corresponding supplemented vitamin cultures
b - $p \leq 0.001$ relative to LLCMK control cultures (0E)
c - $p \leq 0.01$ relative to BL6 control cultures (0E)
d - $p \leq 0.001$ relative to BL6 control cultures (0E)

4.6.4 DISCUSSION

Vitamin E is believed to play an important role in a number of aspects of eicosanoid metabolism (95,196,503). Depending on the system, vitamin E did, or did not, have an effect (503). In one study (503), it was found that microsomes isolated from vitamin E supplemented animals synthesized far greater amounts of PGs than microsomes isolated from vitamin E-deficient animals. On the other hand, studies by ElAttar and Lin (148) have shown that a combination of vitamin E and C resulted in significant reduction of PGE_2 concentration in both the fibroblast and SCC-25 cell lines. In this study BL6 cells supplemented with 5:25μg/ml and 10:25μg/ml combined vitamin E succinate and Asc resulted in a non significant increase in PGE_2, while at 10:50 and 20:25μg/ml a significant increase in PGE_2 levels occurred relative to control cultures (0E). This study therefore does not support the findings of ElAttar and Lin. However, ElAttar and Lin (148) propose that the effects they observed of combined vitamin E succinate and Asc on endogenous PGE_2 were a reflection of the activities of both the PLA_2 and COX enzymes. The synthesis of PGE_2 is usually limited by the availability of free AA, which is liberated from membrane phospholipids by PLA_2 (549). This suggests that the observed increase in PGE_2 levels in BL6 cells in this study, following combined vitamin E succinate and Asc

could be as a result of the increased PLA_2 and COX activity as found in Section 4.2 and 4.5. Furthermore, another finding (Section 4.3) was that of marked increases in percentage AA composition in the membrane and stroma fraction of the substrate AA in combined vitamin supplemented BL6 cells.

In LLCMK cells, supplementation with varying levels of combined vitamin E succinate (5-20μg/ml) and Asc (25-50g/ml) resulted in an increase in PGE_2 levels compared with control cultures (0E), although this was only significant at the 10:50μg/ml level. Studies on the effects of combined vitamin E succinate and Asc supplementation on COX activity in LLCMK cells (Section 4.5) have shown that supplementation increases COX activity but inhibits PLA_2 activity. Furthermore, combined vitamin supplementation of LLCMK cells resulted in an increase in percentage AA composition in the membrane fraction only, suggesting the free AA levels in LLCMK cells may be low. This in turn suggests that the observed increase in PGE_2 levels in LLCMK cells was a result of increased COX activity and not PLA_2.

In this study, PGE_2 concentrations in the LLCMK control cultures (0E) were significantly higher than the PGE_2 levels in the BL6 control cultures. It has been suggested that the metastatic potential of BL6 cells is inversely related to the ability of these cells to synthesize PGE_2 (226,300,308,316,318), and that the highly metastatic BL6F10 as used in this study synthesizes smaller amounts than the less metastatic BL6F1 cells (308). Furthermore, PGE_2 levels have been suggested to be dependent on tissue specificity (300). The general conversion of AA to PGs varies with cell type and effector molecule, although each cell type appears programmed to produce certain types of PGs (283), hence it was of vital importance to determine the quantity and type of other PGs present in the cells used in this study.

Since Fulton (300) states that in the B16 melanoma system, besides PGE_2, the most common arachidonate metabolites are PGD_2, $PGF_{2\alpha}$ and PGI_2, the levels of these PGs in BL6 cells were determined as was the effect of combined vitamin E succinate and Asc supplementation on the levels of these particular PGs. No quantitation of the above-mentioned PGs was carried out in LLCMK cells, since combined vitamin E succinate and Asc supplementation was shown to have little effect on cell growth of this cell line and in view of the cost factor obtaining the assay kits, these assays were omitted.

4.7 COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID AND PGD_2, $PGF_{2\alpha}$ AND PGI_2 LEVELS

4.7.1 INTRODUCTION

AA can interact with prostaglandin endoperoxidase synthase (PES), a membrane-bound multi-enzyme complex (222,232). The ubiquitous PES catalyzes the first step in the formation of PGs and TXs from AA. This enzyme has two activities, a COX which converts AA to PGG_2 and a peroxidase which reduces PGG_2 to form PGH_2 (234-237,249). PGH_2 is the precursor of all PGs (270) as PGH_2 is at a pivotal stage in the divergent pathways leading to the synthesis of various types of PGs and TXs (237). Although the products are derived from a common intermediate, differences in the effects of inhibitors on each, demonstrate that separate receptors and enzymes are involved in the formation of each product from the PGH_2 intermediate (271). PGH_2 is converted to a combination of the prostanoids by the numerous synthase enzymes which are dependant on the cell or tissue (238,239).

4.7.2 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON PGD_2 AND $PGF_{2\alpha}$ LEVELS IN BL6 CELLS

4.7.2.1 MATERIALS AND METHODS

MATERIALS

Refer to 3.6.1.1

The prostaglandin D_2 [^3H] assay kit and prostaglandin $F_{2\alpha}$ [^3H] assay kits were purchased from Amersham Int., Amersham, UK.

METHODS

4.7.2.2 Cell culture procedures

The methods given in 2.2.31 and 2.2.3.2 were repeated.

4.7.2.3 Homogenization of cells and separation of cellular components

The method described in 3.1.2.2 was repeated.

4.7.2.4 Extraction and isolation of prostaglandins

Extraction and isolation of PGD_2 and $PGF_{2\alpha}$ was carried out using the method described in 4.6.2.3, except that dried PG extract was reconstituted in 500μl of assay buffer and duplicate aliquots (100μl) were used for the determination of PGD_2 and $PGF_{2\alpha}$.

4.7.2.5 Prostaglandin D [^{3}H] assay system

The assay is based upon competition between unlabelled PGD_2 and a fixed quantity of tritium-labelled PGD_2 for binding to a limited quantity of an antibody which has high specificity and affinity for PGD_2 (551). Preparation of samples for scintillation counting, using a Beckman L528 00 model scintillation counter, was as described in the assay kit. One hundred microlitre aliquots of sample were pipetted into appropriately labelled tubes. One hundred microlitres tracer was added, followed by the addition of 100μl of antiserum and 100μl of assay buffer. All the tubes were vortex mixed and incubated overnight at 2-8°C. Following incubation all tubes were placed in an ice-water bath to equilibrate for 10 minutes. Thereafter, 500μl of charcoal suspension was added to each tube and vortex mixed whereafter the tubes were left to stand in an ice-water bath for 10 minutes again. All tubes were centrifuged at 2 000g for 10 minutes at 4°C. The resulting supernantant material was immediately decanted into scintillation vials containing 10ml of emulsifier scintillation plus™ scintillation coctail. The concentration of unlabelled PGD_2 (pg PGD_2/tube) in the sample was then calculated from a standard curve recorded in Appendix 7.

4.7.2.6 Prostaglandin $F_{2\alpha}$ [^{3}H] assay system

The assay is based upon competition between unlabelled $PGF_{2\alpha}$ and a fixed quantity of ^3H-$PGF_{2\alpha}$ for binding to a limited quantity of an antibody which has high specificity and affinity for $PGF_{2\alpha}$ (552). Preparation of samples for scintillation counting was as described in the assay kit. A 100μl aliquots of sample were pipetted into appropriatetly labelled tubes. One hundred microlitres of tracer was added to each tube followed by the 100μl of antiserum and 100μl of assay buffer. All tubes were vortex mixed and incubated overnight at 2-8°C. Following incubation period all tubes were placed in

an ice-bath and allowed to equilibrate for 10 minutes. To each tube a 500μl aliqout of charcoal suspension was added. The tubes were vortex mixed and allowed to incubate in an ice-bath for 10 minutes again. All tubes were centrifuged at 2 000**g** for 10 minutes at 4°C. Resulting supernatant solutions were decanted into scintillation vials containing 10ml of emulsifier plus™ scintillation cocktail. The concentration of unlabelled $PGF_{2\alpha}$ (pg $PGF_{2\alpha}$/tube) in the sample was then calculated from a standard curve recorded in Appendix 8.

4.7.3 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ON PGI_2 LEVELS IN BL6 CELLS

4.7.3.1 MATERIALS AND METHODS

MATERIALS

Sulphuric acid (H_2SO_4) was purchased from Holpro Chemical Co., South Africa. 6-Keto-prostaglandin $F_{1\alpha}$ enzyme immunoassay (EIA) system was purchased from Amersham Int., Amersham, UK.

METHODS

4.7.3.2 Cell culture procedures

Methods used in 2.2.3.1 and 2.2.3.2 were repeated.

4.7.3.3 Homogenization of cells and separation of cellular components

The method described in 3.1.2.2 was repeated.

4.7.3.4 Extraction and isolation of PGI_2

Extraction of PGI_2 was carried out using the method described in 4.6.2.3.

4.7.3.5 6-Keto-prostaglandin $F_{1\alpha}$ enzyme immunoassay (EIA) system

Prostacyclin, also known as PGI_2, is an unstable vinyl ether formed from the PG endoperoxide,

PGH_2, which undergoes spontaneous hydrolysis to 6-keto-prostaglandin $F_{1\alpha}$. Due to this spontaneous hydrolysis to PGI_2 , the quantitation of 6-keto-prostaglandin $F_{1\alpha}$ is accepted by many researchers as a measure of PGI_2 formation (553).

The assay is based on the competition between unlabelled 6-keto-prostaglandin $F_{1\alpha}$ and a fixed quantity of peroxidase labelled 6-keto-prostaglandin $F_{1\alpha}$ for a limited number of binding sites on a 6-keto-prostaglandin $F_{1\alpha}$ specific antibody (553). Preparation of samples for PGI_2 determination was carried out as described in the assay kit, and the resultant solution read at 450nm in a EAR 400 plate reader. Fifty microlitre aliquots of samples were pipetted into appropriate wells in a microtitre plate. Fifty microlitres of antiserum was added to each well, followed by shaking for 30 minutes on a microtitre plate shaker at room temperature. Following incubation, 50μl of 6-keto-prostaglandin $F_{1\alpha}$ peroxidase conjugate was added to each well, and the microtitre plate further incubated at room temperature by shaking for 1 hour on a microtitre plate shaker. Thereafter, wells were aspirated and washed four times with 400μl wash buffer. To the washed wells was added 150μl enzyme substrate followed by further incubation at room temperature by shaking for 15minutes. A 100μl aliquot of 1M sulphuric acid was added into each well and the optical density determined in a plate reader at 450nm within 30 minutes. The concentration of unlabelled 6-keto-prostaglandin $F_{1\alpha}$ (pg $PGF_{1\alpha}$/well) in a sample was determined from a standard curve recorded in Appendix 9.

4.7.4 RESULTS

Basal PGE_2 levels in BL6 cells were significantly ($p \leq 0.001$) higher than PGD_2, $PGF_{2\alpha}$ and PGI_2 levels. From the Table below it can be seen that basal levels in decreasing order of magnitude are as follows: PGE_2; PGD_2; $PGF_{2\alpha}$ and PGI_2.

TABLE 24: Comparison of prostaglandin levels in control cultures of BL6 cells. Results are the mean of three cultures ±SEM.

*Treatment	Prostaglandin levels [pg/10^4 cells]			
	PGD_2	**PGE_2**	**$PGF_{2\alpha}$**	**PGI_2**
0E	0.1853[a] ±0.0307	1.6535 ±0.0712	0.0595[a] ±0.0111	0.0077[a] ±0.0003

* - control cultures (0E) containing 0.1% final volume of ethanol
a - $p \leq 0.001$ relative to PGE_2 levels

TABLE 25: The effect of combined vitamin E succinate and ascorbic acid supplementation on PGD_2, $PGF_{2\alpha}$ and PGI_2 levels in BL6 cells respectively. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	**Prostaglandin levels [pg/10^4 cells]**		
	PGD_2	**$PGF_{2\alpha}$**	**PGI_2**
0E	0.185 ±0.031	0.060 ±0.011	0.0077 ±0.0001
5:25	0.387[a] ±0.069	0.085 ±0.017	0.0078 ±0.0001
10:50	0.220 ±0.034	0.093 ±0.006	0.0093[b] ±0.0004
10:25	0.188 ±0.007	0.059 ±0.003	0.0094[c] ±0.0003
20:25	0.255 ±0.005	0.074 ±0.004	0.0164[d] ±0.0003

a - $p \leq 0.05$ relative to PGD_2 control cultures (0E)
b - $p \leq 0.01$ relative PGI_2 control cultures (0E)
c - $p \leq 0.05$ relative PGI_2 control cultures (0E)
d - $p \leq 0.001$ relative PGI_2 control cultures (0E)

The levels of PGD_2 and $PGF_{2\alpha}$ in BL6 cells (Table 24) were found to be greater with combined vitamin E succinate and Asc supplementation, although the only significant ($p \leq 0.05$) increase was in PGD_2 at 5:25g/ml relative to control cultures (0E). In these cells combined vitamin E succinate and Asc resulted in an increase in the PGI_2 levels, with significant increases recorded at 10:50 ($p \leq 0.01$); 10:25 ($p \leq 0.05$) and 20:25μg/ml ($p \leq 0.001$) relative to the control cultures (0E).

4.7.5 DISCUSSION

The quantity and type of PGs produced by the given cells is critical to the metabolic effect they exert (283). Results from a number of laboratories studying the mechanisms controlling the synthesis of these molecules have clearly shown that at least two critical determinants exist in PG synthesis. These are the activation of PLA_2 and the action PES (228,283). Studies described earlier on PLA_2 and COX activity (Sections 4.2 and 4.5) showed significant increases in PLA_2 and COX activity following combined vitamin E succinate and Asc supplementation. Since COX activity forms an integral part of the PES, which functions to catalyze the conversion of AA to PG precursor PGH_2 (232-239), it would seem that any increase in PLA_2 and COX activity would be expected to increase the overall levels of various PGs.

Results from this study have shown that combined vitamin E succinate and Asc supplementation of BL6 cells resulted in marked increases in PGE_2, PGD_2, $PGF_{2\alpha}$, and PGI_2 levels. PGE_2 concentrations, as described earlier, were significantly increased at 10:50 and 20:25μg/ml combined vitamin E succinate and Asc levels respectively, while PGI_2 levels were significantly increased at 10:50; 10:25 and 20:25μg/ml relative to the respective control cultures (0E). The alteration in PG levels observed within these cells following combined vitamin supplementation may be due to alterations in the individual PG synthetase enzymes. Toivanen (501) and Chan *et al* (554) have shown that vitamins E and C affect PGI synthesis in *in vitro* studies. Table 25 shows a comparison of PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 levels within the BL6 control cultures (0E). Fulton (300) states that in the B16 melanoma system PGE_2, PGD_2, $PGF_{2\alpha}$ and PGI_2 are the major arachidonate metabolites. It is evident from this table that BL6 cells convert AA to PGD_2, PGE_2, $PGF_{2\alpha}$ and PGI_2 however, the PGE_2 concentration was significantly higher than the PGD_2 levels. This observation is contradictory to what has been reported by other researchers (300), although $PGF_{2\alpha}$ and PGI_2 levels found in this study support their findings that PGI_2 levels in BL6 cells are markedly lower in BL6 cells than were the other PGs. Lipid hydroperoxides, which are formed as a result of free radical formation (54,206,211), and hydroperoxide intermediates in PG synthesis (268,237,529,543), inhibit PGI_2 synthesis (501). The low PGI_2 levels may therefore be the result of markedly high levels of lipid peroxidation found in BL6 cells (Section 3.1).

It has been reported that the synthesis of $PGF_{2\alpha}$ and PGE_2 are linked. Studies by Kowaza *et al* (555), on cultured neonatal mouse calvaria reported that $PGF_{2\alpha}$ stimulates AA release and PGE_2 synthesis. This implies that any changes in $PGF_{2\alpha}$ levels will correlate with similar changes in PGE levels. BL6 cells supplemented with combined vitamin E succinate and Asc did in fact show increases in $PGF_{2\alpha}$ levels, with a similar trend being observed for PGE_2 levels.

PGE_2 (285,320,416,447,453) and PGI_2 (286,287,422,447) are thought to exert their cellular effects via a cAMP-adenylate linked system. Therefore it was of interest in this study to determine the effect of combined vitamin E succinate and Asc supplementation on the levels of AC activity and cAMP levels in both LLCMK and BL6 cells.

4.8 COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION ADENYLATE CYCLASE ACTIVITY AND CYCLIC ADENOSINE MONOPHOSPHATE FORMATION

4.8.1 INTRODUCTION

Alterations in the membrane intracellular signalling systems are frequently implicated in the development of neoplasia (346,510). One of the major intracellular signalling systems identified to date is the AC-cAMP linked system (510), which is membrane bound (198,331,383) and composed exclusively of intrinsic membrane-bound proteins (198,332,390).

Like many other membrane-bound enzymes, AC activity is affected by changes in the composition of the phospholipid fatty acyl chains and polar head groups (121). This phenomenon is explained by the ability AC to exist in different conformations, and the fact that alterations in membrane lipid composition cause the enzyme to shift from one conformation to another (121). AC activity is also affected by changes of membrane lipid environment (405,408). Canuto *et al* (409) found that in AH-130 Ascites Hepatoma cells there was an enhanced AC activity which was not only related to membrane fluidity but also to an increase in lipid peroxidation. This sensitivity to alterations in membrane fluidity is believed to be due to the changes in the conformational flexibility of intrinsic proteins (186,198) and also due to alterations in the efficacy of interaction of the various components of the AC system (186,198,390).

Studies using transformed and normal cell lines in culture showed that transformed cells had a lower cAMP level than normal cells, and that the levels of cAMP were inversely related to cell growth (427,456-461). Intracellular cAMP levels are regulated by the AC and/or PDE (386,462) hence alterations in AC, and/or PDE through changes in the membrane fluidity could account for the decrease in cAMP levels observed in tumour cells (386,398,453,462). In addition to regulation by membrane fluidity the AC system appears to be affected by PGs (341,410,412-415) as well as intracellular Ca^{2+} levels (344,426). Since combined vitamin E succinate and Asc supplementation has been shown to influence the cellular concentrations of PGs, particularly PGE_2 (Section 4.6) and Ca^{2+} (Section 4.1) in LLCMK and BL6 cells, this study was undertaken to determine the effect of combined vitamin E succinate and Asc supplementation on AC activity and cAMP levels in these cells.

4.8.2 MATERIALS AND METHODS

MATERIALS

Adenosine 5'-triphosphate disodium salt (ATP), Adenosine 3'-5'-cyclic monophosphate sodium salt (cAMP) and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma Chemical Co., USA. Triethanolamine and aluminium oxide 90 (Woeln N active-neutral) were obtained from Merck, Darmstadt, Germany. (2,5'8-^{3}H) Adenosine 5'-triphosphate ammonium salt and the [^{3}H] cAMP assay kit were purchased from Amersham, Int., PLC, USA. Creatine-kinase and creatine-phosphate-Na.4H_2O (creatine-phosphate) were obtained from Boehringer Mannheim, Germany.

METHODS

4.8.2.1 Cell culture procedures

Methods described in 2.2.3.1 and 2.2.3.2 were repeated, except that the cell pellet was resuspended in 1ml of 0.1M Tris HCl buffer, pH 7.5 containing 4mM EDTA.

4.8.2.2 Homogenization of cells and separation of cellular components

The method described in 3.1.1.2 was used with the following changes. The Dounce homogenizer was rinsed with 1ml of Tris HCl buffer. After centrifugation the supernatant fractions were retained for cAMP determinations and membrane fractions were resuspended in 2ml Tris HCl buffer, pH 7.5.

4.8.2.3 Protein Determination

Protein determinations of the membrane fractions were carried out using the modified method of Lowry *et al* (cited in 514). The protein standard curve is recorded in Appendix 5.

4.8.2.4 Adenylate cyclase activity assay

AC activity of the membrane fraction of the cells was determined using the modified method of Schultz and Jakobs (383) and Salomon (385). The assay involves measuring the rate of conversion of [α-^{32}P]ATP to [^{32}P] cAMP. To 20μl 1M Tris HCl buffer, 20μl [α-^{32}P] ATP and a 30μl aliquot of

the standard component mixture was added. The standard component mixture contained 500μl 1M triethanolamine buffer, 500μl 100mM $MgCl_2$ solution, 1ml 10mM 3-IBMX (PDE inhibitor), 100μl cAMP solution, 100μl ATP solution, 16.4mg creatine-phosphate, 4mg creatine kinase, 10mg BSA and 800μl of milli Q water to give a final volume sufficient for 100 assays. After mixing thoroughly, the tubes were allowed to equilibrate thermally at 37°C for five minutes. A 30μl aliquot of the membrane suspensions was added to the tubes, vortex mixed and incubated for a period of one minute. The reactions were stopped at 15 second intervals by adding 400μl 125mM $ZnCl_2$ solution and 500μl 125mM Na_2CO_3 solution to determine the optimum reaction time.

The samples were centrifuged for 5 minutes at 10 000g in a Eppendorf 5203 centrifuge. After centrifugation, 800μl of the supernatant was transferred to alumina columns which had been prepared by pouring dry neutral alumina into plastic syringes to a height of 2cm. These columns were then washed with 10ml 0.1M Tris HCl buffer before the sample was applied. After the sample had drained, 2ml of the Tris HCl buffer was applied, followed (after draining) by another 2ml of this buffer. The entire eluant was collected in scintillation vials containing 10ml emulsifier scintillator plus™ scintillation cocktail. The columns were regenerated by washing with 10ml of the the same Tris HCl buffer.

AC activity was calculated as catalytic activity relative to the mass of protein expressed as the formation of 1pmol cAMP per minute (1unit) per mg protein.

4.8.2.5 Cyclic adenosine monophosphate extraction

The respective stroma fractions of the LLCMK and BL6 cells 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.

4.8.2.6 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 BL6 cells, respectively. The assay is based on the competition between unlabelled cAMP and a fixed quantity of [^{3}H] cAMP for binding to a protein which has a specificity and affinity for cAMP (556).

The samples were heated in a boiling water bath for 5 minutes to coagulate protein. After centrifugation, at 3 000g, in a Eppendrof 5203 centrifuge, the cAMP in the supernantant was assayed using a cyclic AMP[^{3}H] assay system. The tubes, as required, were placed in racks in an ice bath which was kept at 0^{0}C. Fifty microlitres of the samples were added to appropriate assay tubes. Labelled cAMP (50μl) was added to every assay tube followed by 100μl of the binding protein. All the tubes were vortex mixed for 5 seconds. The ice bath containing the tubes was placed in a cold room at 4°C and left for 2 hours. At least 15 minutes before the end of the incubation time, 20ml of ice cold distilled water was added to the charcoal reagent which was strirred continously during use. At the end of the 2 hour incubation period, 100μl of the charcoal suspension was added to each tube. The tubes were centriifuged at 3 000g for 4 minutes. A 200μl aliquot of the resultant supernantant was removed, without disturbing the sediment, and placed in scintillation vials containing 10ml emulsifier scintillator plus™ scintillation cocktail. The amount of labelled protein-cAMP complex formed is inversely related to the amount of unlabelled cAMP present in the assay sample. The cAMP standard curve is recorded in Appendix 10.

4.8.3 RESULTS

4.8.3.1 Adenylate cyclase activity

AC activity (Table 26) in the LLCMK control cultures (0E) was significantly ($p \leq 0.01$) higher than the AC levels detected in the BL6 control cultures (0E).

In the LLCMK cells (Table 26) supplemented with varying levels of combined vitamin E succinate and Asc respectively no increasing or decreasing trend in AC activity was found. However, at 10:50μg/ml combined vitamin E succinate and Asc supplementation a significant ($p \leq 0.001$) increase in AC was observed relative to control cultures (0E). On the other hand, in BL6 cells (Table 26), significantly ($p \leq 0.01$) higher levels of AC activity were detected following combined vitamin E succinate and Asc supplementation compared to control cultures (0E).

TABLE 26: The effect of combined vitamin E succinate and ascorbic acid supplementation on adenylate cyclase activity in LLCMK and BL6 cells. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	Adenylate cyclase activity (U/mg protein)	
	LLCMK CELLS	BL6 CELLS
0E	58.30[a] ±2.15	51.80 ±0.25
5:25	61.05 ±0.98	61.02[c] ±1.15
10:50	79.16[b] ±1.52	64.72[c] ±0.56
10:25	58.21 ±0.58	63.44[c] ±0.83
20:25	54.66 ±0.76	93.71[d] ±3.05

a - $p \leq 0.01$ relative to BL6 control cultures (0E)
b - $p \leq 0.001$ relative to LLCMK control cultures (0E)
c - $p \leq 0.01$ relative to BL6 control cultures (0E)
d - $p \leq 0.01$ relative to BL6 control cultures (0E)

4.8.3.2 Cyclic adenosine monophosphate levels

cAMP levels in the control cultures (0E) of LLCMK cells are significantly ($p \leq 0.01$) higher than the cAMP levels found in control cultures (0E) of BL6 cells (Table 27).

At the varying levels of combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml), significantly ($p \leq 0.001$) higher levels of cAMP were also detected in LLCMK than the corresponding BL6 cells, however at 20:25μg/ml a significantly ($p \leq 0.05$) lower cAMP level was found relative to the corresponding BL6 cell group (Table 27).

In the LLCMK cells significant increases in cAMP levels were detected at 5:25 ($p \leq 0.001$); 10:50 ($p \leq 0.001$); 10:25 ($p \leq 0.05$) and 20:25μg/ml ($p \leq 0.001$) respectively compared to control cultures (0E). BL6 cells supplemented with combined vitamin E succinate and Asc also resulted in significant increases in cAMP levels at 5:25; 10:50; 10:25 ($p \leq 0.05$) and 20:25μg/ml ($p \leq 0.001$) respectively when compared to control cultures (0E).

TABLE 27: The effect of combined vitamin E succinate and ascorbic acid supplementation on cyclic adenosine monophosphate levels in LLCMK and BL6 cells. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	cAMP concentration pmol/mg protein	
	LLCMK CELLS	BL6 CELLS
0E	6.37[a] ±0.19	4.32 ±0.23
5:25	10.93[bd] ±0.48	6.33[f] ±0.13
10:50	10.86[bd] ±0.21	5.95[f] ±0.12
10:25	7.44[be] ±0.13	6.44[f] ±0.16
20:25	13.75[cd] ±0.46	15.61[g] ±1.17

a - $p \leq 0.01$ relative to BL6 control cultures (0E)
b - $p \leq 0.001$ relative to corresponding BL6 supplemented cells
c - $p \leq 0.05$ relative to corresponding BL6 supplemented cells
d - $p \leq 0.001$ relative LLCMK control cultures (0E)
e - $p \leq 0.05$ relative LLCMK control cultures (0E)
f - $p \leq 0.05$ relative to BL6 control cultures (0E)
g - $p \leq 0.001$ relative to BL6 control cultures (0E)

3.8.4 DISCUSSION

The AC enzyme is a ubiquitous, multicomponent and membrane bound enzyme (198,331,383) which catalyzes the conversion of ATP to cAMP (331,384,385). cAMP acts as a second messenger for many systematic and local hormones and consequently controls many physiological processes at the cellular level (198,386).

When comparing the basal levels of AC and cAMP in LLCMK and BL6 cells, LLCMK cells had a significantly higher AC activity and cAMP levels. These results support numerous other studies which report lower AC activity and cAMP levels in transformed cells compared to their normal counterparts (427,456-461). These results suggest that transformation of a normal cell to tumour cells produce changes in the plasma membrane which alter AC activity, resulting in decreased activity.

Supplementation of BL6 cells with vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) resulted

in a significant increase in AC activity at each combined vitamin concentration combination. Changes in membrane fluidity is characteristic of tumour cells (123), hence the ability of vitamin E to alter membrane fluidity through its physicochemical interactions (95,125,127,498) could account for the observed increase in AC activity in BL6 cells. Furthermore Asc would increase the action of the vitamin E due to the regeneration capacity of vitamin C. In addition, various PGs are known to influence AC activity (410,411). Hence the marked increases PGE_2 levels found following supplementation of combined vitamin E succinate and Asc (Section 4.6) could also be a factor causing an increase in AC acitivty in BL6 cells.

The increase in cAMP levels in BL6 cells as a result of vitamin supplementation were positively correlated with increases in AC activity found in these cells (Table 26). In LLCMK cells, significant increases in cAMP levels were also detected at each combined vitamin concentration set compared to control cultures (0E) which also correlated with AC activity at 5:25 and 10:50μg/ml respectively, however at 10:25 and 20:25μg/ml no such correlation existed.

The effect of combined vitamin E succinate and Asc supplementation on the relationship between the second messengers and AA metabolites described in this chapter and the possible influence this may have on cell growth and metabolism will now be discussed.

CHAPTER 5

COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION IN RELATION TO OXIDATION STATE, SECOND MESSENGERS AND CELL GROWTH

Cell metabolism is a collective term for the processes by which a cell regulates its myriad of chemical reaction sequences, and in doing so, controls its internal environment (176). The cells motif for metabolism is to extract energy and reducing power from the cell environment and to synthesize the building blocks for macromolecules (392). The pathway outlined in Figure 9 (repeated on page 139) is just one of the numerous pathways in this highly integrated network. It is however, a pathway that is vital to metabolism as it produces secondary messengers which control various metabolic processes as well as the cell cycle.

The micronutrients, which include the vitamins, minerals and some trace elements are dietary components essential to normal metabolic function (25). A number of studies clearly indicate a relationship between diet and cancer incidence in human populations. Furthermore, differences in cancer rates have been associated with these particular differences in diet (7,25,80,227). One of the most widely explored areas of cancer research is that of anticarcinogenesis, a concept first put forward by Crabtree (cited in 80). Evidence suggests that natural foods contain factors that protect against cancer. Two such micronutrients obtained from the diet are the antioxidants E and C which have received much attention in preventing, enhancing and inhibiting various forms of cancer. Vitamin E supplementation has been shown to have an inhibitory effect on numerous cell lines (49,91,92,94,96,97,148,477) while vitamin C has been reported to have both an inhibitory and a stimulatory effect on the growth of various tumour cell lines (48,64,67,76,478-480).

Vitamin E and Asc have been proposed to act synergistically, where vitamin C represents a pool of antioxidant potential regenerating oxidized vitamin E (103,135,167,168,174) in turn prolonging the action of vitamin E. Since studies by Prasad and Rama (92), Kline *et al* (97) and Prasad and Edwards-Prasad (94) showed significant inhibition of cell growth in various cell lines following vitamin E succinate supplementation one would expect a more pronounced inhibitory growth effect following combined vitamin E succinate and Asc due to the sparing effect of vitamin E. Hence of principal concern in this study has been the effect of combined vitamin E succinate and Asc supplementation on the metabolism of the non-malignant LLCMK and malignant BL6 cells. The particular pathway under scrutiny in these cells is outlined in Figure 9. This study does consider AA

AA supplementation, but the emphasis is on combined vitamin E succinate and Asc supplementation. Chapter 2 investigated the effect of nutrient supplementation on both LLCMK and BL6 cell growth, while Chapter 3 determined the effect of nutrient supplementation on free radical formation and lipid peroxidation levels within these cells. In Chapter 4 the effect of combined nutrient supplementation on certain AA metabolites within these cells was determined. This chapter will relate LLCMK and BL6 cell growth to the free radical formation, lipid peroxidation levels, and 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 combined vitamin E succinate and Asc supplementation on cell growth between experiments. Consequently, relationships between cell growth and free radical formation, lipid peroxidation, and AA 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, free radical formation, lipid peroxidation and AA metabolites relationships with respect to the pathway under consideration. It must be noted that since supplementation of combined vitamin E succinate and Asc to LLCMK cells resulted in variable and less significant effects on cell growth and second messenger metabolism with no real correlation between the two, attention will be placed on the BL6 cells.

