VITAMIN E SUPPLEMENTATION AND SECONDARY METABOLITES: INTERACTIONS AND EFFECTS ON MELANOMA GROWTH

THESIS

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PAULO OTTINO

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ABSTRACT

The present study was undertaken to determine the effects and possible mechanism of action of vitamin E succinate on malignant murine melanoma (BL6) and non-malignant monkey kidney (LLCMK) cell growth *in vitro*. Studies revealed that supplementation of 5, 7 and 10μg/ml vitamin E succinate significantly inhibited BL6 cell growth, while in LLCMK cells no significant increase or decrease in growth was observed.

The actual mechanism by which vitamin E succinate inhibits BL6 cell growth is at present unclear. Studies have suggested a radical or oxidant involvement in a number of degenerative diseases such as cancer, and that supplementation of antioxidant vitamin's such as vitamin E may function to reduce cancer cell growth by quenching free radical species and preventing lipid peroxidation. In addition to its antioxidant role in a cell, vitamin E is believed to modulate the activities of various enzymes and metabolites in the eicosanoid pathway.

Hence, this study investigated the effects of vitamin E succinate supplementation on free radical and lipid peroxidation levels, as well as the activities of various enzymes and metabolites in the eicosanoid pathway. Throughout this study, emphasis was placed on BL6 melanoma cells since the magnitude of the relationship between LLCMK growth and the levels of various enzymes and metabolites in the eicosanoid pathway varied considerably from one experiment to another and did not show the consistent trend found with the BL6 cells.

A decrease in cell growth was found to be accompanied by a concomitant increase rather than a decrease in the levels of free radicals and lipid peroxidation, suggesting that the growth inhibitory effects of vitamin E succinate on BL6 cells *in vitro* was not due to its antioxidant properties associated with the vitamin E component, but rather due to one or more of its other potential roles within the cell. This proposal was further strengthened by findings that vitamin E succinate, a non-physiological antioxidant in its esterified form, did not undergo significant cleavage to free vitamin E in the BL6 cells.

Vitamin E succinate is believed to modulate membrane bound enzyme activities through physicochemical interactions with membrane lipids and changes in membrane fluidity. Hence, this study investigated the role of vitamin E succinate in modulating the activity of various enzymes and

secondary messengers in the eicosanoid pathway. Supplementation of 1-10μg/ml vitamin E succinate resulted in an overall increase in phospholipase A_2 activity while cyclooxygenase and adenylate cyclase activities were found to be significantly increased at vitamin E succinate concentrations of 7 and 10μg/ml respectively. A significant increase in 5-LOX activity was observed at 10μg/ml supplementation. The suggestion that vitamin E succinate modulates membrane bound enzyme activities was further strengthened by uptake and cellular distribution studies, which showed significantly higher levels of vitamin E succinate in membrane fractions of BL6 cells when compared with stroma fractions. Another factor which could account for elevated PLA_2, 5-LOX and COX activities in BL6 cells as a result of vitamin E succinate supplementation, was that of intracellular calcium levels. Supplementation of BL6 cells with 1-7μg/ml vitamin E succinate resulted in an overall increase in intracellular calcium levels. These changes in calcium levels however were positively correlated with changes in PLA_2 activity only.

Since the rate of prostaglandin synthesis is controlled by phospholipase A_2 activity, and net prostaglandin production is dependant on cyclooxygenase activity, the effects of vitamin E succinate supplementation on prostaglandin levels in BL6 cells was determined. Vitamin E succinate supplementation resulted in a significant decrease in prostaglandin D_2 levels at vitamin E succinate concentrations of 3, 5, 7 and 10μg/ml respectively, while prostaglandin $F_{2\alpha}$ levels were significantly decreased at 1-10μg/ml vitamin E succinate. The increases in prostaglandin E_2 and I_2 levels were inversely related to BL6 cell growth suggesting that both prostaglandins may act as negative regulators of BL6 cell growth. When comparing prostaglandin E_2 levels to prostaglandin I_2 levels in BL6 cells, significantly higher levels of prostaglandin E_2 were found, suggesting that vitamin E succinate effects were mediated primarily through an increase in prostaglandin E_2 levels. Furthermore, prostaglandin E_2 levels are believed to modulate adenylate cyclase activity. It is therefore reasonable to conclude that the increased adenylate cyclase activity found in BL6 cells was dependant on prostaglandin E_2 levels, since increases in prostaglandin E_2 levels at 7 and 10μg/ml vitamin E succinate correlated with an increase in adenylate cyclase activity and cyclic adenosine monophosphate levels.

Thus it appeared that the observed inhibitory effects of vitamin E succinate supplementation on BL6 cell growth was not due to the antioxidant properties associated with the vitamin E component of the vitamin E succinate molecule, but was rather mediated in part through a cascade effect initiated by phospholipase A_2 activation and archidonic acid release. This initial effect then appeared to result in an increase in cyclooxygenase activity and activation of a prostaglandin E_2-adenylate cyclase-cyclic adenosine monophosphate linked system, ultimately altering cyclic adenosine monophosphate levels

and inhibiting BL6 cell growth. This was confirmed when BL6 cells were supplemented with indomethacin, a cyclooxygenase inhibitor. Supplementation with the inhibitor resulted in vitamin E succinate having no inhibitory effects on BL6 cell growth. Furthermore, when comparing the levels of prostaglandin E_2, adenylate cyclase activity and cyclic adenosine monophosphate in indomethacin treated cultures to non-indomethacin treated cultures, markedly lower levels of these metabolites were found in the indomethacin treated cultures.

The cause of the increase in free radical and lipid peroxidation levels in BL6 cells following vitamin E succinate supplementation was further investigated. Cyclooxygenase enzymes are believed to generate free radical species and contribute to lipid peroxidation levels during catalytic activity. Markedly lower levels of free radicals and lipid peroxidation in indomethacin treated cultures were found when compared with vitamin E succinate treated cultures alone, suggesting that the increases in free radical and lipid peroxidation levels in BL6 cells supplemented with vitamin E succinate were indirectly due to an increase in cyclooxygenase activity in these cells.

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ABBREVIATIONS

AA	Archidonic acid
AC	Adenylate cyclase
5'-AMP	5'-adenosine monophosphate
ATP	Adenosine triphosphate protein
BL6	Malignant melanoma cells
Ca^{2+}	Calcium
cAMP	Cyclic adenosine monophosphate
COX	Cyclooxygenase
$cPLA_2$	Cytosolic phospholipase A_2
DNA	Deoxyribonucleic acid
FA	Fatty acid
FCS	Foetal calf serum
G-proteins	Guanine nucleotide proteins
GDP	Guanine diphosphate proteins
GTP	Guanine triphosphate proteins
HPETE	Hydroperoxy-eicosatetranoic acid
HPLC	High performance liquid chromatography
INDO	Indomethacin
kDa	Kilodalton
6-keto $PGF_{1\alpha}$	6-Keto Prostaglandin $F_{1\alpha}$
LLCMK	Monkey kidney cells
LOX	Lipoxygenase
MDA	Malondialdehyde
MEM	Minimal essential media
MW	Molecular weight
NBD	Nitroblue diformazan
NBT	Nitroblue tetrazolium
NDGA	Nordihydroguaidaretic acid
OH^-	Hydroxy radical
PBS	Phosphate buffered saline
PDE	Phosphodiesterase

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 synthase	Prostaglandin H synthase
PGH_2	Prostaglandin H_2
PGI_2	Prostaglandin I_2
PLA_2	Phospholipase A_2
pPDE	Soluble phosphodiesterase
sPDE	Particulate phosphodiesterase
PUFA	Poly unsaturated fatty acid
R·	Pentadienyl radical
ROO·	Lipid peroxyl radical
ROOH	Lipid hydroperoxide
SEM	Standard error of the mean
$sPLA_2$	Soluble phospholipase A_2
TBA	Thiobarbaturic acid
TLC	Thin layer chromatography
α-TOH	Alpha tocopherol
TOH	Tocopherol
TOT	Tocotrienols
TX	Thromboxane

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LITERATURE REVIEW

1.1 CANCER

Cancer can be defined as the uncontrolled growth of abnormal cells. The transformation of normal cells to cancer cells is actively induced by a variety of agents which are either physical or chemical in nature (1-6). These agents can be classified into 4 distinct groups: chemical, physical, biological and genetic (1). Groups of these abnormal proliferating cells (tumours) can arise in any part of the body, however not all tumours are potentially lethal. Tumour cells that remain localised in certain tissues are unable to invade surrounding tissue and are referred to as benign tumours, while those that spread from their site of origin through the bloodstream and lymphatic system and destroy surrounding tissue and organs, are referred to as malignant (2,3). The availability of highly sophisticated molecular and biochemical tools over the past few years has led to considerable progress being made in understanding the stages of tumour development. Both epidemiological and experimental studies indicate that tumour development occurs through three distinct stages (figure 1). These include initiation, promotion and progression stages (1,5,7-11).

INITIATION	PROMOTION	PROGRESSION
-Permanent alteration of cell phenotype. -Somatic gene mutation.	- Conversion → Promotion (Stage 1) (Stage 2) * Partially reversible * Reversible	- Additional genetic alterations?

Figure 1: Multistage carcinogenesis model of tumour development (5).

1.1.1 INITIATION

The first step in the multistage carcinogenesis model is the initiation step. During this stage, a permanent and hereditable alteration of the cells genetic material may occur (1,5,7-11). Most tumour

initiating agents are electrophilic in character, or are metabolically converted to electrophilic agents capable of binding to deoxyribonucleic acid (DNA) and other macromolecules within the cells (5,7,10,11). The initiation phase requires a single application of a carcinogen at sub-threshold level (5,7,8), although the efficiency of this step is dependant to a large extent on cell division and DNA synthesis (1,5,8).

1.1.2 PROMOTION

The promotion step of carcinogenesis involves both the selection and clonal proliferation of cells that have undergone initiation (1,5,7-9). This stage occupies a large portion of the latent period of carcinogenesis, and is distinguishable from initiation and progression phases by its operational reversibility (1,8,11). Furthermore, the promotion stage of the multistage carcinogenic model can be modulated by a variety of factors such as frequency of administration of a promoter (1,11), age of the individual and composition of the diet (1). Promoting agents are classified into stage one promoters (incomplete promoters) which are active in tumour initiation or tumour promotion, and stage two promoters (complete promoters) which are active in both initiation and promotional stages of carcinogenesis (5). Most tumour promoting agents are believed to be membrane active agents, which exert their effects on the cell through mediation of receptor linked mechanisms (1,7,9,12). Furthermore tumour promoting agents may increase the risk of cancer development, by increasing the proliferation rate of normal cells, in turn increasing the probability of propagating a genetic error within these cells (1,9).

Although numerous tumour promoters exist in nature, the events triggering the transformation of a normal cell to a tumour cell can be simple, often resulting from damage to the DNA of a critical target gene in a particular cell (13,14). Over the past few years, these target genes have been identified and are referred to as oncogenes (13). Almost all oncogenes arise from the cellular genes, and several lines of evidence suggest that these oncogenes are altered versions of normal cellular genes called proto-oncogenes (13-15). When uninterrupted, proto-oncogenes play an important role in the control of both cellular proliferation and differentiation of normal cells (13-16). To date, oncogenes have been detected in only 15 to 20% of all human tumours, although other as yet unidentified growth promoting oncogenes may exist in human tumours (13). Certain tumours may arise due to the loss of critical sections of genetic information (13). Recent studies have proposed the existence of so-called suppressor genes (anti-oncogenes) (13,14), which when absent result in tumour

cells becoming aberrant (13). This notion has further been encouraged by the finding that fusing a normal cell with a tumour cell results in the tumour cell regaining the genes it lost during its evolution towards malignancy (13).

1.1.3 PROGRESSION

In the final stage of carcinogenesis, benign tumour cells progress into a highly malignant rapidly growing neoplasm (1,7-9). During tumour progression a second discrete and inheritable event occurs through direct chemical interaction with the DNA, or transposition of genetic material (1,9). These alterations in genomic structure are directly related to the increased growth rate, invasivness, metastatic capability and biochemical changes within the cells (8,9,11). One of the most important aspects of tumour progression is the ability of tumour cells to undergo metastatic dissemination. This process involves detachment of tumour cells from a primary tumour, infiltration into blood or lymphatic system, and transport to distant organs. On reaching distant organs, tumour cells infiltrate tissues and begin to proliferate. The ability of tumour cells to metastasise is one of the primary causes of death in cancer patients (17-19).

In conclusion, due to the complexity of various processes involved in cancer development, numerous factors, including properties of a particular tumour cell as well as host factors, may all contribute to this process. Further studies of these factors may contribute to the overall knowledge of cancer development and eventually lead to the development of treatments with far greater success rates.

1.1.4 CHEMOPREVENTION

With the ever increasing number of new cases of cancer occurring each year, and the limited success of established preventative and therapeutic strategies, one cannot escape the need for continued efforts to develop new and improved therapeutic procedures (11,20,21). Advances in our understanding of the various stages involved in carcinogenesis should enable researchers to develop effective treatment strategies. Several new therapeutic approaches are currently under investigation. These include chemoprevention, which involves the systematic use of specific natural or synthetic agents to block or reverse the carcinogenic process prior to the development of invasive cancer (11,20-23). Chemopreventers are found in all classes of foods and include non-nutritional components such as vitamins, polyphenols and other food ingredients (20-22).

Over the past few years numerous studies have shown that a variety of non-nutritive components of the diet can protect against cancer development, and furthermore that their protective effects are due to their antioxidant properties (11,22). In addition to their roles as antioxidants, these agents may exhibit several other modes of action, which are tumour specific, site oriented and complement the total beneficial potentials of chemopreventers (22). Few studies have been carried out to evaluate the role of these antioxidants in carcinogenesis, however their potential roles as chemopreventive agents has further been strengthened by the fact that free radicals may contribute to the initiation and promotional stages of carcinogenesis (8,9). Vitamin E is one such antioxidant whose role in chemoprevention is currently being investigated (24,25).

Since vitamin E and its role in the neoplastic process is the subject of this thesis, the general role of vitamin E in cellular metabolism will be considered.

1.2 VITAMIN E

1.2.1 HISTORY

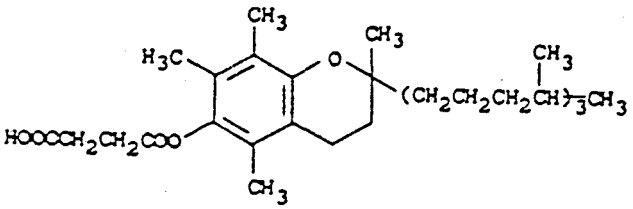
Vitamin E, the generic term for a group of lipid-soluble tocol and tocotrienol (TOT) derivatives possessing varying levels of vitamin E activity, was discovered in 1922 by Evans and Bishop during a course of experiments examining the relationship between the estrous cycle in female rats and the effects of dietary changes on its duration. These studies revealed that female rats fed diets deficient in vitamin E suffered a loss of fertility through resorption of foetuses, unless the diet was supplemented with small amounts of fresh lettuce, wheat germ or dried alfalfa leaves (26-32). Following its isolation in pure form, Evans gave the vitamin a name descriptive of its metabolic function. With the aid of Calhoun, a Greek professor from the University of California, they derived the name tocopherol (TOH) from the Greek words Tokos (offspring) and Phero (to bring forth) (26,28,32).

1.2.2 STRUCTURE

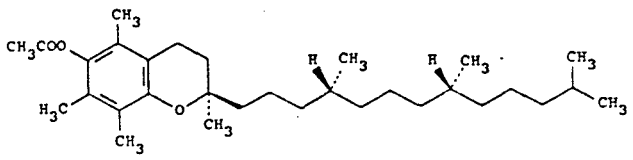
At present eight forms of vitamin E are known to exist in nature. These include α-, β-, γ- and δ-TOTs, which contain saturated phytol side chains, and the α-, β-, γ- and δ-TOHs which possess 3 double bonds in their side chains (25,26,32,33,34). Individual TOHs and TOTs differ according to

the position and number of methyl substituents on the aromatic ring (32,33,35,36).

The functional form of vitamin E, α-TOH, is a 6-hydroxychroman derivative containing methyl groups at carbons 2,5,7,8 and a phytyl chain at carbon two (26,32,34,35,36). Although α-TOH from natural sources is the most biopotent form of vitamin E (27,32,35-38), both TOTs and α-, β-, δ-TOHs do contribute some vitamin E activity (27,38). (In this thesis when vitamin E is referred to the compound α-TOH is understood if not otherwise stated). At present, the two principle sources of vitamin E being used commercially are the acetate and succinate derivatives of d-α-TOH (26,36,39-41). α-TOH esters are widely used in pharmaceutical formulations and dietary supplements due to their relative stabilities and resistance to biological oxidations (36,40-42). The most commonly used α-TOH esters (figure 2) are the non-polar acetate and more polar succinate esters such as α-TOH succinate, although numerous other polar esters such as phosphate, nicotinate and polyethylene glycol 1 000 succinate are also available (36). Polar α-TOH esters are frequently used in *in vitro* studies due to their stability and solubility in growth media, their ability to cross cellular membranes and their slow conversion to α-TOH, resulting in intracellular levels of α-TOH remaining high for longer periods (36,42).



α-Tocopherol succinate



α-Tocopherol acetate

Figure 2: Commercially available α-Tocopherol esters (43).

1.2.3 METABOLISM

Natural TOHs are essential macronutrients in mammals (34,44,45). The term "essential" implies that these vitamins are not synthesised by the body, and therefore have to be acquired through the diet (26,44). In humans and animals, TOHs and TOTs are primarily obtained through the ingestion of plant materials (44). Recommended daily allowances [RDA's] for vitamin E range from 7 to 13mg (10 to 20IU) (28,32,46,47), while the average daily intake of this vitamin from a balanced diet is approximately 7 to 9 mg (11 to 14IU) (28,32,46,47). Vitamin E, although the least toxic of the fat-soluble vitamins, can become toxic at doses above 1200IU (805mg/day). Symptoms of toxicity include nausea, diarrhoea, intestinal cramps, skin reactions, myopathy, gonadal dysfunction and altered vitamin K metabolism (32,46).

The general overview of vitamin E metabolism is shown in figure 3. In nature α-TOH is found as free unesterified RRR-α-TOH (40). The absorption of this fat-soluble vitamin depends on pancreatic function, biliary secretion, micellar formation and transport across intestinal membranes (26,34,41,48). Most pharmaceutical preparations of vitamin E are supplied in the form of α-TOH esters (36,37,40-42). These esterified TOHs are hydrolysed during the absorption process via the action of pancreatic enzymes (34,48), resulting in the release of free α-TOH which is absorbed by the intestine (26,34,40,41,48).

1.2.4 PHYSIOLOGICAL PROPERTIES

All TOH's are light-sensitive viscous oils with the following characteristics. They are insoluble in water but soluble in aprotic solvents, stable to heat and alkali in the absence of oxygen, unaffected by acids up to 100^{0}C and slowly oxidised by atmospheric oxygen (26).

1.2.5 DEFICIENCY DISEASES

Despite many years of intensive research, and its wide acceptance as an essential nutrient, vitamin E's status in humans remains unclear. The search for a clinical correlation between TOH deficiency and deficiency diseases in humans has been based to a large extent on nutritional deprivation experiments in animals (26). In many instances vitamin E deficiency in animals results in diminished reproductive capabilities, muscular dystrophy, exudative diathesis, megaloblastosis, gastrointestinal

degeneration, pulmonary degeneration and nephrosis (26,37). Attempts to demonstrate clinical signs of human TOH deficiency have been unsuccessful due to the existence of extensive stores within the body (28,32,49), and the continual regeneration of biologically active α-TOH by various components within the cells (32). Deficiencies in vitamin E do however occur in humans suffering from malabsorption syndromes and in premature infants (26,32,49). Furthermore these disorders bear superficial resemblance to vitamin E deficiencies observed in animals. Studies have suggested that alterations in vitamin status in humans may be associated with the development of various cancers (24,27,28,46,50,51). To date numerous epidemiological studies have consistently related low intake of vitamin E with increased susceptibility to cancer cell development (52-56).

1.2.6 FUNCTIONS OF VITAMIN E

Since its discovery, vitamin E has been plagued with exaggerated claims about its possible role in human and animal health, although no single primary metabolic role for vitamin E has been discovered as yet. Researchers believe that most of its proposed action within a cell, such as tumour prevention, may be secondary to its primary role as an antioxidant (32,57,58).

1.2.6.1 Vitamin E's antioxidant role

Although oxidation of cellular elements serves an important regulatory role, such as prostaglandin (PG) synthesis, this process is often destructive resulting in disruption of various cellular functions and causing leakage of intracellular contents (37,59). The oxidation of polyunsaturated fatty acids (PUFA) and corresponding inhibition by antioxidants has recently received much attention, since free radical oxidation damage has been implicated in the etiology of a variety of human diseases such as cancers (8,60-71).

Antioxidants are defined as a group of compounds protecting biological systems against the potentially harmful effects of processes or reactions that cause excessive damage (64,72). These antioxidants constitute an important endogenous defence mechanism against oxidative cell and tissue damage caused by toxic and carcinogenic chemicals (36). One antioxidant studied from this perspective is vitamin E (36,62,63,66-68,73), a major soluble antioxidant whose role in the cell is to prevent peroxidation of PUFAs in mammalian membranes (36,37,62,64,68,69,72-80). In addition to its role as a chain-breaking antioxidant, recent studies have demonstrated that α-TOH can quench singlet oxygen, either physically through an electron transport mechanism or chemically through the

formation of various oxidative products (36,81). Furthermore, α-TOH has close to optimal properties for trapping peroxyl radicals (39,82-84). These highly efficient antioxidant properties are believed to be due to the stereoelectronic effect of the oxygen atom in the fully methyl substituted chroman ring of the vitamin (39,78,82-84), which not only makes the α-TOH react more rapidly with peroxyl radicals but gives greater stability to the resulting α-TOH peroxyl radical (39,78). The nature of the phytyl chain attached to the chroman moiety is believed to be important in determining antioxidant effectiveness of the vitamin *in vivo* (39,77,78,82). The phytyl side chain is required to give the vitamin appropriate lipophilicity and solubility in biomembranes (77,78,82,83,85), and furthermore positions the vitamin so that the reactive centre is present where it is needed most (77,82,85).

Figure 3 illustrates the reactions involved in the inhibition of lipid peroxidation by vitamin E. α-TOH traps lipid peroxide radicals via a two-step mechanism. In the first step (reaction 1), a peroxyl radical abstracts a hydrogen from the α-TOH molecule to yield an unusually stable tocopheroxyl radical (α-TO·) (36,39,72,74,78,79). This α-TO· reacts rapidly with another peroxyl radical via a simple coupling reaction to yield a non-radical adduct (reaction 2) (36,39,72,74,78).

$$ROO\cdot + \alpha\text{-TOH} \rightarrow ROOH + \alpha\text{-TO}\cdot \quad (1)$$

$$ROO\cdot + \alpha\text{-TO}\cdot \rightarrow \alpha\text{-T(O)OOR} \quad (2)$$

Figure 3: Inhibition of lipid peroxidation by α-Tocopherol (modified from 74).

Although an effective antioxidant, α-TOH only affords protection when its levels within the cell exceed a critical or threshold level (36). In general, approximately one molecule of α-TOH exists for every 500-1 000 PUFAs in membranes (36,59,86). Clearly these levels are extremely low and the question that may arise is how such low levels of α-TOH protect such large numbers of PUFAs (86). It has been suggested that various pathways may exist to maintain cellular α-TOH concentrations in membranes. This prevention of α-TOH consumption is often referred to as the "sparing effect" and is defined as the ability of one antioxidant to slow or prevent depletion of another. Like other biological antioxidants, α-TOH is believed to function through redox cycles, which deliver reducing equivalents for antioxidant reactions and link antioxidant function to cellular metabolism (36). Recently, numerous studies have shown a synergistic inhibition of oxidative damage with a combination of vitamin E and selenium (87,cited in 88); vitamin E and enzymatic reductants such as selenium-dependant glutathione reductase (88,89); vitamin E and vitamin C (36,66,67,78,80,90-96), as well as vitamin E and β-carotene (36,97). These antioxidant interactions strongly indicate a

requirement for optimal intake of all antioxidant nutrients, since even a marginal deficiency in one of these nutrients would result in a subsequent decrease in the bioactivity of other antioxidant nutrients even though recommended levels are consumed (62).

1.2.6.2 Vitamin E and cellular membranes

All cells are enveloped by membranes, and although they differ widely in chemical composition they all contain lipids, nature's solvent for vitamin E. Numerous observations have recently suggested that membranes are the cellular structures which become impaired during vitamin E deficiency (98). Vitamin E is believed to function as a chain-breaking antioxidant, neutralising free radicals and preventing peroxidation of lipids within the membranes (26,60,86,90,99). This rather specific property of the vitamin appears to reside in the ability of these membranes to bind vitamin E. Furthermore it has been shown that even when the diet has a sufficiency of various other synthetic antioxidants capable of preventing oxidative damaged in membranes, vitamin E appears to have an advantage over these antioxidants, due to its easier access and longer retention within membranes (26). α-TOH is believed to play an important physiological role in the stabilisation of biological membranes containing high levels of PUFAs through a direct interaction between phytyl side chains of α-TOH and the fatty acyl chains of PUFAs, particularly those of arachidonic acid. In this model, the methyl groups at C_4 and C_8 of the phytyl chain of α-TOH fit into pockets created by the *cis* double bonds of the arachidonyl residues (26,86,98,100), resulting in the hydroxyl group on the chromanol ring of vitamin E and the polar group of a membrane phospholipid lying at the same end of the complex, where they are thought to participate in polar interactions at membrane surfaces (86,98). This association between methyl groups of α-TOH and pockets created by *cis* double bonds of PUFAs, enables close packing of the vitamin with these PUFAs, resulting in stabilisation of membranes and protection from oxidative damage (26,86,98).

Vitamin E, through its membrane stabilising effect, guarantees that metabolic interactions based on and coupled with the membrane surface can occur undisrupted, in turn contributing to normal functioning of the cell (86). This suggests that vitamin E deficiency diseases observed at nutritional levels could be explained by membrane instability through absence of vitamin E or metabolic failure of pathways located in or coupled with membranes.

1.2.6.3 Vitamins E and nitrosamines

Nitrosamines, products of the reaction of nitrite with amines under conditions of low pH (101), have been shown to induce a wide variety of tumours in most species of animals (58,101-103). Precursors of these n-nitroso-compounds, namely nitrates, nitrites and secondary or tertiary amines, are widely distributed in nature. Primary sources of these nitrites include water, vegetables and cured meat products (101-103). Nitrates present in these food sources are converted by bacteria in food and saliva to nitrites (103), which in turn react with primary, secondary and tertiary amines to form nitrosamines (101,103). Although daily intake of nitrosamine precursors varies with the dietary habits of the individual (102), levels of nitrite in the body fluids are high regardless of whether or not a person consumes foods high in nitrite content (101). This suggests that a reduction in nitrosamine formation or activity may be a promising avenue for reducing exposure to potential human carcinogens (102). One compound with a potential inhibitory effect on nitrosamine formation is vitamin E (58,101-105). This antioxidant vitamin is believed to prevent nitrosamine formation in hydrophobic environments such as fatty tissue and membranes (101,103,104). Numerous animal studies have demonstrated a potential inhibitory role for vitamin E in nitrosamine formation and eventual cancer development (58).

1.2.6.4 Vitamin E and immunocompetence

Considerable evidence suggests that a decline in immune function could increase the susceptibility of an individual to tumour cell development (60,106,107), and furthermore that numerous antioxidant vitamins such as vitamin E may play an important role in the defence against such diseases (26,57,58,60,106-108). Vitamin E deficiency is believed to result in a decreased immune response (108). The dietary levels of vitamin E which optimally affect the immune response are usually higher than what is available in human or animal diets (57), hence supplementation of vitamin E holds promise for therapeutic applications in cancer treatment (57,108). Numerous studies have shown that supplementation of pharmacological doses of vitamin E significantly increases humoral and cell-mediated immune responses and phagocytic functions in laboratory animals, farm animals and humans (cited 57). The mechanism of immunostimulation by vitamin E is largely unknown, however studies suggest that the antioxidant properties of this vitamin may play an important role in protecting one or more of the cell types of the immunopoietic system (57,108,109) against peroxidative damage by free radicals, peroxides and superoxides (57). Recently however, a more indirect role for vitamin E in immunostimulation has been proposed. This theory suggests that vitamin E functions as a potent

immune modulator by down-regulating production of immune suppressive PG (57,106,108,110). PG production results in immunosuppression through the generation of cyclic adenosine monophosphate (cAMP), a known suppressor of immunological cellular activities (108). Studies by Meydani *et al* (106) have shown that α-TOH acetate enhanced immune response in aged mice and that these effects were associated with a decrease in PG synthesis. Further studies by Watson (cited in 58) demonstrated that vitamin E modulates retrovirus-induced immune dysfunctions by down-regulating prostaglandin E_2 (PGE_2) production in macrophage cells and up-regulating interleukin-2 production. The mechanism by which vitamin E functions to inhibit PG synthesis is at present unclear, but studies suggest that inhibition of PG production may be due to an inhibition of both the phospholipase A_2 (PLA_2) and cyclooxygense (COX) enzymes (cited in 110), or PLA_2 enzyme alone (111). Since vitamin E may function as an immunostimulant, the potential use of vitamin E in immuno-therapeutic treatment of cancer may be an effective approach to treatment of this disease.

1.2.6.5 Vitamin E and gene expression

The P53 gene was discovered in 1979 as a host cell protein binding to DNA virus sv40 (112). This gene is commonly referred to as the "GUARDIAN OF THE GENOME" due to the fact that it becomes active when DNA damage occurs. In essence, the P53 gene determines whether or not a damaged cell repairs itself or commits suicide (112-115). When a cell suffers DNA damage, the P53 gene produces a 53 kDa protein which attaches to other genes and regulates their activities (112,113). Studies revealed that P53 proteins function to switch on a growth-arresting gene called CIP/WAF1 (114). Activation of this gene results in the production of a protein which inhibits CdK_2 kinase activity, an important factor in initiating cell division. This suggests that the P53 genes may help to check uncontrolled growth of damaged cells either by inhibiting cell growth directly (112) or stimulating apocytosis and programmed cell death (112-115).

This may have important consequences for cancer development, since most cancer cells examined to date have mutated P53 genes. These mutant P53 genes render them unable to 'commit suicide' in response to DNA damage, and as a result proliferate out of control (112-114). Studies by Yamada *et al* (116) have found no P53 mutations in 19 primary lesions of gastric cancers, while in metastatic lesions, mutant P53 gene expression occurred. Tumour cells containing the mutant P53 genes are the most aggressive tumours and are particularly likely to spread and cause death (113). Recent studies by Schwartz *et al* (112) have shown that vitamin E succinate supplementation may inhibit squamous

cell carcinoma growth by stimulating the expression of wild type P53 gene (suppressor gene) and diminishing expression of mutant P53 gene. The mechanism(s) by which vitamin E may function to alter the levels of these genes is unclear, however three possibilities have been suggested. These include a) direct stimulation of a cancer repressor gene; b) preventing mutation of P53 to an oncogenic form by promoting DNA repair during the transformation step; c) preventing mutation of other protooncogenes that function together with mutant P53 (112).