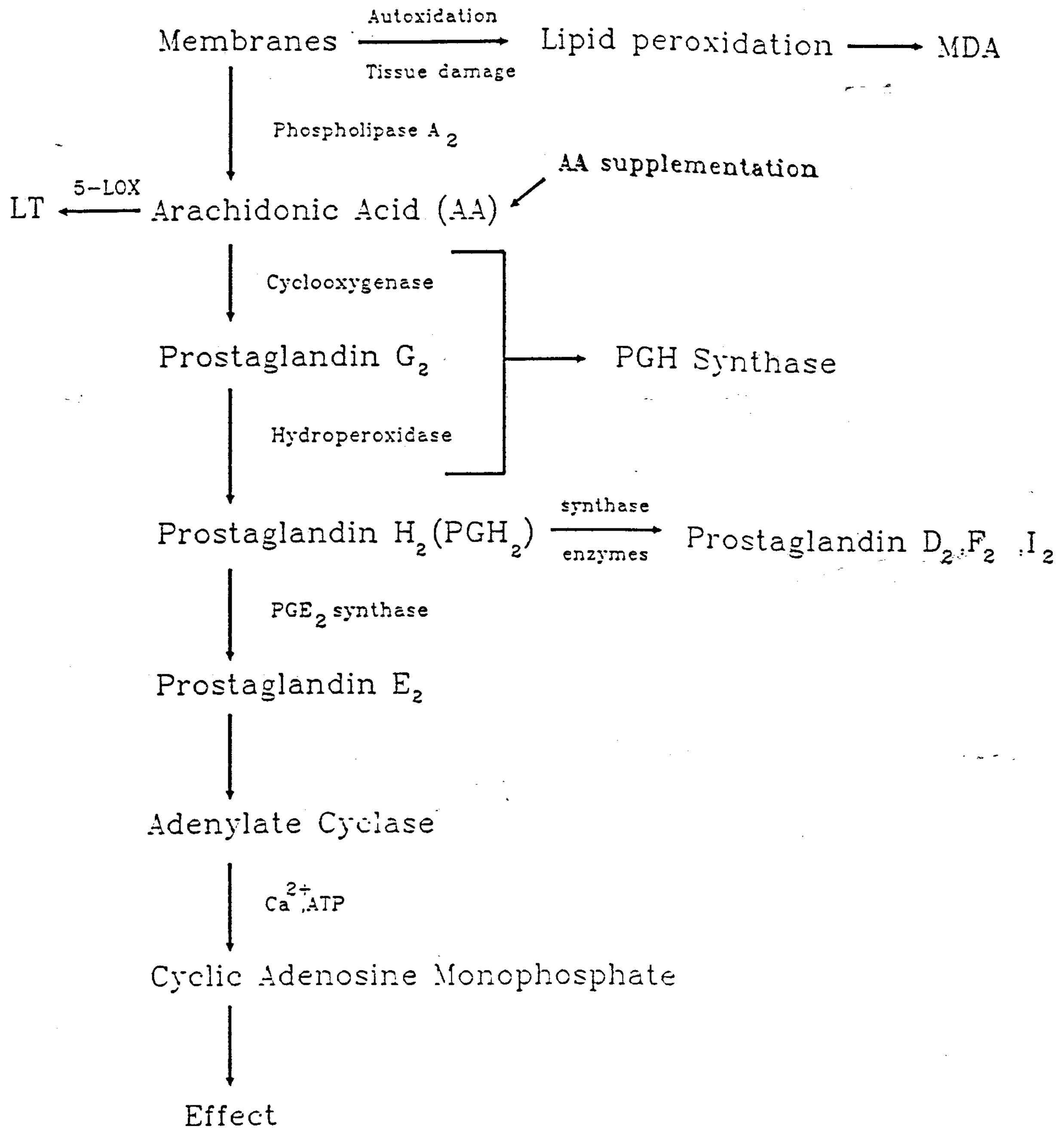


Figure 9: Schematic representation of the AA cascade pathway investigated in this study.

Lipid peroxidation is initiated by free radical attack on membrane PUFAs which produces large amounts of toxic radical products that have been implicated in carcinogenesis (219). Studies have revealed that the inhibitory effect on cell proliferation is related to lipid peroxidation (543;557-559). PUFAs generate lipid peroxides in tissue culture and may represent only one of several pathways in which fatty acid metabolism can alter cell proliferation (557). Thus of concern here is the relationship between cell growth, free radical formation, lipid peroxidation levels and combined vitamin E succinate and Asc supplementation.

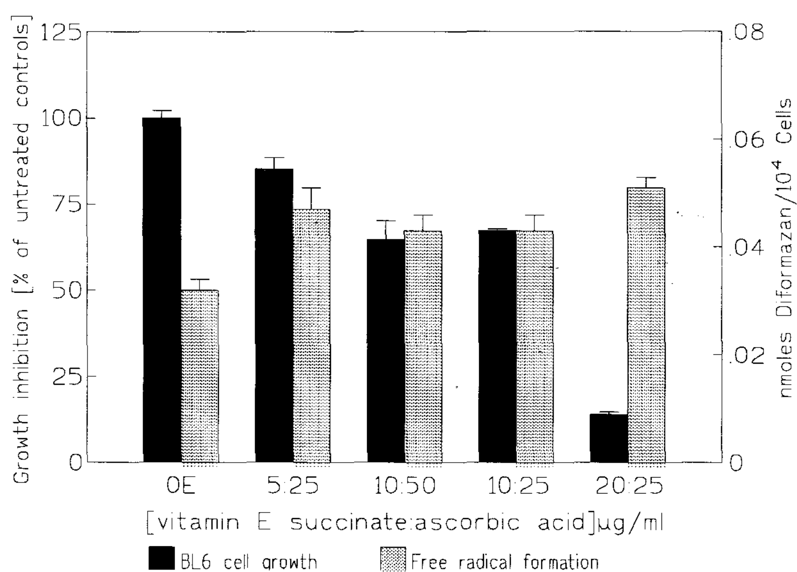


Figure 21: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and free radical formation.

Combined vitamin E succinate and Asc supplementation of LLCMK cells did not significantly affect cell growth or free radical formation. Supplementation of the combined vitamins to the BL6 cells, however, resulted in a significant decrease in cell growth, together with a marked increase in free radical formation (Figure 21). A similar inverse relationship would be expected of cell growth and lipid peroxidation levels in BL6 cells as lipid peroxidation is mediated by free radicals. This was in fact found in BL6 cells (Figure 22).

Supplementation of combined vitamin E succinate and Asc to LLCMK cells did not result in an inverse relationship between cell growth and lipid peroxidation levels, although a significant increase in lipid peroxidation levels following combined vitamin E succinate and Asc was observed at 5:25 and 20:25μg/ml respectively and this correlated with a slight decrease in cell growth. Goldring *et al* (214) suggest that the levels of lipid peroxidation are inversely related to the rate of cellular growth

irrespective of whether they are malignant or not. Furthermore, these peroxidation products, or breakdown products therefrom may influence the way in which proliferative signals cross membranes. This however, was not the case with LLCMK cells in this study, while BL6 cells on the other hand exhibited an inverse relationship. These results were further substantiated by studies of Ottino and Duncan (560). This suggests that free radical formation and lipid peroxidation levels are negative regulators of BL6 cell growth and furthermore that combined vitamin E succinate and Asc enhance free radical formation and lipid peroxidation levels.

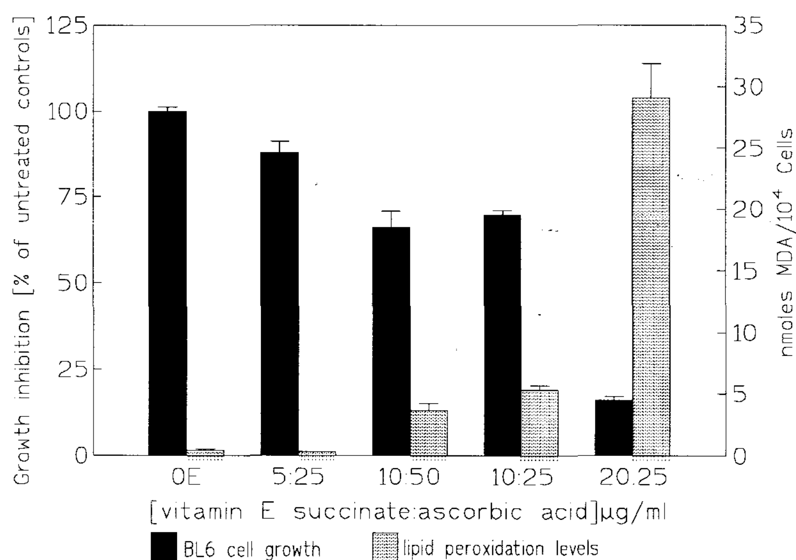


Figure 22: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and lipid peroxidation levels.

Lipid peroxidation is the process of oxidative turnover of PUFAs in which both enzymic and non-enzymic (529,543) lipid peroxidation are initiated by free radicals. Recent investigations on the metabolism of AA by COX and LOX have revealed that intermediate peroxy compounds and hydroxyl radicals are produced in these reactions (237,242,268,529). Since COX activity is known to produce peroxy compounds and hydroxyl radicals adding to the overall lipid peroxidation state, a study was undertaken to determine a relationship between cell growth, and the enzyme activities following combined vitamin E succinate and Asc supplementation. In this experiment, LLCMK cell growth was significantly deceased when supplemented with combined vitamin E succinate and Asc. This decrease in cell growth was inversely related to a significant increase in COX activity with the exception at 5:25µg/ml where COX activity decreased slightly. Combined vitamin E succinate and

Asc supplementation of BL6 cells also resulted in a significant decrease in cell growth, together with a significant increase in COX activity (Figure 23). This suggests an inverse relationship between BL6 cell growth and COX activity, and furthermore supplementation of combined vitamin E succinate and Asc markedly increases the activity of the COX enzyme.

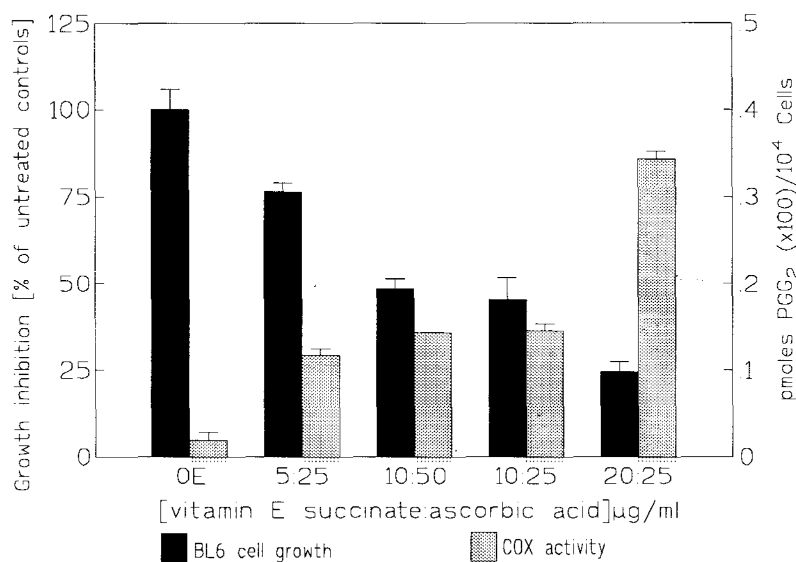


Figure 23: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and cyclooxygenase activity.

In another experiment supplementation of combined vitamin E succinate and Asc of the LLCMK cells did not significantly effect cell growth or 5-LOX activity, although at 5:25μg/ml and 10:50μg/ml respectively, an inverse relationship between cell growth and 5-LOX activity could be seen. In BL6 cells supplementation of combined vitamin E succinate and Asc resulted in an overall significant decrease in cell growth (Figure 24). This significant decrease in growth was accompanied by an overall significant increase in 5-LOX activity. Since investigations on the metabolism of AA by LOX and COX have revealed that intermediate peroxy compounds and hydroxyl radicals are produced in these reactions (237,242,268) and since the activities of both LOX and COX were increased in this study, this would account for the increases in free radicals and lipid peroxidation levels observed in this study as a result of vitamin supplementation.

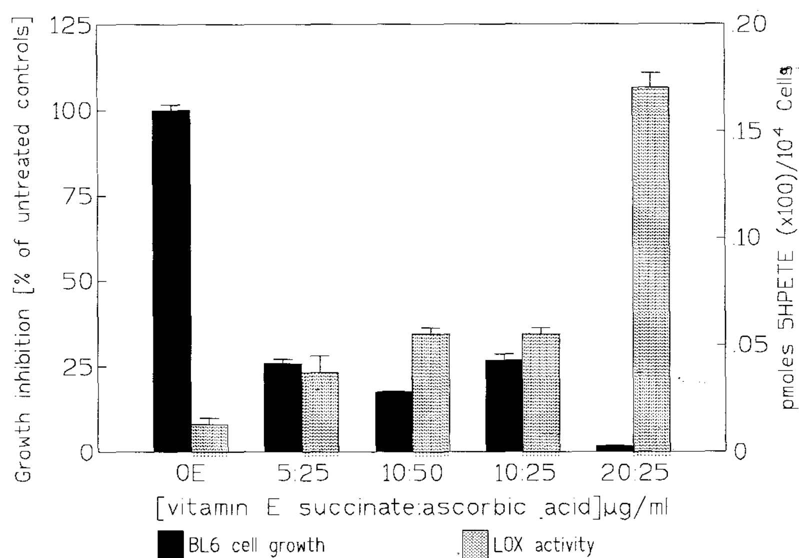


Figure 24: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and 5-lipoxygenase activity.

Another factor which needed to be taken into consideration in this study was the effect of combined vitamin E succinate and Asc on intracellular Ca^{+} levels, since it has been suggested that Ca^{2+} controls cell proliferation (329,335,356) and it is known that Ca^{2+} affects PLA_2, LOX and AC activity. In LLCMK cells, combined vitamin E succinate and Asc supplementation did not result in any trend between cell growth and Ca^{2+} levels while in BL6 cells (Figure 25) cell growth decreased significantly with the exception at 5:25μg/ml where a slight increase in cell growth was observed. Ca^{2+} levels increased significantly following combined vitamin E succinate and Asc supplementation, although at 5:25μg/ml a decrease in Ca^{2+} was detected. Thus, combined vitamin E succinate and Asc supplementation results in an inverse relationship between BL6 cell growth and cellular Ca^{2+}levels.

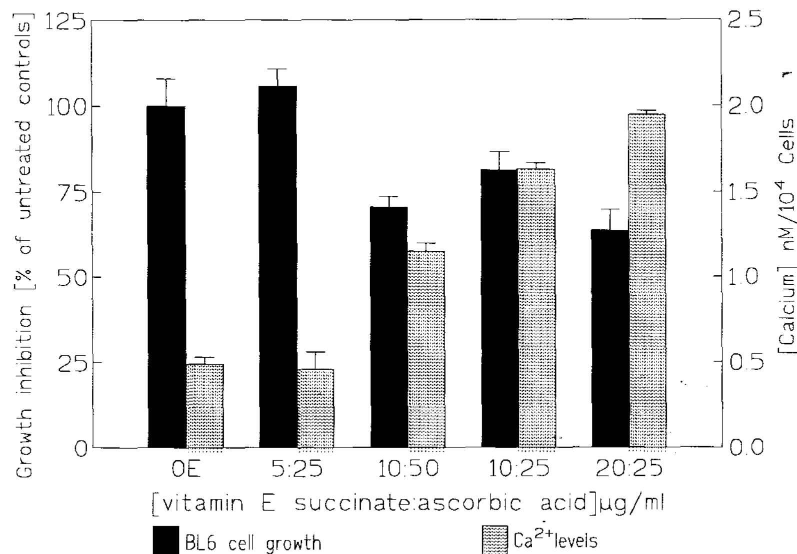


Figure 25: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and Ca^{2+} levels.

As already mentioned Ca^{2+} exerts an important regulatory role in modulating the activity of LOX enzyme (533,535). It may also affect cell metabolism indirectly via the activation of PLA_2 and release of AA (347,348,350,352). Hence an additional factor which needed to be investigated was the effect of combined vitamin E succinate and Asc supplementation on PLA_2 activity. With the exceptions of the 5:25μg/ml and 10:25μg/ml combined vitamin E succinate and Asc supplemented LLCMK cultures, no relationship between cell growth and PLA_2 activity was observed. However, with respect to BL6 cells (Figure 26), a significant decrease in cell growth together with a significant increase in PLA_2 activity following combined vitamin E succinate and Asc supplementation was observed. This suggests that combined vitamin E succinate and Asc stimulates the PLA_2 enzyme. The activation of PLA_2 and subsequent release of AA from phospholipid stores is an important rate limiting substrate of COX activity and eicosanoid metabolism (229,238,347-350). Therefore as a result of the increase in PLA_2 activity an increase in free EFA levels particularly AA and subsequent stimulation of eicosanoid metabolism could be expected.

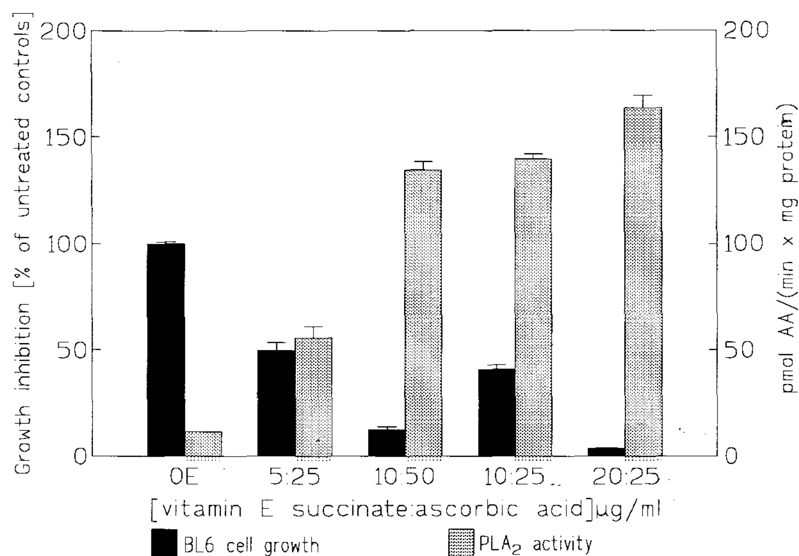


Figure 26: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and PLA_2 activity.

In order to establish whether any changes in AA composition of the cells under investigation was the result of increased PLA_2 activity or a possible increase of AA uptake, investigations of the effect of combined vitamin supplementation on uptake of AA was undertaken. Supplementation of combined vitamin E succinate and Asc together with ^3H AA in LLCMK cells resulted in a significant increase in both cell growth and ^3H AA uptake. Inhibition of BL6 cell growth at all combined vitamin concentration groups was found. ^3H AA uptake was inversely correlated to BL6 cell growth (Figure 27). Further investigation on the effect of combined vitamin E succinate and Asc supplementation together with AA on AA composition and cell growth of these two cell lines also resulted in an overall significant decrease in BL6 cell growth and an accompanying increase in total percentage AA composition (Figure 28). Total percentage AA composition was inversely related to BL6 cell growth. A similar trend was observed in the LLCMK cells.

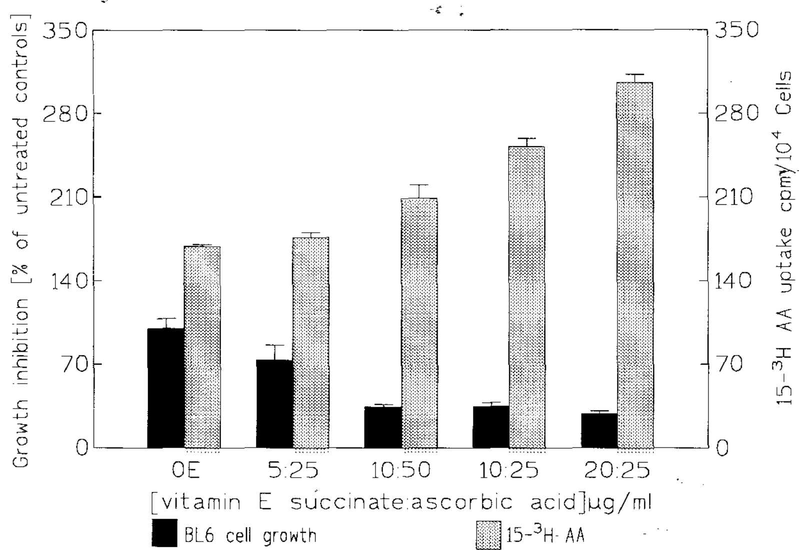


Figure 27: BL6 cell growth and uptake of ^{3}H arachidonic acid with combined vitamin E succinate and ascorbic acid supplementation.

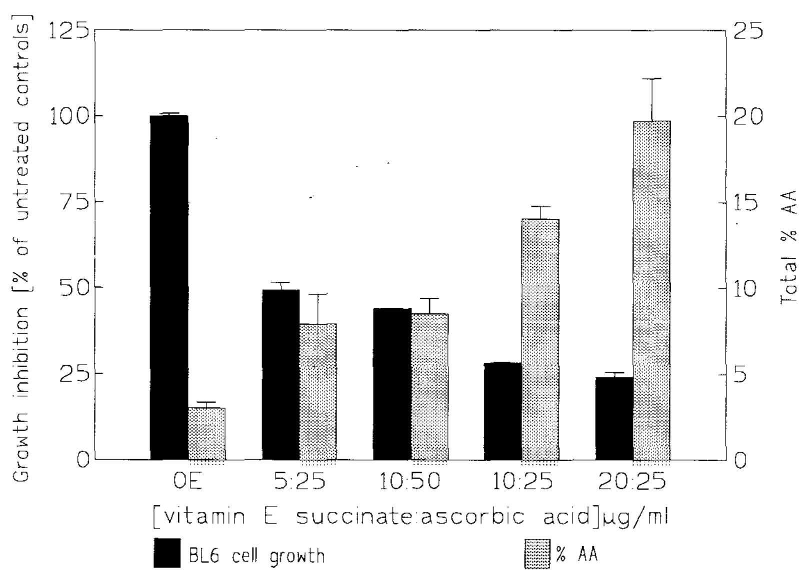


Figure 28: BL6 cell growth and total percentage arachidonic acid composition with combined vitamin E succinate and ascorbic acid supplementation.

This suggests that the effects of combined vitamin E succinate and Asc supplementation on AA uptake into the BL6 cells could be partly responsible for the inverse relationship between PLA_2 activity and cell growth. Furthermore, the magnitude of AA uptake was far greater in the membrane fraction while AA levels were far more noticeable in the stroma fraction following vitamin supplementation hence the increased AA levels observed in this study are likely to be a combined effect of AA uptake and increased PLA_2 activity.

Summarizing the results discussed so far, it appears that supplementation of combined vitamin E succinate and Asc in BL6 cells results in a significant decrease in cell growth compared to control cultures and furthermore that these decreases are inversely correlated to the levels of free radicals, lipid peroxidation and Ca^{2+} as well as the activities of COX, 5-LOX and PLA_2 together with total percentage and cell stroma AA composition.

Since PG synthesis is regulated by the activation of PLA_2 and subsequent release of AA, and furthermore the net production is dependent on the COX activity (229,238), it was necessary to determine the effects of combined vitamin E succinate and Asc supplementation on PG synthesis.

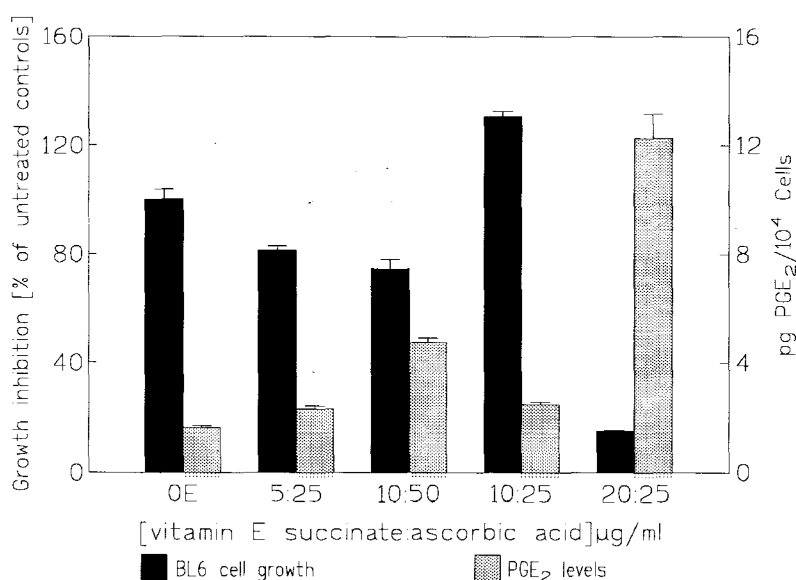


Figure 29: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and prostaglandin E_2 levels.

Upon combined vitamin E succinate and Asc supplementation, the PGE_2 levels found in LLCMK cells were positively correlated with cell growth, with the exception at 20:25μg/ml where an inverse relation was shown. However, upon combined vitamin E succinate and Asc supplementation of BL6 cells, the PGE_2 levels were found to be inversely related to BL6 cell growth (Figure 29) with an exception at 10:25μg/ml. The precise role of PGE_2 in tumourgenicity and metastasis is still unclear (300), although some researchers suggest that in BL6 cells (226,288,300), PGE_2 is negatively correlated with metastatic potential. The quantity and type of PG produced within a cell is essential to the metabolic effect it has (280) and in view of melanoma cells being unique in that they synthesize more than one prostaglandin (300), it was of importance for the study to determine whether combined vitamin E succinate and Asc supplementation affected the levels of other PGs within the BL6 cells.

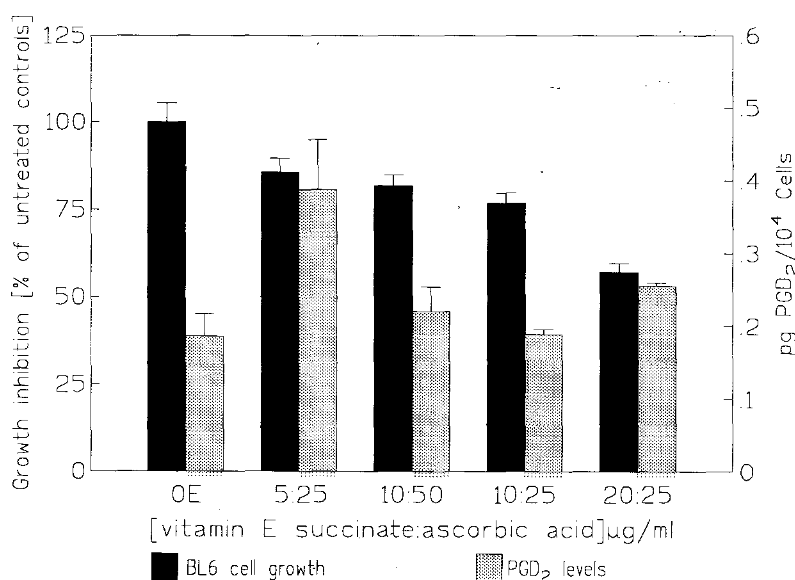


Figure 30: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and prostaglandin D_2 levels.

Combined vitamin E succinate and Asc supplementation of BL6 cells resulted in an overall increasing trend in PGD_2 (Figure 30) and $PGF_{2\alpha}$ (Figure 31) levels. With regards to PGD_2 levels, this increase was inversely related to BL6 cell growth as were $PGF_{2\alpha}$ levels with the exception at 10:25μg/ml where the $PGF_{2\alpha}$ level decreased and there was a decrease in cell growth.

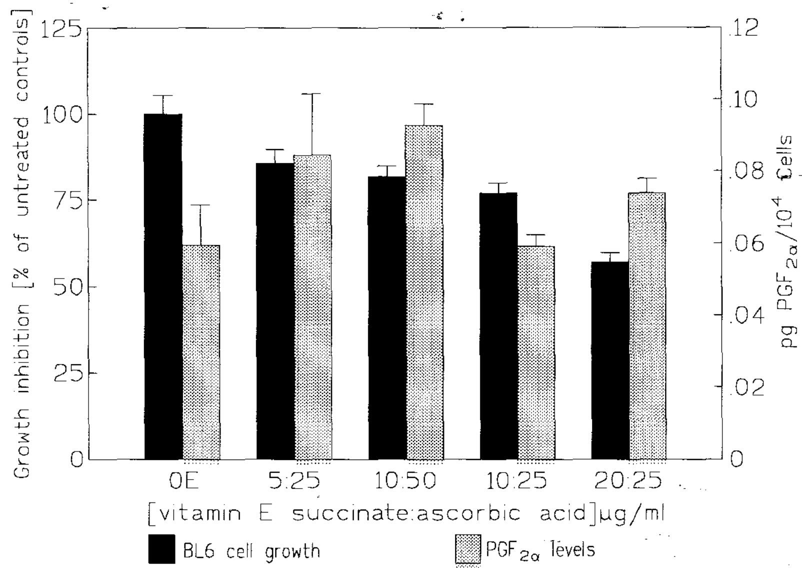


Figure 31: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and prostaglandin $F_{2\alpha}$ levels.

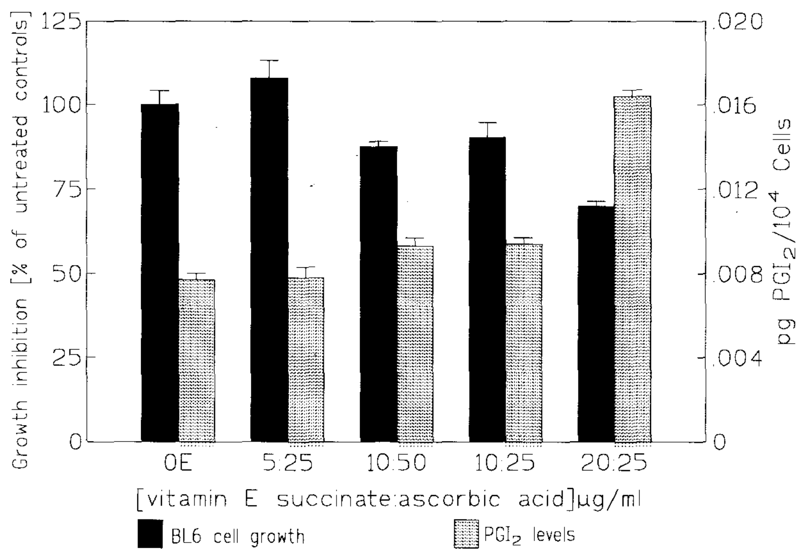


Figure 32: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and prostaglandin I_2 levels.

In the experiment examining the effect of combined vitamin E succinate and Asc supplementation on PGI_2 levels, a significant increase in this PG was found when compared to control cultures. Hence an inverse relationship also existed between BL6 cell growth and PGI_2 levels, with the exception at 5:25μg/ml where cell growth increased with increased PGI_2 levels (Figure 32). These results suggest that the effects of combined vitamin E succinate and Asc supplementation on BL6 cell growth is mediated in part by increasing the levels of PGE_2, PGD_2, $PGF_{2\alpha}$ and PGI_2. PGs are known to exert many of their effects through changes in the levels of cAMP (415,416,431,447) with the PGs of the D, E and I series capable of increasing cAMP formation in numerous systems (561) and cAMP in turn influencing the cell cycle (328).

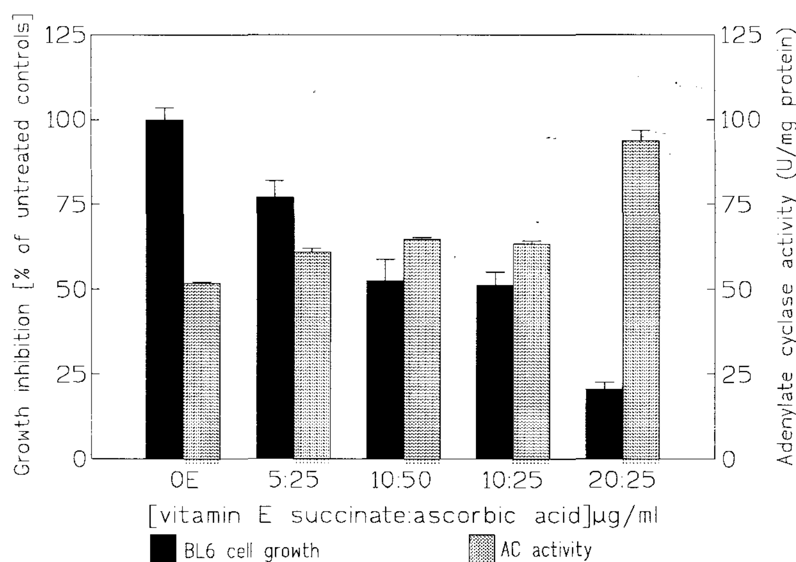


Figure 33: The effect of combined vitamin E succinate and ascorbic acid supplementation on BL6 cell growth and adenylate cyclase activity.

Combined vitamin E succinate and Asc supplementation of the LLCMK cells, significantly inhibited cell growth, while having no affect on AC activity. Combined vitamin E succinate and Asc supplementation, however resulted in a significant decrease in BL6 cell growth, together with a significant increase in AC activity (Figure 33). A similar inverse relationship was also observed when comparing cAMP levels and cell growth in BL6 cells (Figure 34). Hence, as also suggested by another study done on BL6 cells (394,446) it would seem that as more ATP is converted to cAMP, cAMP may in turn negatively regulate cell growth. Thus results from these studies suggest that combined vitamin E succinate and Asc supplementation induce AC activity and hence cAMP levels

which may act as a negative regulator of BL6 cell growth.

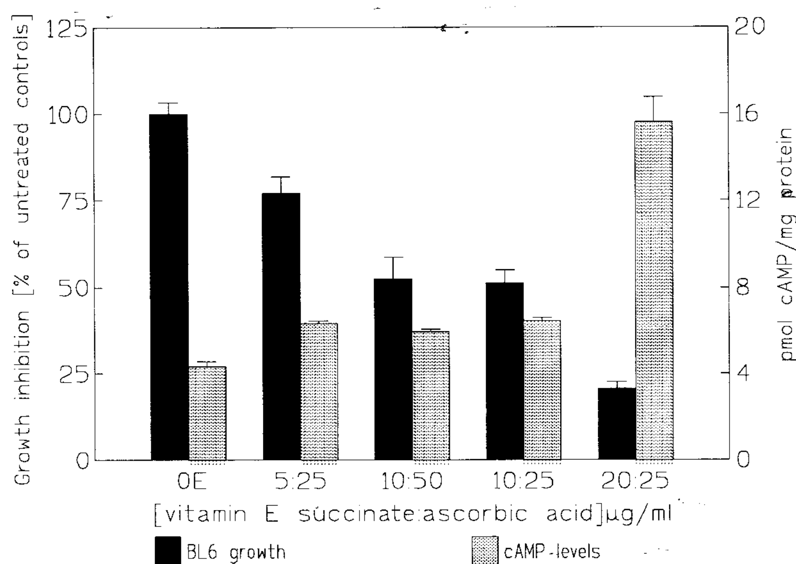


Figure 34: The relationship between cyclic adenosine monophosphate and BL6 cell growth with combined vitamin E succinate and ascorbic acid supplementation.

In conclusion, no definite relationship was determined between LLCMK cell growth and the second messengers shown in Figure 9, as a result of combined vitamin E succinate and Asc supplementation. At certain combined vitamin concentrations an inverse relationship between LLCMK cell growth and second messengers existed, but the magnitude of this relationship varied significantly between individual experiments. However, the combined vitamin E succinate and Asc supplementation to BL6 cells exhibited a definite inverse relationship between cell growth and the secondary messengers shown in Figure 9. A primary effect of combined vitamin supplementation in the BL6 cells, appeared to be on the uptake of AA from the medium and increased activity of the key regulatory enzyme, PLA_2. Thus, the percentage AA composition of the BL6 cells is affected and this in turn appears to influence the cellular levels of the AA metabolites in the eicosanoid pathway.

Combined vitamin E succinate and Asc supplementation of the BL6 cells in this study also resulted in marked increases in both free radical and lipid peroxidation levels while it has been proposed that vitamin C acts synergistically with vitamin E, regenerating oxidizing vitamin E in turn lowering the free radical and lipid peroxidation levels within the cell, results from this study, however, clearly indicate a prooxidant rather than an antioxidant effect. This increase in free radical and lipid

peroxidation levels within the cell appears to be due to the increased activities of both LOX and COX since intermediate oxygen containing radicals are produced in the conversion of AA to the various products by LOX and COX (237,242,268,529).

In an attempt to confirm that the inhibition of BL6 cell growth following combined vitamin E succinate and Asc supplementation was mediated via the proposed cascade effect and whether an association between free radical formation, lipid peroxidation levels and COX activity exists as proposed, a potent PLA_2 inhibitor, dexamethasone, was used. Secondary messenger levels and enzyme activities were then assayed following combined vitamin E succinate and Asc supplementation, in the presence of dexamethasone.

CHAPTER 6

COMBINED NUTRIENT AND DEXAMETHASONE SUPPLEMENTATION OF BL6 CELLS

6.1 INTRODUCTION

In the last two decades a significant link between cancer development, cancer progression, invasiveness and prostaglandins has been reported. Although, some of these reports are conflicting the exact role of PGs in cancer development and progression is not yet clarified (562). PGs act mainly by their specific receptors and by interfering with DNA, RNA, protein synthesis, cell membranes and cell communication (562). PGs which are synthesized from AA by cellular enzymes, are important bioactive substances and modulate diverse cellular functions in ubiquitous cells. As the concentration of free AA is low (339,366), it is well recognized that AA release is a rate limiting step of PG synthesis (555) and the inhibition of AA release could suppress the formation of these mediators. Therefore an inhibition of PLA_2 could result in the suppression of important lipid mediators which offers an attractive therapeutic approach to the design of novel agents for the treatment of various diseased states (563). The glucocorticoid steroids are extremely effective inhibitors of the synthesis of AA metabolites via the inhibition of PLA_2. Dexamethasone (Figure 35), a potent PLA_2 inhibitor, is the most widely used of the glucocorticoid steroids (564). The mechanism of dexamethasone inhibition is still uncertain. Initially dexamethasone was thought to exert its action by inducing the formation of PLA_2 inhibitory proteins known as lipocortins (565,566), however numerous other mechanisms have been proposed.

Figure 35: Structure of Dexamethasone (1).

Since the aim of this study was to determine whether the inhibition of BL6 cell growth that was observed is due to the AA flux in these cells and subsequent effects on the PGE_2-AC-cAMP linked pathway outlined in Figure 9 (page 139) nutrient supplemented BL6 cells were subjected to dexamethasone treatment.

6.2 THE EFFECT OF COMBINED VITAMIN E SUCCINATE, ASCORBIC ACID AND DEXAMETHASONE SUPPLEMENTATION ON CELL GROWTH

6.2.1 INTRODUCTION

As discussed earlier in Section 4.6, PGs have been reported by some to promote cell proliferation and by others to inhibit. Studies have shown that the addition of PGs to cultured cells resulted in a dose dependent inhibition of cell proliferation (9). Cell inhibition was most frequently noted with PGs of the E series and was positively correlated with changes in cAMP, hence the PGE effect was thought to be mediated through the activation of AC. PG synthesis is regulated in turn by the activation of PLA_2 and subsequent release of AA, and furthermore, the net PG production is determined by COX activity (229,238). Dexamethasone is a potent PLA_2 inhibitor, preventing the release of AA from the sn-2 position of the phospholipid stores (357,563,564). In an attempt to determine whether the activation of PLA_2, resulting in the cascade effect was responsible for the inhibition of BL6 cell growth, dexamethasone together was combined vitamin E succinate and Asc was supplemented to BL6 cells.

6.2.2 MATERIALS AND METHODS

MATERIALS

Materials used were the same as in 2.2. Dexamethasone (65mg/g) was purchased from Sigma Chemical Co., USA.

METHODS

6.2.2.1 Preparation of vitamin E succinate, ascorbic acid and dexamethasone stock solutions

Stock solutions of vitamin E succinate (20mg), Asc (5mg) and dexamethasone (10mM) were freshly

prepared in absolute ethanol and milli Q water respectively and diluted 1:1000 in media containing 10% (v/v) FCS to give a final concentration of 20μg/ml vitamin E succinate, 50μg/ml Asc and 10μM dexamethasone respectively, in 0.1% final concentration of ethanol.

The concentration of dexamethasone required to inhibit PLA_2 activity at all concentrations tested was determined by preliminary range finding experiments, and was shown to be most effective at 10μM dexamethasone. At this concentration total inhibition of PLA_2 activity was found.

6.2.2.2 Cell culture procedures

For experiments Section 6.3*, 3 x 10^5 BL6 cells were seeded into 5 sets of 5 25cm^2 flasks. To four sets of these flasks, 10ml of MEM basal medium containing 10% FCS, 10μM dexamethasone and varying levels of combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) was added. The sixth set of flasks received 10ml 10% (v/v) FCS media containing 0.1% final volume of ethanol and 10μM dexamethasone, and were referred to as control cultures (0E). In experiments 6.4*, 6.5* and 6.6*, BL6 cells were seeded into 5 sets of 3 75cm^2 flasks. To 4 of the 5 sets of flasks was added 30ml medium containing 10% (v/v) FCS containing 10μM dexamethasone and varying levels of combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml). The fifth set of flasks received 30ml of media containing 0.1% (v/v) ethanol and 10μM dexamethasone and were referred to as the controls (0E). The flasks were incubated at 37°C for the duration of the experiment with one media change during this period. Experimental cells were harvested as described in 2.2.3.2.

* Numbers refer to analytical experiments performed on these cultured cells further on in this chapter.

6.2.2.3 Statistical analysis

The results obtained were analysed 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 analysed by this method.

6.2.3 RESULTS

The BL6 cell growth in the relevant experiments, in which varying levels of combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) were supplemented, together with dexamethasone is

represented in Table 28. Relevant to this discussion, is the mean of growth inhibition of the percentage of untreated controls of all the experiments. Growth inhibition as a percentage of untreated cells for each individual experiment will be discussed relative to particular cell metabolites in subsequent sections.

The overall mean growth values, recorded in Table 28, indicated that combined vitamin E succinate and Asc, together with dexamethasone supplementation resulted in a slight stimulatory effect (non-significant), on BL6 cell growth.

TABLE 28: The effect of combined vitamin E succinate and ascorbic acid supplementation, together with dexamethasone on BL6 cell growth. Results are the mean of five* or three cultures ± SEM.

[vitamin E succinate:ascorbate] µg/ml	Growth inhibition [% of untreated controls]					
	Sect. 6.3	**Sect. 6.3**	**Exp 6.4**	**Exp 6.5**	**Exp 6.6**	**Mean**
0E	100 ±0.54	100 ±0.57	100 ±1.31	100 ±1.18	100 ±1.57	100 ±0.21
5:25	101.83 ±5.36	113.09 ±5.36	104.83 ±5.54	97.52 ±4.25	141.89[a] ±7.80	114.25 ±6.36
10:50	108.34 ±2.95	101.55 ±5.38	96.05 ±2.74	120.16[c] ±2.06	83.33 ±0.90	103.25 ±5.19
10:25	99.83 ±2.47	120.16 ±2.5	103.07 ±4.49	106.81 ±4.36	96.75 ±2.03	103.81 ±3.65
20:25	100.28 ±2.27	111.56 1.78	98.15 ±5.62	98.42 ±8.01	105.41 ±5.41	103.21 ±2.13

a - $p \leq 0.001$ relative to the respective control cultures 0E
b - $p \leq 0.01$ relative to the respective control cultures (0E)
c - $p \leq 0.05$ relative to the respective control cultures (0E)

In some cases, BL6 cell growth in individual experiments was significantly increased following combined vitamin E succinate and Asc supplementation with dexamethasone relative to respective control cultures (0E).

6.2.4 DISCUSSION

Recently it has been found that dexamethasone may have direct apoptotic or antiproliferative effects

on myeloma cell (567), while Reyes-Moreno *et al* (568) reported that dexamethasone arrested cell progression at the G1/Go phase of the cell cycle in mouse mammary tumours. Results from this study do not support the above finding since combined vitamin E succinate and Asc supplementation of BL6 cells together with the pharmacological dose of dexamethasone resulted in a general increase in cell growth. However, glucocorticoids have shown both inhibitory and stimulatory growth effects *in vivo* and *in vitro* depending on the normal and transformed cell lines investigated and little is known about the mechanism of this cellular response (568). In fact in this study the overall mean of growth of BL6 cells with combined vitamin E succinate and Asc supplementation as well as with dexamethasone resulted in a substantial increase in BL6 cell growth when compared to combined vitamin supplementation alone (Table 2a). Since it is known that the concentration of free AA in cells is low and it is well recognized that AA release is a rate limiting step of PG synthesis (229,238,555) this suggests that the increase in cell growth observed in dexamethasone treated BL6 cells may be the result of decreased PLA_2 activity which would lead to the decrease in AA liberation and hence PG production. As already mentioned PG synthesis is inversely related to cell growth, thus a decrease in PG synthesis would ultimately lead to the observed increase in BL6 cell growth.

Certain enzymes and their relevant metabolites which are associated with this cascade effect were selected for further investigation of the effects of combined nutrient and dexamethasone supplementation in BL6 cells.

6.3 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION TOGETHER WITH DEXAMETHASONE ON FREE RADICAL AND LIPID PEROXIDATION FORMATION

6.3.1 INTRODUCTION

As indicated previously the mechanism(s) by which vitamin E succinate inhibits tumour cell proliferation is unclear although, it has been suggested that the anticarcinogenic effect of vitamin E and C may be in their ability to scavenge free radicals and prevent lipid peroxidation (22,24,36,54,57,63,71,122,128,477). However earlier studies (Section 3.2) have shown significant increases in both free radical formation and lipid peroxidation levels following combined vitamin E succinate and Asc supplementation. Furthermore, Cilliard *et al* (569,570) have suggested that high concentrations of the vitamins E and C may reverse the antioxidant effect of these vitamins to a prooxidant one.

Lipid peroxidation is the process of oxidative turnover of PUFAs and both enzymic and non-enzymic (529) lipid peroxidation is initiated by free radicals. Since lipid peroxidation of PUFA can be initiated enzymatically through the catalytic action of COX enzyme (123,258,529), it was suggested earlier that the observed increase in free radical and lipid peroxidation levels in BL6 cells is a result of the increased COX activity obtained (Section 4.5) within these cells. COX activity is in turn partly regulated by the availability of its substrate, free AA.

In an attempt to demonstrate this relationship, BL6 cells were supplemented with the PLA_2 inhibitor, dexamethasone.

6.3.2 MATERIALS AND METHODS

MATERIALS

Materials used were the same as in 3.2.1 and 6.2.2.

METHODS

6.3.2.1 Cell culture procedures

The same procedures were used as those outlined in 2.2.3.2 and 6.2.2.2.

6.3.2.2 Free radical formation

The same procedure was used as that in 3.1.2.2.

6.3.2.3 Lipid peroxidation determination

The same procedure was used as described in 3.1.2.3.

6.3.3 RESULTS

Treatment of BL6 cells (Table 29) with 10μM dexamethasone and varying levels of combined vitamin E succinate (5-20μg/ml) and Asc (25-50μg/ml) resulted in a decrease of free radical levels when

compared with control cultures (0E), although no significance was recorded.

TABLE 29: The effect of dexamethasone treatment on free radical and lipid peroxidation levels in BL6 cells supplemented with varying levels of combined vitamin E succinate and ascorbic acid. Results are the mean of five cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	Free radical formation nmoles Diformazan/ 10^4 Cells	Lipid peroxidation nmoles MDA/10^4 cells
0E	0.026 ±0.001	0.032 ±0.001
5:25	0.022 ±0.004	0.026 ±0.001
10:50	0.023 ±0.003	0.012[a] ±0.002
10:25	0.020 ±0.003	0.012[a] ±0.004
20:25	0.019 ±0.004	0.001[a] ±0.002

a - $p \leq 0.001$ relative to control cultures (0E)

Lipid peroxidation in BL6 cells following dexamethasone treatment was generally decreased. This decrease in lipid peroxidation levels was significant ($p \leq 0.001$) at 10:50; 10:25 and 20:25μg/ml following 10μM dexamethasone and combined vitamin supplementation when compared with control cultures (0E).

6.3.4 DISCUSSION

The activity of the COX enzyme has been shown to be dependent on the presence of lipid hydroperoxides, and these hydroperoxides are continually required during the catalytic mechanism (237,242,258,259,264). Furthermore, it was found that various lipid hydroperoxides could stimulate COX activity, and the levels of PGG_2, the reaction product of the enzyme (237,242,259).

The lipid hydroperoxides can decompose into TBA reactive materials such as MDA (269,570). This suggests that an increase in COX activity will result ultimately in the increase in MDA levels through the production of lipid peroxides. The conversion of PGG_2 to PGH_2 by PG hydroperoxidase is also

known to produce oxygen containing free radicals, suggesting that any compound capable of increasing the COX activity will also lead to increased hydroperoxidase activity of the PES and indirectly increase the levels of free radicals within these cells.

In this study, supplementation of BL6 cells (Table 29) with dexamethasone resulted in a decrease in both lipid peroxidation and free radical levels when compared with respective control cultures (0E). When comparing free radical and lipid peroxidation levels in combined vitamin E succinate and Asc supplemented cells not treated with dexamethasone (Table 13) with the dexamethasone treated cells, a substantial decrease was also found in the dexamethasone treated cells. These results suggest that the earlier findings of an increase in free radical and lipid peroxidation levels in BL6 cells supplemented with combined vitamin E succinate and Asc were possibly due to a stimulation of COX activity, since dexamethasone treatment would limit this activity as a result of an inhibition of release of its substrate AA, by PLA_2

To substantiate this, the effect of dexamethasone supplementation on COX activity was determined.

6.4 EFFECT OF DEXAMETHASONE AND COMBINED NUTRIENT SUPPLEMENTATION ON CYCLOOXYGENASE ACTIVITY

6.4.1 INTRODUCTION

The initial step of PG biosynthesis is an oxygenative cyclization to produce PGG_2, which contains both an endoperoxide and a hydroperoxidase moiety. The enzyme responsible for this reaction is referred to as a fatty acid COX, the same enzyme also catalyzes the second step of PG biosynthesis to PGH_2 from PGG_2 (237). Because AA is the precursor for the biosynthesis of PGs, inhibition of AA release could suppress the formation of these mediators. Therefore an inhibition of PLA_2 could result in the suppression of the lipid mediators.

6.4.2 MATERIALS AND METHODS

MATERIALS

Materials used were the same as in 4.1.2 and 6.2.1.

METHODS

6.4.2.1 Cell culture procedures

The same procedures were used as in 2.2.3.2 and 6.2.2.2.

6.4.2.2 Homogenization of cells and separation of cellular components

The same procedures were carried out as in 3.2.1.2 and 4.1.2.2.

6.4.2.3 Determination of cyclooxygenase activity

The procedure used for determination of COX activity outlined in 4.1.2.3 was repeated.

6.4.3 RESULTS

TABLE 30: The effect of dexamethasone treatment on cyclooxygenase activity in BL6 cells supplemented with varying levels of combined vitamin E succinate and ascorbic acid. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	Cyclooxygenase activity pmoles PGG_2 (x10)/10^6 Cells
0E	0.0510 ±0.003
5:25	0.0540 ±0.002
10:50	0.0012[a] ±0.0001
10:25	0.0009[a] ±0.0001
20:25	0.0009[a] ±0.0001

a - $p \leq 0.001$ relative to control cultures (0E)

Treatment of BL6 cells (Table 30) with 10μM dexamethasone and varying levels of combined vitamin E succinate and Asc resulted in a significant ($p \leq 0.001$) decrease in COX activity, with the exception

of 5:25μg/ml where a slight increase in COX activity was found when compared to control cultures (0E).

6.4.4 DISCUSSION

While glucocorticoids inhibit PG synthesis from AA at the level of PLA_2, they have also known to inhibit PG synthesis at the COX enzyme level (562). In these studies, supplementation of BL6 cells (Table 30) with the glucocorticoid dexamethasone resulted in an overall significant decrease in COX activity when compared to control cultures (0E). Furthermore, when comparing COX activity in BL6 cells following combined vitamin E succinate and Asc supplementation alone (Table 22) to dexamethasone treated cells a marked decrease in COX activity was observed in dexamethasone treated cells. Although the mechanism in which dexamethasone inhibits the stimulatory effect of combined vitamin E succinate and Asc on COX activity in BL6 cells is still unclear, this data suggests that the observed decrease in COX activity in dexamethasone treated cells is due at least partly to the inhibition of PLA_2 activity and subsequent AA release. As the concentration of free AA is low, it is well recognized that AA release is a rate limiting step in PG synthesis (229,239,555). A direct inhibition of the COX enzyme itself is also possible. The observed decrease in free radical and lipid peroxidation levels in dexamethasone treated cells therefore appears to be due to the decreased COX activity in BL6 cells.

6.5 EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION TOGETHER WITH DEXAMETHASONE ON PGE_2 LEVELS

6.5.1 INTRODUCTION

Eicosanoids are not stored but are synthesized on demand, therefore inhibitors of the various metabolizing enzymes have instantaneous effects on eicosanoid levels (571). Each PG has its own range of biological activities, hence the determination of the role of PGs in tumour growth is a perplexing task. Although there has been a great deal of interest in the involvement of AA metabolites in cancer, there is uncetaintity about which metabolites are the most important and how they contribute to specific steps in cell transformation, tumour growth and metastasis (571). The quantity and type of PG produced within a cell is essential to the metabolic effect (284) and the fact that PGE_2 is negatively correlated with metastatic potential in BL6 cells, it was necessary in this study to determine what effect dexamethasone treatment had on PGE_2 levels in combined vitamin E succinate

and Asc supplemented BL6 cells.

6.5.2 MATERIALS AND METHODS

Materials used were the same as in 4.6.2 and 6.2.1.

METHODS

6.5.2.1 Cell culture procedures

The methods used in 2.2.3.2 and 6.2.2.2 were repeated.

6.5.2.2 Homogenization of cells and separation of cellular components

The methods described in 3.2.1.2 and 4.6.2.2 were repeated.

6.5.2.3 Extraction and isolation of prostaglandins

The same procedure was used as that described in 4.6.2.3.

6.5.2.4 Prostaglandin E_2 assay system

The same assay system was used as that described in 4.6.2.4. The PGE_2 standard curve is represented in Appendix 6.

6.5.3 RESULTS

Treatment of BL6 cells with 10μM dexamethasone (Table 31) and varying levels of combined vitamin E succinate and Asc resulted in no increasing or decreasing trend of PGE_2 levels in BL6 cells when compared with control cultures (0E).

TABLE 31: The effect of dexamethasone treatment on PGE_2 levels in BL6 cells supplemented with varying levels of combined vitamin E succinate and ascorbic acid. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] μg/ml	pg PGE_2 / 10^4 Cells
0E	1.675 ±0.088
5:25	2.311 ±0.451
10:50	2.732 ±0.481
10:25	1.681 ±0.320
20:25	1.471 ±0.142

6.5.4 DISCUSSION

Studies by Kowaza *et al* (555) demonstrated that supplementation of dexamethasone to osteoblast-like cells resulted in the inhibition of PGE_2 release. In the present study supplementation of 10μM dexamethasone to combined vitamin E succinate and Asc supplemented BL6 cells (Table 31) resulted in no increasing or decreasing trend in PGE_2 levels. However, when comparing PGE_2 levels following combined vitamin E succinate and Asc supplementation alone (Table 23) with dexamethasone treated cells (Table 31) a marked decrease in PGE_2 levels was observed. ElAttar and Lin (148) have suggested that endogenous PGE_2 levels are a reflection of the activities of the enzymes PLA_2 and COX. The synthesis of PGE_2 is usually limited by the availability of free AA which is liberated from membrane phospholipids by PLA_2 on agonist stimulation (549). This suggests that the observed decrease in PGE_2 levels in BL6 cells may be due to the decreased PLA_2 and COX activity following dexamethasone supplementation.

PGE_2 (285,320,416,447,453) is known to exert its effects via a cAMP-AC linked system. PGE_2 is reported to have stimulatory (415) and inhibitory effects on AC activity, while cAMP levels within the cells are positively correlated to AC activity (462). Consequently, AC activity and cAMP levels were determined following dexamethasone supplementation.

6.6 THE EFFECT OF COMBINED VITAMIN E SUCCINATE AND ASCORBIC ACID SUPPLEMENTATION TOGETHER WITH DEXAMETHASONE ON ADENYLATE CYCLASE ACTIVITY AND CYCLIC ADENOSINE MONOPHOSPHATE LEVELS

6.6.1 INTRODUCTION

Alterations in the membrane intracellular signalling systems are frequently implicated in the development of neoplasia (345,510). The major intracellular signalling system identified to date is the AC-cAMP linked system (510). AC, the catalytic protein converts ATP to cAMP (331,383,384,385). The activity of the AC enzyme may be influenced by a number of factors within a cell (405,424,465). Numerous studies have shown that PGs function to stimulate AC activity (286,405,416,453,465), and furthermore that numerous biological functions appear to be mediated through cAMP levels (315,416,427,447,453,465). cAMP when added to various cells in culture was found to affect the growth of these cells in either a positive or negative manner depending on the cell type (328,429). *In vitro* studies have shown higher levels of cAMP in normal cell lines when compared to transformed cell lines, and furthermore an inverse relationship existed between cAMP levels and cell growth (328,427,429,442,445,459,462).

6.6.2 MATERIALS AND METHODS

MATERIALS

Materials used are the same as in 4.8.2 and 6.2.1.

METHODS

6.6.2.1 Cell culture procedures

The methods described in 2.2.3.2 and 6.2.2.2 were repeated.

6.6.2.2 Homogenization of cells and separation of cellular components

The methods described in 3.1.1.2 and 4.8.2.2 were used.

6.6.2.3 Protein determination

Protein was determined as outlined in Section 4.3.2.

6.6.2.4 Adenylate cyclase activity assay

The method used for AC determination was the same as described in 4.8.2.4.

6.6.2.5 Cyclic adenosine monophosphate extraction

The same procedure was used as that in 4.8.2.5.

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

The same procedure was used as that described in 4.8.2.6.

6.6.3 RESULTS

TABLE 32: The effect of dexamethasone treatment on adenylate cyclase activity and cyclic adenosine monophosphate levels in BL6 cells supplemented with varying levels of combined vitamin E succinate and ascorbic acid. Results are the mean of three cultures ± SEM.

[vitamin E succinate:ascorbate] µg/ml	Adenylate cyclase activity U/mg protein	cAMP levels pmol/mg protein
0E	25.991 ±1.202	0.200 ±0.007
5:25	20.093[b] 0.921	0.060[a] 0.001
10:50	17.923[b] 0.483	0.036[a] ±0.003
10:25	14.954[a] 2.201	0.045[a] 0.009
20:25	13.712[a] 2.451	0.0285[a] ±0.006

a - $p \leq 0.001$ relative to respective control cultures (0E)
b - $p \leq 0.01$ relative to respective control cultures (0E)

Treatment of BL6 cells with 10μM dexamethasone and varying levels of combined vitamin E succinate and Asc resulted in a significant ($p \leq 0.001$) decrease in both AC activity and cAMP levels, when compared with respective control cultures (0E).

6.6.4 DISCUSSION

The intracellular levels of cAMP are regulated in part by AC activity (434,435) hence any change in AC activity would be expected to bring about similar changes in cAMP levels. In these studies, significant decreases in both AC activity and cAMP levels (Table 31) in BL6 cells were observed following dexamethasone treatment. Furthermore, when comparing AC activity and cAMP levels in cells supplemented with combined vitamin E succinate and Asc alone (Table 26) with dexamethasone treated cells, substantial decreases in both AC activity and cAMP levels were found in the dexamethasone treated cells. PGs function to stimulate AC activity and, since a decrease in PGE_2 levels were observed following dexamethasone treatment in BL6 cells (Section 6.5) it is likely that the decrease in AC activity and subsequent cAMP levels were as a result of the decreased PGE_2 levels.

Data from these studies suggest that the inhibitory effects of vitamin supplementation on BL6 cell growth are a result of a stimulatory effect of combined vitamin E succinate and Asc on PLA_2 activity, resulting in a cascade effect throughout the eicosanoid pathway. The following discussion will summarize the effect in individual experiments of combined vitamin E succinate and Asc supplementation together with dexamethasone.

6.7 GENERAL DISCUSSION ON THE RELATIONSHIP BETWEEN ARACHIDONIC ACID METABOLITES AND CELL GROWTH

Results from Chapter 4 on the effect of combined vitamin E succinate and Asc supplementation on AA metabolites and second messengers in BL6 cells showed that combined vitamin supplementation significantly increased the activity of COX activity, PGE_2, AC activity and cAMP levels within the cells. Relating these enzyme activities and second messengers to BL6 cell growth in Chapter 5, it was evident that an inverse relationship exists between the levels of COX activity, AC activity, PGE_2 and cAMP and cell growth. In this Chapter the effects of dexamethasone supplementation on the relationship between COX activity, PGE_2 levels, AC activity and cAMP levels as well as the effect these have on BL6 cell growth were examined.

A comparison of the relationship between COX activity and BL6 cell growth following vitamin supplementation and dexamethasone treatment revealed that COX activity decreased significantly while BL6 cell growth showed no increasing or decreasing trend (Figure 36). Looking at the relationship between PGE_2 levels and BL6 cell growth following dexamethasone supplementation it can be seen that cell growth and PGE_2 levels were affected in a similar way (Figure 37). As PGE_2 stimulates AC activity (320,282), the relationship between AC and growth in BL6 cells supplemented with dexamethasone should be similar to that of PGE_2 levels and BL6 cell growth. In this study supplementation of BL6 cells with combined vitamins and dexamethasone resulted in no significant change in BL6 cell growth, while AC activity was significantly decreased compared to control cultures (0E) (Figure 38). A similar effect to that observed for AC activity and BL6 cell growth was found when comparing cell growth and cAMP levels (Figure 39).

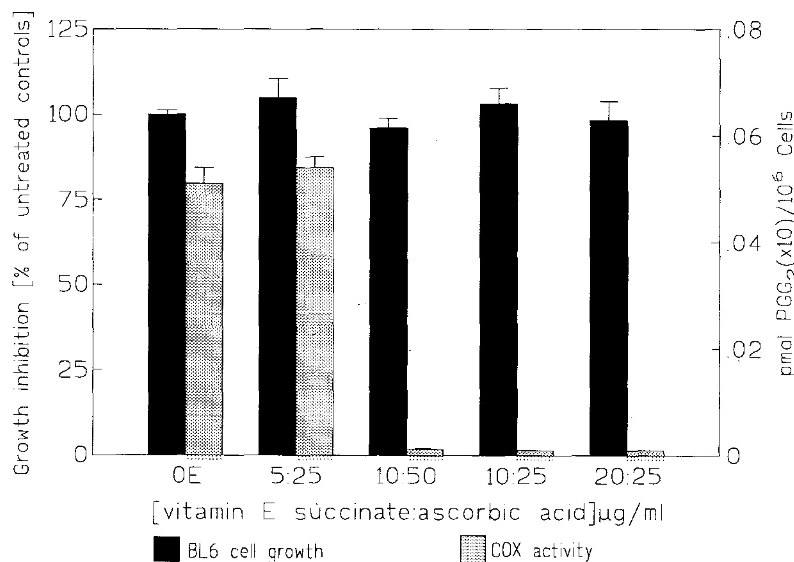


Figure 36: The effect of combined vitamin E succinate and ascorbic acid supplementation together with dexamethasone on BL6 cell growth and cyclooxygenase activity.

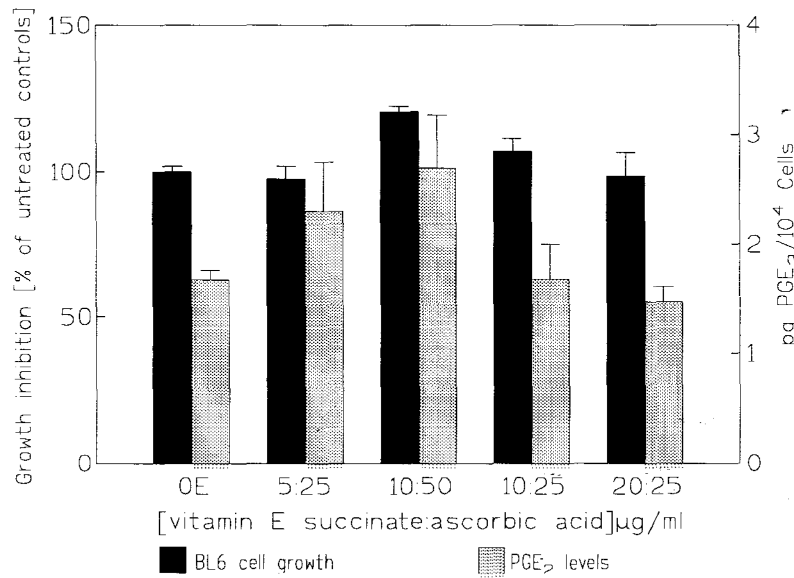


Figure 37: The effect of combined vitamin E succinate and ascorbic acid supplementation together with dexamethasone on BL6 cell growth and prostaglandin E_2 levels.

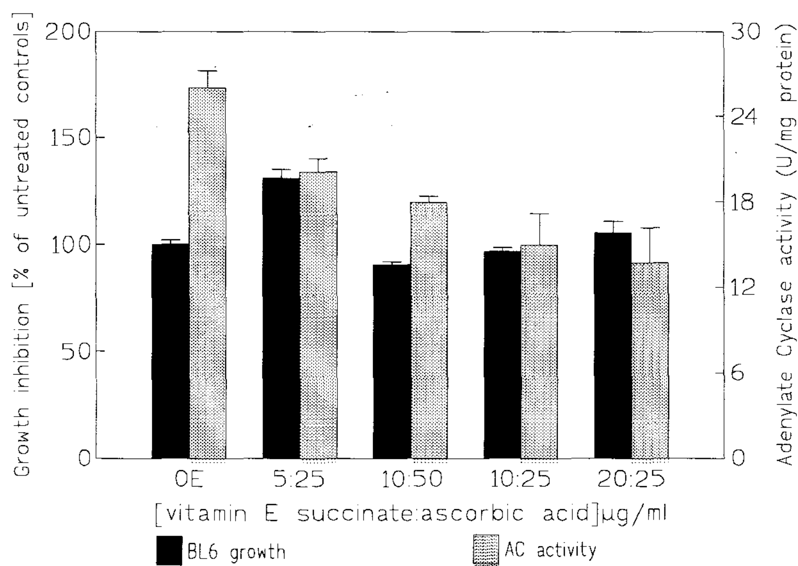


Figure 38: The effect of combined vitamin E succinate and ascorbic acid supplementation together with dexamethasone on adenylate activity and BL6 cell growth.

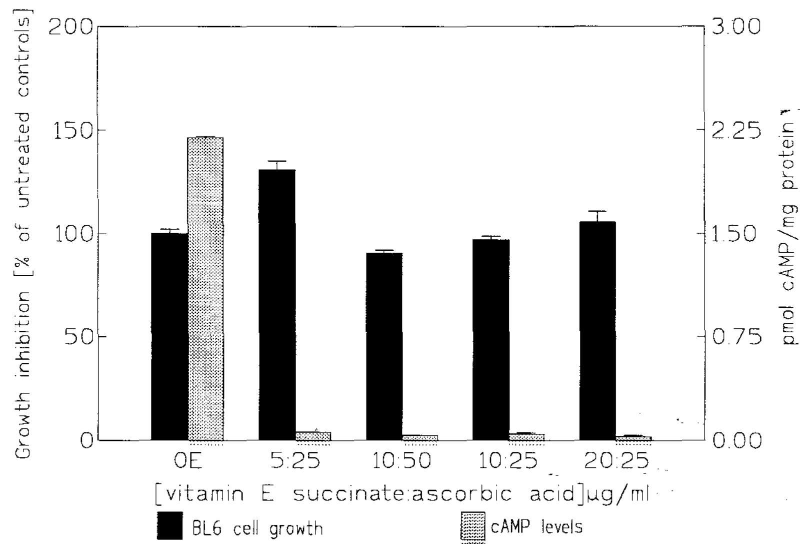


Figure 39: The effect of combined vitamin E succinate and ascorbic acid supplementation together with dexamethasone on BL6 cell growth and cyclic adenosine monophosphate levels.