1.2.6.6 Vitamin E and cancer

The geographical distribution of various cancers, the changing patterns in migrant populations, and the varying incidence of specific tumours by socioeconomic groups, all point to diet and nutrition as important factors in the possible control and prevention of human cancers (46). Although there is no conclusive evidence that antioxidant nutrients administered in large doses have antineoplastic effects, the possibility exists that megadoses of vitamins may affect tumour incidence in mammals (50,51). One antioxidant nutrient currently receiving attention as a possible inhibitor of cancer is vitamin E. The anticarcinogenic effects of vitamin E have both been suggested (22,24,42,46,52,54,55,58,104, 105,110,112,117-130) and disputed (24,25,52,104,105,119,120,129,131) by numerous researchers. Several animal (24,46,58,117,118,120,122) and human epidemiological studies (52,54,55,105,119-121,130) have shown that vitamin E may protect against cancer incidence.

The mechanism(s) by which vitamin E and its derivatives (vitamin E succinate) exert anticarcinogenic effects both *in vivo* and *in vitro* are unclear, however discrete modulatory effects have been explained in part by biochemical strategies utilized by these vitamins. It has been suggested that anticarcinogenic effects of vitamin E may be due to its antioxidant properties and ability to scavenge free radicals (22,24,46,54,58,105,109,112,117-122,127). Furthermore, the effect of vitamin E and its derivatives on tumour cell growth could be mediated through various metabolic pathways. One such pathway is the metabolism of arachidonic acid (AA) or the AA cascade (figure 4). This pathway converts AA, released via PLA_2 activity, primarily to PGs through the action of prostaglandin H synthase (PGH synthase) and adenylate cyclase (AC) enzymes. This pathway is influenced by PLA_2 activity and subsequent mobilization of the precursor fatty acid AA, as well as the calcium (Ca^{2+}) requirement of a number of enzymes. Furthermore membrane stability and oxidative damage to membrane structures may play an important role in modulating the activity of these enzymes. Since this study involves an investigation of the influence of vitamin E supplementation on oxidative damage, as well as possible

influences on various metabolites mentioned above, their interrelationships, and possible role in tumour cell growth, a review of this pathway and its metabolites is necessary.

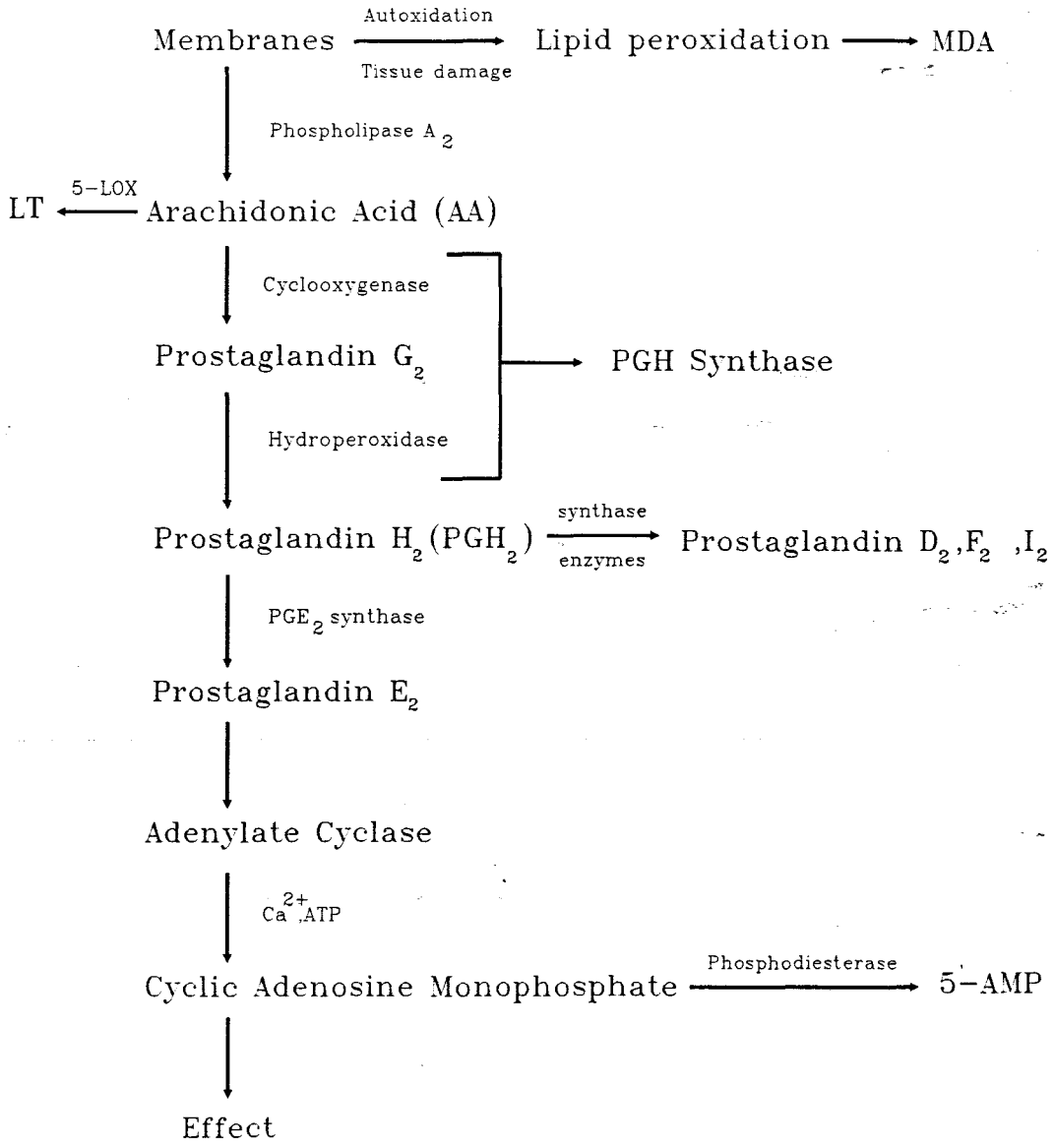


Figure 4: Schematic representation of the AA cascade pathway examined in this project. (AMP-Adenosine monophosphate, ATP-Adenosine triphosphate, LT-Leukotrienes, LOX-Lipoxygenase, MDA-Malondialdehyde).

1.3 FREE RADICALS AND LIPID PEROXIDATION

Although an aerobic lifestyle is advantageous in many ways, the utilization of oxygen by cells results in the production of highly reactive oxygen-containing species called free radicals (60,61,75). A free radical is defined as a molecular species capable of independent existence and possessing one or more unpaired electrons (8,61,62,64,65,132). In biological systems free radical species can be formed in one of three ways. These include homolytic cleavage (65,133), heterolytic fission (65) and electron transfer reactions (65,133). Except in unusual circumstances, free radical production in mammalian cells generally occurs via electron transfer reactions. These reactions can be mediated both enzymatically through the action of various enzymes and non-enzymatically through the redox chemistry of various transitional metal ions. Arguably the most common free radical generated *in vivo* is the hydroxyl radical ($OH^{\cdot}$), which is derived from the breakdown of hydrogen peroxides (H_2O_2) in the presence of transitional metal ions (65). These radicals are extremely reactive oxidising agents which react with numerous macromolecular structures causing extensive damage (5,61,65,75). Under normal circumstances the major source of free radicals in mammalian cells is through electron leakage from the electron transport chains located in the endoplasmic reticulum and mitochondria.

Of all the major classes of biomolecules attacked by $OH^{\cdot}$ radicals, membrane lipids are probably the most susceptible, due to the high levels of PUFAs (65). The oxidative destruction of PUFAs is known as lipid peroxidation and is extremely damaging as a result of the self-perpetuating chain reactions they cause (65,75,133). Figure 5 illustrates the 3 features which characterise the lipid peroxidation process. During the initiation step (reaction 1) PUFAs are attacked by free radical species ($In^{\cdot}$), resulting in abstraction of a hydrogen atom from precursor PUFA (RH) and formation of delocalized pentadienyl radical ($R^{\cdot}$). The propagation step (reaction 2) normally begins with the rapid addition of molecular oxygen to $R^{\cdot}$, to form lipid peroxyl radical ($ROO^{\cdot}$), followed by a rate limiting step (reaction 3), whereby the $ROO^{\cdot}$ abstracts a hydrogen from another PUFA, in turn generating lipid hydroperoxide (ROOH) and another $R^{\cdot}$. This propagation step results in a chain reaction being set in motion which proceeds through reactions 2 and 3 for a number of cycles (39,65,72,75,133,134). The third and final step (reaction 4) is the termination step, which results in 2 $ROO^{\cdot}$ radicals reacting together to form non radical products (39,72,75,134).

Initiation:	$In^{\cdot} + RH$	→	$InH^{\cdot} + R^{\cdot}$	Reaction 1
Reaction:	$R^{\cdot} + O_2$	→	$ROO^{\cdot}$	Reaction 2 (Fast)
	$ROO^{\cdot} + RH$	→	$R^{\cdot} + ROOH$	Reaction 3
Termination:	$2ROO^{\cdot}$	→	Non-radical products	Reaction 4

Figure 5: Chain sequence for free radical autoxidation (134).

Since some free radical production is inevitable in cells, and lipid peroxidation is the major consequence of free radical action, several finely balanced enzymatic and non-enzymatic (vitamin and mineral) antioxidant defence mechanisms have evolved to protect cells (62). This suggests that even slight alterations in antioxidant balance, through exposure of cells to sources which overwhelm antioxidant defences, or inadequate uptake of nutrients such as vitamin E, could contribute to a prooxidant state and the development of various cancers.

1.3.1 FREE RADICALS, LIPID PEROXIDATION AND CANCER

Substantial evidence has implicated free radicals, particularly those derived from oxygen, in both the initiation (8,135,136) and promotion (5,7-9,135-137) stages of carcinogenesis. Numerous tumour initiators have been shown to produce oxygen radicals during their action. Furthermore in recent years it has become clear that free radicals may be involved in the enzymatic activation of various chemical carcinogens. One example is the reaction of peroxyl radicals with 7,8-dihydroxy-7,8-dihydro-benzo-(a)-pyrene which results in the formation of dihydrodiolepoxide, the ultimate carcinogenic form of benza-(a)-pyrene (8). Many classes of tumour promoters are believed to act through an oxidant mechanism (7-9,12).

Although evidence at present suggests a link between the generation of free radical species and tumour initiation (8,136) and promotion (5,7-9,136,137), the molecular mechanisms involved in alteration of a normal cell to a malignant cell are unclear. One potential mechanism by which free radicals may modify phenotypic expression is through interaction with a variety of macromolecules within the cell (5,7-9,132). DNA is one potential target for free radicals. Damage to DNA by these radical species

can cause mutations that may be carcinogenic (5,7-9,62,121). Recently lipid peroxidation, a consequence of free radical attack of PUFAs, has been linked to events promoting carcinogenesis (138,139). It has been suggested that peroxidation products such as malondialdehyde (MDA) (7,138-140) and 4-hydroxynonenal (135,138) are potential mutagens capable of binding to DNA and causing DNA damage and mutagenesis. Furthermore ROO· derived from peroxidation of PUFAs, and hydroxyl radicals have been implicated in protein damage. Free radical protein damage is often complex and irreversible, and frequently leads to large scale structural changes such as protein unfolding, polypeptide chain scission and crosslinking (132). These structural alterations of proteins within and around the cell may play an important role in transformation of normal cells to tumour cells.

1.4 CELL MEMBRANES

Biological membranes are essential for cellular life and function as compartmentalizing structures, separating tissues from cells, and cells into organelles. This compartmentalising effect of membranes not only brings together molecules taking part in metabolic reactions, but also regulates the concentration of ions and molecules within these compartments. Furthermore cell membranes allow specific and spatial organisation of macromelecular complexes, which function as sensors of external signals and enable the cells to change their behaviour in response to these signals (98,141). Despite their varying functions, cell membranes have a common structure. The currently accepted model of membrane structure was put forward by Singer and Nicholson in 1972. They proposed a fluid mosaic model for the gross structural organisation of biological membranes (98,142,143). In this model, biological membranes are believed to be a dynamic, irregular lipid mixture of phospholipids and cholesterol with a heterogenous set of globular proteins embedded across the membranes to varying degrees (98,141-145). Phospholipids are the most abundant lipids found in cellular membranes (141,143). These lipid molecules are amphipathic due to their polar head groups and non polar hydrocarbon tails. This amphipathic nature allows phospholipids to spontaneously form bilayers in aqueous solutions (98,141,143). Apart from their role in bilayer arrangement, phospholipids may be closely associated with integral proteins or loosely associated with peripheral proteins (98,141-143,145). These proteins have important cellular functions as receptors (141,142,144,146-148), transporters (142,146,148) and enzymes (144,146-148). The protein content of membranes varies among different membranes and appears to be directly related to the functional role of these membranes (142). Besides their self-sealing properties, lipid bilayers have numerous other properties

which are believed to be important for maintenance of cellular functions (141). The most important of these is fluidity, which is believed to be important for the functioning of those proteins whose actions depend on mobility within the plane of the membrane (147). Changes in membrane fluidity through physical and chemical perturbation are believed to alter numerous membrane functions such as receptor characteristics (146,147,149), transport (144-146,150-152), and activities of membrane-bound enzymes (144-149,153-157).

1.4.1 TUMOUR CELL MEMBRANES

Membrane fluidity is now believed to play an important role in processes ranging from passive permeability and lateral diffusion to cell recognition, differentiation and malignant transformation (158). Cell membranes isolated from tumours show significant alterations in membrane composition (159-161), structural organisation (143,159-162), and functional properties (159,160). One important characteristic of tumour cell membranes is that they generally exhibit decreased membrane fluidity (39,159,161). These changes in physical (membrane fluidity) and chemical properties of tumour cell membranes are believed to be due to a free radical-mediated mechanism involving lipid peroxidation (159). Numerous studies have shown that oxidative modification of PUFAs by lipid peroxidation results in a significant decrease in membrane fluidity (increased membrane rigidity) (150-152,156,159,163,164). This peroxidation-induced alteration in membrane fluidity is believed to be due to a decrease in unsaturated:saturated fatty acid ratio, a change in chain length percentage distribution of fatty acids, and possibly covalent crosslinks between adjacent lipid radicals formed in membranes (151,152).

1.5 PROSTAGLANDIN H SYNTHASE AND PROSTAGLANDINS

PG synthesis is characterised by three distinct phases. These are; a) activation of specific PLA_2 and release of the precursor fatty acid AA; b) sequential conversion of AA by PGH synthase to prostaglandin G_2 (PGG_2) and then prostaglandin H_2 (PGH_2); c) isomerisation or reduction of PGH_2 to biologically important prostanoids *eg* prostaglandin D_2 (PGD_2), E_2 (PGE_2), $F_{2\alpha}$ ($PGF_{2\alpha}$), I_2 (PGI_2) and thromboxane A_2 (TXA_2) (165,166). The rate of synthesis of PGs is believed to be regulated by PLA_2 activity and AA release, while net production of PGs is dependant on the levels of the PGH synthase enzyme (166).

1.5.1 STRUCTURE AND PROPERTIES OF PROSTAGLANDIN H SYNTHASE ENZYME

The PGH synthase (Ec 1.14.99.1) is found in virtually all mammalian tissues (167-170), and functions primarily to catalyse the first committed step in the AA cascade pathway that produces PGs, thromboxanes (TXs) and prostacyclins (165,166,171-174). This enzyme is a haemoprotein which has two activities, a COX activity which catalyses the bis-dioxygenation of AA to hydroperoxy endoperoxide PGG_2 and a peroxidase activity which reduces PGG_2 to its 15-hydroperoxy analogue PGH_2 (165-169,173-179). Both activities require heme (167-169,174,176,178,179) and are believed to reside in a single polypeptide of MW 69 000 (167).

The COX reaction requires fatty acid substrates containing at least three methylene-interrupted *cis*-double bonds (165,167,180) and the continued presence of a hydroperoxide activator (165,168,169, 175,176,181-183) during catalysis. The most common COX substrate *in vivo* is AA (165), although a variety of hydroperoxides can trigger the COX activity (168,182) of which lipid peroxides generated by LOX activity are the most potent (182). Numerous non-steroidal anti-inflammatory drugs compete with substrate AA for binding to the cyclooxygenase active site and result in irreversible inactivation of the cyclooxygenase enzyme (167,174). The peroxidase activity of PGH synthase on the other hand is not inhibited by non-steroidal anti-inflammatory drugs and exhibits less substrate specificity (165,171,179,184,185). PG hydroperoxidases, like most peroxidases, utilize a wide variety of hydroperoxides and reducing substrates (171,177,184,185).

Both the COX and peroxidase activities of PGH synthase enzyme are irreversibly inactivated following several thousand turnovers (approximately 1 in 5 000) (171,186). It has been proposed that this inactivation results from the attack of a free radical species (oxidising agent) formed by the hydroperoxidase activity. Such a deactivation process can be partially prevented but not reversed by a variety of reducing agents, resulting in an increased number of enzyme turnovers (171,184,185,187). The presence of a highly efficient hydroperoxide reducing activity appears essential for the protection of COX capacity of PGH synthase. At present two isoforms of PGH synthase are believed to occur, a PGH synthase-1 (COX-1) enzyme and a PGH synthase-2 (COX-2) enzyme (170,174,188-194). These two enzymes, although similar at the protein level, differ markedly at the gene level (194). The COX-1 enzyme is believed to be constitutively expressed (174,188-190,192,194) and is characterised by a 72 kDa, membrane bound, N-glycosylated heamoprotein (170,174). Most of the current knowledge on PGH synthase enzyme structure and function has come

from studies with the COX-1 enzyme (174). It is generally believed that this enzyme is located in the endoplasmic reticulum and/or nuclear membrane (170,172-174,195), although other studies have shown the presence of COX-1 in plasma membranes (196) and non-membrane bound lipid bodies (197). Figure 7 illustrates the predicted topology of the cyclooxygenase enzyme in the endoplasmic reticulum with its various functional domains. In this model the terminal amino (NH_2) and carboxyl (COOH) groups are separated by a single transmembrane spanning region (see figure 6). The COOH-terminal end of the protein is believed to be cytoplasmic and contains both the peroxidase and COX domains. The NH_2-terminal portion of the protein on the other hand has three glycosylation domains and possibly an epidermal growth factor-like domain (173,198).

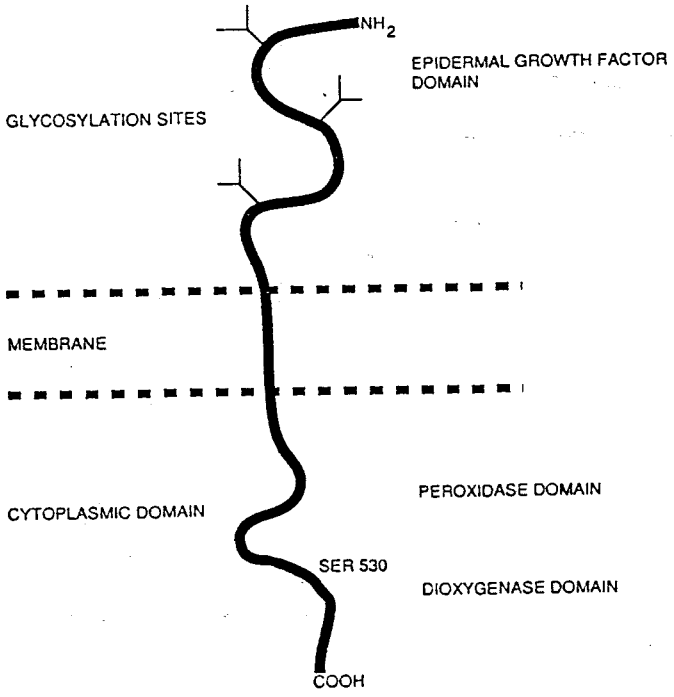


Figure 6: Predicted topology of cyclooxygenase enzyme in the endoplasmic reticulum (173).

The COX-2 enzyme is not expressed constitutively but rather induced rapidly and transiently by a variety of factors such as reactive oxygen species (199), PGE_2 (189,192), $PGF_{2\alpha}$, PGD_2 (189), interleukin-1β (191) and lipopolyssacharides (188,192). COX-2, like COX-1, is located in the endoplasmic reticulum and nuclear membrane (174). Comparison of the primary amino acid sequences of COX-1 and COX-2 have shown dissimilar signal peptides in these enzymes and a unique C terminal segment of 18 amino acids, present only in the COX-2 enzyme (170,174).

1.5.2 MECHANISM OF PROSTAGLANDIN H SYNTHASE ACTION

AA, or 5,8,11,14-eicosatetranoeic carboxylic acid, is a 20 carbon PUFA which contains 4 carbon double bonds at C 5,8,11 and 14. This PUFA is a precursor fatty acid for the two-series PGs (200). The conversion of AA to PGG_2 (175,178,181-183,186,201) and PGG_2 to PGH_2 (175,178,179, 181,183-187,201,202) has been extensively studied and is shown schematically in figure 7.

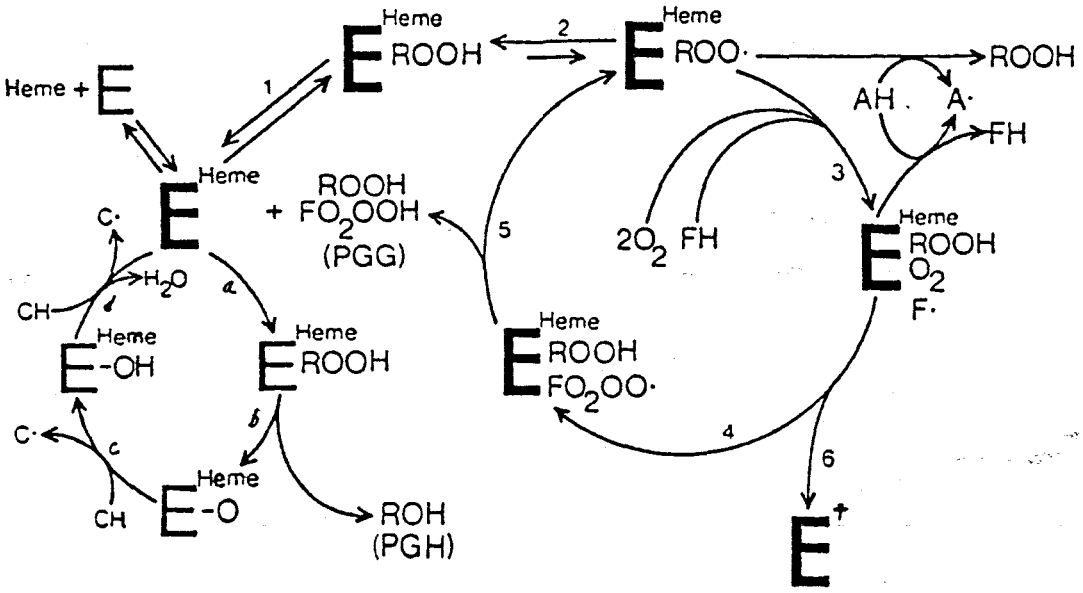


Figure 7: Proposed mechanism for prostaglandin H synthase enzyme (168). (Steps 1-6 represent the COX activity while steps a-d represent those catalyzed by the peroxidase activity. FH- PUFA, F- Lipid radical, CH- Reducing reagent).

When the COX reaction is initiated, ROOH interacts with the enzyme (E^{HEME}) to form an enzyme-bound hydroperoxy radical (E^{HEME}-ROOH-F·0_2) (175,181). Next the FA radical reacts with oxygen at carbon 11 resulting in cyclisation and the addition of another oxygen molecule at C15 (173,175,181,202). To complete the cycle, the resulting 15-hydroperoxy radical (E^{HEME}-ROOH-FO_2·) abstracts a hydrogen from the enzyme-bound ROOH to yield the end product PGG_2 and initiates another cycle (175,186). The self-catalyzed destruction of the COX enzyme is represented in figure 7 by reaction 6. This self-catalyzed inactivation is a separate feature intrinsic to COX catalysis (181) and is believed to result from the intramolecular collapse of the enzyme (175).

The peroxidase enzyme converts PGG_2 to PGH_2 by reducing the hydroperoxide group on C15 of PGG_2 to a hydroxyl group (177,179,184,186). Peroxidase cosubstrates (reducing agents) significantly increase COX activity prior to self-catalyzed inactivation (175,181). During peroxidase activity (steps a to d in figure 7), non-productive oxidised forms of the COX enzyme (E^{HEME}-O) occur. Various reducing agents convert these inactive oxidised forms of the COX back to an active reduced form (E^{HEME}), and in turn stimulate PGH synthase activity (181). This suggests that vitamin E with its ability to act as a reducing agent could increase the activity of the PGH synthase enzyme by preventing the formation of these oxidised forms of the COX enzyme.

1.5.3 PROSTAGLANDINS

In the early 1930s studies by Kurzok and Lieb revealed that human semen contained a factor which caused the uterine tissue to contract or relax violently. At about the same time van Euler characterised the biological effects and the chemical nature of this unknown factor and described it as an acidic lipid which he later named PG (203,204). It is now well established that PGs are a series of closely related 20 carbon unsaturated fatty acids (203,205-207), derived both enzymatically and non-enzymatically from AA (204,205).

1.5.4 BIOSYNTHESIS OF PROSTAGLANDINS

PGs are not stored to any great extent in mammalian tissues (203,208-212), but are rapidly synthesised upon physiological simulation (208,209,212). This process involves release of the precursor fatty acid AA and subsequent oxidation by PGH synthase to endoperoxide PGH_2 (see section 1.5.2). PGH_2 is an important intermediate in that it occupies a pivotal position in the divergent pathways (figure 8) which lead to the synthesis of various PGs and TXs (165,167,169,173,208,209).

The amount and type of PG synthesised by a cell depends largely on the cell or tissue type (165,166,169,200,205,209,213), although most PG synthesising cells predominantly synthesise one type of PG due to the existence of a single PGH_2 metabolising enzyme (165,169). The formation of biologically active PGs from PGH_2 occurs through the action of specific synthase enzymes called PGD synthase, PGE synthase, PGF synthase and PGI synthase respectively (165,169,173).

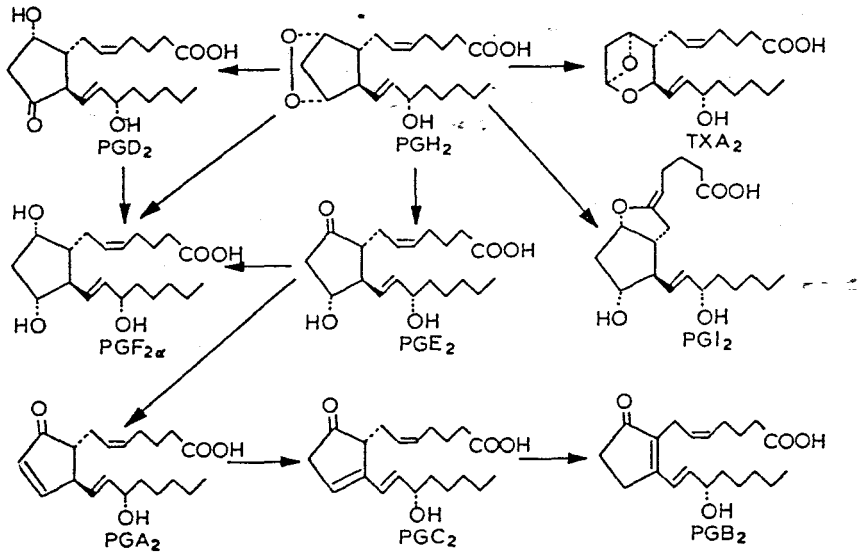


Figure 8: Divergent biosynthetic pathways of Prostaglandin and Thromboxane synthesis (169).

1.5.5 PROSTAGLANDIN SYNTHASE ENZYMES

1.5.5.1 Prostaglandin D synthase

This enzyme is found predominantly in the cytosol of the cell (169,195,204), where it functions to convert PGH_2 to PGD_2 (165,169,173,176,195). This conversion involves the isomerisation of the 9,11-endoperoxide moiety of PGH_2 to 9 α-hydroxy and 11-keto groups (figure 8) (165,169).

1.5.5.2 Prostaglandin E synthase

PGE synthase catalyses the conversion of PGH_2 to PGE_2, and is involved in the isomerisation of 9,11 endoperoxide to 9-keto and 11α-hydroxyl groups (see figure 8). This enzyme is membrane bound (195) and requires coenzyme glutathione for activity (169). The formation of PGE_2 from $PGF_{2\alpha}$ may also occur in the presence of the enzyme 9-hydroxy dehydrogenase (204).

1.5.5.3 Prostaglandin F synthase

Figure 8 depicts three possible ways in which synthesis of $PGF_{2\alpha}$ can occur. In the first mechanism, PGF synthase enzyme catalyses the reductive cleavage of the 9,11-endoperoxide of PGH_2 to form

$PGF_{2\alpha}$, although at present the nature of the enzyme and reducing agent remain unclear (169,214). The second pathway involves the conversion of PGE_2 to $PGF_{2\alpha}$. This reaction is catalysed by the enzyme 9-keto reductase and involves the reduction of the keto group at C-9 in PGE_2 to a hydroxyl group ($PGF_{2\alpha}$) (169,200,204,214). The reduction of the 11-keto group of PGD_2 via the action of 11-keto-reductase is a third possible mechanism through which PGF synthesis may occur (169,205,214).

1.5.5.4 Prostaglandin I synthase

The formation of PGI_2 from PGH_2 is catalysed by PGI synthase and involves the isomerisation of 9,11-endoperoxide of PGH_2 into a 6,9-epoxide and an 11α-hydroxyl group (see figure 8) (169). PGI synthase is a membrane bound enzyme (176,196) which is inactivated by a variety of lipid hydroperoxides (169,176,215-217) and oxygen-containing free radicals generated during PGH synthase activity (200).

1.5.6 FUNCTIONS OF PROSTAGLANDINS

PGs are cell-to-cell messengers produced by eukaryotic cells in response to external stimuli (213). Over the past few years, PGs have attracted a great deal of attention due to their versatility and wide range of biological effects (203,204,207,210). These effects may be specific within a cell, although in general PGs effects are based on certain broad processes such as regulation of blood pressure, reproduction, neural and cardiovascular systems (203,207,210). Furthermore these wide-ranging effects are believed to be mediated via a common mechanism involving alterations in cAMP levels (203,207,210,218). Evidence suggests that PG production is related to cell growth (210,219), and that cells in the quiescent state are the most active PG producers (219).

1.5.6.1 Prostaglandin E_2 Functions

PGE_2 is known to be a potent vasodilator (173,200,203) and bronchodilator (173,200,203,204) *in vivo*. In addition to this, E_2 PGs are believed to regulate the cell kinetics of the gastrointestinal epithelium cells (220), and are capable of increasing cAMP levels in certain cells via the activation of the adenylate cyclase enzyme (210,218,221-224).

1.5.6.2 Prostaglandin D_2 Functions

The biological function of PGD_2 is at present unclear (225) although PGD_2 has been described as an antithrombotic agent (176,225,226) and neuromodulator (169,225). Furthermore PGD_2 has been shown to inhibit DNA (225-230), RNA and protein synthesis in various cell lines (225,229,230).

1.5.6.3 Prostaglandin $F_{2\alpha}$ Functions

$PGF_{2\alpha}$ is a potent vasoconstrictor (173,200,203) and bronchoconstrictor (173,200,204). These effects are in contrast to PGE_2 effects, and have important implications in certain systems such as the cardiovascular (203,210) and pulmonary systems (173,210).