In summary, as described in earlier sections combined vitamin E succinate and Asc supplementation of BL6 cells resulted in significant increases in the levels of COX activity, PGE_2 levels, AC activity and cAMP levels, and furthermore these increases were found to be inversely related to BL6 cell growth. Treatment of nutrient supplemented BL6 cells with the PLA_2 inhibitor, dexamethasone had no effect on cell growth or PGE_2 levels while COX and AC activity as well as cAMP levels decreased. This provides further evidence that the inhibitory effects of combined vitamin E succinate and Asc supplementation on BL6 cell growth observed in earlier studies (Chapter 5) were probably a result of the ability of combined vitamin E succinate and Asc to increase PLA_2 activity, resulting in a cascade effect which brings about an increase in endogenous levels of PGE_2 and this in turn affects cell proliferation by an interaction with an AC-cAMP linked system.

CHAPTER 7

GENERAL DISCUSSION

Fundamental differences regarding cellular function and composition are reported between non-malignant and malignant cells (see Chapter 1). The normal and tumour cells referred to in these reports usually originate from the same tissue types. In contrast, in this study 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. Cell growth is one of the major physiological features distinguishing tumour cells from normal cells. This is due to the fact that tumour cells usually have less control of proliferation rates (19). In this case both maintenance and control cultures of the BL6 melanoma cells consistently showed higher rates of proliferation than non malignant LLCMK control and maintenance cultures.

The treatment of metastatic cancer with chemotherapeutic drugs has always presented problems owing to the non specificity of the drugs used. Researchers are therefore continuing to search for agents which are selectively toxic to tumour cells. One of the foremost approaches is that of chemoprevention. Attention has been focused on nutritional compounds which are generally non-toxic to normal cells, even in high doses, but may interfere with the proliferation of malignant cells. The potential of these natural substances as a preventative measures against neoplasia development is of paramount importance as numerous sources clearly indicate that natural foods contain factors that protect against cancer. Thus a relationship between diet and cancer incidence is clearly indicated in human populations (7,80). 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 (7). The beneficial effects of Asc in the prevention (31) and management (31,52) of neoplasms has been extensively reported, while vitamin E has been shown to induce differentiation and growth inhibition in certain animal and human tumour cells in culture (49,96). Furthermore, emphasis has been placed on supplementation with more than one nutrient to tumours in the hope of enhancing the effects of the individual vitamins.

Oxygen, a necessary element for the life of aerobic cells, is also the source of active states of oxygen, including radicals, which can disrupt cell structure and alter cell function (3,199,572).

Numerous studies over the years have led researchers to suggest that free radicals and products of free radical reactions may be involved in the aetiology of cancer (572). Substantial evidence has implicated free radicals in both tumour initiation and promotion (3,4,8-11,55,133,211,214). These findings have led to widespread interest in compounds capable of reacting with chain propagating radical species resulting in the formation of species no longer capable of hydrogen abstraction. Termination of chain propagation involves a variety of compounds in which attention has been focused in particular on vitamin E - a major lipid-soluble antioxidant present in all membranes (103,110,121) - and Asc, a major water-soluble antioxidant present in the cytosol (33,40,49,52,54,56). Both vitamins function to scavenge free radicals and prevent lipid peroxidation, *in vivo* and *in vitro* (45,46,50,112,143) and vitamin E is said to be the first line of defence against free radicals. Furthermore, *in vivo* and *in vitro* experiments (29,156-168,174) demonstrate that vitamin C can act synergistically with vitamin E, bringing about a sparing effect of vitamin E. This sparing effect results in the slow depletion of vitamin E. Since tumour incidence is linked with nutrition (7) and an inverse relationship is believed to exist between serum vitamin E (123,139,146,151), serum Asc (43,67) and cancer incidence, this study was directed towards determining the effect of combined vitamin E succinate and Asc supplementation on the growth of malignant (BL6) melanoma cells and non malignant monkey kidney (LLCMK) cells. Furthermore, certain metabolic responses to combined vitamin E and Asc supplementation were studied. Attention should be drawn to the fact that although the vitamins E and C may act individually within the cells bringing about the observed effects, it was the synergistic action of the vitamins which formed the main concern of this study. Growth studies involving vitamin E succinate and ascorbic acid on various cell lines have been examined, however the individual effects of vitamin E succinate and ascorbic acid on the enzymes in the arachidonic acid pathway have yet to be explored.

With reference to overall mean growth, supplementation of combined vitamin E succinate and Asc resulted in a significant decrease in BL6 cell growth, while LLCMK cells showed no increase or decrease in cell growth. This selective cytotoxicity of combined vitamin E succinate and Asc towards malignant BL6 cells may be indicative of their value in the treatment of malignant cells and furthermore suggests that the contribution of vitamin E succinate to Asc is important for maximum inhibition of BL6 cells. Vitamin E succinate (49,92,97) and Asc (43,49,59,61,471,472) on their own have been shown to inhibit growth of other cell lines, but the effect of combined vitamin E succinate and Asc has not previously been reported. The actual mechanism by which combined vitamin E succinate and Asc inhibits BL6 cell growth is not yet known. This study considered the possibility that the inhibitory effects of combined vitamin E succinate and Asc on BL6 cell growth was mediated

via the synergistic antioxidant properties associated with the vitamin E component molecule and Asc. Supplementation of BL6 cells with combined vitamin E succinate and Asc however, resulted in a marked increase in free radicals as well as in lipid peroxidation levels. Studies by Cilliard *et al* (569,570) have shown that the antioxidant effect of vitamin E and C can be reversed to a prooxidant effect, particularly when the vitamin concentrations are increased. The increases in both free radical and lipid peroxidation levels were inversely related to BL6 cell growth. These findings support Goldring *et al* (214), who reported that tumour cell growth is inversely related to lipid peroxidation levels, and that any agent capable of increasing lipid peroxidation levels within a cell could inhibit tumour cell growth (573).

Although the vitamin E molecule is a naturally-occurring antioxidant, vitamin E succinate is a non-physiological antioxidant which requires esterase activity in order to liberate the free alcohol, vitamin E, with antioxidant properties (93,97,490,491). Liberation of the free vitamin E from vitamin E succinate must occur in order for Asc to act synergistically with vitamin E bringing about the sparing effect. Since no species-specific hydrolysis of vitamin E succinate has been demonstrated (490), an attempt was made to demonstrate vitamin E succinate cleavage in BL6 cells. Supplementation of BL6 cells with combined vitamin E succinate and Asc resulted in no increasing or decreasing trend of vitamin E succinate levels in the cells while, vitamin E levels on the other hand increased markedly with increasing concentration of vitamin E succinate, although, no significance was recorded. These results suggest that vitamin E succinate is cleaved in these cells and that vitamin E and Asc may indeed act synergistically, resulting in the sparing effect of vitamin E. The inability of combined vitamin E succinate and Asc to reduce free radical formation and lipid peroxidation levels within BL6 cells may therefore not be due to their ineffectiveness as antioxidants but rather the presence of other contributing factors which influence the oxidation state within the BL6 cells.

Vitamin E has numerous other roles, within cells, besides its primary role as an antioxidant. Vitamin E is suggested to alter membrane stability or function by interacting with the unsaturated EFA portion of phospholipids (125,127), stabilizing the membrane which may be enhanced by the presence of Asc due to its sparing effect of vitamin E. In an attempt to substantiate this proposal, BL6 cells were supplemented with ^{3}H vitamin E succinate and ^{14}C ascorbic acid, and the relative uptake and cellular distribution of the vitamins determined. In this study, supplementation of ^{3}H vitamin E succinate and ^{14}C ascorbic acid resulted in a significant increase in both ^{3}H vitamin E succinate and ^{14}C ascorbic acid uptake in BL6 cells. With respect to distribution of vitamin E succinate, significant increases in vitamin E succinate levels were detected, in both membrane and stroma fractions. Asc, on the other

hand, was significantly higher in the stroma fraction relative to corresponding membrane fractions. It would therefore appear that combined vitamin E succinate and Asc effects on BL6 cell growth are not mediated via their synergistic antioxidant role but may rather have a modulatory effect on membrane functions. Studies have shown that vitamin E supplementation stabilizes membranes and alters membrane-bound enzymes (125,127). Vitamin E has been suggested to play a role in a number of aspects of eicosanoid metabolism (196,218,503), altering the activity of key regulatory enzymes such as COX and PLA_2 (196,503) and LOX (543). Hence, this study considered the possibility that the inhibitory effects of combined vitamin E succinate and Asc supplementation on tumour cell growth were mediated to a certain extent through an effect on AA composition in BL6 cells, resultant second messenger levels in the eicosanoid pathway 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 or indirect influence of combined vitamin E succinate and Asc supplementation.

Metabolism of AA by LOX has revealed that intermediate peroxy compounds and hydoxyl radicals are produced in these reactions (529), and these radicals in turn are known to stimulate COX (237,259). Regarding COX and 5-LOX enzymes, supplementation of combined vitamin E succinate and Asc to BL6 cells resulted in a significant increase in both COX and 5-LOX activity at all combined vitamin concentrations. Furthermore, COX activity was found to be approximately 2-fold higher than that of 5-LOX activity. Hemler *et al* (259) found that the lipid hydroperoxides formed by LOX were the most potent activators of COX, which could have accounted for the higher COX activity. This result is also indicative of the fact that the combined vitamin E succinate and Asc inhibitory effects on BL6 cell growth are more likely to be due to changes in COX activity rather than to 5-LOX, and that the products of COX could play an important regulatory role in tumour cell growth.

Although vitamin E is believed to stabilize membranes and alter the activities of membrane-bound enzymes (96,125,127) with Asc enhancing this function of vitamin E, the observed effects of combined vitamin E succinate and Asc supplementation on BL6 cells may be more complex than simply membrane stabilization. Many of the key enzymes involved in AA esterification and release are dependent on Ca^{2+}, since it has a role in COX and LOX activity (339), and hence AA metabolites. The availability of Ca^{2+} within a cell is believed to be a key regulator of cell proliferation (329,335,356). Although it has been reported that BL6 melanoma cells have low Ca^{2+} content (535), these studies showed that an overall increase in Ca^{2+} levels was detected following combined vitamin

E succinate and Asc supplementation with a corresponding decrease in BL6 cell growth. Consequently, another factor accounting for elevated activities of COX and LOX as a result of vitamin supplementation may be an increased availability of Ca^{2+}.

The role of Ca^{2+} in modulating the activity of the PLA_2 enzymes should also be considered. In particular, $cPLA_2$, the enzyme present in the cytosol, translocates to membranes in response to changes in intracellular Ca^{2+} levels (348,353,358,359). PLA_2 functions to release AA - an important rate limiting substrate of 5-LOX, COX and eicosanoid metabolism (237,238,347-350,354,366). Due to PLA_2's possible role in modulating 5-LOX and COX activity, and the resulting inverse relationship between the activity of these enzymes and BL6 cell growth as previously discussed, the effect of combined vitamin E succinate and Asc supplementation on PLA_2 activity was determined. PLA_2 activity was significantly increased at all combined vitamin concentrations following supplementation. Comparing Ca^{2+} levels and PLA_2 activity it was clear that a positive correlation existed between the Ca^{2+} and PLA_2 activity, indicating that PLA_2 activity in BL6 cells may also be Ca^{2+}-dependent.

Tumour cells have been reported to have altered EFA composition in their membranes, which in turn modulates the activity of membrane-associated enzymes, such as PLA_2 and AC (190). Since it would seem that the PLA_2 activity is regulated in part by the EFA composition of the phospholipid bilayer in which it resides (515), and since we have already established a positive correlation between Ca^{2+} levels and PLA_2 activity, it was important to determine AA content within BL6 cells. Due to a significant increase in PLA_2 activity in vitamin supplemented cells an increase in free EFA composition - particularly AA - could be expected, and was found to be the case. It was established that an inverse relationship existed between BL6 cell growth and percentage AA composition upon combined vitamin E succinate and Asc supplementation. This effect was the result of an increased uptake of AA by the BL6 cells from the medium and an increased release of AA from membrane phospholipids by PLA_2 (317).

Since AA was elevated in vitamin-supplemented cells, and AA is the substrate for COX - which is also elevated as a result of vitamin supplementation - it was important to investigate the effect of vitamin E succinate and Asc on the products of COX, the prostaglandins. PG-producing cells predominantly synthesize one type of PG due to the existence of a single PGH_2 metabolizing enzyme (229). Several studies have indicated that BL6 cells are unusual in that PGD_2 is the major metabolite, with lesser amounts of $PGF_{2\alpha}$, PGE_2 and PGI_2 (300,309). However, when comparing the levels of PGs in BL6 cultures in this study, it was evident that in this case BL6 cells convert AA to PGE_2,

PGD_2, $PGF_{2\alpha}$ and PGI_2 in descending order of magnitude, with PGI_2 levels in BL6 cells being found to be markedly lower than PGE_2, PGD_2 and $PGF_{2\alpha}$ levels. Supplementation of combined vitamin E succinate and Asc resulted in a significant increase in PGE_2 and PGI_2 levels. PGD_2 and $PGF_{2\alpha}$ levels were markedly increased, although no significance was recorded. It has been suggested that AA metabolism can be diverted towards PGE_2 synthesis when elevated levels of lipid hydroperoxides occur. Furthermore, ElAttar and Lin (148) suggest that the observed stimulatory or inhibitory effects of combined vitamin E succinate and Asc on endogenous PGE_2 levels was a reflection of the increased or decreased activities of both the PLA_2 and COX enzymes respectively. Combined vitamin E succinate and Asc supplementation in the present study in fact resulted in a significant increase in both PLA_2 and COX activities in BL6 cells. Furthermore the resultant increase in PGE_2 levels was inversely related to BL6 cell growth, as was total percentage AA composition. Hence the amount of PGE_2 synthesized appeared to be dependent on the level of its precursor and its synthesizing enzyme, COX (317). Therefore, it is reasonable to assume that the growth inhibitory effects of combined vitamin E succinate and Asc supplementation was due, at least in part, to the increase in PGE_2 synthesis resulting from the cascade effect throughout the eicosanoid pathway.

PGE_2 in turn stimulates AC activity in a variety of cells, including tumour cells, although tumours can vary in the degree of cAMP response (282). cAMP acts as a second messenger for PGE_2-mediated modulation of biological activity within the cell (337). Combined vitamin E succinate and Asc supplementation of the BL6 cells significantly increased AC activity and cAMP levels. It is therefore reasonable to conclude that the increased AC activity and cAMP levels found are likely to be a result of increased PGE_2 synthesis. Other studies have shown that supplementation of E-series PGs to various malignant and non-malignant cell lines inhibits the growth of these cells, and furthermore that this inhibition of cell growth is positively correlated with changes in cAMP levels (415,420).

With respect to the non-malignant LLCMK cells in general, vitamin supplementation, exhibited no significant trends or relationship between cell growth and cell oxidation states or various metabolites and second messengers in the AA pathway. Furthermore, with regard to those experiments showing an inverse relationship between LLCMK growth and the levels of second messengers and activity of various enzymes in this pathway, no definite conclusion could be made since the magnitude of this relationship varied considerably from one experiment to the other. The malignant cell line (BL6 melanoma) on the other hand, as outlined, exhibited consistent trends and relationships following combined vitamin supplementation, hence this discussion has focused on the BL6 cells.

Results from these studies therefore suggest that the inhibitory effect of combined vitamin E succinate and Asc on BL6 cell growth did not involve their synergistic antioxidant properties. The most important regulator of cell proliferation is most likely to be the final metabolite, cAMP, which is significantly increased in BL6 cells with decreased cell growth and is known to be directly involved in the cell cycle and an important regulator of cell growth with intracellular cAMP levels rising with the entrance of cells into the quiescent state (447). However, in a sense this is an indirect effect of combined vitamin E succinate and Asc supplementation, as it initially directly affects AA levels in BL6 cells, resulting in the cascade effect through the eicosanoid pathway. This effect in turn is expressed through PGE_2 and subsequent AC activity.

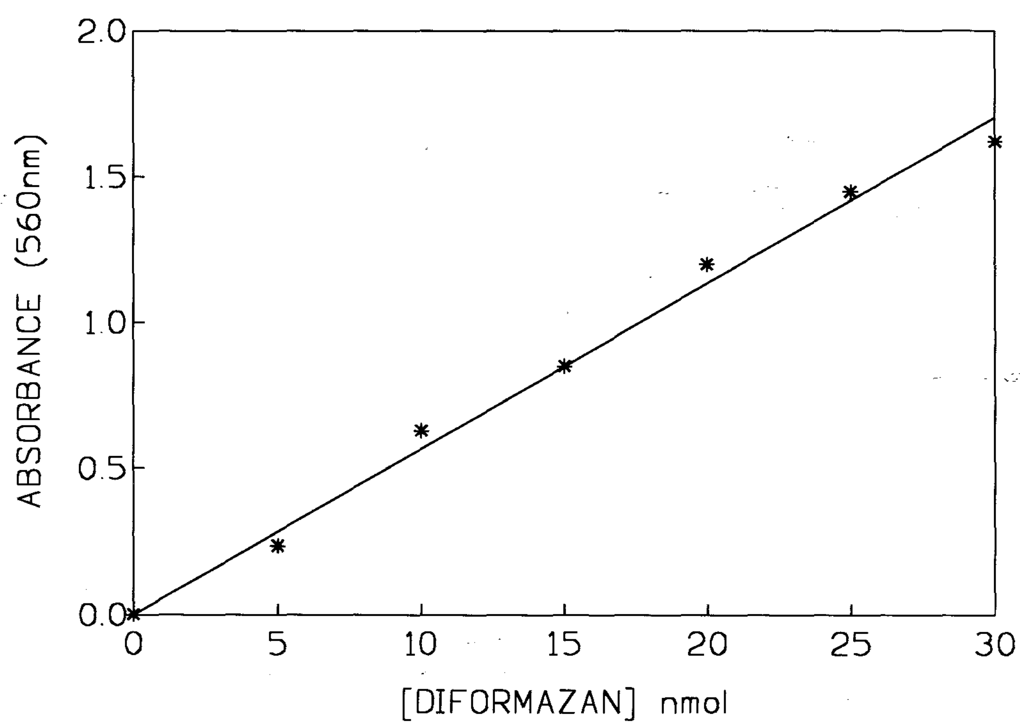
In an attempt to provide further evidence that combined vitamin E succinate and Asc mediated its growth inhibitory effects on BL6 cell growth through the initial activation of PLA_2 and subsequent cascade effect and that a direct link exists between free radical, lipid peroxidation levels and COX activity, BL6 cells were supplemented with PLA_2 inhibitor, dexamethasone. Supplementation of BL6 cells with 10μM dexamethasone and combined vitamin E succinate and Asc resulted in an increase in growth when compared to control cultures although, no significance was recorded. Analysis of COX activity following dexamethasone and combined vitamin E succinate and Asc supplementation in BL6 cells showed a significant decrease in COX activity compared to control cultures, while supplementation with dexamethasone and combined vitamin E succinate and Asc resulted in no significant increases or decreases in PGE_2 levels, while AC activity and cAMP levels were significantly decreased. These results provide further evidence that vitamin E succinate and Asc growth-inhibitory effects on BL6 cell growth were a result of the combined vitamins' ability to increase endogenous PGE_2, which in turn affects cell proliferation through interacting with the AC-cAMPlinked system. With respect to free radical and lipid peroxidation in BL6 cells, dexamethasone supplementation resulted in a non significant decrease in free radical formation, while lipid peroxidation levels were significantly decreased. Therefore it is reasonable to conclude that the observed increase in free radical and lipid peroxidation levels was due the indirect effect of combined vitamin E succinate and Asc to increase COX activity within BL6 cells as a result of increased release of the enzyme substrate, AA.

It can be concluded that in BL6 murine melanoma cells, combined vitamin E succinate and Asc supplementation in pharmacological concentrations mediates an alteration in AA metabolism. This results in a cascade effect throughout the eicosanoid pathway, which stimulates AC activity thus increasing cAMP production. The significant increase in cAMP levels is believed to be responsible

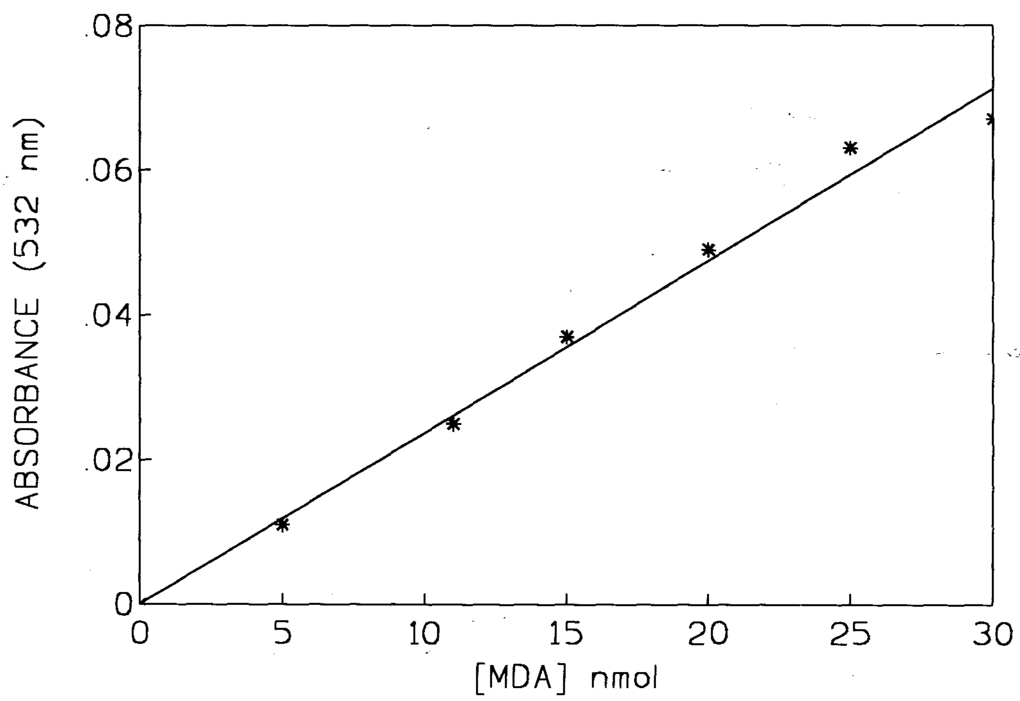
for the inhibitory effect of combined vitamin E succinate and Asc supplementation on BL6 cell growth. However, cAMP may not be the sole factor involved in the process, since other second messengers such as Ca^{2+}, AA, PGE_2 etc may play a more direct role.

It is clear from the studies that vitamin E succinate and Asc may have an important role in controlling cell proliferation of malignant cells, while having little effect on non malignant cells. However, caution should be exercised in extrapolating these results to all other cells since a certain amount of cell specificity in terms of these effects is likely to occur. The extension of these findings to chemoprevention of cancer will also require further investigation.

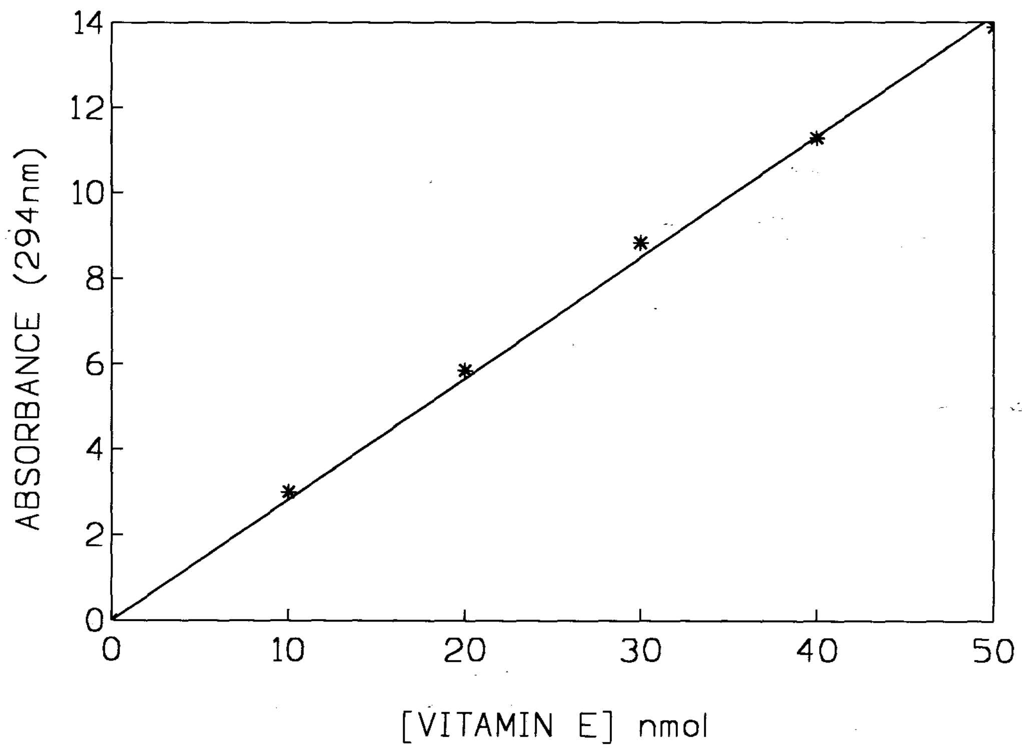
APPENDICES



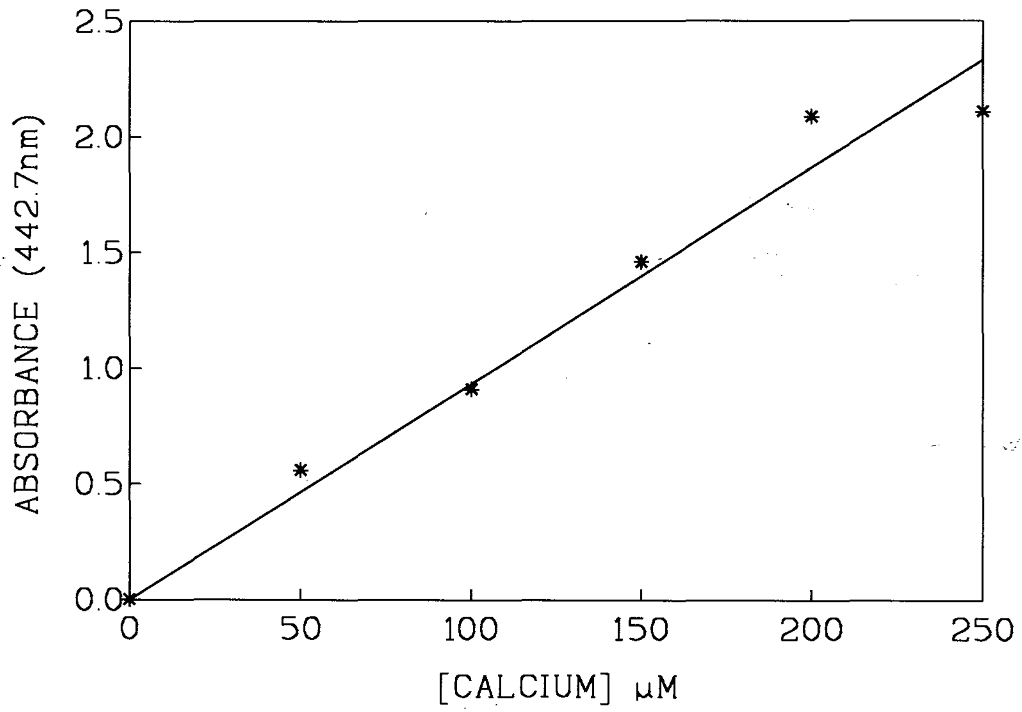
APPENDIX 1: Diformazan standard curve.



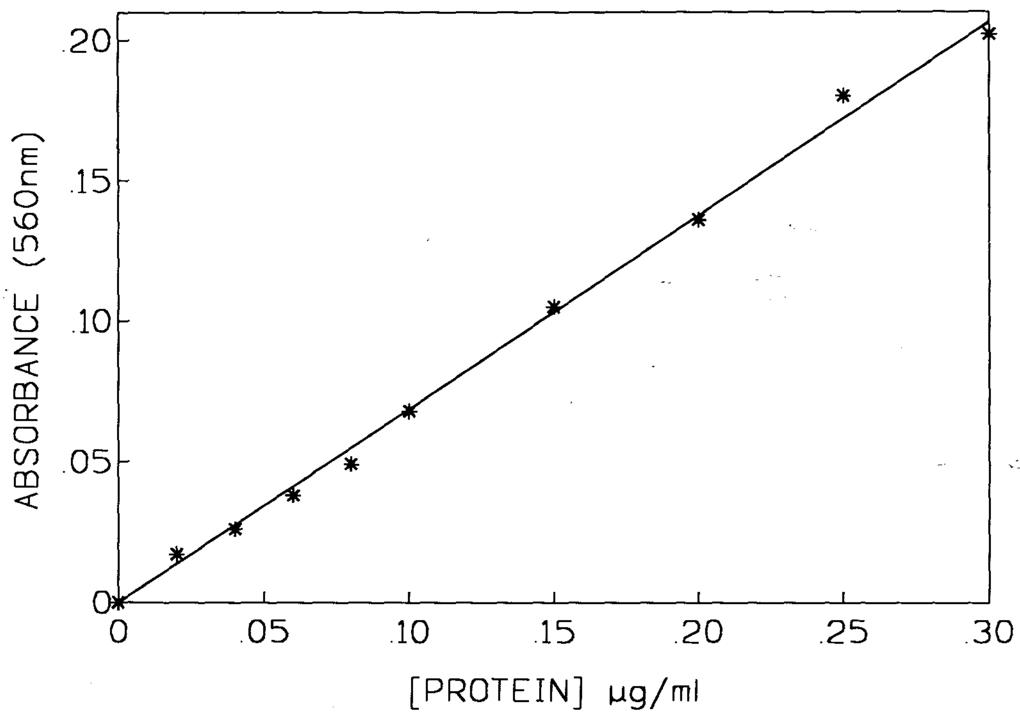
APPENDIX 2: Malondialdehyde standard curve.



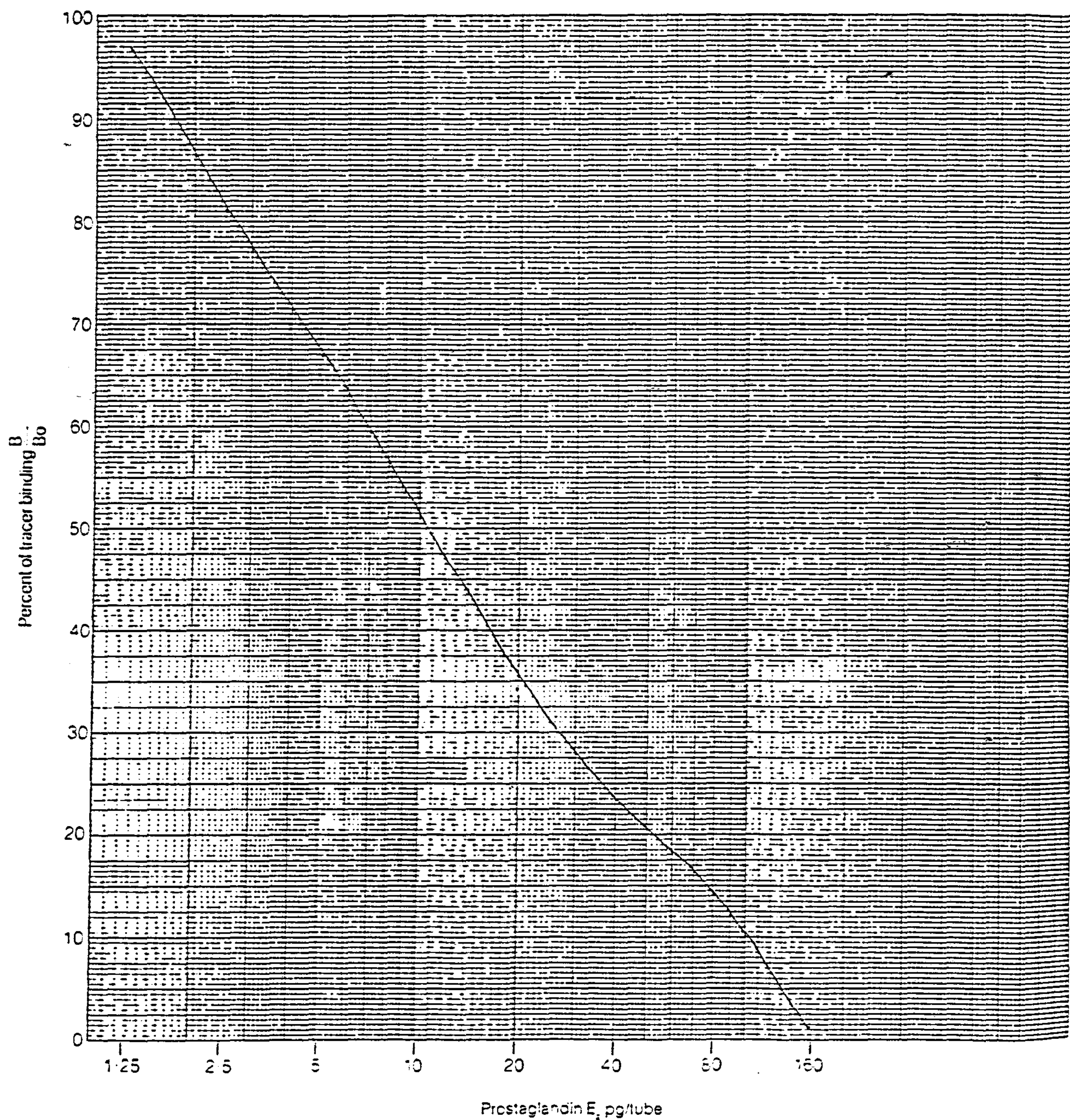
APPENDIX 3: Vitamin E standard curve.



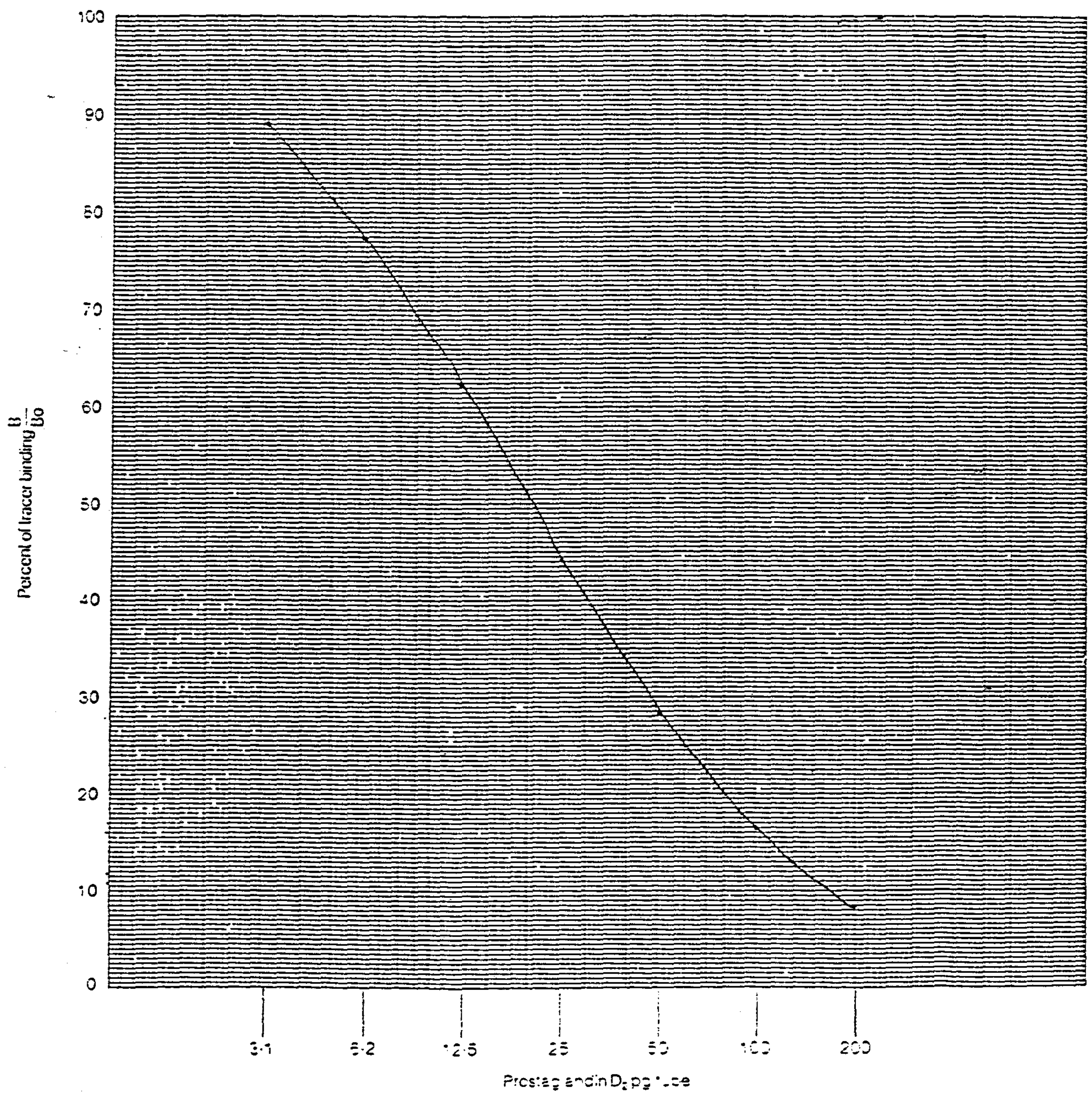
APPENDIX 4: Calcium standard curve.



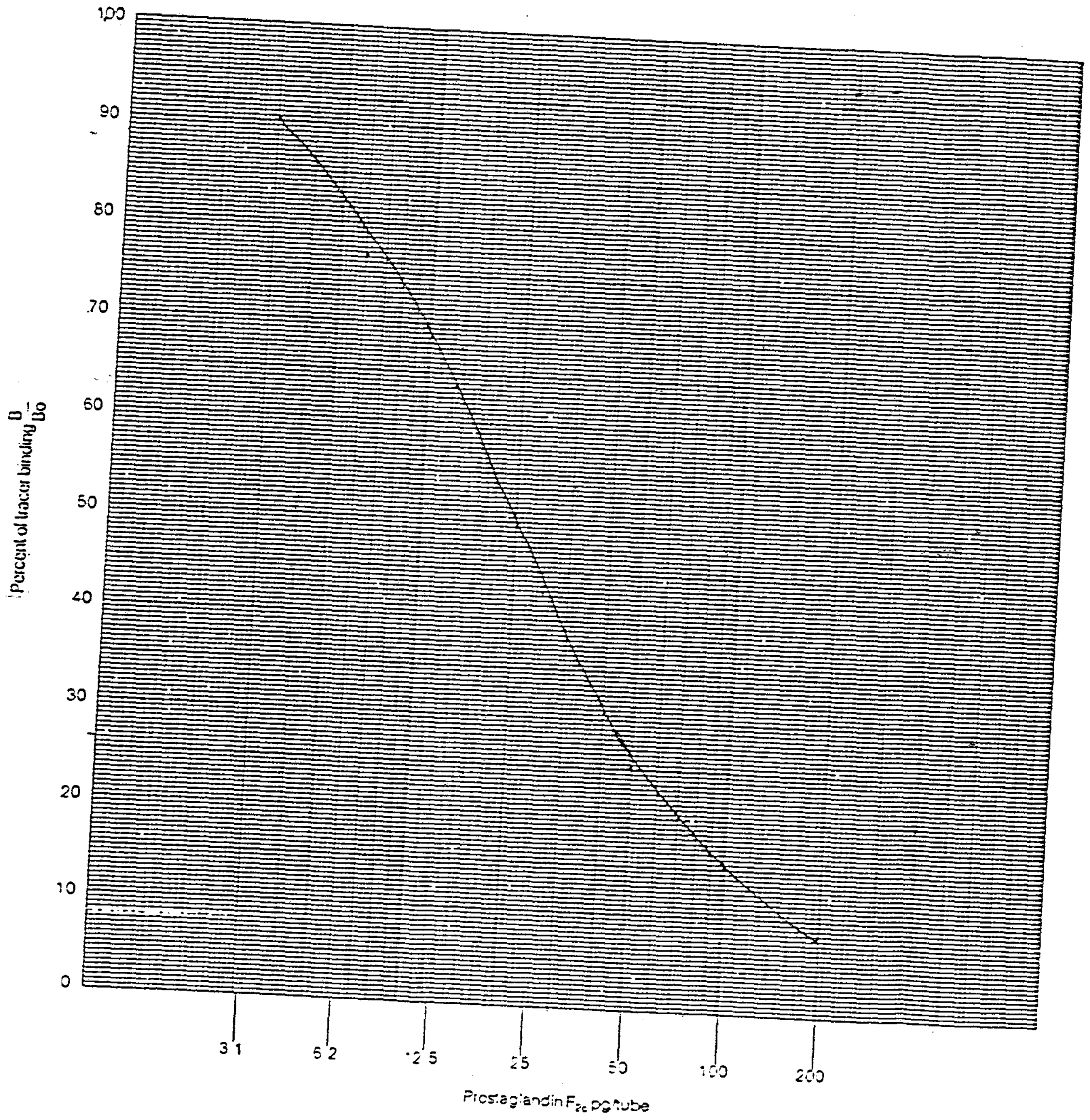
APPENDIX 5: Protein standard curve.



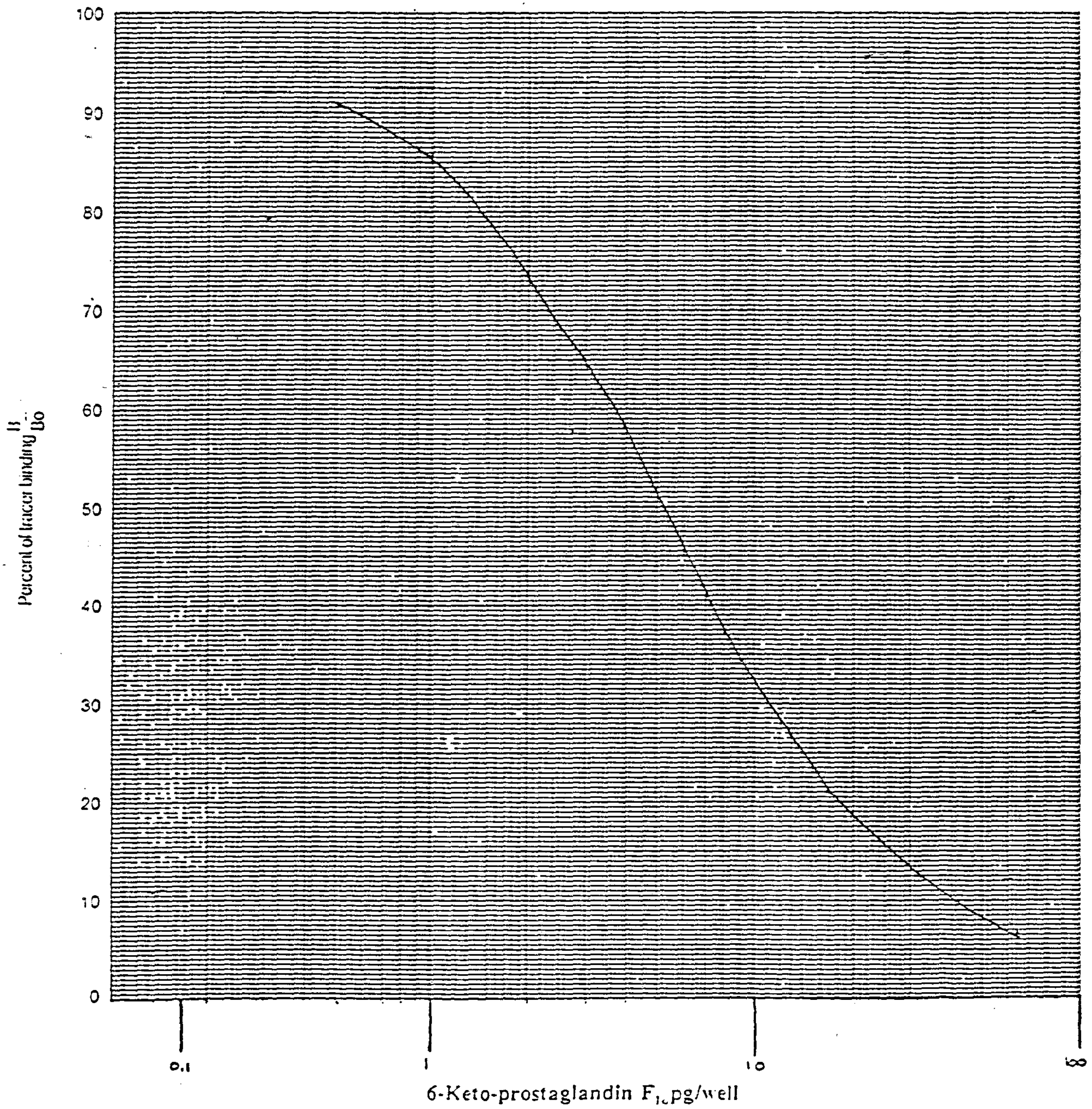
APPENDIX 6: Prostaglandin E_2 standard curve.



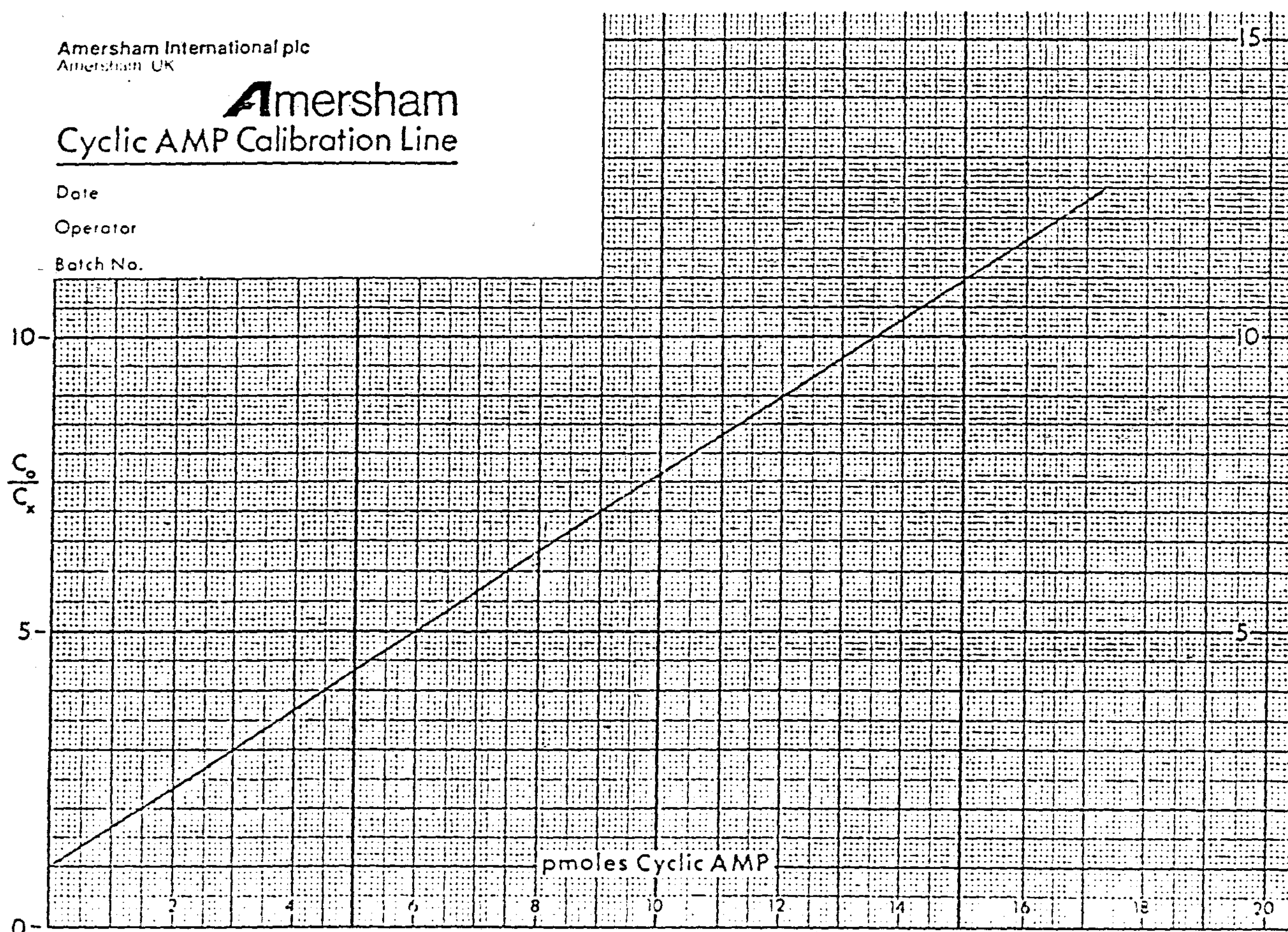
APPENDIX 7: Prostaglandin D_2 standard curve.



APPENDIX 8: Prostaglandin $F_{2\alpha}$ standard curve.



APPENDIX 9: Prostaglandin I_2 standard curve.



APPENDIX 10: Cyclic adenosine monophosphate standard curve.

REFERENCES

1. Berkow R. and Fletcher A.J., eds. (1987) *The Merck Manual* 15ed: 1206. Merck Sharp and Dohme Research Laboratories, New Jersey.
2. Cairns J. (1975) The cancer problem. *Nature.* **255**, 197-200.
3. Witz G. (1991) Active oxygen species as factors in multistage carcinogenesis. *Proc. Soc. Exp. Biol. Med.* **198**, 675-682.
4. Pitot HC and Dragan YP. (1991) Facts and theories concerning the mechanisms of carcinogenesis. *FASEB J.* **5**, 2280-2286.
5. Bertram JS, Kolonel LN and Meyskens FL Jr. (1987) Rationale and strategies for chemoprevention of cancer in humans. *Cancer Res.* **47**, 3012-3031.
6. Benedict WF and Jones PA. (1982) Inhibition of transformation and oncogenic progression by ascorbic acid: A possible role in chemoprevention. In: Molecular Interrelations of Nutrition and Cancer. (Ed: Arnott MS, van Eys J and Wang Y-M) **34**, 351-356.
7. Singh VN and Gaby SK. (1991) Premalignant lesions: role of antioxidant vitamins and β-carotene in risk reduction and prevention of malignant transformation. *Am. J.Clin.Nutr.* **53**, S386-S390.
8. Slaga TJ. (1983) Multistage skin carcinogenesis and specificity of inhibitors.In: Modulation and Mediation of Cancer by Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. pp 10-23.
9. Honn KV, Bockman RS and Marnett LJ. (1981) Prostaglandins and cancer: A review of tumor initiation through tumor metastasis. *Prostagland.* **21**, 838-864.
10 Kensler TW and Taffe BG. (1986) Free radicals in tumor promotion. *Adv.Free Radical Biol.Med.* **2**, 347-387.
11. Flavin DF and Kolbye AC Jr. (1983) Nutritional factors with the potential to inhibit critical pathways of tumor promotion. In: Modulations and Mediation of Cancer by Vitamins. (Ed:Meyskens FL and Prasad KN) Karger, Basel. pp 24-38.
12. Hunter T. (1985) Oncogenes and growth control. *TIBS.* **10**, 275-280.
13. Weinberg RA. (1988) Finding that anti-oncogene. *Sci. Amer.* **259**, 34-41.
14. Travali S, Koniecki J, Petralia S and Baserga R. (1990) Oncogenes in growth and development. *FASEB J.* **4**, 3209-3214.
15. Hellström KE and Hellström I.(1989) Oncogene-associated tumor antigens as targets for immunotherapy. *FASEB J.* **3**, 1715-1722.
16. Ames BN. (1983) Dietary carcinogens and anticarcinogens. *SCI.* **221**, 1256-1262.

17. Chan AC. (1993) Partners in defence, vitamin E and vitamin C. *Can. J. Physiol. Pharmacol.* **71**, 725-731.

18. Niki E. (1990) Interaction of ascorbate and α-tocopherol. *Ann. New York Acad. Sci.* **498**, 186-199.

19. Roos E and Dingemans KP. (1979) Mechanisms of metastasis. *Biochim. Biophys. Acta.* **560**, 135-166.

20. Sugarbaker EV and Ketcham AS. (1977) Mechanisms and prevention of cancer dissemination: an overview. *Semin. Oncol.* **4**, 19-30.

21. Lippman SM, Benner SE and Hong WI. (1993) Strategies for the control of cancer. *Cancer.* **72**, 984-990.

22. Byers TM and Perry G. (1992) Dietary carotenes, vitamin C and vitamin E as protective antioxidants in human cancers. *Annu. Rev. Nutr.* **12**, 139-159.

23. Pascoe GA, Olafsdottir K and Reed DJ. (1987) Vitamin E protection against chemical-induced cell injury. *Arch. Biochem. Biophys.* **256**, 150-159.

24. Tengerdy RP. (1990) The role of vitamin E in immune response and disease resistance. *Ann. New York Acad. Sci.* **587**, 24-33.

25. Hoffman FA. (1985) Micronutrient requirements of cancer patients. *Cancer.* **55**, 295-300.

26. Pauling L. (1970) Evolution and the need for ascorbic acid. *Proc. Nat. Acad. Sci.* **67**, 1643-1648.

27. Padh H. (1990) Cellular functions of ascorbic acid. *Biochem. Cell Biol.* **68**, 1166-1173.

28. Nishikimi M and Yagi K. (1991) Molecular basis for the deficiency in humans of gulonolactone oxidase, a key enzyme for ascorbic acid biosynthesis. *Am. J. Clin. Nutr.* **54**, 1203S-1208S.

29. Linder MC. (1985) Nutritional biochemistry and metabolism - With clinical applications. Elsevier Science Publishing Co., New York. p94-99 and p356-360.

30. Sauberlich HE. (1994) Pharmacology of vitamin C. *Annu. Rev. Nutr.* **14**, 371-391.

31. Cameron E, Pauling L and Leibovitz B. (1979) Ascorbic acid and cancer: A review. *Cancer Res.* **39**, 663-681.

32. Harris LJ. (1993) Vitamins. *Ann. Rev. Biochem.* **2**, 253-298.

33. Davies MB, Austin J and Partridge DA. (1991) Vitamin C: Its chemistry and biochemistry. Royal Society of Chemistry Paperbacks. Cambridge. p7-146.

34. Schorah CJ. (1992) The transport of vitamin C and effects of disease. *Proc. Nutr. Soc.* **51**, 189-198.

35. Rose RC. (1988) Transport of ascorbic acid and other water-soluble vitamins. *Biochim. Biophys. Acta.* **947**, 335-366.

36. Bendich A, Machlin LJ and Scandurra O. (1986) The antioxidant role of vitamin C. *Adv.Free Radical. Biol. Med.* **2**, 419-444.

37. Schorah CJ, Sobala GM, Sanderson M, Collis N and Primrose JN. (1991) Gastric juice ascorbic acid: effects of disease and implications for gastric carcinogenesis. *Am. J. Clin. Nutr.* **53**, 287S-293S.

38. Block G. (1991) Epidemiologic evidence regarding vitamin C and cancer. *Am. J. Clin. Nutr.* **54**, 1313S-1314S

39. Anonymous. (1991) Ascorbic acid levels in cells may be higher than first believed. *Nutr. Rev.***49**, 27-28.

40. Padh H. (1991) Vitamin C: Newer insights into its biochemical functions. *Nutr. Rev.* **49**, 65-70.

41. Cameron E and Pauling L. (1973) Ascorbic acid and the glycosaminoglycans. *Onccol.* **27**, 181-192.

42. Harada S-I, Matsumoto T and Ogata E. (1991) Role of ascorbic acid in the regulation of proliferation in osteoblast-like MC3T3-E1 cells. *J. Bone Miner. Res.* **6**, 903-908.

43. Bhattacherjee J, Sarkar N, Basu A and Mitra S. (1984) Study of ascorbate status in murine and human lymphoma. *IRCS Med. Sci.* **12**, 552-553.

44. Bright-See E and Newmark HL. (1983) Potential and probable role of vitamin C and E in the prevention of carcinogenesis. In: Modulation and Mediation of Cancer in Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. p244-257.

45. Stähelin HB, Gey KF and Brubacher G. (1987) Plasma vitamin C and cancer death: The prospective Basel study. *Ann New York Acad Sci.* **498**, 124-131.

46. Birt DF. (1989) Effects of the intake of selected vitamins and minerals on cancer prevention. *Magnesium.* **8**, 17-30.

47. Willet WC and NacMahon (1984) Diet and cancer- an overview. *New Engl. J. Med.* 310, 633-638.

48. Benade L, Howard T and Burk D. (1969) Synergistic killing of Ehrlich Ascites Carcinoma cells by ascorbate and 3-Amino-1,2,4,-triazole. *Oncol.* **23**, 33-43.

49. Prasad KN and Rama BN. (1983) Modification of the effect of pharmacological agents on tumor cells in culture by vitamin C and vitamin E. In: Modulation and Mediation of Cancer by Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. p244-257.

50. Machlin LJ and Bendich A. (1987) Free radical tissue damage: protective role of antioxidant nutrients. *FASEB J.* **1**, 441-445.

51. Peterkofsky B and Prather W. (1977) Cytotoxicity of ascorbate and other reducing agents towards cultured fibroblasts as a result of hydrogen peroxide formation. *J. Cell Physiol.* 90, 61-70.

52. Prasad KN. (1980) Modulation of the effects of tumor therapeutic agents by vitamin C. *Life Sci.* **27**, 275-280.

53. Prasad KN, Sinha PK, Ramanujam M and Sakamoto A. (1979) Sodium ascorbate potentiates the growth inhibitory effect of certain agents on neuroblastoma cells in culture. *Proc. Natl. Acad. Sci.* **76**, 829-832.

54. Niki E. (1991) Action of ascorbic acid as a scavenger of active and stable oxygen radicals. *Am. J. Clin. Nutr.* **54**, 1119S-1124S.

55. Cheeseman KH and Slater TF. (1993) An introduction to free radical biochemistry. *Br. Med. Bull.* **49**, 481-493.

56. Bodannes RS and Chan PC. (1979) Ascorbic acid as a scavenger of singlet oxygen. *FEBS Lett.* **105**, 195-106.

57. Frei B, England L and Ames BN. (1989) Ascorbate is an outstanding antioxidant in human blood plasma. *Proc. Natl. Acad. Sci.* **86**. 6337-6381.

58. Siegel BV and Morton JI. (1984) Vitamin C and immunity: Influence of ascorbate on prostaglandin E_2 synthesis and implications for the natural killer cell activity. *Int. J. Vitam. Nutr. Res.* **54**, 339-342.

59. Bram S, Froussard P, Guichard M, Jasmin C, Augery Y, Sinoussi-Barre F and Wray W. (1980) Vitamin C preferential toxicty for malignant melanoma cells. *Nature.* **284**, 629-631.

60. Park CH. (1983) Growth-modulation of Human Leukemic colony-forming cells *in vitro* by L-ascorbic acid. In: Modulation and Mediation of Cancer by Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. p266-269.

61. Leibowitz B and Schlesser J. (1983) Effect of L-ascorbic acid on Leukemia development and breast cancer in various inbred strains of mice. In: Modulation and Mediation of Cancer by Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. 140-143.

62. Park CH, Amare M, Savin MA and Hoogstraten B. (1980) Growth suppression of Human Leukemic cells *in vitro* by L-ascorbic acid. *Cancer Res.* **40**, 1062-1065.

63. Bright-See E. (1983) Vitamin C and cancer prevention. *Semin. Oncol.* **X**, 294-298.

64. Park CH. (1985) Biological nature of the effect of ascorbic acids on the growth of Human Leukemic cells. *Cancer Res.* **45**, 3969-3973.

65. Creagan ET, Moertel CG, O'Fallon JR, Schutt AJ, O'Connell MJ, Rubin J and Frytak S. (1979) Failure of high-dose vitamin C (ascorbic acid) therapy to benefit patients with advanced cancer. *N. Engl. J. Med.* **301**, 687-690.

66. Fukushima S, Uwagawa S, Shirai T, Hasegawa R and Ogawa K. (1990) Synergism by sodium L-ascorbate but inhibition by L-ascorbic acid for sodium saccharin promotion of rat two-stage bladder carcinogenesis. *Cancer Res.* **50**, 4195-4198.

67. Moertel CG, Fleming TR, Creagam ET, Rubin J, O',Connell and Ames MM. (1985) High-dose vitamin C versus placebo in the treatment of patients with advanced cancer who have no prior chemotherapy. *N. Engl. J. Med.* **312**,137-141.

68. Wittes RE. (1985) Vitamin C and cancer. *N. Engl. J Med.* **312**, 178-179.

69. Park CH, Amare M and Hoogtraten B. (1980) analysis of the growth enhancing effect of L-ascorbic acid on Human Leukemic cells in culture. *Exp. Hematol.* **8**, 853-859.

70. Tsao C. (1991) Inhibiting effect of ascorbic acid on the growth of human mammary tumor xenografts. *Am. J. Clin. Nutr.* **54**, 1274S-1280S.

71. Ghosh J and Das S. (1985) Evaluation of vitamin A and C status in normal and malignant conditions and their possible role in cancer prevention. *Jpn. J. Cancer Res.* **76**,1174-1178.

72. Cameron E and Pauling L. (1976) Supplemental ascorbate in the supportive treatment of cancer. Prolongation of survival times in terminal human cancer. *Proc. Natl. Acad. Sci.* **73**, 3685-3689.

73. Cameron E. (1991) Protocol for the use of vitamin C in the treatment of cancer. *Med. Hypotheses.* **36**, 190-194.

74. Campbell A and Jack T. (1979) Acute reactions to mega ascorbic acid therapy in malignant disease. *Scott. Med. J.* **24**, 151-153.

75. Knock FE, Gascoyne PRC, Sylvester R and Wibel R. (1981) Ascorbic acid as a thiolprive: ability to induce immunity against some cancers in mice. *Physiol. Chem.Phys.* **13**, 325-333.

76. Benedict WF, Wheatley WL and Jones PA. (1982) Differences in anchorage-dependent growth and tumorigenicities between transformed C3H/10T½ cells with morphologies that are or are not reverted to a normal phenotype by ascorbic acid. *Cancer Res.* **42**, 1041-1045.

77. Benedict WF, Wheatley WL and Jones PA. (1983) The use of ascorbic acid for selection of transformed cells with differences in tumorigenicities and anchorage-independent growth: Implications for chemoprevention. In: Modulation and Mediation of Cancer by Vitamins. (Ed Meyskens FL and Prasad KN) Karger, Basel. p114-118.

78. Bornside GH and Tracey JM. (1983) Partial reversal by sodium ascorbate of hyperoxia-induced damage to HEp-2 cell cultures. *In Vitro.* **19**, 355-360.

79. Benedich A and Langseth L. (1995) The health effects of vitamin C supplementation: A review. *J. Am. Coll. Nutr.* **14**, 124-136.

80. Newberne PM and Suphakarn V. (1983) Nutrition and cancer: A review, with emphasis on the role of vitamins C and E and selenium. *Nutr. Cancer.* **5**,107-119.

81. Mason KE. (1977) The first two decades of vitamin E. *Fed. Proc.* **36**, 1906-1910.

82. Scott ML. (1978) Vitamin E. In: Handbook of Lipid Research: The fat soluble vitamins. (Ed: Dedas Luca HF) Plenum Press, New York. p133-210.

83. Mason KE. (1980). The first two decades of vitamin E history. In: Vitamin E: A comprehensive treatise. (Ed: Machlin LJ) Marcel Dekker, Inc. New York and Basel. p1-5.

84. Packer L. (1994) Vitamin E is nature's master antioxidant. *Sci Amer.* **1**, 54-63.

85. Fritsma GA. (1983) Vitamin E and autooxidation. *Am. J. Med. Technol.* **49**, 453-456.

86. Drevon CA. (1990) Absorption, transport and metabolism of vitamin E. *Free Radical Res. Comms.* **14**, 229-246.

87. Kasparek S. (1980) Chemistry of tocopherols and tocotrienols. In: Vitamin E: A comprehensive treatise. (Ed: Machlin LJ) Marcel Dekker, Inc. New York and Basel. p7-65.

88. Morrissey PA, Quinn PB and Sheehy PJA. (1994) Newer aspects of micronutrients in chronic disease: vitamin E. *Proc. Nutr. Soc.* **53**, 571-582.

89. Gould MN, Haag JD, Kenman WS, Tanner MA and Elson CE. (1991) A comparison of tocopherol and tocotrienol for the chemoprevention of chemically induced rat mammary tumors. *Am. J Clin. Nutr.* **53**, 1068S-1070S.

90. Oski FA. (1977) Metabolism and physiologic roles of vitamin E. *Hosp. Practice.* **12**, 79-85.

91. ElAttar TMA and Lin HS. (1995) Vitamin E succinate potentiates the inhibitory effect of prostaglandins on oral squamous carcinoma cell proliferation. *Prostaglandins Leukotrienes EFAs.* **52**, 69-73.

92. Rama BN and Prasad KN. (1983) Study on the specificity of α-tocopheryl (vitamin E) acid succinate effects on melanoma, glioma and neuroblastoma cells in culture. *Proc. Soc. Exp. Biol. Med.* **174**, 302-307.

93. Badamchian M, Spangelo BL, Hagiwara Y, Hagiwara H, Ueyama H and Goldstein AL. (1995) α-Tocopherol succinate, but not α-tocopherol or other vitamin E analogs, stimulates prolactin release from rat anterior pituitary cells *in vitro*. *Nutr. Biochem.* **6**, 340-344.