1.5.6.4 Prostaglandin I_2 Functions

PGI_2 is a potent inhibitor of platelet aggregation (173,176,210,226) and in some systems is thought to act as a vasodilator (173,176,210). In addition to this, PGI_2 is believed to elevate cAMP levels in various cell lines (176,223,224), possibly via a PGI_2 receptor-linked AC enzyme (176). This PGI_2-induced increase in cAMP levels is believed to mediate anti-aggregatory properties of PGI_2 on platelets (176).

1.5.7 PROSTAGLANDINS AND CANCER

PGs and related eicosanoids have been implicated as modulators of tumour metastasis (205,207,209,231-235), tumour promotion (205,207,209-211,234,236), host immunoregulation (205,206,211,233-235,237) and cell proliferation (205,209,211,218,221,222,234,237-241). This suggests that an oversynthesis or imbalance in PG synthesis could promote tumour cell development (210). Some of the earliest studies to demonstrate a link between PGs and carcinogenic tissue were those of Williams in 1966 (cited in 207,210,241), in which elevated levels of PGE_2 and $PGF_{2\alpha}$ were found in tumour tissue and plasma of patients with medullary carcinoma of the thyroid. Since these discoveries, a large body of experimental evidence has suggested that PG levels, in particular PGs of the E-series, were greatly enhanced in a number of tumour cells (205-207,209,210,234,241-244). These prostaglandins are potent immune suppressors, and are believed to facilitate tumour growth by compromising the immune system (206,207,232,234,235,245,246). In contrast, other researchers have

proposed that increases in PG synthesis observed in tumour cells may represent a homeostatic response directed towards limiting tumour cell growth (207,210). PGE_2 has been shown to significantly inhibit the growth of various tumour cell lines, both *in vitro* (247-251) and *in vivo* (252,253). This inhibitory effect is thought to be associated with activation of the AC enzyme and synthesis of cAMP (205,248).

It is well established that tumour cells can induce platelet aggregation (cited in 232). This inherent property may play an important role in guaranteeing the survival of tumour cells and their ability to metastasise (254). Both PGD_2 and PGI_2 are potent inhibitors of platelet aggregation (see section 1.5.6.2 and 1.5.6.4). The metastatic potential of tumour cells is believed to be inversely related to PGD_2 (255-257) and PGI_2 (254) formation in these cells. Furthermore, both PGD_2 (227-230,258-260) and PGI_2 (261) have been shown to significantly inhibit the growth of various tumour cell lines *in vitro*.

1.6 CALCIUM

Calcium ions are the most abundant cations found in vertebrates, making up approximately 20-30g per kilogram of body weight in humans. The majority of this calcium is trapped in bones where it plays both a structural and functional role (262). In this study the association of Ca^{2+} with COX, LOX, PLA_2, AC and phosphodiesterase (PDE) activity will be highlighted.

1.6.1 CHARACTERISTIC PROPERTIES OF CALCIUM

Although $Ca^{2+}$is present in large amounts in the diet, its function for the survival of an organism is so important that cells have developed numerous ways to utilise these large Ca^{2+} deposits (262). To date Ca^{2+} ions, characterised by their charge, coordination number, and unhydrated radius (263), are known to play an important role in processes such as muscular contraction, stimulus secretion coupling, transduction of hormonal information and neuronal conduction (145). More recently the discovery of calcium-dependant regulatory proteins has suggested a role for Ca^{2+} in the modulation of various metabolic processes within the cell (263-265). One such Ca^{2+} binding protein is calmodulin, a ubiquitous, acidic low molecular weight Ca^{2+} binding protein whose role in mammalian cells is to modulate the activity of a large number of enzymes (265,266).

1.6.2 FUNCTIONS OF CALCIUM

1.6.2.1 Calcium and enzymes

PLA_2 (267-274), AC (263,265,266,275), PDE (263,264,275), COX and LOX (276) are all examples of enzymes whose activities are modulated by Ca^{2+} or Ca^{2+}-binding proteins such as calmodulin. The role of Ca^{2+} in regulating PLA_2 activity is believed to be 3-fold, namely through activation of protein kinase C and subsequent phosphorylation of PLA_2, direct stimulation of PLA_2 activity and through a Ca^{2+} requirement of G-proteins involved in PLA_2 activation (267,272). AC enzymes are closely associated with sites of Ca^{2+} entry (263,277) into the cell, where they form a reversible Ca^{2+}-dependant complex with calmodulin during Ca^{2+} influx (263). Furthermore it has been proposed that millimolar concentrations of Ca^{2+} may alter membrane fluidity in a variety of tissues and that these changes in bilayer fluidity may affect the activities of a number of membrane-bound enzymes (cited in 145).

1.6.2.2 Calcium and cell proliferation

Ca^{2+} metabolism is of paramount importance to cell proliferation, and may be involved in the regulation of numerous biochemical activities (278,279). Ca^{2+} availability is the key regulator of cell proliferation in multicellular organisms, and a defect in the Ca^{2+} system is believed to be the common cause of unrestrained proliferation in cancer cells (278).

Ca^{2+} (278,280,281), as well as calmodulin (279,281,282), have been implicated in the control of some of the most important transitions of the cell cycle, suggesting that proliferation rates of both normal and cancer cells may be controlled by the availability of Ca^{2+}/calmodulin within the cell at specific points and times during the cell cycle. Unlike normal cells, malignant cells loose the ability to maintain cytoplasmic Ca^{2+} concentrations below a critical point (283), and as a result hypercalceamia is a common complication of various types of cancers (284-287). One important consequence of this is that Ca^{2+}requirements of neoplastic cells are very low in comparison to normal cells (278). Numerous *in vitro* studies (279,288,289) have shown that various neoplastic cell lines have the ability to proliferate in Ca^{2+}deficient media, whereas non-neoplastic cells require the presence of Ca^{2+} supplemented media for growth. This suggests that the unrestrained growth observed in various cancers may be due to a bypass or overriding of Ca^{2+}control processes present in normal

cells.

1.7 PHOSPHOLIPASE A_2

Over the last few years, knowledge of the diversity of forms and functions of mammalian phopholipases has increased to a great extent, although the importance and functions of various PLA_2 isoforms have yet to be fully defined.

1.7.1 CLASSIFICATION AND PROPERTIES OF PHOSPHOLIPASE A_2

PLA_2 activity has been found in almost every cell where it has been sought (290). The A_2 phopholipases are a heterogenous family of enzymes, classified into low MW 14 kDa secretory PLA_2 ($sPLA_2$) and the more recently identified high MW 60-110 kDa cytosolic PLA_2 ($cPLA_2$) (291-297). The most thoroughly characterised PLA_2s are the 14 kDa $sPLA_2$s. Mammalian group II PLA_2s are found abundantly at inflammatory loci, and are believed to play an important role in both the initiation and progression stages of inflammatory responses (291,298). The 14 kDa PLA_2 enzyme has been biochemically characterised as active at neutral to alkali pHs (293,295) and requires Ca^{2+} in the millimolar range for typical activity (272,293-296,299). Furthermore this enzyme shows no selectivity towards fatty acids at Sn2 position of phospholipids (292-294,299). The 85 kDa $cPLA_2$ is present in the cytosol of many cells and differs from the 14 kDa PLA_2 enzymes, in that it is translocated to the membranes in response to μM Ca^{2+} concentrations and preferentially hydrolyses arachidonic acid esterified at Sn-2 position of phospholipids (291,293,295,297,299). In addition to this, $cPLA_2$s have Ca^{2+} binding sites similar to protein kinase C and GTPase activating protein (299).

Numerous factors influence the activity of PLA_2 enzymes within a cell. Studies with both pancreatic and serum PLA_2s have shown that substitution of Ca^{2+} with various other ions such as Sr^{2+} completely inhibits the activity of these enzymes (300). PLA_2 activity is enhanced when non-ionic phospholipids are hydrolysed as opposed to hydrolysis of neutral phospholipids (268,290,292,300, 301). This increase in PLA_2 activity is due to anionic phospholipids enhancing the affinity of PLA_2 enzymes for Ca^{2+} (268,292). The activity of PLA_2 in human platelets has been shown to be significantly increased when separated from endogenous lipid inhibitors (302,303). Evidence suggests that the endogenous lipid inhibitor may be associated with a mixture of PUFAs (302), and furthermore that PUFAs, in particular AA, inhibit PLA_2 either competitively (304) or non-

competitively (302). This inhibition of PLA_2 activity by AA suggests that a negative feedback mechanism may be operating to regulate PLA_2 activity (304) and the levels of free AA within a cell (274). In contrast to endogenous inhibitors, PLA_2-activating proteins (PLAP) have been identified (293,305) which function to stimulate PLA_2 activity by directly interacting with the enzyme (293,295,305).

1.7.2 PHOSPHOLIPASE A_2 FUNCTIONS

PLA_2 plays a key role in membrane turnover, membrane remodelling, exocytosis (297) and repair of oxidative damage (297,306). In addition to this, PLA_2 plays a crucial role in normal cellular metabolism, where it functions to produce rate-limiting substrate AA for synthesis of important lipid mediators such as eicosanoids (273,291-297,299,304) and platelet activating factor (272,291-294,297,299). Products of PLA_2 catalysis, such as free fatty acids and lysophospho- glycerides, may also function as intracellular or intramembrane signalling molecules (297,300).

1.7.3 REGULATION OF PHOSPHOLIPASE A_2 ACTIVITIES BY G-PROTEINS

Two major routes of receptor-mediated regulation have been implicated in the control of AA mobilisation and release (293). These two routes include direct activation of PLA_2 via a receptor-linked G-protein (272,293,307-318) and indirect activation through a phospholipase C-mediated pathway, increasing Ca^{2+} levels (269,274,293) and activating protein kinase C (267,270,274,293, 299,308,310,312).

Several receptors have now been characterised that are coupled to G-proteins (319). These G-proteins are found in all eukaryotic organisms and are believed to play an important role in linking surface receptors to membrane-bound effector proteins (319,320). Although several G-proteins have been characterised, the G-proteins that are coupled to PLA_2 enzymes have not been identified (307). Numerous studies (308,316-318) however have shown that G-protein-dependant PLA_2 activity exhibits characteristics which deviate from a typical G-protein-coupled effector system (308) in that $\beta\gamma$ subunits stimulate PLA_2 activity while α-subunit inhibit the enzyme by facilitating reassociation of heterotrimeric complex ($\alpha\beta\gamma$) of G-protein.

1.8 ADENYLATE CYCLASE, PHOSPHODIESTERASE AND CYCLIC ADENOSINE MONOPHOSPHATE

The AC enzyme was first discovered in 1957 by Sutherland and Rall (321). This enzyme catalyses the conversion of adenosine triphosphate (ATP) to cAMP (265,321), an important intracellular mediator of the action of various hormones (157,265,322-324) such as biogenic amines, proteins, polypeptides and some PGs (265). PDE enzymes on the other hand catalyse the non-reversible hydrolysis of cAMP to 5`-AMP (324-326). These enzymes share with the adenylate cyclases the important functions of both the delicate control of cyclic nucleotide levels and their myriad important effects within the cell (324,326).

1.8.1 ADENYLATE CYCLASE STRUCTURE AND PROPERTIES

The AC enzyme is a membrane bound enzyme (157,321,323) found in most types of mammalian cells (168,233), lower animals, unicellular organisms, bacteria and probably plants (321). Cloning of these enzymes has revealed that ACs are large polypeptides (figure 9) of 1080-1248 amino acids which cross the membranes 12 times in two cassettes of 6 transmembrane-spanning domains, with each cassette followed by a large cytosolic domain (277,327).

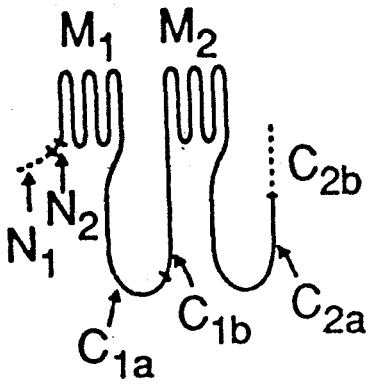


Figure 9: Structural model of adenylate cyclase (modified 327). (N- amino terminal region of proteins; M_1 and M_2 - first and second membrane-spanning domains. C_1 and C_2 -predicated cytoplasmic domains).

Initially the hormone sensitive AC was believed to be composed of three distinct protein components, namely a receptor for binding hormones, a catalytic unit for producing cAMP from ATP, and a

guanine nucleotide binding protein (G-protein), which acts as a transducer between receptor and catalytic subunits (144,145,154,157,265,328-330). More recently however, the hormonally regulated adenylate cyclase system was shown to include 5 components: a stimulatory receptor (Rs); a stimulatory guanine nucleotide binding protein (G_s) composed of 3 subunits α, β and γ; a catalytic moiety of AC; an inhibitory receptor (Ri); and an inhibiting GTP-regulatory protein (Gi), composed of α, β and γ subunits (145). These components are asymmetrically dispersed within the plasma membranes (145,157), such that the receptor components which interact with specific circulating hormones face the external surface of the cell, while the catalytic unit responsible for the formation of cAMP from ATP faces the inside of the intact cells (145,265,331). These components are believed to be able to undergo independent lateral diffusion within the plane of the membrane, interacting functionally and structurally only in the presence of appropriate stimulatory ligands (145,157,265).

1.8.2 PROPERTIES OF ADENYLATE CYCLASE COMPONENTS

1.8.2.1 Receptor component

Receptors for various hormones are individual proteins distinct from adenylate cyclase enzyme. These receptors are located on the external surface of the cell and interact with the membranes via a small hydrophobic region (265). Various hormones which act via the AC enzyme are believed to recognise and bind to specific receptors on the cell surface (332-335), although some receptors are able to bind more than one hormone (265).

1.8.2.2 Nucleotide regulatory proteins

The G-proteins, located on the cytosol-facing half of the membrane (145), play an important role in that they modulate the activity of the hormone-sensitive AC enzyme (265,328,329,336,337) and exert specific regulatory effects on agonist binding to receptors (265,329). The AC enzyme is believed to possess two separate guanine nucleotide regulatory sites. These include a Gi or Ni site which mediates the inhibition of the AC enzyme (154,274,306,311,321,322,337-339) and a Gs or Ns site which stimulates the AC enzyme (154,274,321,322,328,337,338). These regulatory proteins are important in that they functionally couple agonist occupancy with activation of the AC catalytic moiety (329).

1.8.2.3 Catalytic component

This component has a relatively large hydrophobic surface area (145,265), and is believed to have a substrate site for ATP, as well as a binding site for the divalent cation magnesium (Mg^{2+}). Binding of the ATP and Mg^{2+} to the catalytic unit presumably occurs as a Mg^{2+}-ATP complex (265), and is essential for AC activity (274).

1.8.3 MECHANISM OF ADENYLATE CYCLASE ACTION

Guanosine triphosphate (GTP) plays an important role in the regulation of the AC enzyme activity (328). Under conditions where GTP levels are low, hormonal activation of the AC enzyme occurs through a mobile receptor mechanism, whereas in the presence of optimal or elevated levels of GTP, activation occurs via a collision coupling mechanism (145,157). In general however, hormonal activation of AC (figure 10) in the presence of GTP occurs through a collision coupling mechanism (145). The binding of a hormone to its receptor facilitates the interaction of the hormone receptor complex with stimulatory G-protein (Gs). This interaction results in the exchange of guanine diphosphate (GDP) for GTP at Gs, and subsequent release of the α-GTP complex. The α-GTP complex in turn activates the AC catalytic unit, leading to an increase in intracellular levels of cAMP (157,322,340). The adenylate cyclase enzyme is believed to possess a distinct Gi protein which mediates inhibition of AC enzyme (338-340). Stimulation of the inhibitory receptor (Ri) results in Gi coupling to the receptors, and inactivation of the AC enzyme (337,339).

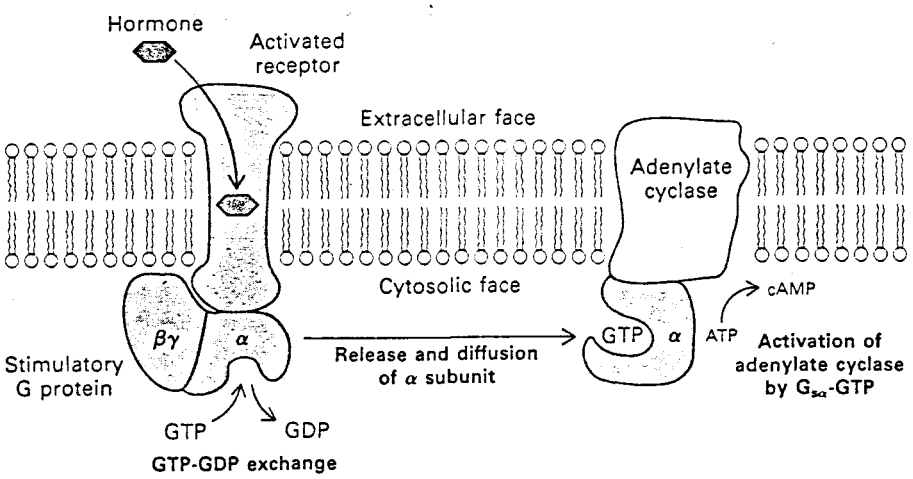


Figure 10: Hormonal activation of adenylate cyclase enzyme (340).

1.8.4 FACTORS INFLUENCING ADENYLATE CYCLASE ACTIVITY

1.8.4.1 Membrane fluidity

Like many integral membrane-bound enzymes, the activity of the AC enzyme is influenced by the nature of its membrane lipid environment (145,149,157). Physical properties of the lipid bilayer, such as the fluidity or rigidity of the membrane, can affect AC activity-presumably by altering the various constraints imposed upon the enzyme by the lipid bilayer (145). In general, an increase in membrane fluidity augments AC activity (145,157,341), whereas a decrease in membrane fluidity inhibits AC activity (157). More recently however, several studies have shown significant increases in adenylate cyclase activity when membrane fluidity decreases (147,149,cited in 154). The ability of membrane fluidity to alter AC activity is believed to be due to its effects on the conformational flexibility of intrinsic proteins (149,157) and/or alterations in the efficacy of the interaction of various components of the AC system (145,149,157).

1.8.4.2 Prostaglandins

In addition to membrane fluidity various PGs are known to influence AC activity (154,342). In general, the actions of these PGs are associated with activation of AC and increased intracellular cAMP levels (154,343,344), although in some cases PGs may inhibit AC activation (154,343). PGs mediate their effects on AC activity through binding to specific receptors on the plasma membranes (335,337,345). These receptors are believed to belong to a superfamily of G-protein coupled receptors (332). Binding of PGs to these receptors activates the receptor and results in the transfer of a signal via G-proteins to an effector component (332,346,347), such as the AC enzyme.

PGE_2 has been reported to have both stimulatory (332,344-349) and inhibitory (337,345,350) effects on AC activity. These dual effects of PGE_2 are believed to be due to PGE_2 receptors coupling to stimulatory Gs (332,345,347) and inhibitory Gi (332,337,345,350) proteins. In addition to PGE_2, prostaglandins I_2 (PGI_2) (333,351), PGD_2 (333) and $PGF_{2\alpha}$ (347) have all been shown to stimulate AC activity. These stimulatory effects of PGD_2, PGI_2 (332) and $PGF_{2\alpha}$ (347) are believed to be mediated via a receptor linked Gs protein.

1.8.4.3 Calcium

Both functional and ultrastructural studies have shown an intimate association between the AC enzyme and sites of Ca^{2+} entry into the cell (cited in 277). The Ca^{2+} binding protein, calmodulin, mediates many of the intracellular actions of Ca^{2+} (266).

1.8.5. CYCLIC ADENOSINE MONOPHOSPHATE

Since the discovery, by Earl Sutherland in the 1960s, that cAMP acts as a secondary messenger for the action of various polypeptide hormones, increased attention has focused on the role of cAMP in the control of many physiological processes at the cellular level (352-354).

1.8.5.1 Biological role of cyclic adenosine monophosphate

Cyclic adenosine monophosphate, the product of AC activity, is a ubiquitous molecule which regulates numerous functions within a cell (355,356). In normal cells cAMP functions to regulate numerous physiological phenomena which are of significance in transformed cells, such as cell morphology, adhesiveness to substratum, motility, growth and synthesis of proteoglycan (355-357). In addition to this, cAMP is believed to regulate the activity of various enzymes (355) in addition to increasing the differential rate of synthesis of various inducible enzymes (355,358) such as β-galactosidase and tryptophanase (358). More recently it has been suggested that cAMP modulates the activity of various oncogenes and growth factors (352).

Accumulated evidence indicates that cAMP plays an important role in the mammalian cell cycle, and that endogenous cAMP levels fluctuate considerably during various stages of this cycle (354,359-361). These fluctuating levels of cAMP are believed to play a significant role in regulating cell cycle activity (360,361). The cell cycle is composed of a regulated program of specific gene activations, transcriptions as well as surges of regulatory components and sequential bursts of enzyme activators (362). These events (figure 11) have been classified into 4 distinct stages, and include the G_1-phase, S-phase, M-phase (360,362) and G_o-phase (quiescent) (360) or probabilistic A state (362).

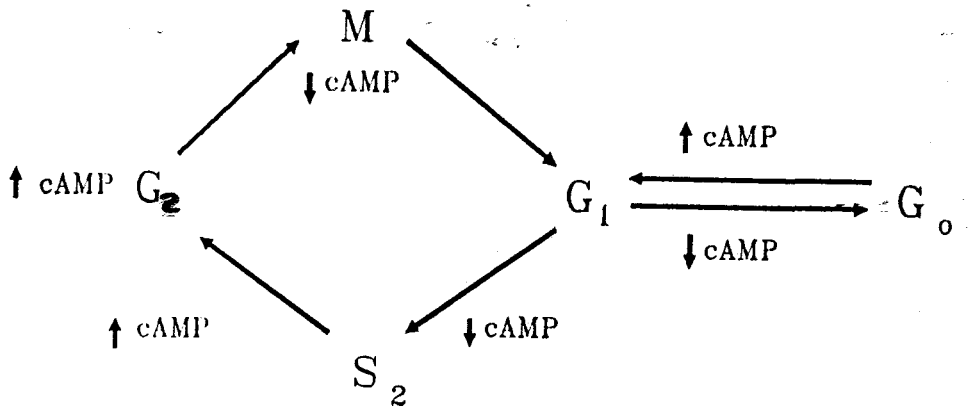


Figure 11: Model depicting various stages of the cell cycle. (G_o- Quiescent or resting phase, G_1- Prepares to replicate chromosomes, S- DNA synthesis, G_2- Preparation for mitosis and cytokinesis, M- Mitosis).

Although the G_o phase is depicted as a unique cell state, it is unclear whether this is in fact the case or whether it forms part of the G_1-phase (360). This G_o stage is believed to act as a transition phase into which the cell enters after completion of each cell cycle. The eukaryotic cell cycle begins with the birth of the cell at cytokinesis or cell division. As the cell ages, it enters the G_1 or Gap-1 phase where it prepares itself to replicate its chromosomes; then goes through an S-phase or DNA synthetic phase during which it replicates its chromosomes; then passes into a second gap or G_2-phase where it prepares for mitosis and cytokinesis; and finally enters the M-phase or mitosis phase, ending its life as an individual cell upon dividing into two new individuals (362).

cAMP exerts its inhibitory effects at numerous points in the cell cycle (figure 11). The G_1 phase is very sensitive to inhibition by shortages of essential nutrients and is most responsive to growth factors and hormones (360,362). Contact-inhibited cells have high levels of cAMP and are usually arrested in the early G_1 or G_o stage of the cell cycle (355). This suggests that a decrease in cAMP levels is an important signal for the transition of cells from a G_o state to a G_1 state, and that preventing a decrease in cAMP levels may inhibit proliferative capacity of a cell by retaining it in a G_o state (360,362,363). Upon release from the G_o state, a relatively prolonged cAMP surge occurs in the G_1 phase (354,360-362). A second surge in cAMP occurs in the late S phase or G_2 of some cells (354,359-362). These cAMP surges function as negative regulators of the cell cycle, inhibiting DNA

synthesis for substantial lengths of time in mid cycle (362). Transformed or malignant cells have cell cycles similar to those of normal cells, however the former cell types have lost the regulator (probably cAMP) which allows them to enter G_0 state and as a result continue to replicate (360).

1.8.5.2 Cyclic adenosine monophosphate and Prostaglandins

PG action is believed to be mediated through changes in the intracellular levels of secondary messengers such as cAMP (334,344,347,364). These effects on cAMP are believed to be stimulatory or inhibitory depending on the cell type (cited in 346). PGs of the D, E and I series are capable of increasing cAMP formation in various systems (345). PGE_2 has been shown to stimulate cAMP production in numerous cell lines such as gastric carcinoma (KATTO III) (248,347), mastocytoma P-815 (334), fibroblasts (365) and mouse embryo limb cell lines (366).

In addition to PGE_2, a number of other PGs such as PGI_2, PGD_2 (333,334,351) and $PGF_{2\alpha}$ (248,347,364,365) have been shown to stimulate cAMP production in various cell lines. PGs are known to influence the growth of not only normal cells but also tumour cells (347). Furthermore several studies (334,347,364,367) have suggested that cAMP mediates the growth inhibitory effects of these PGs. In general PGE_2 is believed to stimulate cAMP formation directly through a PGE_2-receptor-cAMP linked system (366), with the cAMP acting as a secondary messenger for PGE_2-mediated modulation of biological activity in various cells (368).

1.8.6 PHOSPHODIESTERASE

PDE was first discovered in 1958 by Sutherland and Hall (369-371). These enzymes, as previously mentioned, function to catalyse the hydrolysis of the 3'-bond of cyclic nucleotides to a non-cyclic product 5'-monophosphate derivative. Hydrolysis of cAMP by PDE enzymes is the major physiological pathway for the termination of the intracellular effects of cAMP (326).

1.8.6.1 Properties and structure of phosphodiesterase

PDE's occur widely in biological systems and are present in all mammalian tissues, with the exceptions of red blood cells and isolated rat adrenal cells. Since their discovery, numerous forms of the PDE enzyme have been shown to exist under a variety of conditions and from various sources

(326). The pattern and ratio of these PDE enzymes varies according to the cell or tissue type (371), and they are believed to differ in their kinetic behaviour, substrate specificity (326,371), subcellular localisation and possibly function (326). Although various studies (324,cited 326) have demonstrated the existence of multiple forms of the enzyme, it is now believed that two distinct forms of PDE exist (326). These two forms of the enzyme differ in a number of aspects, but primarily in their affinity for two naturally occurring substrates namely cAMP and cyclic guanosine-3',5'-monophosphate (cGMP) (324,353,369,370,372).

The high Km (low affinity) PDE (sPDE) enzyme is largely found in the soluble (cytosolic) portion of cell extracts and has a MW of approximately 400 000 (369,370). This enzyme exhibits a higher affinity for cGMP than for cAMP (324,326,353,369,370,372) and is believed to be sensitive to a calcium-dependant protein activator (326,370-372). The low Km (high affinity) PDE (pPDE) enzyme on the other hand has a MW of approximately 200 000 (369,370) and is mainly associated with various membranes. This enzyme hydrolyses cAMP in a negatively co-operative manner (353,369-371) and has a far higher affinity and specificity for cAMP than does the sPDE enzyme (324,326,353,369,370,372). Furthermore, several hormones as well as intracellular activators such as cAMP are known to stimulate the pPDE enzyme (326,370,372). This suggests that any agent capable of increasing intracellular cAMP levels such as the PGs (see section 1.8.5.2), could mediate their effects on the cell through the induction of the pPDE enzyme. The presence of various forms of the PDE raises the question as to which form of the enzyme is important in controlling intracellular cAMP levels. Because the intracellular levels of cAMP are in the μM range or lower, it has been suggested that only those PDEs with high affinity for cAMP *ie* low Km PDEs or pPDEs, could fulfil this role (326).

1.8.7 Adenylate cyclase, cyclic adenosine monophosphate, phosphodiesterase and cancer

Of the many diseases associated with altered cyclic nucleotide metabolism, cancer has received the most attention (371). Generally malignant or transformed cells in culture have been found to have lower levels of cAMP than normal or untransformed cells (353,357,361,373-377). Since intracellular levels of cAMP are determined by the activity of the AC and PDE enzymes (325,378), lower levels of cAMP in malignant cells may be due to alterations in one or both of these enzymes (354,361,379).

Numerous studies have shown significant decreases in basal (311,323,357,361,374,377,380) AC

activity, as well as a decreased responsiveness to various hormones (353,361,380,381) following transformation. These changes may be due to alterations in the catalytic component of the AC enzyme (353,357). The AC enzyme is believed to have two binding sites for the Mg^{2+}, one at the catalytic site of the enzyme and the other at the G_s site, which functions to stimulate the activity of the enzyme. During transformation, alterations in the G_s site are believed to occur, resulting in Mg^{2+} being unable to enhance AC activity. Furthermore the transformation of normal cells to tumour cells results in a number of changes in the plasma membranes (357,361). Since the adenylate cyclase enzyme is intimately associated with the plasma membrane, these changes could possibly alter AC receptor sites, resulting in decreased AC activity and lower intracellular levels of cAMP (361,374). Lipid peroxidation, as mentioned in section 1.3.1, is believed to be involved in tumour promotion (138,139). Once initiated, this process results in the polymerisation and depletion of membrane PUFAs, decreased membrane fluidity (150-152,156,159,163,169) and inhibition of AC activity (382,383). Although no universal correlation between AC activity and tumour growth rate has been shown in any studies to date, there is a much more consistent pattern during oncogenesis, when AC activity and cAMP levels are found to be elevated (354). At present however, no direct evidence exists to suggest that cAMP is a secondary messenger for the action of any oncogenes (352,354).

As regards PDE activity in normal and malignant cells, the activity is generally found to be lower in malignant cells (325,361), although some studies have reported significant increases (372,374,379). Like normal cells, alterations in PDE activity in malignant cells are regulated by intracellular cAMP levels (325). Furthermore it has been suggested that transformed cells show some selective alterations in the different forms of the enzymes (361). Transformed chicken embryo fibroblasts show significant increases in the ratio of low Km to high Km PDE enzymes when compared to their normal cell counterparts (374). This increase in the ratio of low Km to high Km PDE activity would account for lower levels of cAMP in transformed cells. Numerous *in vitro* studies (355,356,360,361,374,375,384-386) have shown an inverse relationship between intracellular cAMP levels and tumour cell proliferation.

Furthermore many of the characteristic properties of cancer cells, such as abnormal morphology, rapid growth rates, decreased adhesiveness and loss of contact inhibition of growth can be reverted towards those of normal cells, by treatment with dibutyryl cAMP (dcAMP) or agents which increase intracellular cAMP levels (355-357,361). It is reasonable to assume therefore that the abnormal properties of transformed cells could be a consequence of lower intracellular cAMP levels

(357,361,387) as a result of reduced AC activity, although numerous *in vivo* studies (388-392) have reported significant increases in cAMP levels in transformed cells as compared with their normal cell counterparts.

1.9 PROJECT OBJECTIVES

As previously mentioned, the mechanism(s) by which vitamin E and its derivatives exert anticarcinogenic effects *in vitro* are unclear, although it has been suggested that these effects may be due to their antioxidant properties and/or their effect on various metabolic pathways such as the AA cascade pathway shown in figure 4 (repeat of figure on page 13).

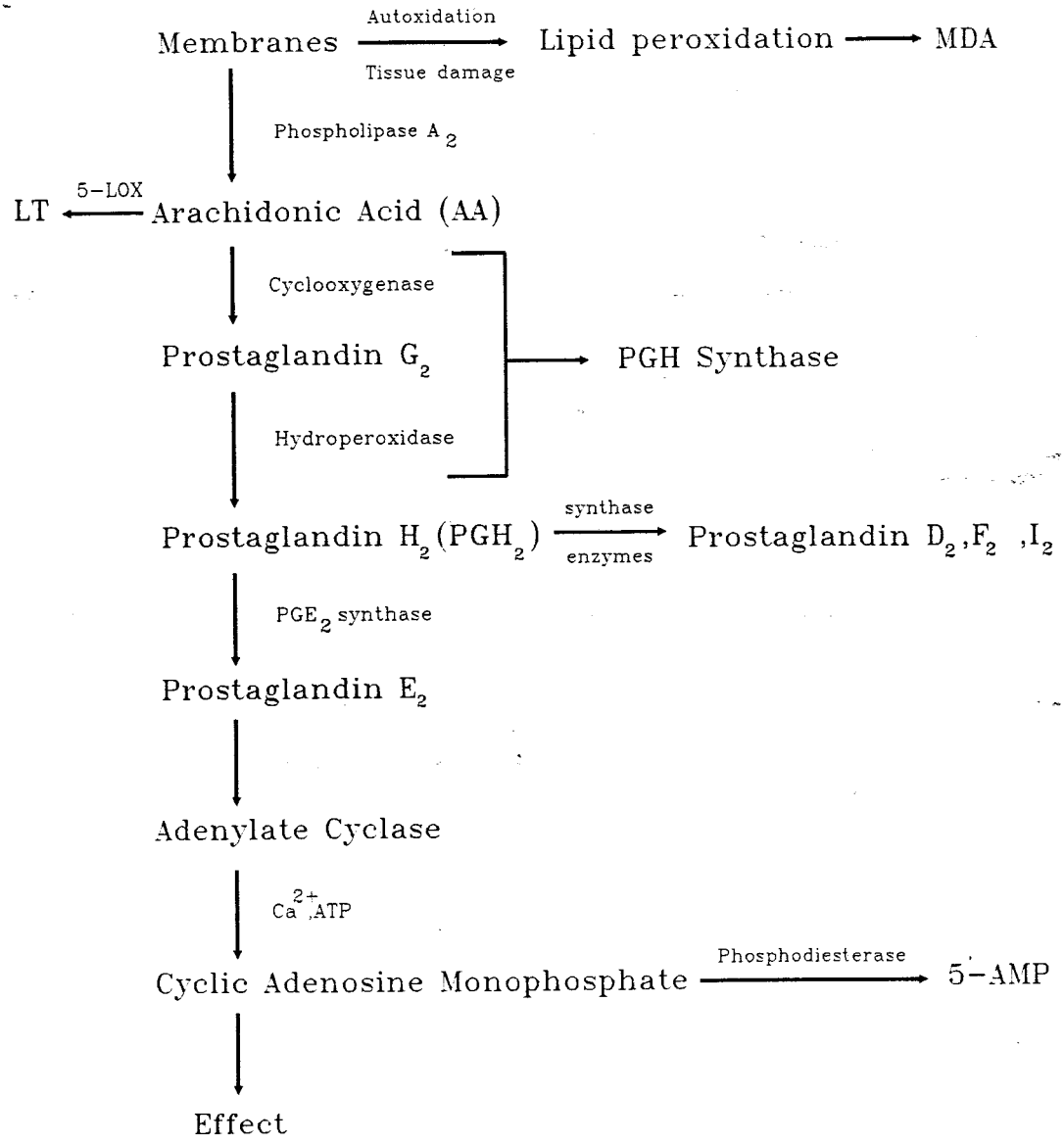


Figure 4: Schematic representation of the AA cascade pathway examined in this project. (AMP-Adenosine monophosphate, ATP-Adenosine triphosphate, LT-Leukotrienes, LOX-Lipoxygenase, MDA-Malondialdehyde).

This pathway shows the metabolites influencing, and sequential steps involved in, the release and conversion of AA to various PGs, with the possible effects of PGE_2, on AC activity, cAMP production and PDE activity.

The objectives of this study were to determine the effect of vitamin E succinate supplementation on the *in vitro* growth of malignant murine melanoma (BL6) and non-malignant monkey kidney (LLCMK) cell lines, and to evaluate the effect of this supplementation on free radical formation, lipid peroxidation and the activity and metabolism of the pathway shown in figure 4. 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.

2

NUTRIENT SUPPLEMENTATION AND CELL GROWTH

2.1 INTRODUCTION

With an ever-increasing number of new cases of cancer expected each year, and the lack of success of established preventative and therapeutic strategies in controlling the high incidence and low survival rates of patients with solid tumours (11,20), there is a need for new and improved therapeutic strategies (11). Over the past few years, the issue of chemoprevention (see section 1.1.4) has received increased attention as a possible, realistic and inexpensive means of reducing cancer incidence (11,20,22). This idea has been designed to work as an adjuvant therapy rather than as a replacement therapy for established strategies (20). A nutrient which has received recent attention as a chemopreventative agent is α-TOH (11,22,24,46,50). Both animal (117,122) and human (22,52-55,105) studies have provided evidence which suggests an inverse relationship between the levels of essential antioxidant nutrients such as α-TOH and the risk of cancer development. It is tempting to speculate that the characteristics of various tumour cells may in part be due to the absence of an essential nutrient such as vitamin E, and that supplementation of this nutrient may reverse the neoplastic state. Vitamin E in combination with various tumour therapeutic agents such as adriamycin, RO20-1724, vincristine, sodium butyrate and chlorozotocin, produce synergistic or additive growth inhibitory effects on both glioma (C6) and neuroblastoma (NB) cells in culture (128). Furthermore, vitamin E succinate in combination with ^{60}Co-γ-irradiation has been found to potentiate cell death and growth inhibition of neuroblastoma cells (393,394) as well as enhance the growth inhibitory effects of PGE_2 (110), Δ^{12}-PGJ_2 and PGJ_2 on human oral squamous carcinoma cells in culture (123).

Tissue culture has been widely used in studies of the functional roles of α-TOH (395). This technique has expanded considerably over the past few years and is now widely accepted in many aspects of biological research. The development of continuous transformed cell lines has attained great importance in research. The advantages of continuous cell lines are their ease of maintenance in simple media and their ability to grow to high cell densities resulting in higher yields (396). In addition to this, the growing of cells in cultures that are no longer organised into tissues, allows for the supplementation of these cells with various nutrients *eg* vitamin E, and the monitoring of the effects of this supplementation on cell growth and various aspects of cell metabolism, without the

problems of the host immune system and non-specific cells generally found *in vivo*. In this study the effects of vitamin E succinate supplementation on the *in vitro* growth of non-malignant LLCMK (monkey kidney) and malignant BL6-F10 (murine melanoma) cell lines were determined.

2.2 MATERIALS AND METHODS

MATERIALS

Highly metastatic murine melanoma BL6-F10 (BL6) and non-malignant monkey kidney (LLCMK) cells were obtained from Highveld Biological Association, South Africa. Basal Minimum Essential Media (MEM), L-Serine, trypsin, succinic acid disodium salt hexahydrates, and (+) α-tocopherol acid succinate (vitamin E succinate) were purchased from Sigma Chemical Co., USA. (U-^{3}H)-vitamin E succinate (^{3}H-vitamin E succinate) (1mCi/ml) was purchased from Amersham International, England. Foetal Calf Serum (FCS) was obtained from Delta Bioproducts South Africa, while sterile disposable tissue culture flasks (25cm^2 and 75cm^2) were purchased from Corning, USA. Sodium Hydrogen Carbonate ($NaHCO_3$), Sodium Chloride (NaCl), anhydrous Sodium Dihydrogen Orthophosphate (NaH_2PO_4), Potassium Dihydrogen Orthophosphate (KH_2PO_4) and Dimethyl Sulphoxide (DMSO) were purchased from Unilab, South Africa. Potassium Chloride (KCl) and Ethylenediamenetetracetic Acid (EDTA) were supplied by Holpro Chemical Co., South Africa. Phenol red, D-glucose and glycine were obtained from BDH Chemicals LTD, England. Sodium Benzylpenicillin (Novo-Pen) and Streptomycin Sulphate (Novo-Strep) was supplied by Novo Industries (Pharmaceutical LTD), South Africa. Haemocytometers were purchased from Neubauer, Germany.

METHODS

2.2.1 PREPARATION OF CULTURE REAGENTS

2.2.1.1 Preparation of cell culture media

Preparation of culture media was carried out as follows. To basal MEM media containing Hanks salts and glutamine but no $NaHCO_3$, was added serine (0.1g/l), glycine (0.06g/l), $NaHCO_3$ (0.75g/l) and 5.0ml of a combined sodium benzylpenicillin and streptomycin sulphate antibiotic solution (one vial of sodium benzylpenicillin (10^6 U) and one vial of streptomycin sulphate ($10^6\mu$g) were combined and

made up to 100ml with milli Q water). Media preparation was always carried out in batches of 10 litres using milli-Q water.

2.2.1.2 Media Filtration

Media was sterilised immediately by positive filtration (figure 12) through a millipore filtration unit (Millipore Corporation, USA) using the following filters: a prefilter, type SM42 "membrane filter" 50K (size 130); a 0.45, 142mm type NA filter (hawp 14250); and a 0.22μm, 142mm type GS filter (GSWP 14250).

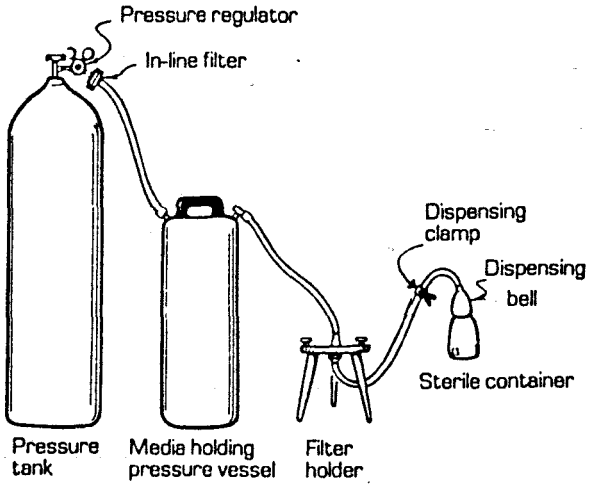


Figure 12: Positive pressure filtration system.

During filtration, the initial 200ml of the medium was discarded while the remainder of the medium was collected into sterile cell culture bottles. Sterilisation of cell culture bottles was carried out by autoclaving for 45 minutes in an Everlight vertical type autoclave. Aseptically dispensed media was then incubated at 37^{0}C for one week to test for contamination.

2.2.1.3 Growth media preparation

Growth media was prepared by filtering FCS through a 0.45μM Millipore filter using a Swinex-25 holder (Millipore Corporation, USA), until the media contained 10% (v/v) FCS. This media was further incubated at 37^{0}C for 48hrs to test for contamination. Media used in the first 24 hour growth

period of cells defrosted from frozen stocks contained 20% FCS.

2.2.1.4 Freezing media preparation

Freezing media was prepared by filtering FCS and DMSO through a 0.45μM millipore filter using a Swinnex-25 holder, until the media contained 20% (v/v) and 10% (v/v) DMSO. All freezing media was stored at -20^{0}C until required.

2.2.1.5 Trypsin preparation

To one litre of milli-Q water was added 8.0g NaCl, 0.4g KCl, 1.0g D-glucose, 0.58g $NaHCO_3$, 0.2g EDTA, 0.02g phenol red indicator, 0.033g trypsin and 10ml of a Novo-Strep:Novo-Pen solution prepared as described in section 2.2.1.1. All trypsin solutions added to culture flasks were filtered through a 0.45μM millipore filter using a Swinnex-25 holder. Trypsin was stored at -20^{0}C until required.

2.2.1.6 Preparation of vitamin E succinate and succinic acid containing media

Stock solutions of vitamin E succinate (1-10mg/ml) were prepared freshly in absolute ethanol and diluted 1:1 000 in media containing 10% (v/v) FCS to give final concentrations of 1, 3, 5, 7, and 10μg/ml vitamin E succinate respectively, in 0.1% final concentration of ethanol. Vehicle controls containing equivalent amounts of succinic acid and absolute ethanol were prepared as follows. Stock solutions of succinic acid 1.89mg/ml were prepared in milli-Q water and diluted 1:1 000 in media containing 10% (v/v) FCS and 0.1% absolute ethanol to give a final succinic acid concentration of 1.89μg/ml. Other concentrations of succinic acid (0.19, 0.57, 0.89, 1.32mg/ml) were prepared by serial dilution of the 1.89mg/ml stock.

2.2.1.7 Preparation of Phosphate Buffered Saline solution

Phosphate Buffered Saline (PBS) pH 6.6, was prepared by the addition of 8.0g NaCl, 0.2g KH_2PO_4, 0.2g KCl and 0.15g of $NaHPO_4{\cdot}2H_2O$ to one litre of milli-Q water.

2.2.2 CELL CULTURE

2.2.2.1 Routine cell culture procedure

All cell culture procedures were conducted on a laminar flow bench which had previously been sterilised by regular swabbing with 70% ethanol and exposure to ultraviolet light. In addition to this, all equipment used under the lamina flow bench was either purchased sterile, autoclaved or swabbed with 70% ethanol prior to use. When not required for experimental purposes, both non-malignant LLCMK and malignant BL6 cells were incubated at 37°C in 75cm^2 flasks containing 30ml of 10% (v/v) FCS supplemented media. Media was changed on a regular basis (approximately once a day) during maintenance of these cell lines. To passage or subculture cells, the growth media was discarded and 10ml of trypsin was added to each flask, followed by incubation at 37°C for 10 minutes. During the incubatory period cells detach from the surface of the flask. These cells were then passaged into three or four flasks followed by the addition of media containing 10% (v/v) FCS.

2.2.2.2 Freezing of cells

Upon reaching confluency, a cell line may be subcultured or stored in a frozen state. This latter procedure involves the harvesting of near-confluent flasks of cells and the storage of these cells frozen in 2.0ml of freezing medium in cryogenic vials under liquid nitrogen.

2.2.3 THE EFFECT OF VITAMIN E SUCCINATE SUPPLEMENTATION ON BL6 AND LLCMK CELL GROWTH

2.2.3.1 Cell culture procedure

Confluent flasks (75cm^2) of BL6 and LLCMK cells were trypsinised with 10ml of trypsin. These flasks were incubated at 37°C until cells detached from the flask surface. The resulting cell suspension was then poured into sterile 15ml centrifugation tubes and spun at 3 000g for 10 minutes. Following centrifugation, the supernatant solution (trypsin) was discarded and resulting cell pellet was resuspended in 2.0ml of MEM basal medium containing 10% (v/v) FCS. Using a sterile pasteur pipette, a suspension of cells was placed onto a haemacytometer and counted. Counts obtained were then used to calculate the volume of cell suspension required to seed 300 000 cells per 25cm^2 flask

or 500 000 per 75cm^2 flask.

In the experiments relevant to 3.2.2 and 3.2.3, for which further analytical procedures and data are described in chapter 3 (the numbering denoting the relevant experiments), 3x10^5 BL6 or LLCMK cells were seeded into 7 sets of 5 25cm^2 flasks. To 5 of the 7 sets of flasks, 10ml of MEM basal medium containing 10% (v/v) FCS and varying levels of vitamin E succinate (1-10μg/ml) was added. The sixth set of flasks each received 10ml of 10% (v/v) FCS medium, and were referred to as control cultures (0), while the seventh set of flasks received 10ml of 10% (v/v) FCS medium containing 0.1% final concentration of ethanol and were referred to as control cultures 0E. In the experiments relevant to 3.3, 4.1, 4.2, 4.3, 4.4, 4.5, 4.6.2, 4.6.4, 4.7 and 4.8, for which further analytical procedures and data are described in chapters 3 and 4 respectively (the numbering denoting the relevant experiments), 5x10^5 BL6 or LLCMK cells were seeded into 7 sets of 3 75cm^2 flasks. To 5 of the 7 sets of flasks was added 30ml of MEM basal medium containing 10% (v/v) FCS and varying levels of vitamin E succinate (1-10μg/ml). The sixth set of flasks received 30ml of medium containing 10% (v/v) FCS and were referred to as control cultures (0) while the seventh set of flasks received 30ml of 10% (v/v) FCS supplemented medium containing 0.1% final volume ethanol and were referred to as control cultures 0E. All flasks were incubated at 37^{0}C for the duration of the experiment with one medium change during this period.

2.2.3.2 Harvesting of cells

When the first set of flasks reached confluency after 5-7 days (determined by viewing under a microscope), cells were harvested under non-sterile conditions. This procedure involved the addition of 5.0ml of trypsin to 25cm^2 flasks and 10ml of trypsin to 75cm^2 flasks, followed by incubation at 37^{0}C until cells had lifted off flask surfaces. The resulting cell suspensions were centrifuged at 3 000g for 10 minutes and cell pellets resuspended in 1.0ml of PBS unless otherwise stated. Cells were counted using a haemocytometer. These cell counts were used as a reflection of cell growth and enabled the effects of vitamin E succinate supplementation on growth of cells to be determined. The cells were then used for further analysis.

2.2.4 THE EFFECT OF SUCCINIC ACID AND VEHICLE TREATMENT ON CELL GROWTH

2.2.4.1 Cell culture procedure

$3x10^5$ BL6 or LLCMK cells were seeded into 6 sets of 5 $75cm^3$ flasks. To 5 sets of these flasks was added 10ml of MEM basal medium containing 10% (v/v) FCS, varying levels of succinic acid (0.19-1.89μg/ml) and 0.1% final concentration of ethanol. The sixth set of flasks received 10ml of medium containing 10% (v/v) FCS and 0.1% final concentration of ethanol. All flasks were incubated at 37°C for the duration of the experiment with one change of medium, and cells harvested as described in section 2.2.3.2.

2.2.5 THE EFFECT OF COMBINED ^{3}H-VITAMIN E SUCCINATE AND VITAMIN E SUCCINATE SUPPLEMENTATION ON CELL GROWTH

2.2.5.1 Cell culture procedure

$5x10^5$ BL6 or LLCMK cells were seeded into 7 sets of three $75cm^2$ tissue culture flasks. To five of these flasks, 30ml of MEM basal medium containing 10% (v/v) FCS and varying levels of vitamin E succinate (1-10μg/ml) were added. The sixth set of flasks received 30ml of medium containing 0.1% final concentration of ethanol and were referred to as control cultures 0E, while the 7th set of flasks received 30ml of MEM basal medium containing 10% (v/v) FCS and were referred to as control culture (0). All the flasks were incubated at 37°C with one change of medium. During media change, 0.1μCi of ^{3}H-Vitamin E acid succinate was added to control culture 0E and all vitamin E succinate supplemented flasks. Control cultures (0), received 30ml of MEM basal medium containing 10% (v/v) FCS. The flasks were incubated at 37°C for the duration of the experiment and the cells harvested as described in section 2.2.3.2. The above experimental procedure is relevant to experiments 3.3 in chapter 3.

2.2.6 STATISTICAL ANALYSIS

Results obtained were analysed using a One Way Analysis of Variance (ANOVA) followed by a Student Newmans-Kuels Multiple Range Test.

2.2.7 RESULTS

2.2.7.1 The effect of vitamin E succinate supplementation on BL6 and LLCMK cell growth

The effect of varying levels (1-10μg/ml) of vitamin E succinate on the *in vitro* growth of malignant BL6 and non-malignant LLCMK cell lines, is shown in tables 1A and 1B respectively. Results shown in these tables are relevant to the individual experiments described in chapters 3 and 4, and will be discussed relative to these studies at a later stage. Of relevance to the discussion in this chapter, is the overall mean growth inhibition results for all the experiments.

The overall mean growth inhibitory effects of vitamin E succinate supplementation on BL6 cell growth indicate that supplementation of 1 and 3μg/ml vitamin E succinate results in no significant change in growth, as compared with control cultures 0E, while at 5, 7 and 10μg/ml respectively a significant ($p \leq 0.001$) decrease in growth occurred. LLCMK cells on the other hand showed no significant increase or decrease in growth over the vitamin concentration tested, although a slight but non-significant decrease was observed at 5, 7 and 10μg/ml vitamin E succinate supplementation. However, LLCMK cell growth in certain individual experiments *ie* Exp's 3.3, 4.1, 4.2 and 4.8, were significantly inhibited by high concentrations of vitamin E succinate, while in others non-significant (Exp's 3.2.2; 3.2.3; 4.5) and significant (Exp's 4.4; 4.7) increases in LLCMK growth occurred following vitamin E succinate supplementation.

2.2.7.2 The effect of succinic acid and vehicle treatments on cell growth

The overall effects of vehicle (ethanol) and combined succinic acid:ethanol treatments on BL6 and LLCMK cell growth are shown in tables 2A, 2B and 3 respectively. In these studies, supplementation with 0.1% ethanol resulted in a marked although non-significant increase in BL6 growth (table 2A) and a non significant decrease in LLCMK growth (table 2B) as compared with control cultures (0), although effects varied considerably within individual experiments. Supplementation with varying levels of succinic acid and equivalent amounts of ethanol (both BL6 and LLCMK cell lines) (table 3) resulted in no significant increase or decrease in growth compared with control cultures 0E, although succinic acid at certain concentrations was shown to stimulate BL6 and LLCMK growth.

Table 1A: The effect of Vitamin E succinate supplementation on BL6 cell growth. (Values recorded in the table represent the mean of 3# or 5* cultures ± SEM).

Vitamin E succinate (μg/ml)	BL6 cells												
	Growth Inhibition (% of untreated control 0E)												
	Exp# 3.2.2	Exp# 3.2.3	Exp* 3.3	Exp* 4.1	Exp* 4.2	Exp* 4.3	Exp* 4.4	Exp* 4.5	Exp* 4.6.2	Exp* 4.6.4	Exp* 4.7	Exp* 4.8	MEAN
0E	100 ±6.1	100 ±6.2	100 ±9.6	100 ±10.6	100 ±16.1	100 ±2.9	100 ±32.4	100 ±2.3	100 ±25.1	100 ±9.0	100 ±19.8	100 ±8.2	100 ±3.43
1	74.4^{c} ±5.21	96.7 ±11.3	108.8^{c} ±6.1	83.1 ±19.3	7.7^{a} ±0.13	77.3^{c} ±4.5	266.2^{a} ±12.4	91.9^{b} ±3.8	112.8 ±9.1	103.3 ±4.8	154.7^{b} ±2.0	85.5 ±4.3	101.1 ±9.2
3	148.3^{a} ±9.6	90.4 ±4.5	67.2^{b} ±4.0	56.5 ±16.1	8.9^{a} ±0.17	47.9^{b} ±8.8	286.4^{a} ±27.5	83.7^{b} ±3.2	112.6 ±9.7	78.6 ±2.6	182.2^{a} ±15.0	81.8 ±7.0	100.2 ±11.4
5	66.3^{b} ±6.3	43.1^{a} ±3.1	66.5^{b} ±0.47	73.4 ±5.5	7.8^{a} ±0.34	41.5^{a} ±3.4	25.1^{c} ±2.8	56.2^{a} ±1.4	109.9 ±5.1	83.8 ±6.0	34.2^{a} ±0.8	57.1 ±6.6	63.1^{a} ±4.5
7	42.77^{a} ±2.4	53.5^{a} ±5.9	69.7^{b} ±3.2	63.2 ±2.6	34.1^{a} ±5.4	64.0^{b} ±9.9	12.0^{c} ±1.4	37.4^{a} ±0.78	97.4 ±14.9	89.3 ±4.9	29.6^{b} ±3.4	80.4 ±6.8	55.0^{a} ±3.9
10	33.3^{a} ±7.5	27.8^{a} ±1.8	21.9^{a} ±0.15	75.9 ±10.9	6.9^{a} ±0.12	57.5^{b} ±9.5	11.5^{c} ±1.4	24.0^{a} ±1.0	79.9 ±6.8	71.4^{c} ±1.5	14.3^{a} ±1.0	75.1^{b} ±4.7	39.1^{a} ±4.0

a= p≤0.001:)
b= p≤0.01:) Relative to control cultures 0E
c= p≤0.05:)

Table 1B: The effect of Vitamin E succinate supplementation on LLCMK cell growth. (Values recorded in the table represent the mean of 3# or 5* cultures ± SEM).

Vitamin E succinate (μg/ml)	LLCMK cells										
	Growth Inhibition (% of untreated control 0E)										
	Exp# 3.2.2	Exp# 3.2.3	Exp* 3.3	Exp* 4.1	Exp* 4.2	Exp* 4.3	Exp* 4.4	Exp* 4.5	Exp* 4.7	Exp* 4.8	MEAN
0E	100 ±11.1	100 ±10.2	100 ±4.6	100 ±4.6	100 ±7.7	100 ±10.3	100 ±7.4	100 ±4.5	100 ±8.2	100 ±11.0	100 ±2.5
1	137.4 ±8.3	98.9 ±13.3	99.7 ±10.8	94.7 ±5.5	122.7^{c} ±10.8	122.4 ±15.8	156.1^{c} ±12.5	86.7 ±13.1	137.3^{a} ±10.8	83.8 ±1.0	112.1 ±4.7
3	129.2 ±6.8	100.9 ±13.6	87.6 ±1.4	97.0 ±10.6	75.5^{c} ±4.1	98.6 ±9.7	145.9^{c} ±6.8	110.9 ±15.6	121.7^{a} ±1.7	85.4 ±3.8	106.0 ±4.3
5	37.2^{b} ±5.5	104.9 ±6.2	86.5 ±2.8	104.5 ±2.9	70.8^{c} ±3.6	94.4 ±8.2	164.2^{c} ±14.0	101.1 ±3.4	120.2^{a} ±3.2	80.9 ±3.9	91.3 ±5.5
7	102.1 ±13.2	118.3 ±3.4	63.7^{b} ±6.7	80.4 ±4.9	29.4^{b} ±1.3	49.7^{c} ±2.5	163.7^{c} ±17.8	99.7 ±5.8	125.5^{a} ±14.9	75.9 ±4.4	90.2 ±6.1
10	126.1 ±30.3	103.1 ±13.0	63.5^{c} ±5.3	70.6^{c} ±3.9	43.8^{a} ±3.2	66.4 ±8.5	116.0 ±7.2	118.0 ±15.8	105.7^{a} ±8.7	57.3^{b} ±3.2	88.6 ±6.5

a= p≤0.001:)
b= p≤0.01:) Relative to control cultures 0E
c= p≤0.05:)

Table 2A: Effect of vehicle supplementation on BL6 cell growth. (Values recorded in the table are the mean of 3# and 5* cultures ± SEM).

Treatments[a]	BL6 cells												
	Growth Inhibition (% of untreated controls 0)												
	Exp# 3.2.2	Exp# 3.2.3	Exp* 3.3	Exp* 4.1	Exp* 4.2	Exp* 4.3	Exp* 4.4	Exp* 4.5	Exp* 4.6.2	Exp* 4.6.4	Exp* 4.7	Exp* 4.8	MEAN
0	100 ±6.6	100 ±5.5	100 ±4.4	100 ±5.7	100 ±12.8	100 ±1.4	100 ±17.2	100 ±1.9	100 ±6.8	100 ±7.1	100 ±16.6	100 ±9.9	100 ±2.2
0E	90.6 ±5.5	99.6 ±6.2	103.7 ±10.0	89.6 ±29.0	197.9 ±5.7	70.5 ±7.2	239.0 ±38.3	146.1 ±3.4	186.6 ±28.0	236.3 ±20.7	96.8 ±19.2	130.1 ±10.7	132.9 ±9.7

a= Cells cultured in presence 0E or absence (0) of ethanol.

Table 2B: Effect of vehicle supplementation on LLCMK cell growth. (Values recorded in the table are the mean of 3# and 5* cultures ± SEM).

Treatments[a]	LLCMK cells										
	Growth Inhibition (% of untreated controls 0)										
	Exp# 3.2.2	Exp# 3.2.3	Exp* 3.3	Exp* 4.1	Exp* 4.2	Exp* 4.3	Exp* 4.4	Exp* 4.5	Exp* 4.7	Exp* 4.8	MEAN
0	100 ±7.2	100 ±5.0	100 ±9.7	100 ±5.7	100 ±10.9	100 ±12.2	100 ±1.4	100 ±10.2	100 ±4.4	100 ±5.0	100 ±2.1
0E	86.1 ±9.5	92.7 ±9.5	81.3 ±3.8	66.7 ±4.9	103.6 ±10.7	130.5 ±10.1	70.4 ±7.2	91.6 ±4.1	89.8 ±7.3	100 ±11.0	90.0 ±3.7

a= Cells cultured in presence 0E or absence (0) of ethanol.

Table 3: The effect of succinic acid and vehicle treatment on BL6 and LLCMK cell growth. (Each value in the table represents the mean 10 determinations ±SEM)

Succinate (μg/ml)	Vehicle concentration (%)	Growth Inhibition (% of untreated controls 0E)	
		BL6	LLCMK
0	0.1	100 ±2.8	100 ±14.8
0.19	0.1	119.1 ±3.0	107.4 ±9.2
0.32	0.1	115.6 ±3.1	75.8 ±10.1
0.89	0.1	95.5 ±10.1	100.4 ±17.2
1.32	0.1	107.4 ±10.9	114.7 ±15.0
1.89	0.1	124.8 ±9.1	117.3 ±15.4

2.2.7.3 The effect of combined ^{3}H-vitamin E succinate and vitamin E succinate supplementation on BL6 and LLCMK growth.

Supplementation of BL6 and LLCMK cells with varying levels (1-10μg/ml) of vitamin E succinate and 0.1μCi of ^{3}H-vitamin E succinate (table 4) resulted in a significant decrease in BL6 growth compared with control cultures 0E, while LLCMK cells showed a non-significant increase at 1, 5 and 7μg/ml vitamin E succinate, and non-significant decrease at 10μg/ml.

Treatment of BL6 and LLCMK cells with 0.1μCi ^{3}H-vitamin E succinate and 0.1% ethanol resulted in an overall non-significant increase in growth compared with untreated controls (0) (table 5).

Table 4: The effect of combined ^{3}H-vitamin E succinate and vitamin E succinate supplementation on BL6 and LLCMK cell growth. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	Ethanol concentration (%)	^{3}H-vitamin E succinate (μCi)	Growth Inhibition (% of untreated controls OE)	
			BL6 cells	LLCMK cells
0E	0.1	0.1	100 ±2.2	100 ±11.7
1	0.1	0.1	54.2[a] ±4.5	103.5 ±7.9
3	0.1	0.1	59.3[a] ±5.6	100.2 ±12.7
5	0.1	0.1	66.6[a] ±6.4	106.6 ±6.9
7	0.1	0.1	66.5[a] ±7.4	116.9 ±6.9
10	0.1	0.1	63.3[a] ±6.6	90.7 ±3.8

a= $p \leq 0.01$: Relative to BL6 control cultures 0E.

Table 5: The effect of combined ^{3}H-vitamin E succinate and vehicle supplementation on BL6 and LLCMK cell growth. (Each value in the table represents the mean of 3 determinations ± SEM).