94. Prasad KN and Edwards-Prasad J. (1982) Effects of tocopherol (vitamin E) acid succinate on morphological alterations and growth inhibition in melanoma cells in culture. *Cancer Res.* **42**, 550-555.

95. Fariss MW. (1990) Oxygen toxicity: Unique cytoprotective properties of vitamin E succinate in hepatocytes. *Free Radical Biol. Med.* **9**, 333-343.

96. Torelli S, Masoudi F and Prasad KN. (1988) Effect of alpha tocopheryl succinate on cyclic AMP- dependent protein kinase activity in murine B-16 Melanoma cells in culture. *Cancer Lett.* **39**,129-136.

97. Kline K, Cochran GS and Sanders BG. (1990) Growth-inhibitory effects of vitamin E succinate on retrovirus-transformed tumor cells *in vitro*. *Nutr. Cancer.* **14**, 27-39.

98. Sakagami H, Asaka K, Abe E, Miyaura C, Suda T and Konno K. (1981) Effect of DL-α-tocopherol (vitamin E) on the differentiation of Mouse Myeloid Leukemia cell. *J. Nutr. Sci. Vitaminol.* **27**, 291-300.

99. Horwitt MK. (1980) Therapeutic uses of vitamin E in medicine. *Nutr. Rev.***38**, 105-113.

100. Bendich A and Machlin LJ. (1988) Safety of oral intake of vitamin E. *Am. J. Clin. Nutr.* **48**, 612-619.

101. Pehr K and Forsey RR. (1993) Why dont we use vitamin E in dermatology? *Can. Med. Assoc. J.* **149**, 1247-1251.

102. Watson RR and Leonard TK. (1986) Selenium and vitamins A, E, and C: Nutrients with cancer prevention properties. *Perspect. Practice.* **86**, 505-510.

103. Liebler DC. (1993) The role of metabolism in the antioxidant function of vitamin E. *Crit. Rev. Toxicol.* **23**, 147-169.

104. Dutta-Roy AK, Gordon MJ, Campbell FM, Duthie GG, and James WPT. (1994) Vitamin E requirements, transport, and metabolism: Role of α-tocopherol-binding proteins. *J. Nutr. Biochem.* **5**, 562-570.

105. Thellmen CA and Shireman RB. (1985) *In vitro* uptake of [^{3}H]α-toccopherol from low density lipoprotein by cultured Human Fibroblasts. *J. Nutr.* **115**, 1673-1679.

106. Bieri JG, Corash L and Hubbard VS. (1983) Medical uses of vitamin E. *New Engl. J. Med.* **308**, 1063-1069.

107. Gallo-Torres HE. (1980) Absorption. In: Vitamin E: A comprehensive treatise. (Ed: Machlin LJ) Marcel Dekker, Inc., New York and Basel. p170-193.

108. Burton GW. (1994) Vitamin E: molecular and biological function. *Proc. Nutr. Soc.* **53**, 251-262.

109. Behrens WA and Madere R. (1987) Mechanisms of absorption, transport and tissue uptake of RRR-α-tocopherol and D-γ-tocopherol in the white rat. *J. Nutr.* **117**, 1562-1569.

110. Packer L. (1991) Protective role of vitamin E in biological systems. *Am. J. Clin. Nutr.* **53**, 1050S-1055S.

111. Tengerdy RP. (1980) Effect of vitamin E on immune responses. In: Vitamin E: A comprehensive treatise. (Ed: Machlin LJ) Marcel Dekker, Inc. New York and Basel. p429-445.

112. Murphy ME, Scholich H, Wefers H and Sies H. (1989) α-Tocopherol in microsomal lipid peroxidation. *Ann. New York Acad. Sci.* **570**, 480-486.

113. Knekt P. (1991) Role of vitamin E in the prophylaxis of cancer. *Ann. Med.* **23**, 3-12.

114. Knekt P, Maatela AA, Aaran R-K, Nikkari T, Hakama M, Hakulinen T, Peto R and Teppo L. (1991) Vitamin E and cancer prevention. *Am. J. Clin. Nutr.* **53**, 283S-286S.

115. Machlin LJ. (1980) Epilogue. In: Vitamin E: A comprehensive treatise. Marcel Dekker, Inc. New York and Basel. p637-645.

116. Schwartz J, Shklar G and Trickler D. (1993) p53 in the anticancer mechanism of vitamin E. *Oral Oncol., Eur. J. Cancer.* **29B**, 313-318.

117. Milner J. (1995) Flexibility: the key to p53 function ? *TIBS.* **20**, 49-51.

118. Friend S. (1994) p53: A glimpse at the puppet behind the shadow play. *SCI.* **265**, 334-335.

119. Culotta E and Koshland DE. (1993) p53 sweeps through cancer research. *SCI.* **262**, 958-1961.

120. Douglas K. (1994) Making friends with death-wish genes. *New Sci.* **143**, 30-34.

121. Spector AA and Yorek MA. (1985) Membrane lipid composition and cellular function. *J. Lipid Res.* **26**, 1015-1035.

122. Palamanda JR and Kehrer JP. (1993) Involvement of vitamin E and protein thiols in the inhibition of microsomal lipid peroxidation by glutathione. *Lipids.* **28**, 427-431.

123. Galeotti T, Borrello S and Minotti G. (1986) Membrane alterations in cancer cells: The role of oxy radicals. *Ann. New York Acad Sci.* **488**, 468-480.

124. Liebler DC, Kling DS and Reed DJ. (1986) Antioxidant protection of phospholipid by α-tocopherol. *J. Biol. Chem.* **261**, 12114-12119.

125. Lucy JA. (1972) Functional and structural aspects of biological membranes: A suggested structural role for vitamin E in the control of membrane permeability and stability. *Ann. New York Acad. Sci.* **203**, 4-11.

126. Urano S, Yano K and Matsuo M. (1988) Membrane-stabilizing effect of vitamin E: Effect of α-tocopherol and its model compounds on fluidity of lecithin liposomes. *Biochem. Biophys. Res. Commun.* **150**, 469-475.

127. Diplock AT and Lucy JA. (1973) The biochemical modes of action of vitamin E and selenium: A hypothesis. *FEBS Lett.* **29**, 205-210.

128. Di Mascio P, Murphy ME and Sies H. (1991) Antioxidant defence systems: the role of carotenoids, tocopherols, and thiols. *Am. J. Clin. Nutr.* **53**, 194S-200S.

129. Molenaar I, Hulstaert CE and Hardonk MJ. (1980) Role in function and ultrastructure of cellular membranes. In: Vitamin E: A comprehensive treatise. (Ed: Machlin LJ) Marcel Dekker, Inc. New York and Basel. p372-390.

130. Balasubramaniyan N, Subrsmanian S and Govindasamy S. (1994) Status of antioxidant systems in human carcinoma of uterine cervix. *Cancer Lett.* **87**, 187-192.

131. Gey KF, Brubacher GB and Stähelin HB. (1987) Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. *Am. J. Clin. Nutr.* **45**, 1368-1377.

132. Dormandy TL. (1983) An approach of free radicals. *Lancet.* **2**, 1010-1040.

133. Sun Y. (1990) Free radicals, antioxidant enzymes, and carcinogenesis. *Free Radical Biol. Med.* **8**, 583-599.

134. Mukai K, Morimoto H, Okauchi Y and Nagaoka S.(1993) Kinetic study of reactions between tocopheroxyl radicals and fatty acids. *Lipids.* **28**, 753-756.

135. Bowry VW and Ingold KU. (1995) Extraordinary kinetic behaviour of the α-tocopheroxyl (vitamin E) radical. *J. Org. Chem.* **60**, 5456-5467.

136. Krinsky NI. (1992) Mechanism of action of biological antioxidants. *Proc. Soc. Exp. Biol. Med.* **200**, 248-254.

137. Terao J and Matsushita S. (1986) The peroxidizing effect of α-tocopherol on autoxidation of methyl linoleate in the bulk phase. *Lipids.* **21**, 255-260.

138. Burton GW and Ingold KU. (1981) Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants *in vitro*. *J. Am. Chem. Soc.* **103**, 6472-6477.

139. Burton GW, Joyce A and Ingold KU. (1983) Is vitamin E the only lipid-soluble, chain-breaking antioxidant in human blood plasma and erythrocyte membranes. *Arch. Biochem. Biophys.* **221**, 281-290.

140. Yamamoto K and Niki E. (1988) Interaction of α-tocopherol with iron: antioxidant and prooxidant effects of α-tocopherol in the oxidation of lipids in aqueous dispersions in the presence of iron. *Biochim. Biophys.* Acta. **958**, 19-23.

141. McCay PB and King MM. (1980) Vitamin E: Its role as a biological free radical scavenger and its relationship to the microsomal mixed-function oxidase system. In: Vitamin E: A comprehensive treatise. (Ed: Machlin LJ) Marcel Dekker, Inc. New York and Basel. p289-313.

142. Miki M, Tamai H, Mino M, Yamamoto Y and Niki E. (1987) Free radical chain oxidation of rat red blood cells by molecular oxygen and its inhibition by α-tocopherol. *Arch. Biochem Biophys.* **258**, 373-380.

143. Burton GW and Ingold KU. (1989) Vitamin E as an *in vitro* and *in vivo* antioxidant. *Ann.New York Acad. Sci.* **570**, 7-22.

144. Packer L and Landvik S. (1989) Vitamin E: Introduction to biochemistry and health benefits. *Ann. New York Acad. Sci.* **570**, 1-6.

145. Borek C. (1990) Vitamin E as an anticarcinogen. *Ann New York Acad. Sci.* **570**, 417-420.

146. Knept P. (1988) Serum vitamin E level and risk of female cancers. *Int. J. Epid.* **17**, 281-286.

147. Schwartz J and Shklar G. (1992) The selective cytotoxic effect of carotenoids and α-tocopherol on Human Cancer Cell Lines *in vitro*. *J. Oral Maxillofac.Surg.* **50**, 367-373.

148. ElAttar TMA and Lin HS. (1992) Effect of vitamin C and vitamin E on prostaglandin synthesis by Fibroblasts and Squamous carcinoma cells. *Prostaglandins Leukotrienes EFAs.* **47**, 253-257.

149. Helson L, Verma M and Helson C. (1983) Vitamin E and Human Neuroblastoma. In: Modulation and Mediation of cancer by Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. p258-265.

150. Zamora R, Hidalgo FJ and Tappel AL. (1991) Comparative antioxidant effectiveness of dietary β-carotene, viitamin E, selenium and coenzyme Q_{10} in rat erythrocytes and plasma. *J.Nutr.* **121**, 50-56.

151. Palan PR, Mikhail MS, Basu J and Romney SL. (1991) Plasma levels of antioxidant β-carotene and α-tocopherol in uterine cervix dysplasias and cancer. *Nutr. Cancer.* **15**, 13-20.

152. Paganelli GM, Biasco G, Brando G, Santucci R, Gizzi G, Villani V, Cianci M, Miglioli M and Barbara L. (1992) Effect of vitamin A, C, and E supplementation on rectal cell proliferation in patients with Colorectal Adenomas. *Reports.* **84**, 47-51.

153. Cook MG and McNamara P. (1980) Effect of dietary vitamin E on dimethylhydrazine-induced Colonic Tumours in mice. *Cancer Res.* **40**, 1329-1331.

154. Sarria A and Prasad KN. (1984) dl-α-Tocopheryl succinate enhances the effect of γ-irradiation on Neuroblastoma Cells in culture. *Proc. Soc. Exp. Biol. Med.* **175**, 82-92.

155. Menkes MS, Comstock GW, Vuilleumier JP, Helsing KJ, Rider AA and Brookmeyer R. (1986) Serum beta-carotene, vitamins A and E, selenium, and the risk of lung cancer. *N. Engl. J. Med.* **315**, 1250-1254.

156. Thurnham DI. (1990) Anti-oxidant vitamins and cancer prevention. *J. Micron. Anal.* 7, 279-299.

157. Garland M, Willet WC, Manson JE and Hunter DJ.(1993) Antioxidant micronutrients and breast cancer. *J. Am. Coll. Nutr.* **12**, 400-411.

158. Nikbin S, Meydani M, Verdon CP, Shapiro AA, Blumberg JB and Hayes KC. (1986) Vitamin E supplementation suppresses prostaglandin E_2 synthesis and enhances the immunne response of aged mice. *Mech. Ageing Dev.* **34**, 191-201.

159. Srinivason V, Jacobs AJ, Simpson SA and Weiss JF. (1983) Radioprotection by vitamin E: Effects on hepatic enzymes, delayed type hypersensitivity, and postirradiation survival of mice. In: Modulation and Mediation of Cancer by Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. p119-131.

160. Das S. (1994) Vitamin E in the genesis and prevention of cancer. *Acta Oncologica.* **33**, 615-619.

161. Nomura AMY, Stemmermann GN, Heilbruin LK, Salkeld RM, Vuilleumier JP. (1988) Serum vitamin levels and the risk of cancer of specific sites in men of Japanese ancestry. *Cancer Res.* **45**, 2368-2372.

162. Mukai K, Nishimurs M and Kikuchi S. (1991) Stopped-flow investigation of the reaction of vitamin C with tocopheroxyl radical in aqueous triton X-100 micellar solutions. *J. Biol. Chem.* **266**, 274-278.

163. McCay PB. (1985) Vitamin E: Interactions with free radicals and ascorbate. *Ann Rev. Nutr.* **5**, 323-340.

164. Borek C. (1988) The role of free radicals and antioxidants in cellular and molecular carcinogenesis *in vitro*. In: Medical, Biochemical and Chemical Aspects of Free Radicals. (Ed: Hayaishi O, Niki E, Kondo M and Yoshikawa T) Elsevier Science Publishers, B.V., Amsterdam.

165. Scarpa M, Rigo A, Maiorino M, Ursini F and Gregolin C. (1984) Formation of α-tocopherol radical and recycling of α-tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes. *Biochim. Biophys. Acta.* **801**, 215-219.

166. Perly B, Smith ICP, Hughes L, Burton GW and Ingold KU. (1985) Estimation of the location of natural α-tocopherol in lipid bilayers by C-NMR spectroscopy. *Biochim. Biophys. Acta.* **819**, 131-135.

167. Doba T, Burton GW and Ingold KU. (1985) Antioxidant and co-antioxidant activity of vitamin C. The effect of vitamin C, either alone or in the presence of vitamin E or a water-soluble vitamin E analogue, upon the peroxidation of aqueous multilamellar phospholipid liposomes. *Biochim Biophys Acta.* **835**, 298-303.

168. Leung H-W, Vang MJ and Mavis RD. (1981) The cooperative interaction between vitamin E and vitamin C in suppression of peroxidation of membrane phospholipids. *Biochim.Biophys. Acta.* **664**, 266-272.

169. Wefers H and Sies H. (1988) The protection by ascorbate and glutathione against lipid peroxidation is dependent. *Eur. J. Biochem.* **174**, 353-357.

170. Niki E, Saito T, Kawakami A and Kamiya Y. (1984) Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. *J. Biol. Chem.* **259**, 4177-4182.

171. Ingold KU, Bowry VW, Stocker R and Walling C. (1993) Autoxidation of lipids and antioxidation by α-tocopherol and ubiquinol in homogenous solution and in aqueous dispersions of lipids: Unrecognized consequences of lipid particle size as exemplified by oxidation of human low density lipoprotein. *Proc.Natl. Acad. Sci.* **90**, 45-49.

172. Kagan VE and Packer L. (1994) Light-Induced generation of vitamin E radicals: Assesing vitamin E regeneration.In: Methods in Enzymology. (Ed: Packer L) Academic Press, New York and London. **234**, p316-320.

173. Kagan VE, Serbinova EA, Forte T, Scita G and Packer L. (1992) Recycling of vitamin E in human low density lipoproteins. *J.Lipid Res.* **33**, 385-397.

174. Buettner GR. (1993) The pecking order of free radicals and antioxidants: Lipid peroxidation, α-tocopherol, and ascorbate. *Arch. Biochem. Biophys.* **300**, 535-543.

175. Singer SJ and Nicolson GL. (1972) The fluid mosaic model of the structure of cell membranes. *SCI.* **175**,720-732.

176. Mathews CK and van Holde KE. (1990) Lipids, membranes, and cellular transport. In: Biochemistry. (Eds: Bowen D and Weisberg S) The Benjamin/Cummings Publishing Company, Redwood City, CA. p298-336.

177. Eze MO. (1991) Phase transitions in phospholipid bilayers: Lateral phase separations play vital roles in biomembranes. *Biochem. Educ.*.**19**, 204-208.

178. Neelands PJ and Clandinin MT. (1983) Diet fat influences liver plasma-membrane lipid composition and glucagon-stimulated adenylate cyclase activity. *Biochem. J.* **212**, 573-583.

179. Farías RN, Roberto B, Morero RD, Siñeriz F and Trucco RE. (1975) Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. *Biochim. Biophys. Acta.***415**, 231-251.

180. Clandinin MT, Cheema S, Field CJ, Carg ML, Venkatraman J and Clandinin TR. (1991) Dietary fat: exogenous determination of membrane structure and cell function. *FASEB J.* **5**, 2761-2769.

181. Sandermann H.Jr. (1978) Regulation of membrane enzymes by lipids. *Biochim. Biophys. Acta.* **515**, 209-237.

182. Stubbs CD and Smith AD. (1984) The modification of mammalian membrane polyunsaturated fatty acid composition in relation to membrane fluidity and function. *Biochim. Biophys. Acta.* **779**, 89-137.

183. Ahmad SN, Alam SQ and Alam BS. (1990) Fatty acid incorporation into membranes of dispersed rat submandibular salivary gland cells and their effect on adenylate cyclase activity. *Arch. Oral Biol.* **35**, 879-883.

184. Murphy MG.(1990) Dietary fatty acids and membrane protein function. *J. Nutr. Biochem.* **1**, 68-76.

185. Yeagle PL. (1989) Lipid regulation of cell membrane structure and function. *FASEB J.* **3**, 1833-1842.

186. Alam SQ and Alam BS. (1986) Effect of essential fatty acid deficiency on acyl group composition, membrane fluidity and adenylate cyclase activity in plasma membranes of rat submandibular salivary glands. *J. Nutr.* **116**, 1620-1630.

187. Spector AA, Mathur SN, Kaduce TL and Hyman BT. (1981) Lipid nutrition and metabolism of cultured mammalian cells. *Prog. Lipid Res.* **19**, 155-186.

188. Jeffcoat R. (1979) The biosynthesis of unsaturated fatty acids and its control in mammalian liver. *Essays Biochem.* **15**, 1-36.

189. Crawford MA. (1983) Background to essential fatty acids and their prostanoid derivatives. *Br. Med. Bull.* **89**, 210-213.

190. Wright S. (1991) Essential fatty acids and the skin. *Brit. J. Dermatol.* **125**, 503-515.

191. Hassam AG, Rivers JPW and Crawford HA. (1977) Metabolism of gamma-linoleic acid in essential fatty acid-deficient rats. *J. Nutr.* **107**, 519-524.

192. Bailey JM and Dunbar LM. (1973) Essential fatty acid requirements of cells in tissue culture: A review. *Exp. Molec. Pathol.* **18**, 142-161.

193. Robinson KM and Botha JH. (1985) Effects of gamma-linoleic acid, dihomo-gamma-linoleic acid and ethanol on cultured human mammary carcinoma cells. *Prostaglandins Leukotrienes Med.* **20**, 209-221.

194. van Dorp DA. (1975) Essential fatty acid metabolism. *Proc. Nutr. Soc.* **34**, 279-286.

195. Huang YS, Horrobin DF, Manku MS and Mitchell J. (1985) Effect of dietary α- and γ-linoleic acid on tissue fatty acids in guinea pigs. *Proc. Soc. Exp. Biol. Med.* **178**, 46-49.

196. Dupont J. (1987) Essential fatty acids and prostaglandins. *Prev. Med.* **16**, 485-492.

197. Lee AG, East JM and Froud RJ. (1986) Are essential fatty acids essential for membrane function. *Prog. Lipid Res.* **25**, 41-46.

198. Houslay MD. (1985) Regulation of adenylate cyclase (EC 4.6.1.1) activity by its lipid environment. *Proc. Nutr. Soc.* **44**, 157-165.

199. Galeotti T, Borrello S, Palombini G, Masotti L, Ferrari MB, Cavatorta P, Arcioni A, Stremmenos C and Zannoni C. (1984) Lipid peroxidation and fluidity of plasma membranes from rat liver and Morris Hepatoma 3924A. *FEBS Lett.* **169**, 169-173.

200. Spector AA and Burns CP. (1987) Biological and therapeutic potential of membrane lipid modification in tumors. *Cancer Res.* **47**, 4529-4537.

201. Wang M-Y and Liehr JG. (1995) Induction by oestrogens of lipid peroxidation and lipid peroxide-derived malonaldehyde-DNA adducts in male Syrian hamsters: role of lipid peroxidation in estrogen-induced kidney carcinogenesis. *Carcinogenesis.* **16**, 1941-1945.

202. Dobretsov GE, Borschevskaya TA, Petrov VA and Vladimirov YA. (1977) The increase of phospholipid bilayer after lipid peroxidation. *FEBS Lett.* **84**, 125-128.

203. Curtis MT, Gilfor D and Farber JL. (1984) Lipid peroxidation increases the molecular order of microsomal membranes. *Arch. Biochem. Biophys.***235**, 644-649.

204. Sawada M, Sester U and Carlson JC. (1992) Superoxide radical formation and associated biochemical alterations in the plasma membrane of brain, heart, and liver during the lifetime of the rat. *J. Cell. Biochem.* **48**, 296-304.

205. Coman DR and Anderson TF. (1955) A structural difference between the surfaces of normal and of carcinomatous epidermal cells. *Cancer Res.* **15**, 541-544.

206. Buechter DD. (1988) Free radicals and oxygen toxicity. *Pharm. Res.* **5**, 253-260.

207. Fridovich I. (1986) Biological effects of the superoxide radical. *Arch. Biochem. Biophys.* **247**, 1-11.

208. Alexander-North LS, North JA, Kiminoyo KP, Buettner GR and Spector AA. (1994) Polyunsaturated fatty acids increase radical formation induced by oxidant stress in endothelial cells. *J. Lipid Res.***35**, 1773-1785.

209. Dean RT, Gieseg S and Davies MJ. (1993) Reactive species and their accumulation on radical-damaged proteins. *TIBS.* **18**, 437-441.

210. Horton AA and Fairhurst S. (1987) Lipid peroxidation and mechanisms of toxicity. *Crit. Rev. Toxicol.* **18**, 27-79.

211. Porter NA, Caldwell SE and Mills KA. (1995) Mechanisms of free radical oxidation of unsaturated fatty acids. *Lipids.***30**, 277-290.

212. Halliwell B. (1995) Oxidation of low-density lipoproteins: questions of initiation, propagation, and the effect of antioxidants. *Am. J.Clin.Nutr.***61**, 670S-677S.

213. Ursini F, Maiorino M and Gregolin C. (1985) The selenoenzyme phospholipid hydroperoxide glutathione peroxidase. *Biochim. Biophys. Acta.* **839**, 62-70.

214. Goldring CEP, Rice-Evans CA, Burdon RH, Rao R, Haq I and Diplock AT. (1993) α-Tocopherol uptake and its influence on cell proliferation and lipid peroxidation in transformed and nontransformed Baby Hamster Kidney Cells. *Arch. Biochem. Biophys.* **303**, 429-435.

215. Salim AS. (1992) Removing oxygen-derived free radicals delays hepatic metastases and prolongs survival in colonic cancer. *Oncol.* **49**, 58-62.

216. Das UN, Begin ME, Huang YS and Horribin DF. (1987) Polyunsaturated fatty acids augment free radical generation in tumor cells in vitro. *Biochem. Biophys. Res. Commun.* **145**, 15-24.

217. Cheeseman KH, Emery S, Maddix SP, Slater TF, Burton GW and Ingold KU. (1988) Studies on lipid peroxidation in normal and tumor tissues. *Biochem. J.* **250**, 247-252.

218. Slater TF. (1991) Free radical studies in relation to cancer and liver regeneration. *South Afri. J. Sci.* **87**. 588-590.

219. Hendrickse CW, Kelly RW, Radley S, Donovan IA, Keighley MRB and Neoptolemos JP. (1994) Lipid peroxidation and prostaglandins in colorectal cancer. *Br. J. Surg.* **81**, 1219-1223.

220. Karmali RA. (1980) Review: Prostaglandins and cancer. *Prostaglandins Med.* **5**, 11-28.

221. Ferreira SH and Vane JR. (1967) Prostaglandins: Their disappearance from and release into the circulation. *Nature.* **216**, 868-873.

222. Hall AK and Behrman HR. (1982) Prostaglandins: biosynthesis, metabolism, and mechanism of cellular action. In: Prostaglandins. (Ed: Lee JB) Elsevier Science Publishers, New York. p1-37.

223. Pike JE. (1971) Prostaglandins. *Sci. Amer.* **225**, 84-92.

224. Hansen HS. (1983) Dietary essential fatty acids and *in vivo* prostaglandin production in mammals. *World Rev. Nutr. Diet.* **42**, 102-134..

225. Turini ME, Basu TK and Clandinin MT. (1990) Prostaglandins- Diet- Cancer: A Review. Nutr. Res. 10, 819-827.

226. Levine L. (1981) Arachidonic acid transformation and tumor production. *Adv. Cancer Res.* **35**, 49-79.

227. Hoffmann P and Mest H.-J. (1987) What about the effects of dietary lipids on endogenous prostanoid synthesis? A state-of the-art review. *Biomed. Biochim. Acta.* **7**, 639-650.

228. Lands WEM. (1991) Biosynthesis of prostaglandins. *Annu. Rev. Nutr.* **11**, 41-60.

229. Smith WL and Marnett LJ. (1991) Prostaglandin endoperoxide synthase: structure and catalysis. *Biochim. Biophys. Acta.* **1083**, 1-17.

230. van Dorp DA. (1967) Aspects of the biosynthesis of prostaglandins. *Prog. Biochem. Pharmacol.* **3**, 71-82.

231. Oliw E, Granström E and Änggård E. (1983) The prostaglandins and essential fatty acids. In: Prostagladins and related substances. (Eds: Neuberger A and van Deenen LLM) Elsevier Science Publishers, New York. **5**, 1-44.

232. Needleman P, Turk J, Jakschik BA, Morrison AR and Lefkowitz JB. (1986) Arachidonic acid metabolism. *Ann. Rev. Biochem.* **55**, 69-102.

233. Otto JC, DeWitt DL, Smith WL. (1993) N-glycosylation of prostaglandin endoperoxide synthases-1 and -2 and their orientations in the endoplasmic reticulum. *J. Biol. Chem.* **268**. 18234-18242.

234. Egan RW, Gale PH, VandenHeuvel WJA, Baptista EM and Kuehl FA. Jr. (1980) Mechanisms of oxygen transfer by prostaglandin hydroperoxidase. *J. Biol. Chem.* **255**, 323-326.

235. Ohki S, Ogino N, Yamamoto S and Hayaishi O. (1979) Prostaglandin hydroperoxidase, an integral part of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* **254**, 829-836.

236. Reiger MK, DeWitt DL, Schindler MS and Smith WL. (1993) Subcellular localization of prostaglandin endoperoxide synthase-2 in murine 3T3 cells. *Arch. Biochem. Biophys.* **301**, 439-444.

237. Yamamoto S. (1983) Enzymes in the arachidonic acid cascade. In: Prostaglandins and related substances. (Eds: Neuberger A and van Deenem LLM) Elsevier Science Publishers, New York. p171-201.

238. DeWitt DL. (1991) Prostaglandin endoperoxide synthase: regulation of enzyme expression. *Biochim Biophys. Acta.* **1083**, 121-134.

239. Smith WL. (1992) Prostanoid biosynthesis and mechanisms of action. *Am. J. Physiol.* **263**, F181-F191.

240. Egan RW, Gale PH, Baptista EM, Kennicott KL, VandenHeuvel WJA, Walker RW, Fagerness PE and Kuehl FA. (1981) Oxidation reactions by prostaglandin cyclooxygenase-hydroperoxidase. *J. Biol. Chem.* **256**, 7352-7361.

241. Egan RW, Gale PH and Kuehl FA. (1979) Reduction of hydroperoxides in the prostaglandin biosynthetic pathway by a microsomal peroxidase. *J. Biol. Chem.* **254**, 3295-3302.

242. Lands WEM and Haneĺ AM. (1983) Inhibitors and activators of prostaglandin biosynthesis. In: Prostaglandins and related substances. (Ed: Neuberger A and van Deenem LLM) Elsevier Science Publishers, New York. **5**, 203-233.

243. Kulmacz RJ. (1989) Topography of prostaglandin H synthase. *J. Biol. Chem.* **264**, 14136-14144.

244. Rollins TE and Smith WL. (1980) Subcellular localization of prostaglandin-forming cyclooxygenase in swiss mouse 3T3 fibroblasts by electron microscope immunocytochemistry. *J. Biol. Chem.* **255**, 4872-4875.

245. Roth GJ, Siok CJ and Ozols J. (1980) Structural characteristics of prostaglandin synthetase from sheep vesicular gland. *J. Biol. Chem.* **255**, 1301-1304.

246. Merlie JP, Fagan D, Mudd J and Needleman P. (1988) Isolation and characterization of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (cyclooxygenase). *J. Biol. Chem.* **263**, 3550-3553.

247. Sigal E. (1991) The molecular biology of mammalian arachidonic acid metabolism. *Am. J. Physiol.* **260**, L13-L28.

248. DeWitt DL and Smith WL. (1988) Primary structure of prostaglandin G/H synthase from sheep vesicular gland determined from complementary DNA sequence. *Proc. Natl. Acad. Sci.* **85**, 1412-1416.

249. Bennet PR, Slater D, Sullivan M, Elder MG and Moore GE. (1993) Changes in amniotic arachidonic acid metabolism associated with increased cyclo-oxygenase gene expression. *Br. J. Obstet. Gynaecol.* **100**, 1037-1042.

250. Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai, K, Kimura S, Kato H, Kondo M and Hla T. (1995) Expression of cyclooxygenase-1 and -2 in human colorectal cancer. *Cancer Res.* **55**, 3785-3789.

251. Goppelt-Struebe M. (1995) Regulation of prostaglandin endoperoxide synthase (cyclooxygenase) isozyme expression. *Prostaglandins Leukotrienes EFAs.* **52**, 213-222.

252. Nanayama T, Hara S, Inoue H, Yokoyama C and Tanabe T. (1995) Regulation of two isozymes of prostaglandin endoperoxide synthase and thromboxane synthase in human monoblastoid cell line U937. *Prostagland.* **49**, 371-382.

253. Feng L, Xia Y, Garcia GE, Hwang D and Wilson CB. (1995) Involvement of reactive oxygen intermediate in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide. *J. Clin. Invest.* **95**, 1669-1675.

254. Pilbeam CC, Raisz LG, Voznesensky O, Alander CB. Delman BN and Kawaguchi H. (1995) Autoregulation of inducible prostaglandin G/H synthase in osteoblast cells by prostaglandins. *J. Bone Miner. Res.* **10**, 406-414.

255. Percival MD, Ouellet M, Vincent CJ, Yergey JA, Kennedy BP and O',Neill. (1994) Purification and characterization of recombinant human cyclooxygenase-2. *Arch. Biochem. Biophys.* **315**, 111-118.

256. Tordjman C, Coge F, Andre N, Rique H, Spedding M and Bonnet J. (1995) Characterization of cyclooxygenase 1 and 2 expression in mouse resident peritoneal macrophages *in vitro*; interaction of non steroidal anti-inflammatory drugs with COX2. *Biochim. Biophys. Acta.* **1256**, 249-256.

257. Marshall PJ, Kulmacz RJ and Lands WEM. (1987) Constraints on prostaglandin biosynthesis in tissues. *J. Biol. Chem.* **262**, 3510-3517.

258. Hemler ME and Lands WEM. (1980) Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. *J. Biol. Chem.* **255**, 6253-6261.

259. Hemler ME, Cook HW and Lands WEM. (1979) Prostaglandin biosynthesis can be triggered by lipid peroxides. *Arch. Biochem. Biophys.* **193**, 340-345.

260. Ogino N, Ohki S, Yamamoto S and Hayaishi O. (1978) Prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* **253**, 5061-5068.

261. Miyamoto T, Ogino N, Yamamoto S and Hayaishi O. (1976) Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. *J. Biol. Chem.* **251**, 2629-2636.

262. Markey CM, Alward A, Weller PE and Marnett LJ. (1987) Quantitative studies of hydroperoxide reduction by prostaglandin H synthase. *J. Biol. Chem.* **262**, 6266-6279.

263. Smith WJ, Eling TE, Kulmacz RJ, Marnett LJ and Tsai A-l. (1992) Tyrosyl radicals and their role in hydroperoxide-dependent activation and inactivation of prostaglandin endoperoxide synthase. *Biochem.* **31**, 3-7.

264. Kulmacz RJ, Pendleton RB and Lands WEM. (1994) Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. *J. Biol. Chem.* **269**, 5527-5536.

265. Samuelsson B. (1972) Biosynthesis of prostaglandins. *Fed. Proc.* **31**, 1442-450.

266. Hamberg M and Samuelsson B. (1967) On the mechanism of biosynthesis of prostaglandins E_1 and $F_{1\alpha}$. *J. Biol. Chem.* **242**, 5336-5343.

267. Samuelsson B. (1965) On the incorporation of oxygen in the conversion of 8,11,14-eicosatrienoic acid to prostaglandin E_1. *J. Am. Soc. Chem.* **87**, 3011-3013.

268. Kukreja RC, Kontos HA, Hess ML and Ellis EF. (1986) PGH synthase and lipoxygenase generate superoxide in the presence of NADH and NADPH. *Circ. Res.* **59**, 612-619.

269. Egan RW, Paxton J and Kuehl FA. Jr. (1976) Mechanism for irreversible self-deactivation of prostaglandin synthetase. *J. Biol. Chem.* **251**, 7329-7335.

270. Ramwell PW, Foegh M, Loeb R and Leovey EMK. (1980) Synthesis and metabolism of prostaglandins, prostacyclin, and thromboxanes: The arachidonic acid cascade. *Semin Perinatol.* **4**, 3-13.

271. Flower RJ, Cheung HS and Cushman DW. (1973) Quantitative determination of prostaglandins and malondialdehyde formed by the arachidonate oxygenase (prostaglandin synthetase) system of bovine seminal vesicle. *Prostagland.* **4**, 325-341.

272 Smith W and Lands WEM. (1972) Oxygenation of polyunsaturated fatty acids during prostaglandin biosynthesis by sheep vesicular gland. *Biochemistry.* **11**, 3276-3285.

273. Hamberg M and Israelsson U. (1970) Metabolism of prostaglandin E_2 in guinea pig liver. *J. Biol. Chem.* **245**, 5107-5114.

274. Hensby CN. (1975) Distribution studies on the reduction of prostaglandin E_2 to prostaglandin $F_{2\alpha}$ by tissue homogenates. *Biochim. Biophys. Acta.* **409**, 225-234.

275. Wong P-Y-K. (1981) Purification and partial characterization of prostaglandin D_2 11-keto reductase in rabbit liver. *Biochim Biophys. Acta.* **659**, 169-178.

276. Reingold DF, Kawasaki A and Needleman P. (1981) A novel prostaglandin 11-keto reductase found in rabbit liver. *Biochim. Biophys. Acta.* **659**, 179-188.

277. Powell WS. (1982) Formation of 6-oxoprostaglandin $F_{1\alpha}$, 6,15-dioxoprostaglandin $F_{1\alpha}$, and monohydroxyicosatetraenoic acids from arachidonic acid by fetal calf aorta and ductus arteriosus. *J. Biol. Chem.* **257**, 9457-9464.

278. Siegal MI, McConnell RT, Abrahams SL. Porter NA and Cuatrecasas P. (1979) Regulation of arachidonate metabolism via lipoxygenase and cyclo-oxygenase by 12-HPETE, the product of human platelet lipoxygenase. *Biochem. Biophys. Res. Commun.* **89**, 1273-1280.

279. Salmon JA, Smith DR, Flower RJ, Moncada S and Vane JR. (1978) Further studies on the enzymatic conversion of prostaglandin endoperoxide into prostacyclin by porcine aorta microsomes. *Biochim. Biophys. Acta.* **523**, 250-262.

280. Polgar P and Taylor L. (1980) Stimulation of prostaglandin synthesis by ascorbic acid via hydrogen peroxide formation. *Prostagland.* **19**, 693-700.

281. Srivastava KC. (1985) Ascorbic acid enhances the formation of prostaglandin E_1 in washed human platelets and prostacyclin in rat aortic rings. *Prostaglandins Leukotrienes Med.* **18**, 227-233.

282. Frazier LW and Yorio T. (1992) Eicosanoids: Their function in renal epithelia ion transport. *Proc. Soc. Exp. Biol. Med.* **210**, 229-243.

283. Polgar P, Douglas WHJ, Terracio L and Taylor L. (1980) Release of arachidonic acid and its conversion to prostaglandins in various diploid cell types in culture. In: Advances in Prostaglandins and Thromboxanes Research. (Eds:Samuelsson B,Ramwell PW and Paoletti R) Raven Press, New York. **6**, 225-229.

284. Kuehl FA. (1974) Prostaglandins, cyclic nucleotides and cell function. *Prostagland.* **5**,325-340.

285. Nakamura A, Chiba T, Yamatani T, Yamaguchi A, Inui T, Morishita T, Kadowaki S, and Fujita T. (1989) Prostaglandin E_2 and $F_{2\alpha}$ inhibit growth of human gastric carcinoma cell line KATO III with simultaneous stimulation of cyclic AMP production. *Life Sci.* **44**, 75-80.

286. Cutler LS, Christian CP and Feinstein MB. (1985) Cytochemical localization of adenylate cyclase in the dense tubule system of human blood platelets stimulated by forskolin, prostacyclin and prostaglandin D_2. *Biochim. Biophys. Acta.* **845**, 4403-410.

287. Thierauch K-H, Dinter H and Stock G. (1994) Prostaglandins and their receptors: II. Receptor structure and signal transduction. *J. Hypertens.* **12**, 1-5.

288. Bennet A. (1984) Prostaglandins and tumor metastasis. In: Icosanoids and Cancer. (Ed: Thaler-Dao H) Raven Press, New York. 223-229.

289. Hubbard WC, Alley MC, NcLemore TL and Boyd MR. (1988) Profiles of prostaglandin biosynthesis in sixteen established cell lines derived from human lung, colon, prostate, and ovarian tumors. *Cancer Res.* **48**, 4770-4775.

290. Levine L, Goldstein SM, Snoek GT and Rigas A. (1984) Arachidonic acid metabolism by cells in culture: Effects of tumor promoters. In: Icosanoids and Cancer. (Ed: Thaler-Dao H) Raven Press, New York. 115-125.

291. Taylor L and Polgar P. (1981) Cell growth and the regulation of prostaglandin synthesis. *Prostagland.* **22**, 723-728.

292. Narumiya S, Ohno K, Fujiwara M and Fukushima M. (1986) Site and mechanism of growth inhibition of growth by prostaglandins. II. Temperature-dependent transfer of a cyclopentenone prostaglandin to nuclei. *J. Pharmacol. Exp. Ther.* **239**, 506-511.

293. Nishizawa EE, Miller WL, Gorman RR, Bundy GL, Svensson J and Hamberg M. (1975) PGD_2 as a potential antithrombotic agent. *Prostagland.* **9**, 109-121

294. Whittle BJR, Moncada S and Vane JR. (1978) Comparison of the effects of prostacyclin PGI_2, $PGDE_1$ and D_2 on platelet aggregation in different species. *Prostagland.* **16**, 373-388.

295. Shimizu T, Mizuno N, Amano T and Hayaishi O. (1979) Prostaglandin D_2, a neuromodulator. *Proc. Natl. Acad. Sci.* **76**, 6231-6234.

296. Sakai T and Yamaguchi N. (1984) Prostaglandin D_2 inhibits the proliferation of human malignant tumor cells. *Prostagland.* **27**, 17-24.

297. Ticconi C, Zicari A, Pontieri G, Salerno A, Mauri A, Piccione E and Pasetto N. (1995) Release of arachidonic acid metabolites by human fetal membranes: Interrelationship between leukotriene B4 and prostaglandin E2. *Prostagland.* **49**, 197-204.

298. Moncada S, Gryglewski R, Bunting S and Vane JR. (1976) An enzyme isolated from arteries transforms prostaglandin endoperoxides to an unstable substance that inhibits platelet aggregation. *Nature.* **263**, 663-665.

299. Christ-Hazelhof E and Nugteren DH. (1981) Prostacyclin is not a circulating hormone. *Prostagland.* **22**, 739-746.

300. Fulton AM. (1988) The role of eicosanoids in tumor metastasis. *Prostaglandins Leukotrienes EFAs.* **34**, 229-237.

301. Stamford IF, Carroll MA, Civiert A, Hensby CN and Bennet A. (1983) Identification of arachidonate metabolites in normal, benign and malignant human mammary tissues. *J. Pharm. Pharmacol.* **35**, 48-49.

302. Tashjian AH, Voelkel EF and Levine L. (1982) Prostaglandins, tumor cells and bone metabolism. *Prostaglandins and Cancer*, 513-523.

303. Bockman R, Bellin A and Hickok N. (1984) Disordered prostaglandin production and cell differentiation/proliferation in cancer. In: Icosanoids and Cancer. (Ed: Thaler-Dao H) Raven Press, New York. 169-183.

304. Young MR and Hoover CS. (1986) Inhibition of spleen cell cytotoxic capacity toward tumor by elevated prostaglandin E_2 levels in mice bearing Lewis Lung carcinoma. *J.N.C.I.* **77**, 425-429.

305. de Asua LJ and Otto AM. (1983) The stimulation of the initiation of DNA synthesis and cell division in Swiss Mouse 3T3 cells by prostaglandin $F_{2\alpha}$ requires specific functional groups in the molecule. *J. Biol. Chem.* **258**, 8774-8780.

306. Kibbey WE, Bronn DG and Minton JP. (1979) Prostaglandin synthetase and prostaglandin E_2 levels in human breast carcinoma. *Prostaglandins Med.* **2**, 133-139.

307. Karmali RA, Horrobin DF, Menezes J and Patel P. (1979) The relationship between concentrations of prostaglandin A1, E1, E2 and E2α and rates of cell proliferation. *Pharmacol. Res. Commun.* **11**, 69-75.

308 Fitzpatrick FA and Stringfellow DA. (1979) Prostaglandin D_2 formation by malignant melanoma cells correlates inversely with cellular metastatic potential. *Proc. Natl. Acad. Sci.* **76**, 1765-1769.

309. Stringfellow DA and Fitzpatrick FA. (1979) Prostaglandin D_2 controls pulmonary metastasis of malignant melanoma cells. *Nature*. **282**, 76-78.

310. Fukushima M, Kato T, Ueda R, Ota K, Narumiya S and Hayaishi O. (1982) Prostaglandin D_2, a potential antineoplastic agent. *Biochem. Biophys. Res. Commun.* **105**, 956-964.

311. Simmet T and Jaffe BM. (1983) Inhibition of B-16 melanoma growth *in vitro* by prostaglandin D_2. *Prostagland.* **25**, 47-54.

312. Bregman MD and Meyskens FL. Jr. (1983) *In vitro* modulation of human and murine melanoma growth by prostanoid analogues. *Prostagland.* **26**, 449-456.

313. Keyaki A, Handa H, Yamashita J, Tokuriki Y, Otsuka S--I, Yamasaki T and Gi H. (1984) Growth-inhibitory effect of prostaglandin D_2 on mouse glioma cells. *J. Neurosurg.* **61**, 912-917.

314. Bhuyan BK, Adams EG, Badiner GJ, Li LH and Barden K. (1986) Cell cycle effects of prostaglandins A_1, A_2, and D_2 in human and murine melanoma cells in culture. *Cancer Res.* **46**, 1688-1693.

315. Thomas DR, Philpott GW and Jaffe BM. (1974) The relationship between concentration of prostaglandin E and rates of cell replication. *Exp. Cell Res.* **84**, 40-46.

316. Santorio MG, Benedetto A and Jaffe BM. (1979) Effect of endogenous and exogenous prostaglandin E on Friend Erythroleukaemia cell growth and differentiation. *Br. J. Cancer.* **39**, 250-267.

317. Stoll KE, Ottino P and Duncan JR. (1994) Interrelationship of ascorbate, arachidonic acid and prostaglandin E_2 in B16 melanoma cells. *Prostaglandins Leukotrienes EFAs.* **50**, 123-131.

318. Santoro MG and Jaffe BM. (1979) Inhibition of Friend Erythroleukaemia-cell tumours *in vivo* by a synthetic analogue of prostaglandin E_2. *Br. J. Cancer.* **39**, 408-413.

319. Foecking MK, Kibbey WE, Abou-Issa H, Matthews RH and Minton JP. (1982) Hormone dependence of 7,12-dimethylbenz[a]anthracene-induced mammary tumor growth: correlation with prostaglandin E_2 content. J.N.C.I. 69, 443-446.

320. Jaffe BM. (1974) Prostaglandins and cancer: An update. *Prostagland.* **6**, 453-461.

321. de Asua LJ, Macphee C, Drummond AH and Otto AM. (1984) Prostaglandins and the proliferation of swiss mouse 3T3 cells: A model system for studying growth control. In: Icosanoids and Cancer. (Eds: Thaler-Dao H) Raven Press, New York. 139-153.

322. Cyran JA, Lysz TW and Lea. MA. (1990) Influence of inhibitors of eicosanoid metabolism on proliferation of rat hepatoma cells and on tumor-host interaction. *Prostaglandins. Leukotrienes EFAs.* **39**, 311-319.

323. Kawamura M and Koshihara Y. (1983) Prostaglandin D_2 strongly inhibits growth of murine mastocytoma cells. *Prostaglandins Leukotrienes. Med.* **12**, 85-93.

324. Honn KV and Meyer J. (1981) Thromboxanes and prostacyclin: Positive and negative modulators of tumor growth. *Biochem. Biophys. Res. Commun.* **102**, 1122-1129.

325. Honn KV, Cicone B and Skoff A. (1981) Prostacyclin: A potent antimetastatic agent. SCI. **212**, 1270-1272.

326. Pozzan T, Rizzuto R, Volpe P and Meldolesi J. (1994) Molecular and cellular physiology of intracellular calcium stores. *Physiol. Rev.* **74**, 595-599.

327. Lipkin M and Newmark H. (1995) Calcium and the prevention of colon cancer. *J. Cell. Biochem.* **22**, 65-73.

328. Whitfield JF, Boynton AL, MacManus JP, Rixon RH, Sikorska M, Tsang B and Walker PR. (1980) The roles of calcium and cyclic AMP in cell proliferation. *Ann. New York Acad. Sci.* **339**, 216-240.

329. Durham ACH and Walton JM. (1982) Calcium ions and the control of proliferation in normal and cancer cells. *BioSci. Rep.* **2**, 15-30.

330. Dawson AP. (1990) Regulation of intracellular Ca^{2+}. *Essays Biochem.* **25**, 1-37.

331. Ross EM and Gilman AG. (1980) Biochemical properties of hormone-sensitive adenylate cyclase. *Ann. Rev. Biochem.* **49**, 533-564.

332. Horrobin DF. (1980) The reversibility of cancer: The relevance of cyclic AMP, calcium, essential fatty acids and prostaglandin E1. *Med. Hypotheses.* **6**, 469-486.

333. Yoneda T, Kitamura M, Ogawa T, Aya, S-I and Sakuda M. (1985) Control of VX_2 Carcinoma cell growth in culture by calcium, calmodulin and prostaglandins. *Cancer Res.* **45**, 398-405.

334. Hickie RA and Kalant H. (1967) Calcium and magnesium content of rat liver and Morris Hepatoma 5123 tc. *Cancer Res.* **27**, 1053-1057.

335. Batra S, Popper LD and Hartley-Asp B. (1991) Effect of calcium and calcium antagonists on ^{45}Ca influx and cellular growth of human prostatic tumor cells. *The Prostate.* **19**, 299-311.

336. Sasaki Y and Hidaka H. (11982) Calmodulin and cell proliferation. *Biochem Biophys. Res. Commun.* **104**, 451-456.

337. Whitfield JF, Bird RP, Chakaravarthy BR, Isaacs RJ and Morley P. (1995) Calcium-cell cycle regulator, differentiator, killer, chemopreventor, and maybe, tumor promoter. *J. Cell. Biochem.* **22**, 74-91.

338. Revillion F, Vandewalle B, Lassalle B and Lefebre J. (1992) cAMP effect on extracellular matrix synthesis in human breast cancer cells. *Cell Prolif.* **25**, 633-642.

339. Peplow PV. (1992) Modification to dietary intake of sodium, potassium, calcium, magnesium and trace elements can influence arachidonic acid metabolism and eicosanoid production. *Prostaglandins Leukotrienes EFAs*. **45**, 1-19.

340. Murthy M, Rao GH, Robinson P and Reddy S. (1995) Influx of extracellular calcium and agonist-coupling appear essential for the activation of thromboxane A_2-dependent phopholipase A_2 in human platelets. *Prostaglandins Leukotrienes EFAs*. **53**, 31-39.

341. Gonich JH, Bonventre JV and Nemenoff RA. (1988) Identification and characterization of a hormonally regulated form of phospholipase A_2 in rat renal mesengial cells. *J. Biol. Chem.* **263**, 16645-16651.

342 Axelrod J. (1990) Receptor-mediated activation of phospholipase A_2 and arachidonic acid release in signal transduction. *Biochem. Soc. Trans.* **18**, 503-507.

343. Xing M and Mattera R. (1992) Phosphorylation-dependent regulation of phospholipase A_2 by G-proteins and Ca^{2+} in HL60 granulocytes. *J. Biol. Chem.* **267**. 25966-25975.

344. Spiro TG. (1983) Calcium in biology. John Wiley and Sons Inc., New York. 6, p3-7 and p68-75

345. MacNeil S, Walker SW, Senior HJ, Pollock A, Brown BL, Bleehen SS, Munro DS and Tomlinson S. (1984) Calmodulin activation of adenylate cyclase in the mouse B16 melanoma. *Biochem. J.* **224**, 453-460.

346. Birnbaumer L, Abramowitz J and Brown AM. (1990) Receptor-effector coupling by G proteins. *Biochim. Biophys. Acta*. **1031**, 163-224.

347. Mayer RJ and Marshall LA. (1993) New insights on mammalian phopholipase A_2(s); comparison of arachidonyl-selectiive and -nonselective enzymes. *FASEB J.* **7**, 339-348.

348. Hara S, Kudo I, Komati T, Takahashi K, Nakatani Y, Natori Y, Ohshima M and Inoue K. (1995) Detection and purification of two 14 kDa phospholipase A_2 isoforms in rat kidney: their role in eicosanoid synthesis. *Biochim Biophys. Acta*. **1257**, 11-17.

349. Ross BM, Kim DK, Bonventre JV and Kish SJ. (1995) Characterization of a novel phospholipase A_2 activity in human brain. *J. Neurochem.* **64**, 2213-2221.

350. Pfeilschifter J, Schalkwijk C, Briner VA and van den Bosch H. (1993) Cytokine-stimulated secretion of group II phospholipase A_2 by rat mesangial cells. *J. Clin. Invest.* **92**, 2516-2523.

351. Ohara O, Ishizaki J and Arita H. (1995) Structure and function of phospholipase A_2 receptor. *Prog. Lipid Res.* **34**, 117-138.

352. Angel J, Berenbaum F, Le Denmat C, Nevalainen T, Masliah J and Fournier C. (1994) Interleukin-1-induced prostaglandin E_2 biosynthesis in human synovial cells involves the activation of phospholipase A_2 and cyclooxygenase-2. *Eur. J. Biochem.* **226**, 125-131.

353. Rosenthal MD, Rzigalinski BA, Blackmore PPF and Franson RC. (1995) Cellular regulation of arachidonate mobilization and metabolism. *Prostaglandin Leukotrienes EFAs*. **52**, 93-98.

354. Guthridge CJ, Stampfer MR, Clark MA and Steiner MR. (1994) Phospolipases A_2 in *ras*-transformed and immortalized human mammary epithelial cells. *Cancer Lett.* **86**, 11-21.

355. Steiner MR, Bomalaski JS and Clark MA. (1993) Responses of purified phospholipases A_2 to phospholipase A_2 activating protein (PLAP) and melittin. *Biochim. Biophys. Acta.* **1166**, 124-130.

356. de Carvalho MS, McCormack FX and Leslie CC. (1993) The 85-kDa, arachadonic acid-specific phospholipase A_2 is expressed as an activated phosphoprotein in Sf9 cells. *Arch. Biochem. Biophys.* **306**, 534-540.

357. Gelb MH, Jain MK and Berg OG. (1994) Inhibition of phospholipase A_2. *FASEB J.* **8**, 916-924.

358. Wijkander J and Sundler R. (1992) Macrophage arachidonate-mobilizing phospholipase A_2: role of Ca^{2+} for membrane binding but not for catalytic activity. *Biochem. Biophys. Res. Commun.* **184**, 118-124.

359. Whatley RE, Satoh K, Zimmeran GA, McIntyre TM and Prescott SM. (1994) Proliferation-dependent changes in release of arachidonic acid from endothelial cells. *J. Clin. Invest.* **94**, 1889-1900.

360. van den Bosch H. (1980) Intracellular phospholipase A. *Biochim. Biophys. Acta.* **604**, 191-246.

361. Leslie CC. (1991) Kinetic properties of a high molecular mass arachidonyl-hydrolyzing phospholipase A_2 that exhibits lysophospholipase activity. *J. Biol. Chem.* **266**, 11366-11371.

362. Saris N-EL, Steinbrückner B, Thurén T, Lalla MLT, van den Bosch H, Kinnunen PKJ and Hoffmann GE. (1992) Characterization of cellular and elevated serum phospholipase A_2 activities with a comparison of two methods. *Scand. J. Clin. Lab. Invest.* **52**, 19-25.

363. Ransac S, Aarsman AJ, van den Bosch H, Gancet C, de Haas GH and Verger R. (1992) Rat platelet phospholipase A_2. *Eur. J. Biochem.* **204**, 793-797.

364. Kannagi R and Koizumi K. (1979) Effect of different physical states of phospholipid substrates on partially purified platelet phospholipase A_2 activity. *Biochim. Biophys. Acta.* **556**, 423-433.

365. de Winter JM, Korpancova J and van den Bosch H. (1984) Regulatory aspects of rat liver mitochondrial phopholipase A_2: effects of calcium ions and calmodulin. *Arch. Biochem. Biophys.* **234**, 243-252.

366. Burgoyne RD and Morgan A. (1990) The control of free arachidonic acid levels. *TIBS.* **15**, 365-366.

367. Axelrod J, Burch RM and Jelsema CJ. (1988) Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: arachidonic acid and its metabolites as second messengers. *TINS.* **11**, 117-123.

368. Lister MD, Deems RA, Watanbe Y, Ulevitch RJ and Dennis EA. (1988) Kinetic analysis of the Ca^{2+}-dependent, membrane-bound-macrophage phospholipase A_2 and the effects of arachidonic acid. *J. Biol. Chem.* **263**, 7506-7513.

369. Ballou LR and Cheung WY. (1983) Marked increase of human platelet phopholipase A_2 activity *in vitro* and demonstration of an endogenous inhibitor. *Proc. Natl. Acad. Sci.* **80**, 5203-5207.

370. Ballou LR and Cheung WY. (1985) Inhibition of human platelet phospholipase A_2 activity by unsaturated fatty acids. *Proc. Natl. Acad. Sci.* **82**, 371-375.

371. Lin L-L, Lin A and DeWitt DL. (1992) Interleukin-1α induces the accumulation of cytosolic phospholipase A_2 and the release of prostaglandin E_2 in human fibroblasts. *J.Biol. Chem.* **267**, 23451-23454.

372. Nakano T, Ohara O, Teraoka H and Arita H. (1990) Glucocorticoids suppress group II phospholipase A_2 production by blocking mRNA synthesis and post-transcriptional expression. *J. Biol. Chem.* **265**, 12745-12748.

373. Schlame M, Horvath I, Török Z, Horvath LI, Vigh L. (1990) Intramembraneous hydrogenation of mitochondrial lipids reduces the substrate availabilty, but not the enzyme activity of endogenous phospholipase A. The role of polyunsaturated phospholpid species. *Biochim. Biophys. Acta.* **1045**, 1-8.

374. Karli UO, Schäfer T and Burger MM. (1990) Fusion of neurotransmitter vesicles with target membrane is calcium independent in a cell-free system. *Proc. Natl. Acad. Sci.* **87**, 5912-5915.

375. Sevanian A, Miuakkassah-Kelly SF and Montestruque S. (1983) The influence of phospholipase A_2 and glutathione peroxidase on the elimination of membrane lipid peroxides. *Arch. Biochem. Biophys.* **223**, 441-452.

376. Flower RJ and Blackwell GJ. (1976) The importance of phospholipase-A_2 in prostaglandins biosynthesis. *Biochem. Pharmacol.* **25**, 285-291.

377. Buckley BJ, Barchowsky A, Dolor RJ and Whorton AR. (1991) Regulation of arachidonic acid release in vascular endothelium. *Biochem. J.* **280**, 281-287.

378. Ando M, Furui H, Suzuki K, Taki F and Takagi K. (1992) Direct activation of phospholipase A_2 by GTP-binding protein in human peripheral polymorphonuclear leukocytes. *Biochem. Biophys. Res. Commun.* **183**, 708-713.

379. Kramer RM, Roberts EF, Manetta J and Putnam JE. (1991) The Ca^{2+}-sensitive cytosolic phospholipase A_2 is a 100-kDa protein in Human Monoblast U937 cells. *J. Biol. Chem.* **266**, 5268-5272.

380. Brooks RC, McCarthy KD, Lapetina EG and Morell P. (1989) Receptor-stimulated phospholipase A_2 activation is coupled to influx of external calcium and not to mobilization of intracellular calcium in C62B glioma cells. *J. Biol. Chem.* **264**, 20147-20153.

381. Hendrickse CW, Radley S, Donovan IA, Keighly MRB and Neoptolemos JP. (1995) Activities of phospholipase A_2 and diacylglycerol lipase are increased in human colorectal cancer. *Br. J. Surg.* **82**, 475-478.

382. Nishizuka Y. (1984) The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature.* **308**, 693-697.

383. Schultz G and Jakobs KH. (1984) Adenylate cyclase. In: Methods in Enzmology. (Ed: Bergmeyer HU, Bergmeyer J and Graβl M) Verlag Chemie, Weinheim. **IV**, p369-378.

384. Weign P, Dutton J and Lurie KG. (1993) An enzymatic fluorometric assay for adenylate cyclase activity. *Analyt. Biochem.* **208**, 217-222.

385. Salomon Y. (1979) Adenylate cyclase assay. In: Advances in Cyclic Nucleotide Research. (Ed: Brooker G, Greengard P and Robisson GA) Raven Press, New York. **10**, 35-55.

386. Hunt NH and Martin TJ. (1980) Hormone receptors and cyclic nucleotides; significance for growth and function of tumors. *Mol. Aspects Med.*3, 59-118.

387. Levine MA, Greene A, Turner RT and Bell NH. (1984) Effects of human erythrocyte guanine nucleotide-binding regulatory protein on parathyroid hormone-responsive adenylate cyclase from canine renal cortex. *Endocrinol.* **115**, 1386-1391.

388. Steer ML and Wood A. (1979) Regulation of human platelet adenylate cyclase by epinephrine, prostaglandin E_1 and guanine nucleotides. *J. Biol Chem.***254**, 10791-10797.

389. Lefkowitz RJ, Stadel JM and Caron MG. (1983) Adenylate cyclase-coupled beta-adrenergic receptors: Structure and mechaanisms of activation and desensitization. *Ann.Rev. Biochem.* **522**, 159-186.

390. Houslay MD and Gordon LM. (1983) The activity of adenylate cyclase is regulated by the nature of its lipid enviroment. In: Current Topics in Membranes and Transport. Academic Press, New York. **188**, 179-231.

391. Witte K and Lemmer B. (1991) Rhythms in second messenger mechanisms. *Pharmac. Ther.* **51**, 231-237.

392. Stryer L. (1988) Hormone Action. In: Biochemistry. W.H. Freeman and Company, New York.

393. Engelhard VH, Esko JD, Storm DR and Glaser M. (1976) Modification of adenylate cyclase activity in LM cells by manipulation of the membrane phospholipid composition *in vivo*. *Proc. Natl. Acad. Sci. USA*. **73**, 4482-4486.

394. Emmelot P and Bos CJ. (1971) Adenyl cyclase in plasma membranes isolated from rat and mouse livers and hepatomas, and its hormone sensitivity. *Biochim. Biophys.Acta*. **249**, 285-292.

395. Tsai BS and Lefkowitz RJ. (1979) Multiple effects of guanine nucleotides on human platelet adenylate cyclase. *Biochim. Biophys. Acta*. **587**, 28-41.

396. Codina J, Hildebrandt JD, Sekura RD, Birnbaumer M, Byran J, Maanclark CR, Iyengar R and Birnbaumer L.(1984) N_s and N_i, the stimulatory and inhibtory regulatory components of adenyl cyclases. *J. Biol. Chem.* **259**, 5871-5886.

397. Anand-Srivastava M, Franks DJ, Cantin M and Genest J. (1982) Presence of "Ra" and "P"-site receptors for adenosine coupled to adenylate cyclase in cultured vascular smooth muscle cells. *Biochem. Biophys. Res. Commun.***108**, 213-219.

398. Anderson WB, Johnson GS and Pastan I. (1973) Transformation of chick-embryo fibroblasts by wild-type and temperature-sensitive Rous sarcoma virus alters adenylate cyclase activity. *Proc. Natl. Acad. Sci.* **70**, 1055-1059.

399. Pertseva M. (1991) The evolution of hormonal signalling systems. *Comp. Biochem. Physiol*. **100A**, 775-787.

400. Pfeuffer T. (1977) GTP-binding proteins in membranes and the control of adenylate cyclase activity. *J. Biol. Chem.* **252**, 7224-7234.

401. Fung BKK, Hurley JB and Stryer L. (1981) Flow of information in the light-triggered cyclic nucleotide cascade of vision. *Proc. Natl. Acad. Sci.* **78**, 152-156.

402. Manning DR and Gilmann AG. (1983) The regulatory components of adenylate cyclase and transducin. *J. Biol. Chem.* **258**, 7059-7063.

403. Hildebrandt JD, Sekura RD, Codina J, Iyengar R, Manclark CR and Birnbaumer L. (1983) Stimulation and inhibition of adenyl cyclases mediated by distinct regulatory proteins. *Nature*. **302**, 706-709.

404. Walton KG and Baldessarini RJ. (1976) Effects on Mn^{2+} and other divalent cations on adenylate cyclase activity in rat brain. *J. Neurochem.* **27**, 557-564.

405. Alam SQ, Alam BS and Ren Y-F. (1987) Adenylate cyclase activity, membrane fluidity and fatty acid composition of rat heart in essentiial fatty acid deficiency. *J. Mol. Cell. Cardiol.* **19**, 465-475.

406. Anand-Srivastava MB and Johnson RA. (1981) Role of phospholipids in coupling of adenosine and dopamine receptors to striatal adenylate cyclase. *J. Neurochem.* **36**, 1819-1828.

407. Murphy MG. (1985) Membrane fatty acids, lipid peroxidation and adenylate cyclase activity in cultured neural cells. *Biochem. Biophys. Res. Commun.* **132**, 757-763.

408. Baba A, Lee E, Ohta A, Tatsumo and Iwata H. (1981) Activation of adenylate cyclase of rat brain by lipid peroxidation. *J. Biol. Chem.* **256**, 3679-3684.

409. Canuto RA, Paradisi L, Muzio G, Maggiora M, Mengozzi G, Garramone A and Dianzani MU. (1995) Changes of adenylate cyclase activity in AH-130 Ascites Hepatoma of Yoshida induced by enrichment with fatty acids. *Biochem. Biophys. Res. Commun.* **213**, 853-860.

410. Keirns JJ, Kreiner PW, Brock WA, Freemann J and Bitensky MW. (1973) Prostaglandins and the adenyl cyclase of the cloudman melanoma. In: The role of Cyclic Nucleotides in Carcinogenesis. (Eds: Schultz J and Gratzner HG) Academic Press, New York. **6**, p181-198.

411. Torikai S and Kurokawa K. (1981) Distribution of prostaglandin E_2-sensitive adenylate cyclase along the rat nephron. *Prostagland.* **21**, 427-437.

412. Lefkowitz RJ, Mullikin D, Wood CL, Gore TB and Mukherjee C. (1977) Regulation of prostaglandin receptors by prostaglandins and guanine nucleotides in frog erythrocytes. *J. Biol.Chem.* **252**, 5295-5303.

413 Sinha AK and Colman RW. (1980) Persistence of increased platelet cyclic AMP induced by prostaglandin E_1 after removal of the hormone. *Proc. Natl. Acad. Sci.* **77**, 2946-2950.

414. Kahn NN and Sinha AK. (1988) Inhibition of protaglandin E_1-induced activation of adenylate cyclase in human blood platelet membrane. *Biochim. Biophys. Acta.* **972**,45-53.

415. Yamaguchi DT, Hahn TJ, Beeker TG, Kleeman CCR and Muallen S. (1988) Relationship of cAMP and calcium messenger systems in prostaglandin-stimulated UMR-106 cells. *J. Biol. Chem.* **263**, 10745-10753.

416. Nakamura A, Yamatanni T, Fujita T and Chiba T. (1991) Mechanism of inhibitory action of prostaglandins on the growth of human gastric carcinoma cell line KATO III. *Gastroent.* **101**, 910-918.

417. Wisner A, Bommelaer-Bayet M-C, Tiberghein C, Renard C-A, David F and Dray F. (1989) Hypothalamic prostaglandin E_2 receptors coupled to an adenyl cyclase. *Eur. J. Pharmacol.* **162**, 89-100.

418. Dominguez JH, Shuler F, Olszowy MW, Brown T and Puschett JB. (1988) Prostaglandin E_2 is an inhibitor of adenylate cyclase in rabbit proximal tubule. *Am. J. Physiol.* **254**,C304-C309.

419. Yamashita L and Sweat FW. (1976) The stimulation of rat liver adenylate cyclase by prostaglandin E_1 and E_2. *Biochem. Biophys. Res. Commun.* **70**. 438-444.