Treatment	Growth Inhibition (% of untreated controls 0)	
	BL6 cells	LLCMK cells
0	100 ±4.6	100 ±31.7
0E[a]	106.3 ±8.7	120.7 ±14.1

a= Control cultures containing 0.1% ethanol and 0.1μCi ^{3}H-vitamin E succinate

2.3 DISCUSSION

Of all the various forms of vitamin E tested for their ability to inhibit tumour cell growth, vitamin E succinate has proven to be the most potent form *in vitro* (42,397). This vitamin has been found to

have several unique biochemical properties both *in vitro* and *in vivo* (398), and is believed to induce differentiation (42,398,399) and inhibit growth and survival (42,110,123,126,127,397-399) of various tumour cell lines *in vitro*. In this study supplementation of 5, 7 and 10μg/ml vitamin E succinate, resulted in a significant decrease in BL6 cell growth. Numerous other studies (42,124,127,399) have shown similar inhibition of BL6 cell growth following supplementation with 5-10μg/ml vitamin E succinate. Furthermore treatment of these cells with vitamin E succinate has been shown to result in morphological changes (42,399), with the cells resembling their non-malignant counterparts (42). Apart from its growth inhibitory effects on BL6 cells, vitamin E succinate supplementation has been shown to inhibit the growth of various other tumour cell lines *in vitro* such as human tongue carcinoma (Scc-25) (110,123), mouse neuroblastoma (NPB_2), rat glioma (127), human neuroblastoma (397) and avian retrovirus-transformed immature lymphoid tumour (C4#1) cells (126).

Although several studies have investigated the effects of vitamin E succinate supplementation on tumour cell growth and proliferation, very few have investigated the *in vitro* effects of vitamin E succinate supplementation on normal or non-transformed cells (126). Studies by Helson and co-workers (397), have shown that supplementation of 40μg/ml d-α-TOH polyethylene glycol succinate (vitamin E-TPGS) to normal human skin fibroblasts in culture resulted in a 50% reduction in growth, while Prasad and Edwards-Prasad (399), studying the effects of vitamin E succinate supplementation on growth and differentiation of mouse fibroblasts in culture (L-cells), showed that 10μg/ml vitamin E succinate inhibited L-cell growth by 50%. In addition to this, Kline *et al* (126) showed that vitamin E succinate at 5 and 10μg/ml respectively was cytotoxic to lymphocytes from the spleen, thymus, bursa and peripheral blood of normal chickens, while lower concentrations (0.1-1μg/ml) of vitamin E succinate enhanced cell growth.

Supplementation of non-malignant LLCMK cells with 1 and 3μg/ml vitamin E succinate in this study resulted in an overall marked increase in growth, while at 5, 7 and 10μg/ml respectively, a non-significant decrease in LLCMK growth occurred. However, in certain experiments (Exp's 3.3; 4.1, 4.2; 4.8) (table 1B), LLCMK growth was significantly decreased upon supplementation of vitamin E succinate, while in other experiments both significant (Exp's 4.4; 4.7) and non-significant (Exp's 3.2.2; 3.2.3; 4.5) increases in LLCMK growth occurred. The reason for these varying effects on LLCMK growth in individual experiments is unclear, although the possibility exists that vitamin E succinate may have varied effects within the same cell line, a common occurrence in cell culture systems.

2. Cell Growth

In an attempt to eliminate the possibility that the observed effects of vitamin E succinate supplementation on LLCMK and BL6 growth were due to the vehicle used, or the succinic acid component of the vitamin E succinate molecule, control experiments using equivalent amounts of ethanol (table 2A and 2B) and succinic acid with an equivalent amount of ethanol (table 3) were set up. In LLCMK cells, supplementation with ethanol or combined succinic acid and ethanol resulted in no significant increase or decrease in LLCMK growth, while BL6 cells showed a marked although non-significant increase in growth. Numerous studies with BL6 cells (42,126,399) have shown that supplementation with succinic acid, with an equivalent amount of ethanol, results in no significant change in growth or morphology of these cells. Thus it is reasonable to assume that the observed effects of vitamin E succinate supplementation on LLCMK and BL6 growth are the result of the vitamin E succinate molecule as a whole rather than the vehicle used or the succinic acid component of the vitamin.

3

VITAMIN E SUCCINATE UPTAKE, METABOLISM AND ROLE IN FREE RADICAL FORMATION AND LIPID PEROXIDATION

3.1 INTRODUCTION

Reactive oxygen species such as the superoxide (SO_2^-), hydroxyl (OH^-), organic peroxide radicals, hydrogen peroxide (H_2O_2) and singlet oxygen, are continuously generated intracellularly in aerobic organisms (400), and if allowed to react uncontrollably are capable of causing extensive damage to macromolecular components within the cell such as DNA, carbohydrates, proteins and lipids (69,119,121,159). More recently, reactive oxygen species have been suggested to play an important role in the stimulation of cell division (400). The close relationship between free radical activity and malignancy has been well documented (reviewed in 71), and at present the basic assumption appears to be that free radical mediated disturbances may play an important role in triggering the transformation of non-malignant cells to malignant cells (71,135).

In normal cells, highly effective antioxidant defence mechanisms have been developed to protect against lipid peroxidation, toxic products of lipid peroxidation and free radical species (65,119,235,400). These protective mechanisms or antioxidant defences can be classified into two main categories, namely the enzymatic defence mechanisms, which include the superoxide dismutase, catalase and glutathione peroxidase enzymes (8,65,72,75,235), and the non-enzymatic cellular antioxidants which include the minerals selenium, copper, zinc and magnesium, and the vitamins A, E and C (60,65, 72,75,119,235,401). Under normal conditions, these antioxidant defence mechanisms provide adequate protection from free radical attack and lipid peroxidation, but, the possibility exists that under certain circumstances the effectiveness of these mechanisms may be decreased or even completely overwhelmed (401). Tumour cells are believed to have defective enzymatic antioxidant defences (139,400). Compared with their normal cell counterparts, it has been found that tumour cells are always low in superoxide dismutase, catalase (8,70,71,159,402) and glutathione peroxidase activity (8,71,159,402). Apart from antioxidant enzymes, tumour cells may be deficient in antioxidant vitamins and minerals. Numerous studies on the relationship between serum vitamin E and incidence of cancer have suggested an inverse relationship between the levels of vitamin E and risk of cancer development (52,54,60,119). As a result, one would expect tumour cells to have higher free radical

and lipid peroxidation levels than their normal counterparts (71,159).

Results from growth studies in section 2.2.3 have shown that vitamin E succinate supplementation significantly reduced the growth of BL6 cells in culture. The actual mechanism by which vitamin E succinate inhibits growth is at present unknown (126). Studies by Rama and Prasad (127) have suggested that the growth inhibitory effects of vitamin E succinate are mediated in part by its antioxidant properties. Vitamin E succinate, however, is a non-physiological antioxidant which requires esterase activity in order to liberated the free alcohol, α-TOH, with antioxidant properties (126,398,403,404). To date no species specific hydrolysis of vitamin E succinate has been demonstrated in murine melanoma cells. Researchers have suggested that esterified forms of vitamin E may undergo hydrolysis via a number of non-specific esterases associated with hydrophobic membranes and membranous structures present in most cells, such as the mitochondria, endoplasmic reticulum and nucleus (404). Concerning the uptake of vitamin E succinate, it appears that vitamin E esters are absorbed into the cell via an unknown transfer mechanism (405).

Since it is unclear whether the growth inhibitory effect of vitamin E succinate on BL6 cells was mediated via an antioxidant mechanism, and because no species specific hydrolysis of vitamin E succinate has been demonstrated in BL6 or LLCMK cells, attempts were made to determine the effect of vitamin E succinate supplementation on free radical and lipid peroxidation levels in BL6 and LLCMK cells, as well as the relative uptake and metabolism of vitamin E succinate by these cells.

3.2 EFFECT OF VITAMIN E SUCCINATE SUPPLEMENTATION ON FREE RADICAL FORMATION AND LIPID PEROXIDATION LEVELS

3.2.1 MATERIALS

Trichloroacetic Acid (98%) (TCA) and glacial acetic acid (99.5%) were obtained from Holpro Chemical Co., South Africa. Butylated Hydroxytoluene (BHT), Nitroblue Diformazan (NBD), Nitroblue Tetrazolium (NBT) and 2-Thiobarbaturic acid (98%) (TBA) were purchased from Sigma Chemical Co., USA. 1,1,3,3,-tetramethoxy-propan (98%) (malondialdehyde-tetramethylacetal) was obtained from Fluka AG, Switzerland.

3.2.2 EFFECT OF VITAMIN E SUCCINATE SUPPLEMENTATION ON FREE RADICAL FORMATION

METHODS

3.2.2.1 Cell culture procedure

Methods described in sections 2.2.3.1 and 2.2.3.2 were used, except that cell pellets were resuspended in 1.0ml PBS (pH 7.4).

3.2.2.2 Nitroblue tetrazolium assay

This is a simple and reliable technique for determining superoxide and other free radicals (406,407). The principle of the assay is based on the ability of free radicals to reduce NBT to insoluble diformazan, which can be extracted with glacial acetic acid and read at 560nm. A modified method of Sagar *et al* (406) and Das *et al* (407) was used for this assay. 1.0ml cell suspensions were incubated with 0.4ml of 0.1% NBT for two hours at 37^{0}C. Termination of the assay and extraction of reduced NBT was achieved by addition of 0.6ml glacial acetic acid. The absorbance of the reduced NBT was measured at 560nm and the resulting absorbance was converted to nmol diformazan using a standard curve (appendix 1) generated from NBD. Final results were expressed as nmol diformazan/10^{6} cells.

3.2.3 EFFECT OF VITAMIN E SUCCINATE SUPPLEMENTATION ON LIPID PEROXIDATION LEVELS

METHODS

3.2.3.1 Cell culture procedure

Methods described in sections 2.2.3.1 and 2.2.3.2 were used, except that cell pellets were resuspended in 1.0ml 0.9% (w/v) saline solution (pH 7.6).

3.2.3.2 Thiobarbaturic acid assay

The TBA assay is one of the most widely used methods for the determination of lipid peroxidation in biological samples (408,409). The principle of the assay is based on the reaction of MDA equivalents with TBA to form a pink complex which can be extracted with butanol and read at 532nm. A modified method of Sagar *et al* (406) and Draper and Hardley (408) was used. To 1.0ml cell suspensions, 1.0ml of 25% TCA and 0.5ml of BHT were added. Cell suspensions were centrifuged at 2 000g for 20 minutes to remove insoluble protein. Following centrifugation, 2.0ml of supernate was removed, and 0.25ml of 0.33% TBA added. Supernatant fractions were boiled for an hour at 95^{0}C. Tubes were allowed to cool and the corresponding pink TBA-MDA complex was extracted with 2.0ml of butanol and read at 532nm. The absorbance values obtained were converted to nmol MDA from a standard curve generated with 1,1,3,3-tetramethoxy propane (appendix 2). Final results were expressed as nmol MDA/10^6 cells.

3.2.4 STATISTICAL ANALYSIS

The results obtained were analysed using a One Way Analysis of Variance (ANOVA), followed by a Student Newmans-Kuels Multiple Range test. Data in all subsequent sections of this chapter were similarly analyzed.

3.2.5 RESULTS

The effect of vitamin E succinate supplementation on free radical levels in BL6 and LLCMK cells is shown in table 6.

In BL6 cells, there was no significant increase or decrease in free radical levels at vitamin E succinate concentrations of 1, 3 and 5μg/ml compared with control cultures 0E, while at 7 and 10μg/ml, a marked increase in free radical levels occurred, with a significant ($p \leq 0.005$) increase occurring at 10μg/ml. Analysis of lipid peroxidation levels in BL6 cells revealed a similar trend to that observed for free radical levels. At vitamin E succinate concentrations of 1 and 3μg/ml, no significant increase or decrease in MDA levels occurred, while at 5, 7 ($p \leq 0.005$) and 10μg/ml ($p \leq 0.001$) respectively, a significant increase in MDA levels occurred, compared with control cultures 0E.

3. Cell Oxidation

Table 6: The effect of vitamin E succinate supplementation on free radical formation and lipid peroxidation levels in BL6 and LLCMK cells respectively. (Values in the table are the mean of 5 determinations ± SEM).

Vitamin E succinate (μg/ml)	BL6 cells		LLCMK cells	
	Free radical formation(nmol diformazan/10^6 cells)	Lipid peroxidation (nmol MDA/10^6 cells)	Free radical formation (nmol diforamzan/10^6 cells)	Lipid peroxidation (nmol MDA/10^6 cells)
0E	2.52 ±0.23	0.91 ±0.08	5.68[c] ±0.47	0.72 ±0.12
1	2.96 ±0.38	0.48 ±0.11	2.92[a] ±0.17	0.61 ±0.17
3	2.22 ±0.37	1.01 ±0.18	4.04 ±0.31	0.59 ±0.14
5	2.98 ±0.43	3.37[b] ±0.34	4.44 ±0.32	1.05[e] ±0.14
7	3.58 ±0.22	3.28[b] ±0.54	5.08 ±0.50	1.07[e] ±0.15
10	4.52[b] ±0.41	4.86[a] ±0.88	6.50[d] ±0.99	0.92[e] ±0.10

a= $p \leq 0.001$: Relative to respective control cultures 0E.
b= $p \leq 0.005$: Relative to BL6 control cultures 0E.
c= $p \leq 0.001$: Relative to free radical levels in BL6 control cultures 0E.
d= $p \leq 0.05$: Relative to free radical levels in BL6 cells supplemented with 10μg/ml vitamin E succinate.
e= $p \leq 0.001$: Relative to lipid peroxidation levels in BL6 cells in relevant groups.

The LLCMK cells on the other hand (table 6) showed a general decrease in free radical formation at 1, 3, 5 and 7 μg/ml vitamin E succinate, with a significant ($p \leq 0.001$) decrease occurring at 1μg/ml, while at 10μg/ml vitamin E succinate, a non-significant increase in free radical levels occurred. Supplementation of LLCMK cells with 5, 7 and 10μg/ml vitamin E succinate resulted in a non-significant increase in lipid peroxidation levels.

Comparing free radical formation and lipid peroxidation levels in BL6 and LLCMK cells, it was evident that lipid peroxidation levels were generally higher in BL6 cells, significantly so at 5, 7 and 10μg/ml vitamin E succinate. Free radical levels on the other hand were found to be generally higher in LLCMK cells, with significantly higher levels occurring in the control ($p \leq 0.001$) and 10μg/ml ($p \leq 0.05$) supplemented cultures.

3.3 ^{3}H-VITAMIN E SUCCINATE UPTAKE IN BL6 AND LLCMK CELLS.

3.3.1 MATERIALS AND METHODS

MATERIALS

Emulsifier scintillator plus™ scintillation cocktail and scintillation vials were purchased from Packard Company. USA. Refer to section 2.2 for the remainder of materials used.

METHODS

3.3.1.1 Cell Culture procedure

Refer to section 2.2.5.1 for method used.

3.3.1.2 Homogenisation and separation into membrane and stroma fractions

After cell enumeration, cell suspensions were poured into a dounce homogeniser and homogenised with 30 plunges of a tight plunger. The dounce homogeniser was rinsed with 1.0ml PBS, pH 6.6. Resulting homogenates were separated into membrane and stroma fractions by differential centrifugation. Cell homogenates were centrifuged (Beckman model J2-21) at 480g for 20 minutes at 4^{0}C to remove undisrupted cells. Supernatant phase from step one was retained and further centrifuged at 4 000g for 20 minutes at 4^{0}C. The supernatant was again retained and separated into membrane (pellet) and stroma (supernate) fractions by centrifugation at 20 000g for 30 minutes at 4^{0}C. The pellet was resuspended in 2.0ml of PBS pH 6.6.

3.3.1.3 Determination of vitamin E succinate uptake

Duplicate aliquots of membrane and stroma fractions were removed and added to scintillation vials containing 10ml of scintillation cocktail. The radioactivity in membrane and stroma fractions was determined by counting in a Beckman (model LS2800) scintillation counter.

3.3.2 RESULTS

Uptake of ^{3}H-vitamin E succinate by BL6 and LLCMK cells (combined membrane and stroma fractions) is shown in figure 13. The levels of ^{3}H-vitamin E succinate in control cultures of LLCMK cells were found to be significantly ($p \leq 0.01$) higher than the levels in BL6 control cultures 0E. Supplementation of LLCMK cells with 3, 5, 7 and 10μg/ml vitamin E succinate resulted in a significant ($p \leq 0.05$) decrease in vitamin E succinate uptake compared with control cultures 0E, while in BL6 cells, supplementation of 1 through to 10μg/ml vitamin E succinate resulted in a significant ($p \leq 0.05$) increase in vitamin E succinate uptake.

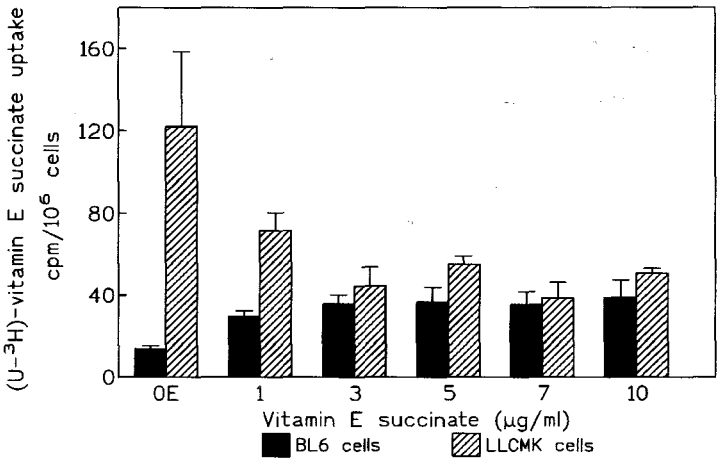


Figure 13: Comparison of total ^{3}H-vitamin E succinate uptake in BL6 and LLCMK cells, and the effect of vitamin E succinate supplementation on these levels. (Each point on the graph represents the mean of 3 determinations $\pm$ SEM).

Table 7 shows the relative uptake of ^{3}H-vitamin E succinate in membrane and stroma fractions of BL6 and LLCMK cells respectively. Supplementation of BL6 cells with 1-10μg/ml vitamin E succinate, resulted in an overall increase in ^{3}H-vitamin E succinate uptake in both membrane and stroma fractions when compared to their respective control cultures 0E. However, ^{3}H-vitamin E succinate uptake in LLCMK stroma fractions was significantly ($p \leq 0.05$) decreased following supplementation with 3, 5, 7 and 10μg/ml vitamin E succinate, while in the membrane fractions uptake was significantly decreased at 3, 7 ($p \leq 0.01$) and 10μg/ml ($p \leq 0.05$) respectively.

3. Cell Oxidation

Table 7: The effect of vitamin E succinate supplementation on ^{3}H-vitamin E succinate uptake in membrane and stroma fractions of BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	^{3}H-vitamin E succinate (μg/ml)	^{3}H-vitamin E succinate uptake (cpm/10^6 cells)			
		Membrane fraction		Stroma fraction	
		BL6 cells	LLCMK cells	BL6 cells	LLCMK cells
0E	0.1	13.81 ±1.42	12.56[a] ±0.28	15.96 ±0.43	73.42 ±9.50
1	0.1	29.47 ±3.00	12.72[a] ±0.87	23.22 ±3.16	71.68 ±8.51
3	0.1	35.72 ±4.48	8.28[be] ±1.14	25.31 ±3.26	36.28[d] ±8.20
5	0.1	36.69 ±7.18	9.82[a] ±0.60	36.07 ±8.33	45.22[d] ±3.55
7	0.1	35.42 ±6.19	7.84[ce] ±0.65	35.62 ±7.5	30.77[d] ±7.06
10	0.1	39.03 ±8.32	9.24[bf] ±0.83	19.93 ±2.64	41.59[d] ±3.1

a= p≤0.001: Relative to ^{3}H-vitamin E succinate levels in LLCMK stroma fractions in relevant groups.
b= p≤0.01: Relative to ^{3}H-vitamin E succinate levels in LLCMK stroma fractions in relevant groups.
c= p≤0.05: Relative to 7μg/ml vitamin E succinate supplemented LLCMK stroma fraction.
d= p≤0.05: Relative to LLCMK stroma control cultures 0E.
e= p≤0.01: Relative to LLCMK membrane control cultures 0E.
f= p≤0.05: Relative to LLCMK membrane control cultures 0E.

Comparing uptake of ^{3}H-vitamin E succinate between membrane and stroma fractions of these cells, non-significant differences were observed in BL6 cells. In contrast, ^{3}H-vitamin E succinate uptake in membrane fractions of LLCMK control and vitamin E succinate supplemented cultures, was significantly lower than the uptake in the corresponding stroma fractions.

3.4 METABOLISM OF VITAMIN E SUCCINATE IN BL6 AND LLCMK CELLS

3.4.1 MATERIALS AND METHODS

MATERIALS

Nitric acid (HNO_3) 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. Neostigmine, ascorbic acid (reduced-99%), iminodiacetic acid and (±) α-TOH (95%) were purchased from Sigma Chemical Co., USA. A reverse phase Bondaclone 10 C^{18} HPLC column (300mm x 3.9mm) was purchased from Phenomex, USA. Guard-Pak precolumns (5μm,ODS) were obtained from Metachemicals, USA. Sodium dihydrogen orthophosphate was supplied by Unilab, SAARCHEM, South Africa.

METHODS

3.4.1.1 Glassware preparation

All glassware was treated by a modified method of Buettner (cited in 410). This procedure involved soaking glassware in 25% (v/v) HNO_3 for 48 hours followed by another 48 hour soak in milli-Q water containing 5g/l of the chelating resin iminodiacetic acid.

3.4.1.2 Preparation of calcium and magnesium free phosphate buffered saline

In order to minimise TOH loss, trace amounts of adventitious catalytic transition metals such as Ca^{2+} and Mg^{2+} were omitted from the assay buffer. This buffer was prepared as described in Kelly *et al* (410) with the exception that neostigmine (10nM), an esterase inhibitor, was included in the buffer.

3.4.1.3 Cell culture procedure

Procedures described in section 2.2.3.1 and 2.2.3.2 were used, except that cell pellets were resuspended in 1.0ml of calcium and magnesium free PBS, containing 10nM neostigmine.

3.4.1.4 Extraction of vitamin E and vitamin E succinate

Extraction of vitamin E and vitamin E succinate was carried out using a modified method of Kelly *et al* (410). Following cell enumeration, cell suspensions were centrifuged (Eppendorf 5403 centrifuge) at 750g for 10 minutes at 4°C, supernatant discarded and the wash repeated with equivalent amounts of PBS and ascorbic acid. To pellets from the final wash was added 1.0ml of PBS buffer, followed by vortex mixing and incubation on ice for 2 minutes. Cell suspensions were then transferred to a precooled dounce homogeniser containing 0.5ml of 95% (v/v) ethanol, vortexed and homogenised 30 times with a tight plunger. The resulting cell homogenates were poured into precooled centrifugation tubes containing ice cold hexane, and the homogeniser rinsed with 0.5ml PBS buffer. Samples were vortex mixed for 1.0 minute, then centrifuged at 300g for 3 minutes to separate the hexane layer. The hexane layer was then transferred by glass pasteur pipette to storage vials. The head space was replaced with nitrogen and the vials stored at -70°C until required.

3.4.1.5 Vitamin E and vitamin E succinate determination

Extracted samples were analysed by Reverse-Phase HPLC, using a modified method of Scalia *et al* (411). Hexane extracts were dried under a stream of nitrogen and reconstituted in 100μl of mobile phase containing methanol: acetonitrile (60:40 (v/v)). Separations were performed on a Bondaclone 10 C^{18} column fitted with a Guard-Pak precolumn (5μm, ODS) and eluted isocratically at a flow rate of 1.5ml/min. 50μl aliquot volumes of the extracts were injected onto the column. Vitamin E and vitamin E succinate were detected using a UV-visible spectrophotometer (Beckman, System Gold programmable detector module, model 166, USA). The identities of the separate compounds were determined by co-chromatography with authentic standards ((±) α-TOH and vitamin E succinate), and the relative quantities of these compounds in the extracts were determined using peak areas and the standard curve shown in appendix 3.

3.4.2 RESULTS

The effect of vitamin E succinate supplementation on cellular vitamin E and vitamin E succinate content in BL6 and LLCMK cells is shown in table 8. Supplementation of BL6 cells with 1-10μg/ml vitamin E succinate resulted in a marked increase in vitamin E succinate levels being detected, with significant increases occurring at 3, 5 ($p \leq 0.05$), 7 and 10μg/ml ($p \leq 0.001$) respectively, compared

with 1μg/ml vitamin E succinate supplemented cultures. In contrast, vitamin E levels in BL6 cells supplemented with 1-10μg/ml vitamin E succinate, were marginally increased but showed no significant trend compared with 1μg/ml supplemented cultures. In LLCMK cells, supplementation with 3-10μg/ml vitamin E succinate resulted in no significant change in the levels of vitamin E succinate compared with 1μg/ml sample. However, with respect to the vitamin E levels in LLCMK cells, vitamin E succinate supplementation resulted in a general increase when compared with the 1μg/ml group, with a significant ($p \leq 0.001$) increase occurring at 10μg/ml.

Table 8: The effect of vitamin E succinate supplementation on vitamin E and vitamin E succinate levels in BL6 and LLCMK cells respectively. (Each value in the graph represents the mean of 3 samples $\pm$ SEM).

Vitamin E succinate (μg/ml)	Vitamin E succinate (nmol/10^6 cells)		Vitamin E (nmol/10^6 cells)	
	BL6 cells	LLCMK cells	BL6 cells	LLCMK cells
0E	ND	ND	ND	ND
1	0.294 $\pm$0.069	0.203[e] $\pm$0.048	0.075 $\pm$0.018	0.016 $\pm$0.002
3	1.149[bc] $\pm$0.129	0.048 $\pm$0.007	0.235 $\pm$0.121	0.073[f] $\pm$0.035
5	0.922[bd] $\pm$0.104	0.229 $\pm$0.075	0.143 $\pm$0.056	0.091 $\pm$0.031
7	2.152[ac] $\pm$0.234	0.452[e] $\pm$0.233	0.055 $\pm$0.004	0.107 $\pm$0.010
10	4.09[ac] $\pm$0.277	0.168 $\pm$0.032	0.159 $\pm$0.037	0.156[g] $\pm$0.034

ND= Not detectable
a= $p \leq 0.001$: Relative to 1μg/ml vitamin E succinate supplemented BL6 cells.
b= $p \leq 0.05$: Relative to 1μg/ml vitamin E succinate supplemented BL6 cells.
c= $p \leq 0.001$: Relative to vitamin E levels in BL6 cells in the relevant groups.
d= $p \leq 0.01$: Relative to vitamin E levels in 5μg/ml vitamin E succinate supplemented BL6 cells.
e= $p \leq 0.01$: Relative to vitamin E levels in LLCMK cells in the relevant groups.
f= $p \leq 0.05$: Relative to vitamin E succinate levels in 3μg/ml supplemented LLCMK cells.
g= $p \leq 0.01$: Relative to 1μg/ml vitamin E succinate supplemented BL6 cells.

Comparison of vitamin E succinate and vitamin E levels in BL6 cells supplemented with 1-10μg/ml vitamin E succinate, revealed generally higher levels of vitamin E succinate in these cells, significantly so at 7 and 10μg/ml. In LLCMK cells a similar trend to BL6 cells was observed, with

significant increases occurring at 1 and 7μg/ml. The only exception was that recorded at 3μg/ml, where significantly higher levels of vitamin E were detected.

3.5 DISCUSSION

Studies by Fariss on vitamin E succinate-mediated cytoprotection against cadmium-induced (412) and oxygen-induced (413) toxicity in freshly isolated rat hepatocytes showed that vitamin E succinate supplementation significantly reduced lipid peroxidation levels. In addition, studies by Pascoe and Reed (cited in 404,405) have shown that vitamin E succinate was more effective than vitamin E in inhibiting the toxic effects of free radical-generating drugs such as adriamycin. The actual mechanism by which vitamin E succinate acts as an antioxidant, quenching free radicals and preventing lipid peroxidation, is unclear, although it has been suggested that vitamin E succinate may act as a carrier for vitamin E, enabling the uptake, release and accumulation of vitamin E at a unique and critical cellular site within the cell (404,405,412,413).

In BL6 and LLCMK cells used in this study, however, supplementation of vitamin E succinate resulted in a marked increase in lipid peroxidation levels, with a significant increase occurring in the BL6 cells. The free radical levels in BL6 cells followed a similar trend to that of lipid peroxidation, while in the non-malignant LLCMK cells, an initial decrease in free radical levels occurred, followed by a general increase in free radical levels. One possible explanation for the ineffectiveness of vitamin E succinate in preventing free radical and lipid peroxidation in BL6 and LLCMK cells is that different forms of vitamin E exhibit varied intracellular compartmental distribution patterns (126), which may be cell specific. This is suggested by findings in BL6 and LLCMK uptake studies, which clearly show that vitamin E succinate was taken up by these cells, and furthermore that in BL6 cells, equivalent amounts of vitamin E succinate were located in both the cytosolic and membrane fractions, while in LLCMK cells the majority of vitamin E succinate was located in the cytosolic portion of the cell.

The theory that vitamin E succinate acts as a carrier for vitamin E, enabling the uptake and release of vitamin E at a unique site within the cell, is not supported by data from these studies. In BL6 cells, supplementation with increasing concentrations of vitamin E succinate did not result in a marked increase in cellular vitamin E content, but rather a cellular accumulation of the intact vitamin E succinate molecule. LLCMK cells showed a general increase in vitamin E levels with increasing concentrations of vitamin E succinate, although the levels of vitamin E succinate were generally

higher than vitamin E levels. These results suggest that the inability of vitamin E succinate supplementation to reduce free radical and lipid peroxidation levels in BL6 and LLCMK cells, may be due to the absence of various esterase enzymes in these cells, capable of removing the succinate component of the vitamin E succinate molecule, and liberating free vitamin E with antioxidant properties.

Vitamin E succinate may have numerous other potential roles within the cell, such as alteration in membrane stability or function through the direct interaction of the lipophilic TOH moiety with unsaturated fatty acids and the ionic succinate moiety with polar phospholipid regions of the membranes (412,413). Several reports indicate that vitamin E succinate supplementation stabilises membranes (414) and alters membrane-bound enzyme activities (124,415,416). In order to explain the lack of antioxidant effect of vitamin E succinate found in this study, alternative pathways needed to be investigated. Since vitamin E has been suggested to play a role in the direct modulation of regulatory proteins in signal transduction pathways, as well as modulating the activity of key regulatory enzymes in the eicosanoid pathway (135,417,418), the effect of vitamin E succinate supplementation on the eicosanoid pathway and its relevant metabolites associated with cell growth were subsequently investigated in BL6 and LLCMK cells.

4

VITAMIN E SUCCINATE SUPPLEMENTATION AND SECONDARY MESSENGER METABOLISM

Studies reported in chapter 2 have shown that supplementation of vitamin E succinate significantly reduced BL6 cell growth while having minimal effects on LLCMK cell growth. Although numerous researchers working with BL6 cells have reported similar growth inhibitory effects upon supplementation of vitamin E succinate (42,124,127,399), the actual mechanism by which vitamin E succinate inhibits growth is unclear. This study initially considered the possibility that toxic effects of vitamin E succinate supplementation on tumour cell growth were mediated to a certain extent through the antioxidant properties of the vitamin. Studies reported in chapter 3 however revealed no antioxidant role for vitamin E succinate in BL6 or LLCMK cells, but rather a prooxidant role, increasing free radical formation and lipid peroxidation levels within these cells. Since lipid peroxidation (159,235) and free radical formation (235) has been linked to COX and LOX activity, and vitamin E is believed to be active in a number of aspects of the eicosanoid metabolism (135,417,418), it was necessary to determine the effect of vitamin E succinate supplementation on various enzymes and metabolites associated with this pathway (figure 4), as well as the various factors influencing the pathway.

4.1 VITAMIN E SUCCINATE SUPPLEMENTATION AND CALCIUM LEVELS

4.1.1 INTRODUCTION

Although characterised only by its charge, coordination number and unhydrated radius (263), Ca^{2+} ions acquire a specific ability to modulate many cellular functions when bound to calcium-dependant regulatory proteins such as calmodulin (263,264,419). Ca^{2+} ions exert a variety of effects on biological processes and are known to participate in membrane-associated functions such as regulation of enzyme activities (145,275,277,419,420), transduction of hormonal information, stimulus secretion coupling, transport systems, neuronal conduction and muscular contraction (145). The ability of Ca^{2+} to regulate metabolic processes within a cell, is believed to be due to the "sticky" nature of Ca^{2+} ions, which enables them to bind to a variety of ligands such as proteins and phosphate groups (264). The

availability 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 (278,279), and this is believed to be a major factor contributing to tumour mortality (284). Since Ca^{2+} levels play an important role in modulating cell proliferation, and earlier studies (chapter 2) have shown significant inhibition of malignant BL6 cell growth following vitamin E succinate supplementation, it was necessary to determine the levels of Ca^{2+} in malignant BL6 and non-malignant LLCMK cells, as well as the effect of vitamin E succinate supplementation on intracellular Ca^{2+} levels.

4.1.2 MATERIALS AND METHODS

MATERIALS

A GBC 909 model Atomic Absorption Spectrophotometer was used for Ca^{2+}determinations. Calcium Chloride ($CaCl_2$) standard was purchased from Merck, Darmstadt Germany.

METHODS

4.1.2.1 Glassware preparation

Refer to section 3.4.1.1 for method used.

4.1.2.2 Cell culture procedure

Methods described in section 2.2.3.1 and 2.2.3.2 were repeated, except that cell pellets were resuspended in 1.0ml of Tris-HCl buffer pH 7.4.

4.1.2.3 Acid digestion and calcium determination

On completion of cell enumeration, suspensions were centrifuged at 3 000g for 10 minutes. The resulting supernatant solutions were discarded and the wash repeatedly with 1.0ml of Tris-HCl buffer. The supernatant fraction was discarded and centrifugation tubes were drained. Cell pellets were resuspended in 0.2ml concentrated HCl and boiled at 100^{0}C for two hours. Following acid digestion,

0.3ml of milli-Q water was added to cell pellets to give a final volume of 1.0ml. This was followed by centrifugation at 3 000g for 10 minutes to remove debris. Ca^{2+} levels in supernatant phases were determined using flame atomic absorption spectroscopy, using a GBC model atomic absorption spectrophotometer. The fuel source was nitric oxide:acetylene; slit width was 0.5nm; wavelength set at 422.7nm; and current 3mA. The absorbance values obtained were converted to μM Ca^{2+}concentrations from a standard curve (appendix 4) generated from standard $CaCl_2$ solution.

4.1.3 STATISTICAL ANALYSIS

The results obtained were analysed using a One Way Analysis of Variance (ANOVA) followed by the Student Newmans-Kuels Multiple Range Test. Data in all subsequent sections of this chapter were analysed by this method.

4.1.4 RESULTS

Supplementation of BL6 cells with 1-10μg/ml vitamin E succinate (table 9) resulted in a non-significant increase in Ca^{2+} levels at 1-7μg/ml respectively, while at 10μg/ml supplementation, a non-significant decrease occurred compared with control cultures 0E.

In LLCMK cells, supplementation with 1 and 3μg/ml vitamin E succinate resulted in a marked increase in Ca^{2+} levels compared with control cultures 0E, with a significant ($p \leq 0.05$) increase occurring at 3μg/ml. In contrast, supplementation of 5, 7 and 10μg/ml vitamin E succinate resulted in a general decrease in Ca^{2+}levels, with a significant ($p \leq 0.001$) decrease occurring at 7 and 10μg/ml respectively. The Ca^{2+} levels in BL6 control and vitamin E succinate-supplemented cultures were generally lower than the levels of Ca^{2+} found in the LLCMK control cultures, significantly so in the control and 1-5μg/ml vitamin E succinate-supplemented cultures.

Table 9: The effect of vitamin E succinate supplementation on calcium levels in BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	Calcium concentration (μg/10^6 cells)	
	BL6 cells	**LLCMK cells**
0E	8.24[a] ±1.57	103.80 ±60.83
1	11.30[c] ±6.00	226.83 ±115.11
3	10.10[c] ±3.60	404.80[b] ±134.09
5	9.79[d] ±3.35	84.92 ±31.51
7	9.68 ±1.44	10.84[a] ±1.82
10	7.30 ±3.30	8.96[a] ±1.29

a= $p \leq 0.001$: Relative to LLCMK control cultures 0E.
b= $p \leq 0.05$: Relative to LLCMK control cultures 0E.
c= $p \leq 0.001$: Relative to calcium levels in LLCMK cells in the corresponding groups.
d= $p \leq 0.05$: Relative to Ca^{2+} levels in 5μg/ml supplemented LLCMK cultures.

4.1.5 DISCUSSION

Cancer is associated more frequently with hypocalcaemia than with hypercalcaemia (421). Pancreatic acinar carcinoma cells have been shown to have lower levels of free Ca^{2+} than their normal cell counterparts (cited in 422). In contrast, other tumour cells have higher levels of Ca^{2+} than their normal cell counterparts (285,287). In this study malignant BL6 control cultures were found to have significantly lower levels of Ca^{2+} than non-malignant LLCMK control cultures. Coman (cited in 285 and 287) postulated that the local invasiveness and ability of tumour cells to metastasis were due to decreased adhesiveness between tumour cells as a result of decreased ability of these cells to bind Ca^{2+}. If this hypothesis were valid, one would expect lower levels of free Ca^{2+}in malignant cells than in non-malignant cells. In another study on BL6 cells, the amount of soluble Ca^{2+} in most cases was below 50% of the total Ca^{2+} within the cell (287).

4. Secondary Messenger Metabolism

Changes in the distribution and levels of intracellular Ca^{2+} are believed to play an important role in the development of neoplasia (422). In BL6 cells used in this study, supplementation of 1 through to 7μg/ml vitamin E succinate resulted in a general although non-significant increase in intracellular Ca^{2+} levels compared to control cultures, while at 10μg/ml, a slight decrease in Ca^{2+}levels occurred. The ability of vitamin E succinate to alter the physical properties of membranes through their physicochemical interaction with membrane lipids (412,413), could account for the observed changes in Ca^{2+} levels, since the only mechanism available for causing large scale changes in intracellular Ca^{2+} levels involves movements across the plasma membranes (264). In non-malignant LLCMK cells, an inconsistent trend in Ca^{2+} levels was found following supplementation with vitamin E succinate. As far as can be ascertained, the effect of vitamin E succinate supplementation on Ca^{2+} levels in LLCMK cells has not been reported to date. As Ca^{2+} levels play an important role in modulating PLA_2 activity (270-277,420), and since marked changes in intracellular Ca^{2+} levels have been shown to occur in BL6 and LLCMK cells supplemented with varying levels of vitamin E succinate, it was necessary to study the effect of vitamin E succinate supplementation on PLA_2 activity in BL6 and LLCMK cells.

4.2 VITAMIN E SUCCINATE SUPPLEMENTATION AND PHOSPHOLIPASE A_2 ACTIVITY

4.2.1 INTRODUCTION

All mammalian cell membranes consist of a lipid bilayer composed primarily of phospholipids and cholesterol (146). Phospholipids constitute the major structural components of cell membranes and play a key role in a number of biological functions such as signal transduction, secondary messenger generation, cell membrane integrity (146,423,424) and activity of membrane-bound enzymes (146,423-425). The fatty acid AA, present at the one and two position on the phospholipid molecule (423) is an important precursor of PG and leukotriene biosynthesis (426-428) in its non-esterified form (429). The release of AA from the Sn_2 position of phospholipids moieties occurs primarily through the activation of PLA_2 enzymes (424-427,430-432). These enzymes are a structurally diverse group, which include low MW Ca^{2+}-dependant and independent intracellular forms (420). Of interest to this study is the membrane-bound 85KDa PLA_2 (cPLA), which is specific for AA at the Sn_2 position, and regulated by intracellular Ca^{2+} levels. This enzyme is activated and becomes membrane-associated at levels of Ca^{2+} found intracellularly (291,293,295,297,299,311,420). Furthermore studies

by Lin *et al* (311) have demonstrated that $cPLA_2$ activity can be triggered by the activation of cell surface receptors, and that receptor-mediated activation of $cPLA_2$ requires synergistic actions of intracellular Ca^{2+} and a phosphorylation of enzyme.

The calcium-dependant PLA_2 enzyme plays a crucial role in the eicosanoid pathway, where it functions to produce rate-limiting precursor substrates for 5-LOX and COX enzymes (273,292-297,299,304). Since this study considered the effect of vitamin E succinate supplementation on the eicosanoid pathway (figure 4), and prostaglandin synthesis is regulated acutely by PLA_2 activity, and indirectly by changes in intracellular Ca^{2+} levels, the effect of vitamin E succinate supplementation on PLA_2 in BL6 and LLCMK cells was determined.

4.2.2 MATERIALS AND METHODS

MATERIALS

1-Palmitoyl-2-(1-C^{14})-Arachidonyl-sn-Glycero-3-Phosphotidylcholine (1-C^{14}-PC) was supplied by New England Nuclear Products, Boston, USA. Taurocholic acid, fatty acid free Bovine Serum Albumin (BSA) and Phosphatidyl Choline (PC) were purchased from Sigma Chemical Co., USA. Chloroform was obtained from BDH Chemicals LTD, England, while Folin-Ciocalteau reagent, Copper Sulphate ($CuSO_4 \cdot 5H_2O$) and sodium tartrate were purchased from Unilab, SAARCHEM, South Africa. Silica gel 60 aluminium Thin Layer Chromatography (TLC) plates were supplied by Merck, Darmstadt, Germany.

METHODS

4.2.2.1 Cell culture procedure

Methods described in 2.2.3.1 and 2.2.3.2 were repeated except that cell pellets were resuspended in 1.0ml of 0.1M Tris-HCl buffer pH 7.4.

4.2.2.2 Homogenisation and separation of membrane and stroma fractions.

The method described in section 3.3.1.2 was used with the following change. The pellet (membrane) fraction was retained and resuspended in 2.0ml 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 433). The absorbance values obtained were converted to mg/ml protein from a standard curve (appendix 5) generated from BSA.

4.2.2.4 Phospholipase A_2 assay

A modified method of Krumhardt and Dupont (425) and Ballou and Cheung (303) was used to determine membrane PLA_2 activity.

A 200μl aliquot of membrane suspension was incubated for 10 minutes at 37^{0}C with 50μl of a reaction mixture containing 0.1M Tris-HCl buffer pH 7.5, 50mM taurocholic acid, 20mM $CaCl_2$, 0.05% (w/v) BSA and 0.03μCi of 1-C^{14}-PC. Following a 10 minute incubation period, a 100μl aliquot of this solution was spotted on a silica gel 60 TLC plate. Plates were developed in a solvent system of chloroform:methanol:water (65:25:4 v/v/v). AA and PC were used as markers. Silica gel in the regions corresponding to the AA and PC were scraped off into scintillation vials containing 10ml of emulsifier scintillation plus™ scintillation cocktail and counted in a Beckman scintillation counter (Model LS 2800). PLA_2 activity was determined as net 1-C^{14}-AA released from 1-C^{14}-PC substrate, and was expressed as pmol AA released per minute per mg membrane protein.

4.2.3 RESULTS

PLA_2 activity in BL6 control and vitamin E succinate-supplemented cultures were significantly ($p \leq 0.001$) lower than the levels of PLA_2 activity in LLCMK cultures (table 10). Supplementation of vitamin E succinate resulted in a significant increase in PLA_2 activity in BL6 cells at 1 ($p \leq 0.05$), 3 ($p \leq 0.001$) and 5μg/ml ($p \leq 0.05$) respectively, while at 7 and 10 μg/ml vitamin E succinate, a non-significant increase in PLA_2 activity was observed compared to control cultures 0E.

Table 10: The effect of vitamin E succinate supplementation on Phospholipase A_2 activity in BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	Phospholipase A_2 activity (pmol AA/min/mg protein)	
	BL6 cells	LLCMK cells
0E	2.48^{c} ±0.15	13.94 ±2.19
1	3.80^{bc} ±0.14	12.46 ±1.18
3	4.98^{ac} ±0.49	13.53 ±1.38
5	4.25^{bc} ±0.51	13.99 ±0.29
7	3.25^{c} ±0.12	14.27 ±0.87
10	3.38^{c} ±0.07	13.05 ±1.75

a= $p \leq 0.001$: Relative to BL6 control cultures 0E.
b= $p \leq 0.05$: Relative to BL6 control cultures 0E.
c= $p \leq 0.001$: Relative to PLA_2 activity in LLCMK cells in corresponding groups.

Supplementation of LLCMK cells with 1-10μg/ml vitamin E succinate resulted in no significant increasing or decreasing trend in PLA_2 activity compared to control cultures 0E, except at 7μg/ml vitamin E succinate, where an increase in PLA_2 activity was observed.

4.2.4 DISCUSSION

The mobilisation of AA is primarily initiated by physiological and/or pharmacological agonists which function to activate one or more intracellular or extracellular PLA_2 enzymes. In unstimulated cultured cells, the basal turnover of phospholipids by PLA_2 exhibits no particular fatty acyl specificity. In contrast, agonist-stimulated fatty acid mobilisation is remarkably specific for AA and a few structurally unrelated PUFAs. Many of the agonists which stimulate AA mobilisation also increase the levels of intracellular free Ca^{2+} (299). The 85KDa PLA_2 enzymes exhibit many prerequisite characteristics for involvement in intracellular signal transduction pathways (299), such as selectivity towards phospholipids containing AA at Sn_2 position, and its activation and translocation to

membranes in response to physiological (submicromolar) concentrations of Ca^{2+} (291,293,295,297, 299).

The activities of PLA_2 assessed in tumour and normal tissue have generally shown increased PLA_2 in tumour tissue compared to normal tissue (427,428). Comparing membrane bound PLA_2 activity in BL6 and LLCMK control cultures in this study, revealed that PLA_2 activity was significantly lower in the malignant BL6 cell line. Since this study was concerned with a Ca^{2+}-dependant PLA_2 enzyme, and earlier studies reported in section 4.1 have shown significantly lower levels of Ca^{2+} in BL6 cells compared to LLCMK cells, it is reasonable to assume that the lower levels of PLA_2 in BL6 control cultures may be due to lower intracellular Ca^{2+} levels. In addition to this, tumour cells have been reported to have altered essential fatty acid (EFA) compositions compared to their normal cell counterparts (423,434,435). In particular, the levels of PUFA AA have been found to be generally lower in tumour cells (434,435). Thus, limited availability of AA required for membrane-bound PLA_2 activity (299,311,420), could also account for the lower PLA_2 activity observed in BL6 cells.

Supplementation of BL6 cells with increasing levels of vitamin E succinate resulted in a general increase in PLA_2 activity relative to control cultures. These increases correlate positively with the corresponding slightly increased intracellular Ca^{2+} levels which were found in earlier studies, suggesting that PLA_2 activity in BL6 cells may be mediated in part by the intracellular Ca^{2+} levels. An exception was at 10μg/ml supplementation, which showed a marked increase in PLA_2 activity and decrease in Ca^{2+} levels. Since tumour cells are believed to have altered membrane fluidities (159-161), and vitamin E succinate supplementation has been suggested to alter membrane fluidity (413,414), it is proposed that the observed increase in membrane bound PLA_2 activity in BL6 cells at 10μg/ml vitamin E succinate supplementation, could be due to changes in the membranes physical properties, although the role of vitamin E succinate in modulating membrane fluidity at 1-7μg/ml vitamin E succinate may also influence PLA_2 activity at these levels of supplementation. While PLA_2 activity increased with vitamin E succinate supplementation in BL6 cells, LLCMK cells showed marginal increases in PLA_2 at 7μg/ml vitamin E succinate only. The reason for this lack of effect in LLCMK cells is unclear, although one factor to consider is that the physical and chemical properties of non-malignant cell membranes are essentially "normal" compared to those of malignant cells, and as a result membrane-bound enzymes are more stable in normal cells and less likely to be affected by vitamin E succinate supplementation. The dependence of 5-LOX and COX activity on Ca^{2+} (276) and AA levels within a cell, and the finding that vitamin E succinate supplementation markedly

altered intracellular Ca^{2+} levels and PLA_2 activity in BL6 cells, led to further investigations of both the activities of 5-LOX and COX in BL6 and LLCMK cells.

4.3 VITAMIN E SUCCINATE SUPPLEMENTATION AND LIPOXYGENASE ACTIVITY.

4.3.1 INTRODUCTION

AA is a precursor of a large variety of potent physiological effectors. This PUFA is the branch point leading to two important pathways, namely the COX pathway which functions to synthesise PGs and thromboxanes, and the LOX pathway which synthesises various leukotrienes and lipoxins (436). To date, three animal LOXs have been identified, and are referred to as 5-, 12- and 15-LOX depending on where oxygen is inserted on the AA moiety (437). Both the 12- and 15-LOX enzymes have been shown to have broad substrate specificities, whereas the 5-LOX enzymes predominantly react with C_{20} fatty acids such as AA. LOX enzymes are non-heme, non-sulphur iron dioxygenases which react with fatty acid substrates containing one or more 1,4-z,z-pentadiene moieties to yield z,e-conjugated hydroperoxides (436,438,439). These hydroperoxides are precursors of specific regulatory molecules, such as leukotrienes and lipoxins in animals (436,439), and jasmonic acid and traumatin in plants (439). Leukotrienes are a group of potent biological mediators which act at nanomolar concentrations in intracellular communications, signal transduction and more importantly host immune defence (440). Since the LOX pathway synthesises many products which affect cell proliferation (159,235), and the recent findings that metabolism of AA by LOX enzymes is a major source of free radical (235) and lipid peroxidation levels within a cell (159,235), the possibility exists that the observed increase in free radical and lipid peroxidation levels in BL6 and LLCMK cells (see section 3.2) supplemented with varying levels of vitamin E succinate were due to an increased LOX activity in these cells. Hence this study was undertaken to determine the effect of vitamin E succinate supplementation on 5-LOX activity in malignant BL6 and non-malignant LLCMK cells.

4.3.1.1 MATERIALS AND METHODS

MATERIALS

ATP and 5 (s)-Hydroperoxy-(6E,8Z,11Z,14Z)-Eicosatetranoic acid (5-HPETE) were purchased from Sigma Chemical Co., USA. Calcium Chloride ($CaCl_2$) was purchased form Merck, Darmstadt,

Germany, while sucrose was obtained from Unilab, SAARCHEM, South Africa.

METHODS

4.3.1.2 Cell culture procedure

The methods described in 2.2.3.1 and 2.2.3.2 were used, except that cell pellets were resuspended in 1.0ml of 10mM Tris-HCl buffer (pH 8.0) containing 1mM EDTA and 0.25M sucrose.

4.3.1.3 Homogenisation and enzyme preparation

Enzyme preparation was carried out using a modified method of Soberman (441). Following cell enumeration, cell suspensions were poured into a dounce homogeniser and homogenised 30 times with a tight plunger. The homogeniser was rinsed with 1.0ml of Tris-HCl buffer. Resulting cell homogenates were poured into precooled centrifugation tubes and centrifuged at 10 000g for 10 minutes at 4°C. Supernatant solutions were retained and stored at -70°C until required.

4.3.1.4 5-Lipoxygenase assay

For routine assay of 5-LOX activity, a modified method of Soberman (441) and Furukawa *et al* (442) was used. The principle of this assay is based on the addition of ^{3}H-AA to the enzyme preparation, separation of product 5-HPETE from substrate ^{3}H-AA by TLC and quantification and determination of radioactive product formed by scraping the relevant sections of the TLC plates. Standard reaction mixtures containing 10mM Tris-HCl (pH 8,0), 4mM ATP, 2mM $CaCl_2$, 20μM 15-^{3}H AA and enzyme solution (75μl) in 200μl final volume, were allowed to react for 10 minutes at room temperature, and terminated by the addition of 500μl 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. A 100μl aliquot of upper organic layer was removed, spotted onto precoated silica gel F_{254} aluminium TLC plates, and dried under a stream of nitrogen. AA and 5-HPETE standards were used as markers. Plates were developed in ethyl ether:petroleum ether:acetic acid (50:50:1 v/v/v) at 4°C. Silica gel in the regions corresponding to AA and 5-HPETE were scraped off into scintillation vials containing 10ml of emulsifier scintillator plus™ scintillation cocktail and counted in a Beckman counter (Model LS 2800). Final results were expressed as pmol 5-HPETE formed per 10^6 cells.

4.3.2 RESULTS

The effect of vitamin E succinate supplementation on 5-LOX activity in BL6 and LLCMK cells is shown in table 11. In BL6 cells, supplementation with 3-10μg/ml vitamin E succinate resulted in a general increase in 5-LOX activity compared with control cultures 0E, with a significant ($p \leq 0.001$) increase occurring at 10μg/ml. Supplementation of LLCMK cells with 1-10μg/ml vitamin E succinate resulted in no significant increase or decrease in 5-LOX activity at 1, 3 and 5μg/ml vitamin E succinate, while at 7 and 10μg/ml respectively, a significant ($p \leq 0.001$) increase in 5-LOX activity occurred. When comparing the levels of 5-LOX activity in control cultures of BL6 and LLCMK cells, lower levels of 5-LOX activity were detected in BL6 cells.

Table 11: The effect of vitamin E succinate supplementation on 5-lipoxygenase activity in BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	5-lipoxygnease activity (pmol 5-HPETE formed/10^6 cells)	
	BL6 cells	LLCMK cells
0E	0.23 ±0.02	0.28 ±0.06
1	0.15 ±0.03	0.29 ±0.06
3	0.31 ±0.03	0.17 ±0.02
5	0.28 ±0.03	0.41 ±0.05
7	0.33 ±0.04	1.07[b] ±0.15
10	0.85[a] ±0.03	1.07[b] ±0.10

a= $p \leq 0.001$: Relative to BL6 control cultures 0E.
b= $p \leq 0.001$: Relative to LLCMK control cultures 0E.

4.3.3 DISCUSSION

The 5-LOX (EC 1.13.11.34) enzyme catalyses the first two steps in leukotriene formation (443-446).

These two steps involve the oxygenation of AA to form 5-hydroperoxy acid (5HPETE) and subsequent conversion of 5-HPETE to leukotriene A_4 (LTA_4) (442,443,446-448). 5-LOX activity in the BL6 control and vitamin E succinate-supplemented cultures in this study, were found to be markedly lower than that in the LLCMK control cultures, which supports evidence that malignant cells generally have lower 5-LOX (449).

Results from this study also indicate that vitamin E succinate supplementation stimulates 5-LOX activity in BL6 and LLCMK cells. Other studies (439,450) however, have suggested that chain breaking antioxidants such as vitamin E inhibit LOX activity, and furthermore that these inhibitory effects occur at high doses of vitamin E. Vitamin E succinate, however, is a non-physiological antioxidant which exhibits no antioxidant properties in its esterified form (126,398,403,404). Since no significant cleavage of vitamin E succinate was shown to occur in BL6 cells (section 3.4), it is proposed that the observed effects of vitamin E succinate supplementation on 5-LOX activity in BL6 cells did not involve antioxidant properties. 5-LOX enzymes exhibit a specific requirement for mM Ca^{2+}concentrations (438,442,443,446), lipid hydroperoxide activators (438,449,451) and precursor fatty acids AA (438) during catalytic activity. This suggests that the observed effects of vitamin E succinate supplementation on BL6 and LLCMK 5-LOX activity may be mediated through changes in the levels of one or more of these cofactors. However, earlier studies on intracellular Ca^{2+} in LLCMK cells have shown significant decreases in Ca^{2+} levels at 7 and 10μg/ml vitamin E succinate, while in BL6 cells, marked decreases occurred at 10μg/ml supplementation. These decreases in intracellular Ca^{2+} levels were inversely related to 5-LOX activity, and suggests that 5-LOX activity in these cells was independent of intracellular Ca^{2+} levels at high concentrations of vitamin E succinate.

4.4 VITAMIN E SUCCINATE SUPPLEMENTATION AND CYCLOOXYGENASE ACTIVITY

4.4.1 INTRODUCTION

The cyclooxygenase enzyme is a membrane-bound multi-enzyme complex (176) located on the smooth ER (170,172-174,195). This enzyme metabolises AA to various eicosanoids (see section 1.5.4 and 1.5.5) and is found in both normal and neoplastic tissues (452). As previously mentioned, vitamin E and other lipid-soluble antioxidants are believed to modulate the eicosanoid pathway at a number

of different points (417,418). It has been suggested that antioxidants either inhibit, stimulate or have no effect on COX activity (417). These effects however depend largely on the type and concentration of the antioxidant used (453) as well as the system being studied (417). In addition to their general role in eicosanoid metabolism, the COX enzymes are believed to be major sources of free radical (235) and lipid peroxidation levels within a cell (159,235). Since preliminary studies outlined in chapter 3 have shown significant increases in free radical and lipid peroxidation levels in BL6 cells following supplementation with vitamin E succinate, this study was undertaken to determine the effects of vitamin E succinate supplementation on COX activity in malignant BL6 and non-malignant LLCMK cells.

4.4.2 MATERIALS AND METHODS

MATERIALS

AA, Prostaglandin B_2 (PGB_2) and Protoporphyrin IX were purchased from Sigma Chemical Co., USA. Tris (Hydroxymethyl)-aminomethane was purchased from Boehringer Mannheim. Precoated Silica gel 60 F_{254} aluminium TLC plates were supplied by Merck, Darmstadt, Germany, while citric acid was obtained from H.W.O. Chemical Co., South Africa. 15-^{3}H AA was purchased from Amersham International, England. Petroleum ether, ethyl ether and anhydrous Sodium Sulphate (Na_2SO_4) were obtained from Unilab, SAARCHEM, South Africa.

METHODS

4.4.2.1 Cell culture procedure

The methods described in 2.2.3.1 and 2.2.3.2 were used, except that the cell pellets were resuspended in 1.0ml of Tris-HCl buffer, and final pellets (membrane fraction) resuspended in 2.0ml of Tris-HCl buffer.

4.4.2.2 Homogenisation and separation into membrane and stroma fractions

The method described in section 3.3.1.2 was used except that cell pellets (membrane fractions) were resuspended in 2.0ml 0.1M Tris-HCl buffer pH 7.4.

4.4.2.3 Cyclooxygenase assay

A modified method of Yamamoto (454) was used to determine COX activity. The principle of this assay is based on the addition of a radioactive substrate (^{3}H-AA) to the enzyme preparation and separation of reaction product PGG_2 by thin layer chromatography (TLC) based upon their affinity for a non-polar solvent. A reaction mixture containing 70μl of 0.2M Tris-HCl buffer pH 8.0, 10μl of 40μM manganese protoporphyrin IX, 5μl of 5nmol AA and 5μl of 15-^{3}H AA (0.42pmol) was vortex mixed to disperse AA. A 10μl aliquot of the membrane suspension was added to the reaction mixture, and allowed to react for 2 minutes at room temperature. Termination of the reaction was carried out by addition of 300μl of a mixture of ethyl ether:methanol:0.2M citric acid (30:4:1 v/v/v) precooled to -20^{0}C. The terminated reaction mixture was transferred to an ice bath, where 0.5g of anhydrous sulphate was added with gentle shaking. A 150μl aliquot of the upper organic phase was removed using a graduated capillary tube, spotted onto precoated silica gel 60 F_{254} aluminum TLC plates and dried under a stream of nitrogen. AA and PGB_2 standards were used as markers since PGG_2 is not available commercially. Plates were developed in TLC tanks containing ethyl ether:petroleum ether:acetic acid (85:15:0.1 v/v/v) in a fridge at 4^{0}C. AA and PGB_2 markers were visualised with iodine vapours and product PGG_2 located between these two markers (455). Silica gel in the regions corresponding to AA, and PGG_2 were scraped off into scintillation vials containing 10ml of emulsifier scintillator plus™ scintillation cocktail and counted in a Beckman scintillation counter (Model LS 2800). Final results were expressed as pmol PGG_2 formed per 10^6 cells.

4.4.3 RESULTS

At vitamin E succinate concentrations of 1 and 3μg/ml, no significant increase or decrease in COX activity in BL6 cells (table 12) was detected, compared with control cultures 0E, while at 5,7 and 10μg/ml, a marked increase in COX activity occurred, with significant increases occurring at 7μg/ml ($p \leq 0.025$) and 10μg/ml ($p \leq 0.001$) respectively.

Treatment of LLCMK cells with varying levels of vitamin E succinate (1-10μg/ml) resulted in significant decreases in COX activity at 1 ($p \leq 0.001$), 5 ($p \leq 0.01$) and 7μg/ml ($p \leq 0.05$) respectively, while at 10μg/ml, a non-significant increase in COX activity occurred when compared with control cultures 0E. Comparing the levels of COX activity in BL6 versus LLCMK cultures (table 12), the trend revealed was that of substantially higher levels of COX activity in BL6 cells.

Table 12: The effect of vitamin E succinate supplementation on cyclooxygenase activity in BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	Cyclooxygenase activity (pmol PGG_2 formed/10^6 cells)	
	BL6 cells	LLCMK cells ($x10^2$)
0E	0.248f ±0.086	0.592 ±0.055
1	0.579^{f} ±0.020	0.293^{c} ±0.017
3	0.018^{h} ±0.005	0.504 ±0.020
5	0.826^{g} ±0.610	0.402^{d} ±0.045
7	1.641^{bg} ±0.188	0.428^{e} ±0.034
10	3.785^{ag} ±0.460	0.669 ±0.131

a= p≤0.001: Relative to BL6 control cultures 0E.
b= p≤0.025: Relative to BL6 control cultures 0E.
c= p≤0.001: Relative to LLCMK control cultures 0E.
d= p≤0.01: Relative to LLCMK control cultures 0E.
e= p≤0.05: Relative to LLCMK control cultures 0E.
f= p≤0.01: Relative to COX activity in LLCMK cells in corresponding groups.
g= p≤0.001: Relative to COX activity in LLCMK cells in corresponding groups.
h= p≤0.05: Relative to COX activity in 3μg/ml supplemented LLCMK cultures.

4.4.4 DISCUSSION

As previously mentioned, vitamin E is believed to be active in modulating the activity of a number of key regulatory enzymes in the eicosanoid pathway, such as the COX enzyme (417,418). In this study, supplementation of BL6 cells with 1, 3 and 5μg/ml vitamin E succinate did not significantly affect COX activity. However, as the vitamin supplementation was increased to 7 and 10μg/ml, a significant increase in COX activity was detected. Although the role of vitamin E in COX reactions has been disputed for some time, it is suggested that vitamin E stimulates COX activity by protecting the enzyme from inactivation resulting from autooxidation (417). In BL6 cells however, vitamin E succinate supplementation was shown to exhibit no antioxidant properties in BL6 cells (see section 3.2). One possible role for vitamin E succinate in modulating the activity of the COX enzyme is

through its physicochemical interaction with and stabilisation of biological membranes (412,413). Membranes as compartmentalizing structures not only bring together molecules taking part in metabolic reactions (98), but also regulate the activities of various membrane-bound enzymes (98,455). In diseased states (*eg* neoplastic states), lipid structures are usually altered, resulting in changes in membrane fluidity and consequent enzyme activities (159,161). Studies by Lai *et al* (414) have shown that vitamin E succinate supplementation stabilises membranes.