420. Negishi M, Ito S and Hayaishi O. (1989) Prostaglandin E receptors in bovine adrenal medulla are coupled to adenylate cyclase via G_i and to phosphoinositide metabolism in a pertussis toxin-insensitive manner. *J. Biol. Chem.* **264**, 3916-3923.

421. Toriyama K, Morita I and Murota S.-I. (1992) The existence of distinct classes of prostaglandin E_2 receptors mediating adenylate cyclase and phospholipase C pathways in osteoblast clone MC3T3-E1. *Prostaglandins Leukotrienes EFAs.* **46**, 15-200.

422. Lapetina EG, Schmitges CJ, ChandraboseK and Cuatrecasas P. (1978) Regulation of phospholipase activity in platelets. In: Advances in Prostaglandin and Thromboxane Research. (Ed: Galli C) Raven Press, New York. **3**, 127-135.

423. van Sande J, Cochaux P, Decoster C, Boeynaems JM and DuMont JE. (1982) Effects of prostaglandins F_α on dog thyroid cyclic AMP level and function. *Biochim. Biophys. Acta.* **716**, 53-60.

424. Cole OF, Elder MG and Sullivan MHF. (1995) Decidual adenylate cyclase and prostaglandin production *in vitro*. *Prostagland.* **49**, 167-174.

425. Cooper DMF and Brooker G. (1993) Ca^{2+}-inhibited adenylyl cyclase in cardiac tissue. *TIPS.* **14**, 34-36.

426. Cooper DMF, Mons N and Karpen JW. (1995) Adenylyl cyclases and the interaction between calcium and cAMP signalling. *Nature.* **374**, 421-425.

427. Sheppard JR. (1971) Restoration of contact-inhibited growth to transformed cells by dibutyryl adenosine 3':5'-cyclic monophosphate. *Proc. Natl. Acad. Sci.* **68**, 1316-1320.

428. Lewin S. (1976) Indirect biological influence of ascorbate on nucleotide-mediated hormonal activities. In: Vitamin C: Its Molecular Biology and Medical Potential. Academic Press, New York. p91-98.

429. Gottesman MM and Fleischmann RD. (1986) The role of cAMP in regulating tumour cell growth. *Cancer Surv.* **5**, 291-305.

430. Chalapowski FJ, Kelly LA and Butcher RW. (1975) Cyclic Nucleotides in cultured cells. In: Advances in Cyclic Nucleotide Research. (Eds: Greengard P and Robison GA) Raven Press, New York. **6**, p245- 326.

431. Pastan I and Johnson GS. (1974) cAMP and the transformation of fibroblasts. *Adv. Cancer Res.***19**, 303-329.

432. Weiss B and Halt WN. (1977) Selective cyclic nucleotide phosphodiesterase inhibitors as potential therapeutic agents. *Annu. Rev. Pharm. Tox.* **17**, 441-477.

433. Anderson WB, Russel TB, Carchman RA and Pastan I. (1973) Interrelationship between adenylate cyclase activity, adenosine 3':5' cyclic monophosphate levels, and growth of cells in culture. *Proc. Natl. Acad. Sci.* **70**, 3802-3805.

434. D'Armiento M, Johnson GS and Pastan I. (1972) Regulation of adenosine 3':5'-cyclic monophosphate phosphodiesterase activity in fibroblasts by intracellular concentration of cyclic adenosine monophosphate. *Proc. Natl. Acad. Sci.* **69**, 459-462.

435. Elks ML and Manganiello VC. (1984) Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, adenosine 3',5'-monophosphate metabolism, and lipolysis in 3T3-L1 adipocytes. *Endocrinol.* **115**, 1262-1268.

436. Wells JN and Hardman JG. (1977) Cyclic nucleotide phosphodiesterases. In: Advances in Cyclic Nucleotide Research. (Eds: Greengard P and Robison GA) Raven Press, New York. **8**, 119-143.

437. Monn E and Christiansen RO. (1971) Adenosine 3',5'-monophosphate phosphodiesterase: Multiple molecular forms. *SCI.* **173**, 540-542.

438. Amer MS and Kreighbaum WE. (1975) Cyclic nucleotide phosphodiesterases: Properties, activators, inhibitors, structure-activity relationships, and possible role in drug development. *J. Pharm.Sci.* **69**, 1-32.

439. Appleman MM, Thompson WJ and Russel TR. (1973) Cyclic nucleotide phosphodiesterases. In: Advances in Cyclic Nucleotide Research. (Ed: Greengard P and Robison GA) Raven Press, New York. **3**, 65-90.

440. Gericke D and Chandra P. (1969) Inhibition of tumor growth nucleoside cyclic 3',5'-monophosphates. Hoppe-Seyler's Z. Physiol. Chem. **350**, 1469-1471.

441. Johnson GS. (1973) Regulation of cell functions in fibroblasts by cyclic AMP. In: The role of cyclic nucleotides in carcinogenesis. (Eds: Schultz J and Gratzner HG) Academic Press, New York and London. **6**, 39-41.

442. Friedman DL. (1976) Role of cyclic nucleotides in cell growth and differentiaiton. *Physiol. Rev.* **56**, 652-708.

443. Chatterjee SK and Kim U. (1975) Adenosine-3',5'-cyclic monophosphate levels and adenosine-3',5'-cyclic monophosphate phosphodiesterase activity in metastasizing and nonmetastasizing rat mammary carcinomas. *J.N.C.I* . **54**, 181-186.

444. Sheppard JR. (1972) Difference in the cyclic adenosine 3',5'-monophosphate levels in normal and transformed cells. *Nature*. **236**, 14-16.

445. Otten J, Johnson GS and Pastan I. (1971) Cyclic AMP levles in fibroblasts: Relationship to growth rate and contact inhibition of growth. *Biochem. Biophys. Res. Commun.* **44**, 1192-1198.

446. Boynton AL and Whitfield JF. (1983) The role of cyclic AMP in cell proliferation: A critical assessment of the evidence. *Adv. Cyclic Nucleotide Res.* **15**, 193-294

447. Yatsunami K, Icchikawa A and Tomha K. (1981) Accumulation of adenosine 3',5'-monophosphate induced by prostaglandin E_1 binding to mastocytoma P-815 cells. *Biochem. Pharmacol.* **30**, 1325-1332.

448. Fulton AM, Laterra JJ and Hanchin CM. (1989) Prostaglandin E_2 receptor heterogeneity and dysfunction in mammary tumor cells. *J. Cell. Physiol.* **139**, 93-99.

449. Michael AE, Abayasekara DRE and Weblry GE. (1993) The luteotrophic actions of prostaglandins E_2 and $F_{2\alpha}$ on dispersed marmoset luteal cells are differentially mediated via cyclic AMP and protein kinase C. *J. Endocrinol.* **138**, 291-298.

450. Peters HD, Peskar BA and Schönhöfer. (1977) Influence of prostaglandins on connective tissue cell growth and function. *Nauyn-Schmiedeberg's Arch. Pharmacol.* **297**, S89-S93.

451. Craven PA, Saito R and DeRubertis. (1983) Role of local prostaglandin synthesis in the modulation of proliferative activity of rat colonic epithelium. *J. Clin. Invest.* **72**, 1365-1375.

452. Ishihara K, Ono S, Takahama Y, Hirayama F, Hirano H, Itoh K, Dobashi K, Murakami S, Katoh Y, Yamaguchi M and Hamaoka T. (1989) Different effect of prostaglandin E_2 on B-cell activation by two distinct B-cell differentiation factors, B151-TRF1/IL--5 and B151-TRF2: selective inhibition of B151-TRF2-induced antibody response through increases in intracellular cyclic AMP levels. *Immunol.* **68**, 154-162.

453. Ballard TA and Elmer WA. (1990) Effect of prostaglandin E_2 on cyclic AMP levels in limb cells of mouse mutant brachypodism. *Devl. Biol.* **142**, 489-492.

454. Fine KM, DuPont J and Mathias MM. (1981) Rat platelet prostaglandin, cyclic AMP and lipid response to variations in dietary fat. *J. Nutr.* **111**, 699-707.

455. Husby G, Strickland RG, Rigler GL, Peake GT, Williams RC. (1977) Direct immunochemical detection of prostaglandin-E and cyclic nucleotides in human malignant tumors. *Cancer.* **40**, 1629-1642.

456. Guerinot F, Delarue J-C, Contesso G and Bohuon C. (1977) adenosine 3',5'-cyclic monophosphate and guanine 3',5'-cyclic monophosphate levels in human breast cancer tissue. *Oncol.* **34**; 261-263.

457. Burger MM, Bombik BM, Breckenridge B and Sheppard JR. (1972) Growth control and cyclic alterations of cyclic AMP in the cell cycle. *Nature.* **239**. 161-164.

458. Hickie RA, Walker CM and Croll GA. (1974) Decreased basal cyclic adenosine 3',5'-monophosphate levels in morris hepatoma 5123 t.c. (h). *Biochem. Biophys. Res. Commun.* **59**, 167-173.

459. Pastan I, Willingham M, Carchman R and Anderson WB. (1973) Cyclic AMP metabolism in normal and transformed fibroblasts. In: The role of Cyclic Nucleotides in Carcinogenesis. (Ed: Schultz J and Gratzner HG) Academic Press, New York. **6**, p47-52.

460. Horrobin DF. (1980) The reversibility of cancer: The relevance of cyclic AMP, calcium, essential fatty acids and prostaglandin E1. *Med. Hypotheses.* **6**, 469-486.

461. Bürk RR. (1968) Reduced adenyl cyclase activity in a polyoma virus transformed cell line. *Nature.* **219**, 1272-1275.

462. Ryan WL and Heidrick HL. (1974) Role of cyclic nucleotides in cancer. *Adv. Cyclic Nucleotide Res.* **4**, 8-116.

463. Lando M, Abemayor E, Verity MA and Sidell N. (1990) Modulation of intracellular cyclic adenosine monophosphate levels and the differentiation response of human neuroblastoma cells. *Cancer Res.* **50**, 722-727.

464. Shinohara K, Fujiki H, Hidaka H, Tseng YK, Murakami H and Omura H. (1985) Effect of reductones on cyclic 3'-5'-adenosine monophosphate phosphodiesterase. *Acta Vitaminol. Enzymol.* **7**, 99-108.

465. Johnson GS and Pastan I. (1971) Change in growth and morphology of fibroblasts by prostaglandins. *JN.C.I.* **47**, 1357-1364.

466. Kreider JW, Rosenthal M and Lengle N. (1973) Cyclic adenosine 3',5'-monophosphate in the control of melanoma cell replication and differentiation. *J.N.C.I.* **50**, 555-558.

467. Kreider JW, Wade DR , Rosenthal M and Densley T. (1975) Maturation and differentiation of B16 melanoma cells induced by theophylline treatment. *J.N.C.I.* **54**, 1457-1467.

468. Minton JP, Wisenbaugh T and Matthews RH. (1974) Elevated cyclic AMP levels in human breast-cancer tissue. *J. Natl. Cancer Inst.* **53**, 283-284.

469. Freshney RI. (1989) Introduction: Principles of sterile technique and cell propagation. In: Animal Cell Culture. (Ed: Freshney RI) IRL Press, Washington DC. p1-11.

470. Biowhitakker (1993) Biotechnology Products Catalog and Technical Manual. p161-234.

471. Poydock ME and Rice DR. (1982) Influence of vitamins C and B_{12} on the survival rate of mice bearing Ascites tumor. *Exp. Cell Biol.* **50**, 88-91.

472. O'Connor MK, Malone JF, Moriaty M and Mulgrew S. (1977) A radioprotective effect of vitamin C observed in Chinese hamster ovary cells. *Br. J. Radiol.* **50**, 587-591.

473. Pauling L, Nixon JC, Stitt F, Marcuson R, Dunham WB, Barht R, Bensch K, Herman ZS, Blaisdell BE, Tsao C, Prender M, Andrews V, Willoughby R and Zuckerkandl E. (1985) Effect of dietary ascorbic acid on the incidence of spontaneous mammary tumors in RIII mice. *Proc. Natl. Acad. Sci.* **82**, 5185-5189.

474. Prasad KN, Edwards-Prasad J, Ramanujam S and Sakamoto A. (1980) Vitamin E increases the growth inhibitory and differentiation effects of tumor therapeutic agents in Neuroblastoma and Glioma cells in culture. *Proc. Soc. Exp. Biol. Med.* **164**, 158-163.

475. Cerutti PA. (1985) Prooxidant states and tumor promotion. *SCI.* **227**, 375-381.

476. Sakamoto A, Chougule PB and Prasad KN. (1983) Retrospective analysis of the effect of vitamin A, C, and E in Human Neoplasia. In: Modulation and Mediation of Cancer by Vitamins. (Ed: Meyskens FL and Prasad KN) Karger, Basel. p330-333.

477. Odeleye OE, Eskelson CD, Mufti SJ and Watson RR. (1992) Vitamin E protection against nitrosamine-induced esophageal tumour incidence in mice immunocompromised by retroviral infection. *Carcinogenesis.* **13**, 1811-1816

478. Bishun N, Basu TK, Metcalfe S and Williams DC. (1978) The effect of ascorbic acid (Vit C) on two tumour cell lines in culture. *Oncol.* **35**, 160-162.

479. Leuchtenberger C and Leuchtenberger R. (1977) Protection of hamster lung cultures by L-cysteine or vitamin C against carcinogenic effects of fresh smoke from tobacco or marihuana cigarettes. *Br. J. Exp. Pathol.* **58**, 625-634.

480. Meadows GG, Pierson HF and Abdallah RM. (1991) Ascorbate in the treatment of experimental transplanted melanoma. *Am. J. Clin. Nutr.* **54**, 1284S-1291S.

481. Stoll KE and Duncan JR. (1995) Phospholipase A_2 activity *in vitro* cultured cells- influence of ascorbate supplementation. *J. Lipid M.* **11**, 261-266.

482. Gardiner NS and Duncan JR. (1988) Enhanced prostaglandin synthesis as a mechanism for inhibition of melanoma cell growth by ascorbic acid. *Prostaglandin Leukotrienes EFAs.* **34**, 119-126.

483. Ottino P and Duncan JR. (1996) The role of adenylate cyclase, cAMP and PGE_2 in the *in vitro* growth regulation of murine melanoma cells following vitamin E supplementation. *Prostaglandins Leukotrienes EFAs.* **54**, 375-383.

484. Oberley TD, Schultz JL, Li N and Oberley LW. (1995) Antioxidant enzyme levels as a function of growth state in cell culture. *Free Radical Biol. Med.* **19**, 53-63.

485. Peskin AV, Koen YM, Zbarski IB and Konstantinov AA. (1977) Superoxide dismutase and glutathione peroxidase activities in tumours. *FEBS Lett.* **78**, 41-43

486. Oberley LW and Buettner GR. (1979) Role of superoxide dismutase in cancer: a review. *Cancer Res.* **39**, 1141

487. Bize IB, Oberley LW and Morris HP. (1980) Superoxide dismutase and superoxide radical in Morris hepatomas. *Cancer Res.* **40**, 3686

488. Diplock AT. (1991) Antioxidant nutrients and disease prevention: an overview. *Am. J. Clin Nutr.* **53**, 189S-193S

489. Cheeseman, KH, Holley AE, Kelly FJ, Wasil M, Hughes L and Burton G. (1995) Biokinetics in humans of RRR-α-tocoppherol: The free phenol, acetate ester and succinate ester forms of vitamin E. *Free Radical Biol Med.* **19**, 591-598.

490. Trevithick JR and Mitton KP. (1993) Topical application and uptake of vitamin E acetate by the skin and conversion to free vitamin E. *Biochem. Molecular Biol. Int.* **31**, 869-878.

491. Carini R, Dianbzani MU, Maddix SP, Slater TF and Cheeseman KH. (1990) Competitive evaluation of the antioxidant activity of α-tocopherol, α-tocopherol polyethylene glycol 1000 succinate and α-Tocopherol succinate in isolated hepatocytes and liver microsomal suspensions. *Biochem. Pharm.* **39**, 1597-1601.

492. Pascoe GA and Reed DJ. (1987) Vitamin E protection against chemical-induced cell injury. *Arch. Biochem. Biophys.* **256**, 159-166.

493. Sagar PS, Das UN, Koratkar R, Ramesh G, Padma M and Sravan kumur G. (1992) Cyotototoxic action of cis-unsaturated fatty acids on human cervical carcinoma (HeLa) cells: relationship to free radicals and lipid peroxidation and its modulation by calmodulin antagonists. *Cancer Lett.* **63**, 189-198.

494. Das UN, Padma M, Sagar PS, Ramesh G and Koratkar R. (1990) Stimulation of free radical generation in human leukocytes by various agents including tumor necrosis factor is a calmodulin dependent process. *Biochem. Biophys. Res. Commun.* **167**, 1030-1036.

495. Drapper HH and Hadley M. (1990) Oxygen radicals in biological systems. In: Methods in Enzymology. (Ed: Packer I and Glazer AN). Academic Press, New York. **186**, pp421-431.

496. Kelley EE, Buettner GR and Burns CP. (1995) Relative α-tocopherol deficiency in cultured cells: Free radical-mediated lipid peroxidation, lipid oxidizability, and cellular polyunsaturated fatty acid content. *Arch. Biochem. Biophys.* **319**, 102-109.

497. Scalia S, Renda A, Ruberto G, Bonina F and Menegatti E. (1995) Assay of vitamin A palmitate and vitamin E acetate in cosmetic creams and lotions by supercritical fluid extraction and HPLC. *J. Pharm. Biomed. Anal.* **13**, 273-277.

498. Fariss MW. (1991) Cadmium toxicity: unique cytoprotective properties of alpha tocopheryl succinate in hepatocytes. *Toxicol.* **69**, 63-77.

499. Pascoe GA and Reed DJ. (1987) Relationship between cellular calcium and vitamin E metabolism during protection against cell injury. *Arch. Biochem. Biophys.* **253**, 287-296.

500. Lai MZ, Duzgunes N and Szoka FC. (1985) Effects of replacements of the hydroxyl group of cholesterol and tocopherol on the thermotropic behaviour of phospholipid membranes. *Biochem.* **24**, 1646-1653.

501. Toivenen TJ. (1987) Effects of selenium, vitamin E and vitamin C on human prostacyclin and thromboxane synthesis *in vitro*. *Prostaglandin Leukotrienes Med.* **26**, 265-280.

502. Brase DA (1972) Effect of rat liver phenylalanine hyroxylase activity by derivatives of vitamin E. *Biochem. Biophys. Res. Commun.* **48**, 1185-1191.

503. Panganamala RV and Cornwell DG. (1982) The effects of vitamin E on arachidonic acid metabolism. *Ann. New York Acad. Sci.* **393** 376-391.

504. Anghileri LJ, Miller ES, Robinette J, Prasad KN and Lagerborg VA. (1971) Calcium metabolism in tumors -its relationship with chromium complex accumulation. II. Calcium, magnesium and phosphorus in human and animal tumors. *Oncol.* **25**, 193-209.

505. Berridge MJ. (1995) Calcium signalling and cell proliferation. *BioEssays.* **17**, 491-500.

506 Vacher P, McKenzie J and Dufy B. (1992) Complex effects of arachidonic acid and its lipoxygenase products on cytosolic calcium in GH_3 cells. *Am. J. Physiol. Soc.* **263**, E903-E912.

507. Rodan SB, Rodan GA, Simmon HA, Walenga RW, Feinstein MB and Raisz LG. (1982) Bone resorptive activity in conditioned rat medium from rat osteosarcoma cell line. In: Prostaglandins and Cancer. Alan R. Liss, New York. p573-578.

508. Martin TJ and Grill V. (1992) Hypercalcemia in cancer. *J. Steroid Biochem. Molec. Biol.* **43**, 123-129.

509. Mundy GR. (1978) Calcium and cancer. *Life Sci.* **23**, 1735-1744.

510. Hill SE, Rees RC and MacNeil S. (1990) A positive association between agonist-induced cyclic AMP production in vitro and metastatic potential in murine B16 melanoma and hamster fibrosarcoma. *Clin. Exp. Metastasis.* **8**, 461-474.

511. Parsons PG, Musk P, Goss PD and Leah J. (1983) Effects of calcium depletion on human cells in vitro and the anomalous behaviour of the human melanoma cell line MM170. *Cancer Res.* **43**, 2081-2087.

512. Dennis EA. (1987) Regulation of eicosanoid production: Role of phosholipases and inhibitors. *Biotechnol.* **5**, 1294-1300.

513. Lin L-L, Lin AY and Knopf JL. (1992) Cytosolic phospolipase A_2 is coupled to hormonally regulated release of arachidonic acid. *Proc. Natl. Acad. Sci.* **89**, 6147-6151.

514. Clarke JM and Switzer RL. (1977) Photometery. In: Experimental Biochemistry. W.H Freeman and Co., New York. p12-13.

515. Krumhardt B and DuPont J. (1991) Rat platelet phospholipase A_2 activity and thromboxane synthesis are concomitantly affected by dietary linoleic acid. *J. Nutr.Biochem.* **2**, 443-447.

516. Vajreswari A and Narayanareddy K. (1992) Effect of dietary fats on some membrane-bound enzyme activities, membrane lipid composition and fatty acid profiles of rat heart sarcolemma. *Lipids.* **27**, 339-343.

517. Spector AA, Kiser RE, Denning GM, Koh S-W-M and DeBault LE. (1979) Modification of the fatty acid composition of cultured human fibroblasts. *J. Lipid Res.* **20**, 536-547.

518. Murphy MG and Byczko Z. (1990) Effects of membrane polyunsaturated fatty acids on adenosine receptor function in intact N1E-115 neuroblastoma cells. *Biochem. Cell Biol.* **68**, 392-395

519. Denizot Y, Najid A and Riguad M. (1993) Incorporation of arachidonic acid in a human cancer gastric tumor cell line (HGT) at various stages of cell proliferation. *Cancer Lett.* **68**, 199-205.

520. Stoll KE. (1993) Nutrient supplementation and secondary metabolites in melanoma cells. PhD Thesis. Rhodes University. South Africa.

521. Calderaro V, Parrillo C, Greco R, Pignalosa P and Rossi F. (1992) Transport of arachidonic acid across mammalian epithelia. *Pharmacol. Res.* **25**, 226-228.

522. Calderaro V, De Simone B, Giiovane A, Quagliuolo L, Servillo L, Giordano C and Balestrieri C. (1991) Mechanism of arachidonic acid transport across rabbit distal colonic mucosa. *Am. J. Physiol.* **261**, G451-G457.

523. Stremmel W, Strohmeyer G and Berk PD. (1986) Hepatocellular uptake of oleate is energy dependent, sodium linked, and inhibited by an antibody to a hepatocyte plasma membrane fatty acid binding protein. *Proc. Natl. Acad. Sci.* **83**, 3584-3588.

524. Goré J and Hoinard C. (1992) Linolenic acid transport in hamster intestinal cells is carrier-mediated. *J. Nutr.* **123**, 66-73.

525. Boyington JC, Gaffney BJ and Amzel LM. (1993) The three-dimensional structure of an arachidonic acid 15-lipoxygenase. *SCI.* **260**, 1482-1486.

526. Yamamoto S. (1992) Mammalian lipoxygenases: molecular structures and functions. *Biochim. Biophys. Acta.* **1128**, 117-131.

527. Maccarrone M, Veldink GA, Vliegenthart JFG and Agrò AF. (1995) Inhibition of soybean lipoxygenase-1 by chain-breaking antioxidants. *Lipids.* **30**, 51-54.

528. Mayatepek E and Hoffmann GF. (1995) Leukotrienes: Biosynthesis, metabolism, and pathophysiologic significance. *Pediatr. Res.* **37**, 1-9.

529. De Vries CEE and Van Noorden JF. (1992) Effects of dietary fatty acid composition on tumor growth and metastasis. *Anticancer Res.* **12**, 1513-1522.

530. Soberman RJ. (1988) 5- and 15(ω-6)-Lipoxygenases from human polymorphonuclear leukocytes. In: Methods in Enzymology. (Ed: Abelson JN and Simon MI) Academic Press, New York. **163**, 344-349.

531. Furukawa M, Yoshimoto T, Ochi K and Yamamoto S. (1984) Studies on arachidonate 5-lipoxygenase of rat basophilic leukemia cells. *Biochim. Biophys. Acta.* **795**, 458-465.

532. Noguchi M, Miyano M, Matsumoto T and Noma M. (1994) Human 5-lipoxygenase asscociates with phosphatidylcholine liposomes and modulates LTA_4 synthetase activity. *Biochim. Biophys. Acta.* **1215**, 300-306.

533. Rådmark O, Zang Y-Y, Hammarberg T, Lind B, Hamberg M, Steinhilber D and Samuelsson B. (1995) 5-Lipoxygenase: structure and stabibity of recombinant enzyme, regulation in Mono Mac 6 cells. In: Advances in Prostaglandins, Thromboxanes, and Leukotrienes Research. (Ed: Samuelsson B) Raven Press, Ltd., New York. 23, 1-10.

534. Rouzer CA and Samuelsson B. (1986) The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase. *FEBS.* **204**, 293-296.

535. Rouzer CA and Kargman S. (1988) Translocation of 5-lipoxygenase to the membrane in human leukocytes challenged with ionophore A23187. *J. Biol. Chem.* **263**, 10980-10988.

536. Smith WL and Lands WEM. (1972) Oxygenation of unsaturated fatty acids by soybean lipoxygenase. *J. Biol. Chem.* **247**. 1038-1047.

537. Hansson G, Malmsten C and Rådmark O. (1983) The leukotrienes and other lipoxygenase products. In: Prostaglandins and related substances. (Ed: Neuberger and Deneemen) Elsevier Science Publishers, New York. p127-161.

538. Goetzl EJ. (1980) Vitamin E modulates the lipoxygenation of arachidonic acid in leukocytes. *Nature.* **288**, 183-185.

539. Buckman DK, Hubbard NE and Erickson KL. (1991) Eicosanoids and linoleate-enhanced growth of mouse mammary tumor. *Prostaglandins Leukotrienes EFAs.* **44**, 177-184.

540. Fujimoto Y, Takai S, Matsuno K, Sumiya T, Nishida H, Sakuma S and Fujita T. (1992) Effect of tert-butyl hydroperoxide on cyclooxygenase and lipoxygenase metabolism of arachidonic acid in rabbit platelets. *Prostaglandins Leukotrienes EFAs.* **47**, 259-264.

541. Panganamala RV, Miller JS, Gwebu ET, Sharma HM and Cornwell DG. (1977) Differential inhibitory effects of vitamin E and other antioxidants on prostaglandin synthetase, platelet aggregation and lipoxidase. *Prostagland.* **14**, 261-271.

542. Yamamoto S. (1982) Purification and assay of PGH synthase from bovine seminal vesicles. In : Methods in Enzymology. 86, 55-73.

543. Fujimoto Y and Fujita T. (1982) Effects of lipid peroxidation on prostaglandin synthesis in rabbit kidney medulla slices. *Biochim. Biophys. Acta.* **710**, 82-86.

544. Smith C, Otto AM and Jiminez de Asua L. (1984) Prostaglandins and the proliferation of cultured animal cells. In: Growth and Maturation Factors. (Ed: G. Guroff). John Wiley and Sons, New York. p132-160.

545. Powell WS. (1987) Rapid extraction of oxygenated metabolites of arachidonic acid from biological samples using octadecylsilyl silica. *Prostagland.* **20**, 947-957.

546. Powell WS. (1987) High-pressure liquid chromatography in the analysis of arachidonic acid metabolites. In: Prostaglandins and Related Substances. (Ed: Benedetto C, Nigam S, McDonald-Gibson RG and Slater TF) IRL Press, Washington. p75-98.

547. Nigam S. (1987) Extraction of eicosanoids from biological samples. In: Prostaglandins and Related Substances (Ed: Benedetto C, Nigam S, McDonald-Gibson RG and Slater TF) IRL Press, Washington. p45-52.

548. Prostaglandin E_2 [^{125}I] Assay Kit, Amersham International, United Kingdom.

549. Sakamoto W, Fujie K and Handa H. (1993) Effect of vitamin E on arachidonic acid-release in rat peritoneal macrophages. *Biochim. Biophys. Acta.* **1170**, 296-300.

550. Fulton AM. (1987) Interactions of natural effector cells and prostaglandins in the control of metastasis. *J.N.C.I.* **78**, 735-741.

551. Prostaglandin D_2 [^{3}H] Assay Kit, Amersham International,United Kingdom.

552. Prostaglandin $F_{2\alpha}$ [^{3}H] Assay Kit, Amersham International, United Kingdom.

553. 6-keto-Prostaglandin $F_{1\alpha}$ enzymeimmunoassay. Amersham International, United Kingdom.

554. Chan AC, Pitchard ET and Choy PC. (1983) Differential effects of dietary vitamin E and antioxidants on eicosanoid synthesis in young rabbits. *J. Nutr.* **113**, 813-820.

555. Kowaza O, Tokuda H, Suzuki A, Kotoyori J, Ito Y and Oiso Y. (1994) Effect of glucocorticoid on prostaglandin $F_{2\alpha}$-induced prostaglandin E_2 synthesis in osteoblast-like cells: inhibitin of phosphoinositide hydrolysis by phospolipase C as well as phospholipase A_2. *Eur. J. Endocrinol.* **131**, 510-515.

556. Cyclic adenosine monophosphate [^{3}H] Assay Kit. Amersham International, United Kingdom.

557. Morisaki N, Sprecher H, Milo GE and Cornwell DG. (1982) Fatty acid specificity in the inhibition of cell proliferation and its relationship to lipid peroxidation and prostaglandin biosynthesis. *Lipids.* **17**, 893-899.

558. Gavino VC, Miller JS, Ikharebha SO, Milo GE and Cornwell DG. (1981) Effect of polyunsaturated fatty acids and antioxidants on lipid peroxidation in tissue culture. *J. Lipid Res.* **22**,763-769.

559. Bègin ME, Ells G and Horribin DF. (1988) Polyunsaturated fatty acid-induced cytotoxicty against tumour cells and its relationship to lipid peroxidation. *J.N.C.I.* **80**, 188-194.

560. Ottino P and Duncan JR. (1996) Effect of α-tocopherol succinate on free radical and lipid peroxidation levels in BL6 cells. In Press.

561. Sonnenberg WK and Smith WL. (1988) Regulation of cyclic AMP metabolism in rat cortical collecting tubule cells by prostaglandins. *J. Biol. Chem.* **263,** 6155-6160.

562. Lupulescu A. (1996) Prostaglandins, their inhibitors and cancer. *Prostaglandins Leukotrienes EFAs.* **54**, 83-94.

563. Tramposch KM, Chilton FH, Stanley PL, Franson RC, Havens MB, Nettleton DO, Davern LB, Darling IM and Bonney RJ. (1994) Inhibitor of phospholipase A_2 blocks eicosanoid and platelet activating factor biosynthesis and has topical anti-inflammatory activity. *J. Pharm. Exp. Ther.* **271**, 852-859.

564. McLeod J and Bolton C. (1995) Dexamethasone induces an increase in intracellular and membrane-associated lipocortin-1 (Annexin-1) in rat astrocyte primary cultures. *Cell. Mol Neurobiol.* **15**,193-205.

565. Vervoordeldonk MJBM, Schalkwijk CG, Vishwanath BS, Aarsman AJ and van den Bosch. (1994) Levels and localization of group II phospholipase A_2 and annexin I in interleukin- and dexamethasone-treated rat mesangial cells: evidence against annexin mediation of the dexamethasone-induced inhibition of group II phospolipases A_2. *Biochim. Biophys. Acta.* **1224**, 541-550.

566. Flower RJ and Blackwell GJ. (1979) Anti-inflammatory steroids induce biosynthesis of a phospholipase A_2 inhibitor which prevents prostaglandin generation. *Nature.* **278**, 456-459.

567. Juge-Morineau N, Francois S, Puthier D, Godard A, Bataille R and Amiot M. (1995) The gp 130 family cytokines IL-6, LIF and OSM but not IL-11 can reverse the anti-proliferative effect of dexamethasone on human myeloma cells. *Br. J. Haematol.* **90**, 707-710.

568. Reyes-Moreno C, Frenette G, Boulanger J, Lavergne E, Govindan MV and Koutsilieris M. (1995). Mediation of glucocoticoid receptor function by transforming growth beta I expression in human PC-3 prostrate cancer cells. *The Prostrate.* **26**, 260-269.

569. Cillard J, Cillard M, Cormier M and Girre L. (1980) α-Tocopherol prooxidant effect in aqueous media: increased autoxidation rate of linoleic acid. *J.A.O.C.S.* **57**, 252-255.

570. Cilliard, Cillard M and Cormier M. (1980) Effect of experimental factors on the prooxidant behaviour of α-Tocopherol. *J.A.O.C.S.* **57**, 255-261.

571. Ara G and Teicher BA. (1996) Cyclooxygenase and lipoxygenase inhibitors in cancer therapy. *Prostaglandins Leukotrienes EFAs.* **54**, 3-16

572. Gey KF. (1993) Prospects for the prevention of free radical disease, regarding cancer and cardiovascular disease. *Br. Med. Bull.* **49**, 679-699.

573. Gutteridge JMC. (1993) Invited review: Free radicals in disease processes: a complication of cause and consequence. *Free Radical. Res. Commun.* **19**, 1126-1128.