In LLCMK cells, a significant decrease in COX activity occurred at vitamin E succinate concentrations of 1, 5 and 7μg/ml respectively. Antioxidants are believed to inhibit COX activity by lowering the concentration of oxygen-centred radicals necessary for COX activity (417). Although vitamin E succinate exhibits no antioxidant properties in its esterified form (126,403,404), earlier studies (see section 3.4) have shown that supplementation of vitamin E succinate to LLCMK cells results in marked increases in cellular α-TOH content. This increase in free α-TOH could account for the observed decrease in LLCMK COX activity. Comparing levels of COX activity in BL6 and LLCMK cells, the activity of the COX enzyme was shown to be significantly higher in BL6 cells. As previously mentioned, neoplastic transformation is believed to alter lipid structures and membrane fluidity in a number of tissues (159,161). Since the COX enzyme is membrane-bound (170,172-174,195,196), such changes in membrane physical properties may account for the differences in COX activity in BL6 and LLCMK cells.

4.5 VITAMIN E SUCCINATE SUPPLEMENTATION AND PROSTAGLANDIN E_2 LEVELS

4.5.1 INTRODUCTION

PGs are a group of closely related 20-carbon compounds, derived both enzymatically and non-enzymatically from the PUFA AA (203,205,207,209). PG synthesis is initiated when active and specific phospholipases release AA from various phospholipid and glyceride stores (165,166,209,417). These compounds are believed to play a regulatory role in a number of systems such as the reproductive, neural and cardiovascular systems (207). More recently PGs have been implicated as modulators of tumour metastasis (205,207,209,231-235), tumour promotion (205,207,209-211,234-236), host immunoregulation (205,206,211,233-235,237) and cell proliferation (205,209,211,218,221, 222,234, 237-241). Of particular interest to this study were the levels of PGE_2, since PGE_2 has been shown to inhibit the growth of various tumour cell lines *in vitro* (247-251) and *in vivo* (252,253),

through activation of an AC-cAMP linked system (205,248). Tumour cells synthesise and secrete large quantities of PGs, in particular E series PGs (205-207,209,210,234,241-244). Increased PGE_2 levels were found in tumours induced by Maloney sarcoma virus in mouse leg muscle tissue compared to normal leg muscle tissue in the same animal (209,210,241). Untransformed baby hamster kidney (BHK) and balb/c 3T3 fibroblasts in culture were shown to secrete low quantities of PGs into the media, whereas transformation of these cells with polyoma virus resulted in a significant increase in PGE_2 release into the media (210).

Vitamin E succinate supplementation has been shown to increase both PLA_2 (section 4.2) and COX (section 4.4) activity in BL6 cells, and to inhibit COX activity in LLCMK cells. Since the rate of synthesis and net production of PGs is controlled by the activities of the PLA_2 and COX enzymes respectively (166,168), it was necessary to study the effect of vitamin E succinate supplementation on PGE_2 levels in malignant BL6 and non-malignant LLCMK cells.

4.5.2 MATERIALS AND METHODS

MATERIALS

PGE_2 -I^{125} radio-immunoassay kit was obtained from Amersham Life Science, Inc, Plc., England. Methyl formate was supplied by Sigma Chemical Co., USA, while Sep-Pak C^{18}-Octadecyclsilyl (ODS) silica cartridges were obtained from Waters Association, USA.

METHODS

4.5.2.1 Cell culture procedure

Methods described in 2.2.3.1 and 2.2.3.2 were repeated, except that cell pellets were resuspended in 2.0ml of PBS buffer pH 7.0.

4.5.2.2 Homogenisation and preparation of samples

After cell enumeration, cell suspensions were poured into a dounce homogeniser and homogenised with 30 plunges of a tight plunger. The dounce homogeniser was rinsed with 2.0ml of water to give

a final volume of 4.0ml homogenate (2.0ml PBS and 2.0ml of water). Resulting homogenates were centrifuged at 1 000g for 10 minutes at 4°C to remove precipitated proteins.

4.5.2.3 Extraction of prostaglandin E_2

Extraction and isolation of PGE_2 was carried out using a modified method of Powell (456,457) and Nigan (458). Following centrifugation, the supernatant solution were poured into 15ml of cold absolute ethanol and shaken for one minute. The pH of the solutions was adjusted to 3.0 with 1N HCl. Sep-Pak C^{18} cartridges were pretreated with 20ml of 80% ethanol, followed by 20ml of distilled water to remove excess ethanol. Acidified samples were passed through the Sep-Pak C^{18} cartridges. Contaminating materials such as inorganic salts and neutral lipids were removed by passing 10ml of water and 10ml of distilled petroleum ether through the columns. PG extracts were eluted using 5.0ml of methyl formate, and dried under a stream of nitrogen at 25°C.

4.5.2.4 Determination of prostaglandin E_2 levels

The principle of this assay is based on the competition between unlabelled methyl-oximate derivative (PGE_2-MOX) and a fixed quantity of ^{125}I-labelled PGE_2 methyl-oximate derivative for binding sites on a PGE_2 antibody, which is specific for the methyl-oximate (459). Prior to their analysis with PGE_2-I^{125} assay kit, PG extracts were converted to methyl oximate derivatives. Dried PGE_2 extracts were reconstituted in 100μl of PBS buffer pH 7.0. To reconstituted samples were added 100μl of methyl oximate reagent, followed by vortex mixing and incubation at 60°C for one hour. Following methyl oximation, samples were diluted to a final volume of 500μl with PBS (pH 7.0) and assayed as described in the assay protocol. To appropriately labelled tubes was added 100μl of sample and 100μl of tracer followed by 100μl of antiserum. Tubes were briefly vortex mixed and incubated for two hours at room temperature. After incubation, 250μl of 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 centrifugation at 1500g for 10 minutes at 4°C. Following centrifugation, the supernatant solution was discarded and radioactivity in each sample was determined using a Packard Auto-Gamma scintillation counter. Picograms of PGE_2/tube were determined from a PGE_2 standard curve (appendix 6). Final results were expressed as pg $PGE_2/10^6$ cells.

4.5.3 RESULTS

The levels of PGE_2 in BL6 and LLCMK cells supplemented with 1-10μg/ml vitamin E succinate are

shown in table 13. In BL6 cells supplemented with 1, 3 and 5μg/ml vitamin E succinate, no significant increase or decrease in PGE_2 levels occurred compared with control cultures 0E. As the vitamin concentrations were increased to 7 and 10μg/ml respectively, a marked increase in PGE_2 levels occurred, with a significant ($p \leq 0.01$) increase occurring at 10μg/ml.

Table 13: The effect of vitamin E succinate supplementation on prostaglandin E_2 levels in BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	PGE_2 levels (pg/10^6 cells)	
	BL6 cells	LLCMK cells
0E	9.60[b] ±0.31	33.11 ±2.63
1	10.80[b] ±0.81	44.70 ±7.60
3	8.20[b] ±0.90	18.30 ±1.90
5	8.90[b] ±0.66	18.00 ±3.10
7	12.0[b] ±0.58	23.00 ±5.50
10	13.53[ab] ±0.56	20.00 ±2.70

a= $p \leq 0.01$: Relative to BL6 control cultures 0E.
b= $p \leq 0.05$: Relative to PGE_2 levels in LLCMK cells in corresponding groups.

Supplementation of LLCMK cells with 1-10μg/ml vitamin E succinate resulted in a non-significant increase in PGE_2 levels at 1μg/ml, followed by a non-significant decrease at 3-10μg/ml. The PGE_2 levels in BL6 control and vitamin E succinate-supplemented cultures were generally lower than PGE_2 levels in LLCMK cultures.

4.5.4 DISCUSSION

Vitamin E is believed to play an important role in several aspects of eicosanoid metabolism (417,418). Depending on the system, vitamin E may have no effect or may enhance PG synthesis (417). In one study (cited in 417), it was found that microsomes isolated form vitamin E-supplemented animals

synthesised far greater amounts of PGs than microsomes isolated from vitamin E-deficient animals. In this study supplementation of malignant BL6 cells with 1, 3 and 5μg/ml vitamin E succinate resulted in no significant increase or decrease in PGE_2 levels, while at 7 and 10μg/ml vitamin E succinate, marked increases in PGE_2 levels occurred relative to control cultures. Studies by El Attar and Lin (460) have shown that supplementation of Scc-25 oral squamous carcinoma cells with 1 and 10μM vitamin E succinate stimulates PGE_2 synthesis, and furthermore suggest that these effects of vitamin E on endogenous PGE_2 levels were a reflection of the activities of both the PLA_2 and COX enzymes. This suggests that the observed increase in PGE_2 levels in BL6 cells following vitamin E succinate supplementation could be due to increased PLA_2 and COX activity observed in earlier sections of this study (see section 4.2 and 4.4). In non-malignant LLCMK cells on the other hand, supplementation of 3-10μg/ml vitamin E succinate resulted in a general decrease in PGE_2 levels compared to control cultures 0E. This decrease was positively correlated with a decrease in LLCMK COX activity (section 4.4), providing further evidence for a direct link between PGE_2 levels and COX activity. Human gingival fibroblasts supplemented with 10 and 100μM vitamin E succinate also showed significant decreases in PGE_2 formation (460).

Over the past few years evidence has been put forward which suggests that increased PG synthesis may be responsible for increasing both the tumorigenicity and metastatic potential of various tumour cells (205,207,210,211,232,236). The precise role of PGE_2 in tumorigenicity is at present unclear (232), although its presence in most tumour cells is believed to contribute to the ability of these cells to metastasise (232,236,239,461). Researchers have suggested that the metastatic potential of BL6 cells is inversely related to the ability of these cells to synthesise PGE_2 (209,232,250,252,255), and furthermore that the highly metastatic BL6-F10 population, as used in this study, synthesise far less PGE_2 than the less metastatic F_1 population (233,255). Since the quantity and type of PG produced by a given cell is vital to its metabolic effect, elucidation of the various PGs produced by a cell is of paramount importance in understanding the metabolic regulation in that cell (213). In terms of eicosanoid biology the BL6 melanoma cells are unusual in that PGD_2 is the major PG produced, while PGE_2 and $F_{2\alpha}$ are synthesised in smaller amounts (232,256,257). It is therefore important to determine the extent of production of the AA metabolites PGD_2, $PGF_{2\alpha}$ and PGI_2 in BL6 cells, and the effect of vitamin E succinate supplementation on the levels of these PGs. The above-mentioned PGs are the major ones produced by BL6 cells (232,233,255). No quantitation of these PGs was carried out in LLCMK cells, since vitamin E succinate supplementation was shown to have little effect on cell growth, and indeed decreased the activity of the COX enzyme, a critical determinant in controlling

PG synthesis (165,166,213). In view of these factors, and the cost of obtaining the assay kits, PGI_2, $F_{2\alpha}$ and D_2 assays were not performed in LLCMK cells.

4.6 VITAMIN E SUCCINATE SUPPLEMENTATION AND PROSTAGLANDIN D_2, $F_{2\alpha}$ AND I_2 LEVELS IN BL6 CELLS

4.6.1 INTRODUCTION

Mammalian tissues do not store PGs to any great extent (203,208-212), but rapidly synthesise them upon stimulation (208,209,212). Induction of PG synthesis occurs when a hormone or protein interacts with specific receptors or protein targets on the cell surface (166). This interaction results in the release of AA and subsequent oxidation by PGH synthase enzyme to endoperoxide PGH_2 (208) (see section 1.5.4). The product of the PGH synthase enzyme, PGH_2, is an important intermediate as it occupies a pivotal position in the divergent pathways which lead to the synthesis of various PGs and thromboxanes (165,167,169,173,208,209). The aim of this part of the study was to quantitate and compare the levels of PGD_2, $PGF_{2\alpha}$ and PGI_2 in BL6 cells with previously determined PGE_2 levels, as well as the effect of vitamin E succinate supplementation on the levels of these PGs.

4.6.2 THE EFFECT OF VITAMIN E SUCCINATE SUPPLEMENTATION ON PROSTAGLANDIN D_2 AND $F_{2\alpha}$ FORMATION IN BL6 CELLS

4.6.2.1 MATERIALS AND METHODS

MATERIALS

^{3}H-PGD_2 and ^{3}H-$PGF_{2\alpha}$ radio-immunoassay kits were obtained from Amersham Life Science, Int., England.

METHODS

4.6.2.2 Cell culture procedure

Methods described in 2.2.3.1 and 2.2.3.2 were repeated except, that cell pellets were resuspended

in 2.0ml of PBS pH 7.0.

4.6.2.3 Homogenisation and preparation of samples

Refer to section 4.5.2.2 for method used.

4.6.2.4 Extraction of prostaglandin D_2 and $F_{2\alpha}$

Isolation and extraction of PGD_2 and $PGF_{2\alpha}$ was carried out using the method described in section 4.5.2.3 with the following changes. The dried PG extracts were reconstituted in 500μl of assay buffer and duplicate 100μl aliquots used for PGD_2 and $PGF_{2\alpha}$ determination.

4.6.2.5 Determination of prostaglandin D_2 levels

The principle of this assay is based on the competition between unlabelled PGD_2 and a fixed quantity of ^{3}H-PGD_2 for binding to a fixed quantity of antibody which is both specific and has a high affinity for PGD_2 (462). A 100μl aliquot of sample was pipetted into appropriately labelled tubes. One hundred microlitres of tracer was added to each vial, followed by a 100μl of antiserum and 100μl of assay buffer. All tubes were vortex mixed and incubated overnight at 2-8^{0}C. Following incubation period, 500μl of charcoal suspension was added to each tube. The tubes were vortex mixed and allowed to incubate in an ice bath for 10 minutes before centrifuging at 2 000g for 10 minutes at 4^{0}C. Resulting supernatant solutions were decanted into scintillation vials containing 10ml of emulsifier scintillator plus™ scintillation cocktail. Radioactivity in each sample was determined using a Beckman (Model LS 2800) scintillation counter. The concentration of unlabelled PGD_2 in the sample was determined from a PGD_2 standard curve (appendix 7), and final results expressed as pg PGD_2 per 10^6 cells.

4.6.2.6 Determination of prostaglandin $F_{2\alpha}$ levels

The principle of this assay is based upon competition between unlabelled $PGF_{2\alpha}$ and a fixed quantity of ^{3}H-$PGF_{2\alpha}$ for binding to a fixed quantity of an antibody which has high specificity and affinity for $PGF_{2\alpha}$ (463). A 100μl aliquot of sample was pipetted into appropriately labelled tubes. One hundred microlitres of tracer was added to each tube, followed by 100μl of assay buffer. All tubes were vortex

mixed and incubated overnight at 2-8°C. After incubation, 500µl of charcoal suspension was added to each tube. The tubes were vortex mixed and allowed to incubate in an ice bath for 10 minutes, before centrifuging at 2 000g for 10 minutes at 4°C. Resulting supernatant solutions were decanted into scintillation vials containing 10ml of emulsifier scintillator plus™ scintillation cocktail. Measurement of radioactivity in each sample was determined using a Beckman (model LS 2800) scintillation counter. The concentration of unlabelled $PGF_{2\alpha}$ in the samples was determined from a $PGF_{2\alpha}$ standard curve (appendix 8), and final results expressed as pg $PGF_{2\alpha}$ per 10^6 cells.

4.6.3 RESULTS

The levels of PGD_2 and $PGF_{2\alpha}$ in BL6 cells supplemented with 1-10µg/ml vitamin E succinate are shown in table 14. PGD_2 levels in BL6 cells were found to be significantly ($p \leq 0.05$) decreased at vitamin E succinate concentrations of 3, 5, 7 and 10µg/ml. compared with control cultures 0E, while $PGF_{2\alpha}$ levels were significantly ($p \leq 0.001$) decreased at all levels of vitamin E succinate supplementation.

Table 14: The effect of vitamin E succinate supplementation on prostaglandin D_2 and prostaglandin $F_{2\alpha}$ levels in BL6 cells. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (µg/ml)	Prostaglandin levels (pg/10^6 cells)	
	PGD_2 Levels	$PGF_{2\alpha}$ Levels
0E	26.80 ±4.31	10.10 ±0.58
1	20.41 ±1.30	6.50[a] ±0.35
3	13.80[b] ±2.30	5.20[a] ±0.64
5	13.30[b] ±1.40	5.20[a] ±0.42
7	15.70[b] ±2.90	4.10[a] ±0.95
10	15.40[b] ±1.82	4.20[a] ±0.34

a= $p \leq 0.001$: Relative to $PGF_{2\alpha}$ levels in control cultures 0E.
b= $p \leq 0.05$: Relative to PGD_2 levels in control cultures 0E.

4.6.4 THE EFFECT OF VITAMIN E SUCCINATE SUPPLEMENTATION ON PROSTAGLANDIN I_2 LEVELS IN BL6 CELLS

4.6.4.1 MATERIALS AND METHODS

MATERIALS

Sulphuric acid (H_2SO_4) was obtained from Holpro Chemical Co., South Africa, while a 6-Keto-$PGF_{1\alpha}$ enzyme immunoassay kit was purchased from Amersham Life Science, Int., England.

METHODS

4.6.4.2 Cell culture procedure

The methods described in section 2.2.3.1 and 2.2.3.2 were used except that cell pellets were resuspended in 2.0ml of PBS buffer pH 7.0.

4.6.4.3 Homogenisation and preparation of samples

Refer to section 4.5.2.2 for the method used.

4.6.4.4 Extraction of prostaglandin I_2

Extraction of PGI_2 was carried out using the method described in section 4.5.2.3, with the following change. Dried PG extracts were resuspended in 500μl of assay buffer, and duplicate 50μl aliquots were used for PGI_2 determination.

4.6.4.5 Determination of prostaglandin I_2 levels

Prostacyclin (PGI_2) is an unstable vinyl ether which undergoes spontaneous hydrolysis to 6-Keto-$PGF_{1\alpha}$. The quantification of 6-Keto-$PGF_{1\alpha}$ is accepted as a measure of PGI_2 formation (464,465). This assay is based on the competition between 6-Keto-$PGF_{1\alpha}$ and a fixed quantity of peroxidase-labelled 6-Keto-$PGF_{1\alpha}$ for binding sites on a 6-Keto-$PGF_{1\alpha}$ specific antibody (464). Preparation of samples for PGI_2 determination was carried out as described in the 6-Keto-$PGF_{1\alpha}$ assay kit. A 50μl

aliquot of sample was pipetted into appropriate wells in a microtitre plate. Fifty microliters of antiserum was added to each well followed by shaking for 30 minutes on a microtitre plate shaker at room temperature. After incubation, 50μl of 6-Keto-$PGF_{1\alpha}$ peroxidase conjugate was added to each well and microtitre plate further shaken for one hour at room temperature. Thereafter, all wells were aspirated and rinsed four times with 400μl of was buffer. To the washed wells was added 150μl of enzyme substrate followed by incubation for 10 minutes at room temperature. Termination of the reaction was carried out by the addition of a 100μl of 1.0M H_2SO_4, and the resultant colour read at 450nm in a EAR 400 plate reader. The concentration of unlabelled 6-Keto-$PGF_{1\alpha}$ was determined from a 6-Keto-$PGF_{1\alpha}$ standard curve (appendix 9), and final results were expressed as pg PGI_2 per 10^6 cells.

4.6.5 RESULTS

In BL6 cells supplementation of 1, 3, 5 and 7μg/ml vitamin E succinate resulted in no significant increase or decrease in PGI_2 levels (table 15), compared with control cultures 0E, while at 10μg/ml, a significant increase in PGI_2 levels was observed.

Table 15: The effect of vitamin E succinate supplementation on prostaglandin I_2 levels in BL6 cells. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	PGI_2 levels (pg/10^6 cells)
0E	0.39 ±0.07
1	0.34 ±0.02
3	0.37 ±0.01
5	0.38 ±0.02
7	0.29 ±0.05
10	0.57[a] ±0.07

a= $p \leq 0.05$: Relative to control culture 0E.

4.6.6 DISCUSSION

In section 4.4, supplementation of BL6 cells with 1-10μg/ml vitamin E succinate resulted in a significant increase in COX activity. Since the COX enzyme forms an integral part of the PGH synthase enzyme, which functions to catalyse the conversion of AA to PGH_2 (166-169,171,173-179), any increase in COX activity would be expected to increase the overall levels of various PGs within a cell. Results from this study have shown that supplementation of vitamin E succinate to BL6 cells results in a significant increase in PGE_2 and PGI_2 levels at 10μg/ml concentrations, while PGD_2 and $PGF_{2\alpha}$ levels were significantly decreased at 3,5,7 and 10μg/ml vitamin E succinate. Synthesis of various biologically active eicosanoids from precursor PGH_2, occurs via the action of PG synthase enzymes specific for each PG (165,169), although the amount and type of PG produced depends largely on the cell or tissue type (165,166,169,200,205,207,209,213), which could account for the fact that an increase in PGD_2 and $F_{2\alpha}$ was not found.

A comparison of PGD_2, $PGF_{2\alpha}$, PGE_2 and PGI_2 levels in BL6 control cultures is shown in figure 14. As previously mentioned, BL6 cells are unusual in terms of eicosanoid biology in that they predominantly synthesise PGD_2 (232,256,257), while PGE_2 and $PGF_{2\alpha}$ are produced in smaller amounts (232,233,255). From this graph, it is evident that BL6 cells convert AA primarily to PGD_2, and also $PGF_{2\alpha}$, PGE_2 and PGI_2 in descending order of magnitude.

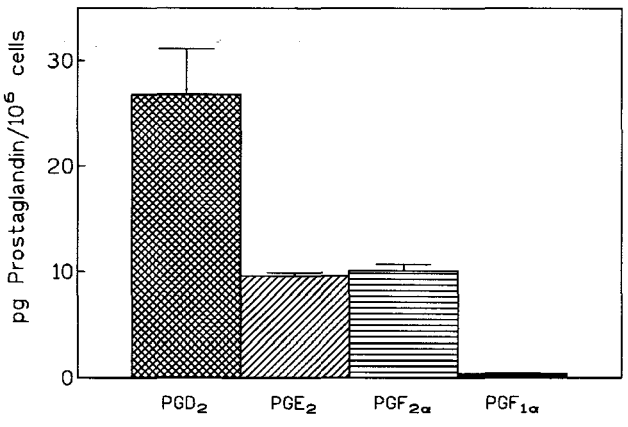


Figure 14: Comparison of prostaglandin levels in control cultures of BL6 cells. Each bar on the graph represents the mean of 3 determinations ± SEM.

4. Secondary Messenger Metabolism

Although most PG-producing cells predominantly synthesise one type of PG due to the existence of a single PGH_2 metabolising enzyme (165,169), in BL6 cells it is clear that the stimulatory effect of vitamin E succinate on COX activity (see section 4.4), an enzyme closely related to the PG synthase enzymes, mainly influences PGE_2 and PGI_2 and not PGD_2 and $PGF_{2\alpha}$ synthesis. Studies by Toivanen (415) and Chan *et al* (466,467), have shown that in various experimental models vitamin E affects PGI_2 synthesis. PGI_2 levels in BL6 cells were found to be significantly lower than the levels of PGD_2, $PGF_{2\alpha}$, and PGE_2. PGI_2 synthase activity is believed to be inactivated by a variety of lipid hydroperoxides (169,176,215-217) and hydroperxide intermediates of PG synthesis (215). Since earlier studies have shown markedly higher levels of lipid peroxidation (section 3.2.3) and COX activity (section 4.4) in malignant BL6 cells compared to non malignant LLCMK cells, this may explain the low PGI_2 concentrations in the BL6 cells.

The synthesis of $PGF_{2\alpha}$ is believed to be linked to PGE_2 synthesis, and studies with neonatal mouse calvaria cells have shown that $PGF_{2\alpha}$ stimulates the synthesis of PGE_2 via the release of AA from membranes (468). This suggests that any change in the levels of $PGF_{2\alpha}$ would result in similar changes in PGE_2 levels within the cell. In BL6 cells however, vitamin E succinate supplementation resulted in a significant decrease in $PGF_{2\alpha}$ levels, while PGE_2 levels remained unchanged over vitamin concentrations of 1-5μg/ml, followed by a significant increase at 7 and 10μg/ml respectively. One factor to consider in this regard is that vitamin E succinate supplementation may modulate the activity of PGE_2 and $PGF_{2\alpha}$ synthase enzymes differently.

Of particular interest to this study were PGE_2 (248,334,345,347,365,366) and PGI_2 (333,334,351), since their actions within a cell are believed to be mediated via a cAMP-AC linked system. Since vitamin E succinate supplementation has been shown to increase both PGE_2 and PGI_2 levels in BL6 cells, and decreases PGE_2 levels in LLCMK cells, it was of interest to this study to determine the effect of vitamin E succinate supplementation on AC activity and cAMP levels in BL6 and LLCMK cells respectively.

4.7 VITAMIN E SUCCINATE SUPPLEMENTATION, ADENYLATE CYCLASE ACTIVITY AND CYCLIC ADENOSINE MONOPHOSPHATE FORMATION.

4.7.1 INTRODUCTION

Changes to the membrane intracellular signalling systems are frequently implicated in the development of neoplasia, and possibly the acquisition of a metastatic phenotype (422,469). One major intracellular signalling system identified to date is the AC-cAMP linked system (422). This system is membrane-bound (157,321), and composed exclusively of intrinsic membrane-bound proteins (145,157,265). Like many membrane-bound enzymes, the activity of the AC system is influenced by the nature of its membrane lipid environment (145,149,157). This sensitivity to alterations in membrane fluidity is believed to be due to changes in the conformational flexibility of intrinsic proteins (149,157) as well as alterations in the efficacy of the interaction of various components of the AC system (145,149,157). Transformation of normal cells to tumour cells is usually accompanied by dramatic changes in the lipid structural organisation of membranes (159,247). Numerous studies (352,356,373,360,374,376,384,386,387,470) have shown an inverse relationship between intracellular levels of cAMP and tumour cell growth. Since intracellular levels of cAMP are regulated by AC and PDE activity (325,378,379) alterations in the activities of either of these enzymes, possibly through changes in membrane fluidity, could account for the lower intracellular levels of cAMP observed in tumour cells.

In addition to membrane fluidity, AC activity is influenced by various PGs (154,342) as well as by intracellular Ca^{2+} levels (266,275,277). Since vitamin E succinate supplementation has been shown to influence the cellular concentrations of both PGE_2 (section 4.5) and to a lesser extent Ca^{2+}(section 4.1) in BL6 cells, the aim of this part of the study was to determine the effects of vitamin E succinate supplementation on AC activity and cAMP levels in these cells and to compare this with effects on non-malignant LLCMK cells.

4.7.2 MATERIALS AND METHODS

MATERIALS

3-Isobutyl-1-Methylxanthine (IBMX), cAMP and ATP disodium salts were purchased from Sigma

Chemical Co., USA. Triethanolamine and aluminium oxide 90 (Woelm N active-neutral) were obtained from Merck, Darmstadt Germany. (2,5',8-^{3}H)-ATP ammonium salt and ^{3}H-cAMP assay kit was supplied by Amersham Life Sciences, Int., England. Creatine-Phosphate-Na_2·$4H_2O$ (Creatine Phosphate) and creatine-kinase were purchased from Boehringer Mannheim Gmbh, Germany.

METHODS

4.7.2.1 Cell culture procedure

The methods described in section 2.2.3.1 and 2.2.3.2 were used, except that cell pellets were resuspended in 1.0ml of 0.1M Tris-HCl buffer pH 7.5 containing 4mM EDTA.

4.7.2.2 Homogenisation and preparation of samples

The method described in section 3.3.1.2 was used with the following changes. The dounce homogeniser was rinsed with 1.0ml of Tris HCl buffer pH 7.5. Following centrifugation, the supernatant fractions were retained for cAMP determination and membrane fractions (pellet) resuspended in 2.0ml of Tris-HCl buffer pH 7.5 for determination of adenylate cyclase activity.

4.7.2.3 Protein determination

Protein determinations of membrane and stroma fractions were carried out using a modified method of Lowry *et al* (cited in 433) and absorbance values converted to mg/ml protein from the standard curve in appendix 5.

4.7.2.4 Adenylate cyclase assay

Adenylate cyclase activities of membrane samples were determined using the method of Schultz and Jakobs (321) and Salomon (471). In this assay, the formation of ^{3}H-cAMP from ^{3}H-ATP is measured. GTP and forskolin were omitted from the reaction mixture and were substituted with 20μl of 0.1M Tris-HCl buffer pH 7.5 to obtain the desired volume. The standard component reaction mixture contained 500μl 1M triethanolamine buffer, 500μl 100mM Mg_2Cl_2 solution, 1ml 10mM 3-IBMX (PDE inhibitor), 100μl 10mM cAMP solution, 100μl 10mM ATP solution, 16.4mg creatine-

phosphate, 4mg creatine kinase, 10mg BSA and 500μl of water, to give a final volume sufficient for 100 assays. Constant time course assays were performed over a period of 10 minutes to determine optimal incubation periods. Maximal AC activity was detected after a 2 minute incubation period. The AC activity related to the mass of protein (specific activity), was expressed as the formation of 1pmol cAMP per minute per mg protein.

4.7.2.5 Cyclic adenosine monophosphate assay

Following protein determinations, the remaining supernatant fractions were boiled for 5 minutes to precipitate proteins, and centrifuged at 3 000g for 3 minutes. The resulting pellet (protein) was discarded and supernatant fractions retained for cAMP determination using a commercially available ^{3}H-cAMP assay kit.

The principle of this assay is based on the competition between unlabelled cAMP and a fixed quantity of ^{3}H-cAMP for binding to a protein which has high specificity and affinity for cAMP (472). The tubes, as required, were placed in racks in an ice bath. Fifty microliters of the samples were added to the appropriate assay tubes. To these tubes was added 50μl of labelled cAMP and 100μl of binding protein. All tubes were vortex mixed for 5 seconds, followed by transfer to an ice bath in a cold room at 4^{0}C, where they were incubated for 2 hours. At least 15 minutes prior to the end of incubation, 20ml of ice cold milli-Q water was added to charcoal reagent and stirred continuously during use. After incubation, a 100μl of the charcoal suspension was added to all tubes and centrifuged at 3 000g for 4 minutes. Following centrifugation, and without disturbing the charcoal sediment, 200μl of supernatant phase was removed and placed into scintillations vials containing 10ml of scintillator plus™ scintillation cocktail. Radioactivity in each sample was determined by counting in a Beckman (LS 2899) scintillation counter. The amount of unlabelled cAMP in the assay sample was determined from a standard curve (appendix 10), and final results were expressed as pmol cAMP/mg protein.

4.7.3 RESULTS

LLCMK cells (Table 16) supplemented with 1-10μg/ml vitamin E succinate showed no significant increase or decrease in AC activity or cAMP levels, compared with control cultures 0E. BL6 cells on the other hand showed no significant increase or decrease in AC activity at vitamin E succinate concentrations of 1, 3 and 5μg/ml, but at 7 ($p \leq 0.01$) and 10 ($p \leq 0.001$), a significant increase in AC

activity occurred. Supplementation of BL6 cells with 1 and 3 μg/ml vitamin E succinate resulted in a decrease in cAMP levels, with a significant decrease ($p \leq 0.01$) occurring at 3μg/ml. As the vitamin concentration was increased to 5 ($p \leq 0.05$), 7 and 10μg/ml ($p \leq 0.001$) respectively, a significant increase in cAMP levels was noted.

Table 16: The effect of vitamin E succinate supplementation on adenylate cyclase activity and cyclic adenosine monophosphate levels in BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations $\pm$ SEM)

Vitamin E succinate (μg/ml)	BL6 cells		LLCMK cells	
	Adenylate cyclase activity (U/mg protein)	cAMP Levels (pmol/mg protein)	Adenylate cyclase activity (U/mg protein)	cAMP Levels (pmol/mg protein)
0E	66.09 $\pm$1.87	30.84 $\pm$0.09	85.98 $\pm$4.01	24.96 $\pm$1.17
1	73.64 $\pm$1.37	24.97 $\pm$1.75	90.92 $\pm$3.37	24.98 $\pm$2.16
3	67.05[d] $\pm$1.47	14.51[b] $\pm$1.44	103.32 $\pm$4.40	17.85 $\pm$2.58
5	70.01[e] $\pm$2.89	40.2[cf] $\pm$4.94	105.13 $\pm$19.04	21.47 $\pm$2.98
7	81.41[b] $\pm$2.60	61.48[af] $\pm$1.65	72.29 $\pm$3.10	20.94 $\pm$0.21
10	84.00[a] $\pm$0.77	52.27[af] $\pm$5.35	67.10 $\pm$1.15	18.42 $\pm$0.35

a= $p \leq 0.001$: Relative to respective BL6 control cultures 0E.
b= $p \leq 0.01$: Relative to respective BL6 control cultures 0E.
c= $p \leq 0.05$: Relative to cAMP levels in BL6 control cultures 0E.
d= $p \leq 0.01$: Relative to AC activity in 3μg/ml supplemented LLCMK cultures.
e= $p \leq 0.05$: Relative to AC activity in 5μg/ml supplemented LLCMK cultures.
f= $p \leq 0.001$: Relative to cAMP levels in LLCMK cells in respective groups.

Comparing the levels of AC activity in BL6 and LLCMK cells, the observed trend was that of lower AC activity in the control, 1, 3, and 5μg/ml vitamin E succinate supplemented BL6 cultures, while at 7 and 10μg/ml, AC activity was markedly higher in BL6 cultures. cAMP levels in BL6 cells were

also significantly higher in the 5, 7 and 10μg/ml vitamin E succinate supplemented cultures, while at 1 and 3μg/ml, lower levels of cAMP were detected.

4.7.4 DISCUSSION

The AC enzyme is a membrane-bound enzyme (157,321,323) whose function in the cell is to catalyse the conversion of ATP to cAMP (265,321). Comparing the basal levels of AC activity in BL6 and LLCMK cells, the trend observed was that of higher AC activity in LLCMK cells. Studies by Emmelot and Bos (323) on plasma membranes isolated from liver tumours (rat hepatoma 484A) have shown decreased basal AC activity compared with their normal cell counterparts. Decreased AC activity was also reported in various cell lines transformed by polyoma virus (380).

Supplementation of BL6 cells with 1-10μg/ml vitamin E succinate resulted in a general increase in AC activity, with a significant increase occurring at 7 and 10μg/ml respectively. Like many integral membrane-bound enzymes, the activity of the AC enzyme is modulated by the nature of its membrane lipid environment (143,146,147,149,157). Hence the ability of vitamin E succinate to alter membrane fluidity through its physicochemical interaction with membranes (412,413), could account for the observed increase in AC activity in BL6 cells following vitamin E succinate supplementation. In LLCMK cells on the other hand, a general increase in AC activity occurred at 1, 3 and 5μg/ml vitamin E succinate, followed by a non-significant decrease at 7 and 10μg/ml respectively. In addition various PGs are known to influence the activity of the AC enzyme (154,310). Studies reported in section 4.5, have shown that supplementation of vitamin E succinate, particularly at higher concentrations, results in a significant increase in PGE_2 levels in BL6 cells, while in LLCMK cells, a decrease in PGE_2 levels occurs. These changes in PGE_2 levels in BL6 and LLCMK cells were positively correlated with changes in AC activity.

Since the intracellular levels of cAMP are controlled in part by the activity of the AC enzyme (380,473), any change in AC activity would be expected to bring about similar changes in cAMP levels. In this study supplementation of BL6 cells with 5, 7 and 10μg/ml vitamin E succinate resulted in a significant increase in cAMP levels, compared with control cultures 0E. This increase was positively correlated with an increase in AC activity in BL6 cells (table 16). In contrast, cAMP levels in LLCMK cells were generally unaffected by vitamin E succinate supplementation, which correlated with the non-significant effect of vitamin E succinate supplementation on AC activity in these cells.

Another finding was that cAMP levels in control cultures of BL6 cells were marginally higher than the levels in LLCMK control cultures. If cAMP levels were regulated by AC activity, one would have in fact expected lower cAMP levels in BL6 cells. Numerous studies with tumorigenic and non-tumorigenic cell lines have shown also markedly higher levels of cAMP in non-tumorigenic cells compared to tumorigenic cells (352,355,356,360,372,373,375,384,386,387). One factor to consider is that the levels of intracellular cAMP are determined in large part by its rate of synthesis, catalyses by AC enzyme, and its rate of hydrolysis catalyses by PDE enzymes (325,378,379,473). A lower PDE activity in malignant compared to non-malignant cells could therefore account for the higher levels of cAMP observed in control cultures of BL6 cells. Studies by Chatterjee and Kim (473) have in fact shown that spontaneously metastasising rat mammary tumours have higher levels of cAMP than non-metastasising tumours, and they suggested that this increase in cAMP was due to decreased PDE activity in non-metastasising tumours.

Since a close relationship exists between AC, cPDE activity and cAMP levels (378), it was therefore relevant to determine the effect of vitamin E succinate supplementation on PDE activity in BL6 and LLCMK cells.

4.8 VITAMIN E SUCCINATE SUPPLEMENTATION AND PHOSPHODIESTERASE ACTIVITY

4.8.1 INTRODUCTION

PDEs are a group of hydrolytic enzymes which function to catalyse the hydrolysis of the 3' bond of cyclic nucleotides to a non-cyclic product 5'-monophosphate derivative (473). Although numerous forms of the PDE enzyme are believed to exist in cells from various sources (324), it is now believed that two distinct forms of PDE occur, namely a low Km PDE and a high Km PDE (326,371). Both the low Km membrane-bound or particulate (pPDE) and high Km soluble PDE (sPDE) enzymes play an important role in regulating the intracellular levels of cAMP, although the pPDE is the major enzyme involved in controlling cAMP levels (370).

Various hormones as well as intracellular activators such as cAMP are known to stimulate the activity of the low Km form of the enzyme (326,370,372). As changes in the membrane intracellular signalling systems are frequently implicated in the development of neoplasia (159,247), it is reasonable to assume

that the levels of PDE activity would also be altered in tumourigenic cells. SV40 transformed 3T3 cells exhibit low Km PDE activity only, while non-transformed 3T3 cells were found to contain both low and high Km PDE activity (325). Perkins et al (474) studying particulate and soluble PDE activity in malignant human glial tumour cells found that the activities of these enzymes were markedly lower than those found in normal cells. The aim of this part of the study was to determine the activity of both the low Km and high Km forms of the PDE enzymes in malignant BL6 and non-malignant LLCMK cells, as well as the effect of vitamin E succinate supplementation on the activities of these enzymes.

4.8.2 MATERIALS AND METHODS

MATERIALS

Ethylene-bis (oxy-ethylenenitrilo) Tetraacetic Acid (EGTA), leupeptin, phenylmethylsulfonylflouride, pepstatin, triton X-100, brij 30 (polyethylene 4-Lauryl ether) and snake venom (Naja hannah, King Cobra) were purchased from Sigma Chemical Co., USA. Ion exchange resin Super Q-650c was obtained from TOSOH Corp., Japan.

METHODS

4.8.2.1 Preparation of homogenising buffers A and B.

Homogenising buffers A and B were prepared as described in Elks and Manganiello (475).

4.8.2.2 Preparation of anion exchange resin

Anion exchange resin was prepared as described in Elks and Manganiello (475).

4.8.2.3 Cell culture procedure

The method described in section 2.2.3.1 and 2.2.3.2 were used, except that cell pellets were resuspended in 1.0ml of homogenising buffer A.

4.8.2.4 Homogenisation and preparation of soluble and particulate phosphodiesterase enzymes

Preparation of soluble and particulate fractions of PDE were carried out using a modified method of Elks and Manganiello (475). After cell enumeration, suspensions were poured into a dounce homogeniser and homogenised with 30 plunges of a tight plunger. The dounce homogeniser was rinsed with 1.0ml of buffer A. Following homogenisation, cell homogenates were centrifuged (Beckman model L8-80M) at 100 000g for 40 minutes at 4°C. Supernatant fractions (sPDE fractions) were retained and stored at -70°C until required. Resulting pellet fractions were resuspended in 2.0ml of homogenising buffer B and incubated overnight at 4°C, followed by centrifugation at 100 000g for 4 minutes at 4°C. Supernatant fractions (pPDE fractions) were retained, and stored at -70°C until required.

4.8.2.5 Phosphodiesterase assay

The principle of this assay involves the conversion of ^{3}H-cAMP to ^{3}H-5'-AMP by PDE in the presence of an excess of 5'-nucleotidase to produce ^{3}H-adenosine as the final product (474). Soluble and particulate PDE activity was determined using a modified method of the one step assay procedure described by Thompson *et al* (476). Standard reaction mixtures containing 5μl of a 100mM Tris-HCl buffer pH 8.0 containing 0.1μCi 8-^{3}H-cAMP, 1.2 μmole $MgCl_2$, 20μl of a cAMP solution calculated to alter total cAMP concentrations from 0.1μM to 0.1mM, 5μl of PDE preparation (soluble or particulate), and 5μl of King Cobra venom (A nucleotidase which functions to convert 5'-AMP formed by PDE to ^{3}H-adenosine) were allowed to react for 10 minutes at 30°C. Reactions were terminated by addition of 0.8ml of anion exchange resin slurry and allowed to stand for 10 minutes. Following the 10 minute equilibration period, samples were centrifuged at 2 000g for 10 minutes. 0.5ml aliquot of supernatant liquid was removed and counted by liquid scintillation using 10ml of emulsifier scintillator plus™ scintillation cocktail. Assay blanks were prepared with heat inactivated enzyme. PDE activity was expressed as pmoles ^{3}H-adenosine per 10^6 cells.

4.8.3 RESULTS

The effect of vitamin E succinate supplementation on PDE activity (combined soluble and particulate fractions) is recorded in figure 15. In BL6 cells supplementation of 1-10μg/ml vitamin E succinate did not result in any significant increase or decrease in total PDE activity compared to control cultures 0E

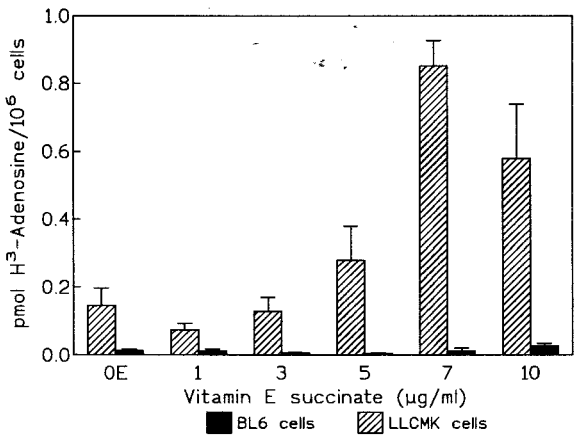


Figure 15: Total phosphodiesterase activity in BL6 and LLCMK cells respectively. Each bar on the graph represents the mean of 3 determinations ± SEM.

Table 17: The effect of vitamin E succinate on phosphodiesterase activity in soluble and particulate fractions of BL6 and LLCMK cells respectively. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (µg/ml)	Particulate PDE activity (x10³) (pmol ³H-Adenosine/10⁶ cells)		Soluble PDE activity (x10³) (pmol ³H-Adenosine/10⁶ cells)	
	BL6 cells	LLCMK cells	BL6 cells	LLCMK cells
0E	7.67 ±2.44	95.80[e] ±21.2	5.31 ±1.67	50.50 ±2.64
1	5.64 ±2.88	20.10 ±8.10	5.95 ±2.46	54.30 ±23.40
3	1.31 ±1.08	58.90 ±29.10	5.08 ±1.01	69.70 ±25.70
5	3.64 ±2.38	142.7 ±33.60	3.83 ±2.50	148.50 ±54.80
7	5.98 ±1.10	396.40[b] ±41.50	6.77 ±2.29	453.00[c] ±37.50
10	19.00[ad] ±4.53	200.00[c] ±7.28	6.57 ±4.05	385.20[c] 161.20

a= p≤0.05: Relative to BL6 control cultures 0E.
b= p≤0.001: Relative to LLCMK control cultures 0E.
c= p≤0.05: Relative to LLCMK control cultures 0E.
d= p≤0.05: Relative to sPDE activity in BL6 cells supplemented with 10µg/ml vitamin E succinate.
e= p≤0.05: Relative to sPDE activity in LLCMK control cultures 0E.

(figure 15), while LLCMK cells on the other hand showed a general increase in PDE activity at 5,7 and 10μg/ml vitamin E succinate, with a significant increase occurring at 7 ($p \leq 0.001$) and 10 μg/ml ($p \leq 0.01$) respectively. PDE activity in BL6 control cultures was found to be significantly ($p \leq 0.01$) lower than the PDE activity in BL6 control cultures.

Comparing PDE activity in soluble and particulate fractions (table 17) of BL6 and LLCMK control cultures, it is apparent that the trend is towards higher levels of PDE activity in particulate fractions, significantly so in LLCMK control cultures. This trend was in general reversed as a result of vitamin E succinate supplementation. BL6 cells, supplemented with 7 and 10μg/ml vitamin E succinate showed a non-significant increase in sPDE activity compared to control cultures 0E. In respect of pPDE activity in BL6 cells, supplementation with 1-7μg/ml vitamin E succinate resulted in an overall decrease in activity, while at 10μg/ml a significant ($p \leq 0.05$) increase in pPDE activity occurred.

Supplementation of LLCMK cells with 1-10μg/ml vitamin E succinate resulted in a non-significant decrease in pPDE activity at 1 and 3μg/ml respectively, compared to control cultures 0E. As the vitamin concentration was increased to 5, 7 and 10μg/ml, a marked increase in pPDE activity occurred, with a significant increase occurring at 7 ($p \leq 0.001$) and 10μg/ml ($p \leq 0.05$) respectively. sPDE activity was generally increased in LLCMK cells supplemented with 1-10μg/ml vitamin E succinate, however this increase was significant at 7 and 10μg/ml supplementation only.

4.8.4 DISCUSSION

cAMP is believed to regulate many basic biological processes within a cell, suggesting that any alteration in the intracellular levels of these compounds might disrupt normal physiological functions and in turn lead to a diseased state (371). Many of the abnormal properties of transformed cells are believed to be due to changes in intracellular cAMP levels (325,378,379).

The PDE enzymes play a key role in regulating intracellular levels of cAMP (325,378,379). A comparison of total PDE activity in BL6 and LLCMK cells revealed that PDE activity of the BL6 control cultures was significantly lower than PDE activity in LLCMK control cultures. Studies on PDE activity in normal and malignant cells have generally found lower levels of PDE activity in tumour cells (325,361). The transformation of normal cells to tumour cells is believed to alter the ratio of the pPDE to sPDE enzymes (374,473). This may have important consequences for the cell,

since the pPDE enzyme is believed to regulate intracellular levels of cAMP (326). Studies by Clarke *et al* (cited in 379 and 473), reported that neoplastic cells have higher ratios of pPDE to sPDE activity, compared to their normal counterparts. Results from this study however indicate that BL6 control cultures have lower ratios of pPDE to sPDE activity compared to LLCMK control cultures. The reason for this is at present unclear.

Of interest to this study was the pPDE activity due to its ability to regulate intracellular cAMP levels and respond to various hormones and intracellular cAMP levels. Supplementation of BL6 cells with vitamin E succinate resulted in a non-significant decrease in pPDE activity compared to control cultures 0E. An exception was at 10μg/ml supplementation, where a significant increase in pPDE occurred. pPDE is a membrane-bound enzyme (369,370) and since tumour cell membranes generally have altered physical and chemical properties (159,161,247,436), and the fact that vitamin E succinate has been shown to stabilise membranes (414), it is proposed that the observed decreases in pPDE activity following vitamin E succinate supplementation, could be due to changes in the physical properties of the tumour cell membranes. However, it is important to remember that alterations in PDE activity may be more complicated than merely altering the physical properties of membranes, since PDE enzymes form part of an extremely complex system of enzymes the characteristics of which differ from one tissue to another and even between cell types (371). In LLCMK cells, supplementation of vitamin E succinate resulted in a marked increase in pPDE activity at 5, 7 and 10μg/ml, with a significant increase occurring at 7 and 10μg/ml respectively. A similar increase in sPDE activity was observed in LLCMK cells supplemented with 5, 7 and 10μg/ml vitamin E succinate, although this increase was significant at 7μg/ml only. The reason for this increase in sPDE and pPDE activity in LLCMK cells is unclear, although factors to consider are the intracellular levels of cAMP and Ca^{2+} (263,266,275) both of which are known to regulate PDE activity.

When relating AC activity (section 4.7) and total PDE activity (figure 15) to cAMP levels in BL6 cells, it was clear that the observed increase in cAMP levels detected in BL6 cells (see section 4.7) supplemented with 5, 7 and 10μg/ml vitamin E succinate was due to marked increases in AC activity rather than to changes in PDE activity, since supplementation of 5-10μg/ml vitamin E succinate resulted in a marked increase in AC activity, significantly so at 7 and 10μg/ml supplementation, while having no stimulatory effect on PDE activity. In LLCMK cells on the other hand, supplementation of 3-10μg/ml vitamin E succinate resulted in a non-significant decrease in cAMP levels. This decrease in cAMP levels at 7 and 10μg/ml vitamin E succinate in LLCMK cells was positively correlated with

a decrease in AC activity and inversely related to PDE activity. This suggests that in LLCMK cells, the observed decrease in cAMP levels at 7 and 10μg/ml vitamin E succinate was due to increased PDE activity and decreased AC activity in these cells.

4.9 Summary

Of main concern in this chapter was the effect of vitamin E succinate supplementation on the metabolism of the AA cascade pathway in BL6 and LLCMK cells that is outlined in figure 4. In BL6 cells, supplementation of 1-10μg/ml vitamin E succinate resulted in a general increase in PLA_2 activity. Calcium, a known regulator of membrane-bound PLA_2 activity, was positively correlated with changes in PLA_2 activity, suggesting a possible link between the activity of this enzyme and intracellular Ca^{2+} levels in BL6 cells. Analysis of 5-LOX and COX activity in BL6 cells revealed significant increases in COX activity at 7 and 10μg/ml vitamin E succinate supplementation, while 5-LOX activity was increased significantly at 10μg/ml only. This increase in COX activity at 7 and 10μg/ml vitamin E succinate correlated with an increase in the levels of PGE_2, AC activity and cAMP. Furthermore, this increase in COX activity was positively correlated with an increase in free radical formation and lipid peroxidation levels in BL6 cells (chapter 3). A similar relationship between 5-LOX activity, free radical formation and lipid peroxidation levels was observed in BL6 cells supplemented with 10μg/ml vitamin E succinate. Another factor considered in these studies was the role of PDE in regulating intracellular cAMP levels following vitamin E succinate supplementation. Results from these studies revealed no significant increase or decrease in PDE activity in BL6 cells supplemented with 1-10μg/ml vitamin E succinate, suggesting that intracellular cAMP levels in BL6 cells were regulated in part by AC activity and not PDE activity.

Supplementation of LLCMK cells with 1-10μg/ml vitamin E succinate resulted in no significant increase or decrease in PLA_2 activity, while intracellular Ca^{2+}levels were increased at 1, 3 and 5μg/ml vitamin E succinate and decreased at 7 and 10μg/ml supplementation. COX activity in LLCMK cells was decreased at vitamin E succinate concentrations of 1-7μg/ml, while at 10μg/ml a non-significant increase in activity was observed. 5-LOX activity on the other hand was significantly increased at 5, 7 and 10μg/ml vitamin E succinate. This increase in 5-LOX activity correlated with increased free radical formation and lipid peroxidation levels in these cells (chapter 3). In LLCMK cells, PGE_2 levels were decreased at all levels of vitamin E succinate supplementation. This decrease in PGE_2 levels correlated with a decreased in COX activity, with the exception of the 10μg/ml vitamin E succinate

supplementation, where a non-significant increase in COX activity occurred. Supplementation of LLCMK cells with 1-10μg/ml vitamin E succinate resulted in a general increase in AC activity at 1, 3 and 5μg/ml vitamin E succinate, followed by a non-significant decrease at 7 and 10μg/ml respectively. This decrease in AC activity was inversely related to PDE activity in LLCMK cells, suggesting that the observed decrease in intracellular cAMP levels at 7 and 10μg/ml vitamin E succinate supplementation in LLCMK cells was due to a decreased AC activity and increased PDE activity within these cells.

The effect of vitamin E succinate supplementation on free radical and lipid peroxidation levels (chapter 3) as well as its effects on the levels of secondary messengers and metabolites (chapter 4) will now be discussed in relation to its possible effects on cell growth (chapter 2).

5

VITAMIN E SUCCINATE SUPPLEMENTATION IN RELATION TO CELL OXIDATION STATE, SECONDARY MESSENGERS AND CELL GROWTH

The geographical distribution of various cancers, the changing patterns in migrant populations, and the varying incidence of specific tumours in various socioeconomic groups, all point to diet and nutrition as important factors in the possible control and prevention of human cancers (46,105). The human diet contains a wide variety of naturally occurring mutagens and carcinogens, as well as numerous antimutagens and anticarcinogens (477). As already discussed (section 1.2.6.6), one dietary nutrient receiving recent attention as a possible anticarcinogen is vitamin E. Numerous epidemiological studies (52,54,55,105,119-121,130) over the past few years have revealed a link between dietary levels of vitamin E and tumour incidence. Since vitamin E's discovery, exaggerated claims have made about its possible role in human health, although no single primary role for this vitamin has as yet been discovered. It has been suggested that vitamin E's action within a cell may be secondary to its primary role as an antioxidant (26,32,57,58). Of interest to this study was the effect of vitamin E succinate supplementation on cell growth and free radical and lipid peroxidation levels in BL6 and LLCMK cells, as well as its effects on the AA cascade (figure 4), and the various metabolites affecting this pathway.

In chapter 2 the effect of vitamin E succinate supplementation on BL6 and LLCMK cell growth was determined, while chapter 3 investigated the effect of vitamin E succinate supplementation on free radical and lipid peroxidation levels in these cells. Finally chapter 4 investigated the effects of vitamin E succinate supplementation on various AA metabolites and secondary messengers shown in figure 4. In this chapter an attempt will be made to relate the effects of vitamin E succinate supplementation on BL6 and LLCMK cell growth, to free radical and lipid peroxidation levels as well as to the levels of various secondary messengers and factors influencing the AA pathway. Due to some variability in the effect of vitamin E succinate supplementation on cell growth in the different experiments performed, the relationship between various metabolites relative to cell growth will be discussed for each individual experiment. Furthermore, since supplementation of vitamin E succinate to LLCMK cells resulted in variable and less significant effects on cell growth and non-significant trends or relationships between various metabolites and secondary messengers in the AA pathway (figure 4), emphasis in this chapter was placed on the BL6 cell line. It must be noted however, that although

studies have consistently shown significant decreases in BL6 cell growth at 5-10μg/ml vitamin E succinate, the effects at 1 and 3μg/ml were inconsistent, with some studies showing inhibitory and others stimulatory effects. The reason for this variability is unclear although results from various other studies have shown that the effective concentration range of vitamin E succinate in BL6 cells lies between 5 and 10μg/ml respectively (42,127).

Lipid peroxidation, a consequence of free radical attack on PUFAs, has been linked to events promoting carcinogenesis (138,139). Recently however, numerous studies (235,478-482) have suggested an inverse relationship between lipid peroxidation levels and cell growth. The prime concern of this study was to establish whether such an inverse relationship existed between free radical formation, lipid peroxidation levels and cell growth following vitamin E succinate supplementation. In LLCMK cells, supplementation of vitamin E succinate resulted in a general although non-significant increase in growth, with the exception of the 5μg/ml supplemented cultures, where a significant decrease in growth occurred. Free radical levels on the other hand were generally decreased at all vitamin concentrations tested when compared with control cultures 0E. The only exception was that recorded in the 10μg/ml supplemented cultures, where a marked increase in free radical levels occurred. This decrease in free radical levels was inversely related to LLCMK cell growth at 1 and 3μg/ml vitamin E succinate.

When comparing free radical levels to BL6 cell growth (figure 16), the general trend that was apparent was that when BL6 cell growth was decreased, free radical levels were increased and *vice versa*. Studies by Yoshikawa *et al* (483) have also shown an inverse relationship between free radical levels and tumour cell growth. Vitamin E succinate supplementation did not significantly affect cell growth or lipid peroxidation levels in LLCMK cells but in BL6 cells, resulted in a significant decrease in growth and increase in lipid peroxidation levels at vitamin E succinate concentrations of 5, 7 and 10μg/ml respectively (figure 17). Hence upon vitamin E succinate supplementation, free radical and lipid peroxidation levels found in the BL6 cells were inversely related to BL6 cell growth (484). Begin *et al* (478) studying the contribution of lipid peroxidation in killing breast carcinoma cells, also demonstrated an inverse relationship between intracellular levels of thiobarbaturic acid-reactive materials, and tumour cell growth. Further studies by Cheesman *et al* (481) have shown lower levels of lipid peroxidation in highly undifferentiated rapidly proliferating Novikoff hepatoma tissue, compared with normal rat liver tissue.

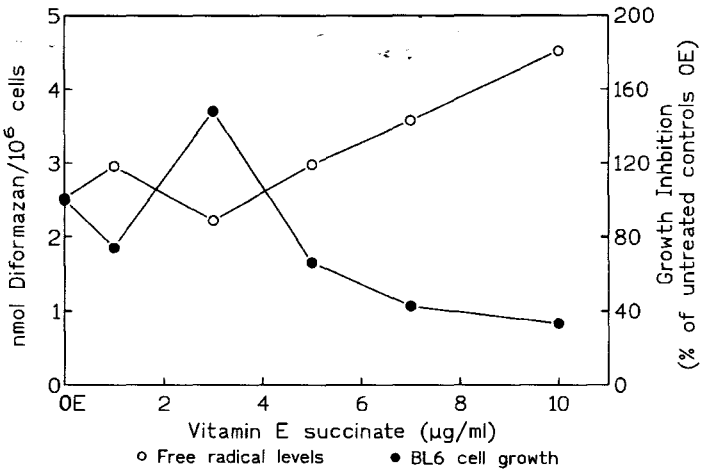


Figure 16: Effect of vitamin E succinate supplementation on free radical levels and BL6 cell growth.

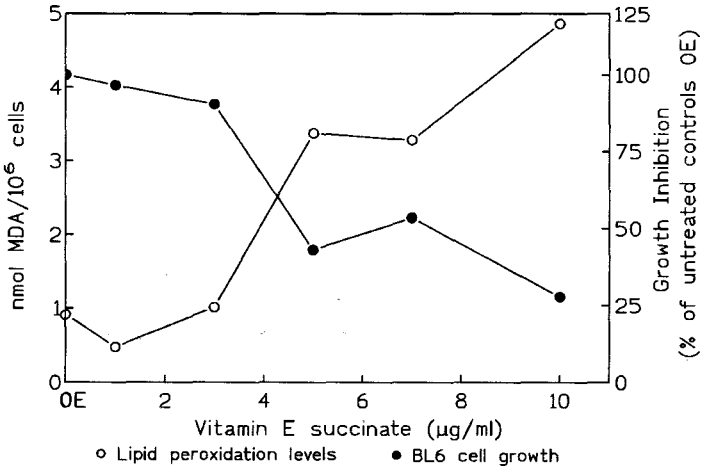


Figure 17: Effect of vitamin E succinate supplementation on lipid peroxidation levels and BL6 cell growth.

Lipid peroxidation, can be initiated both enzymatically through the catalytic action of LOX and COX enzymes (159,235), or non-enzymatically as by products of free radical chain reactions (135,159, 235). Since the catalytic action of COX and LOX enzymes are believed to contribute to free radical formation (235) and lipid peroxidation levels (159,235) in a cell, an investigation of the relationship between the activity of these enzymes and cell growth was necessary. Supplementation of LLCMK

cells with vitamin E succinate resulted in a significant decrease in COX activity, with the exception of the 10μg/ml supplemented cultures, where a non-significant increase occurred. This decrease in COX activity was inversely related to LLCMK growth. With respect to BL6 cells (figure 18), supplementation of vitamin E succinate resulted in a general increase in COX activity, with a significant increase occurring at 7 and 10μg/ml respectively. The only exception was that recorded at 3μg/ml supplementation, where a marked decrease in COX activity was detected. This general increase in COX activity was inversely related to BL6 growth at vitamin concentrations of 5, 7 and 10μg/ml respectively.

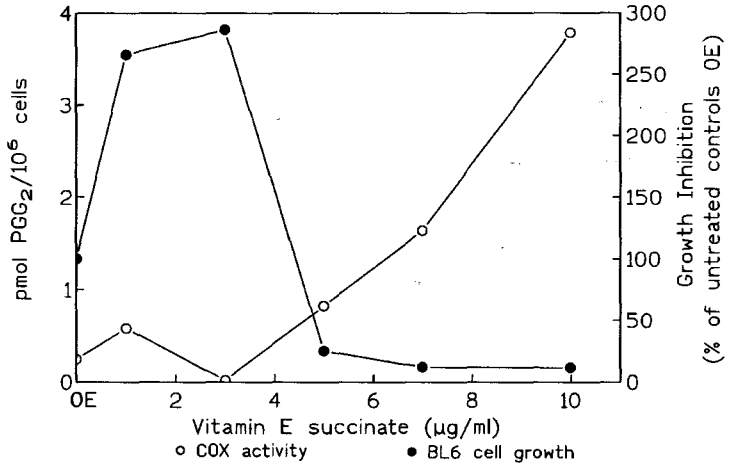


Figure 18: Effect of vitamin E succinate supplementation on cyclooxygenase activity and BL6 cell growth.

Relevant to the 5-LOX studies, vitamin E succinate supplementation of BL6 and LLCMK cells resulted in an overall decrease in growth with a significant decrease occurring in the BL6 cells. 5-LOX activity in LLCMK cells was generally increased following vitamin E succinate supplementation, significantly so at 7 and 10μg/ml supplementation. In BL6 cells (figure 19), supplementation of 3-10μg/ml vitamin E succinate resulted in a general increase in 5-LOX activity, with a significant increase occurring at 10μg/ml supplementation.

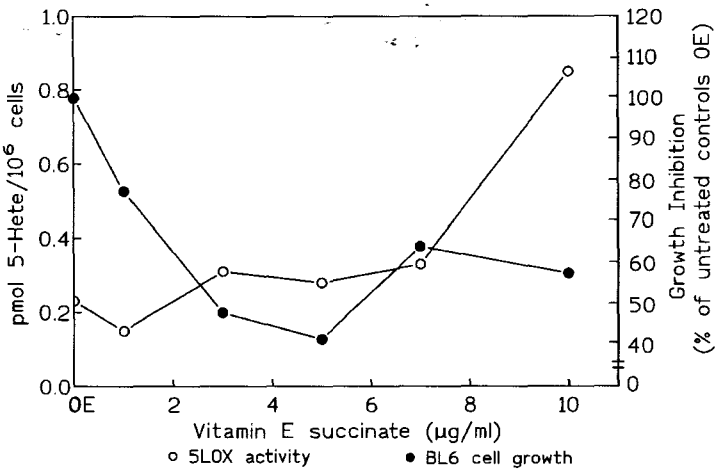


Figure 19: Effect of vitamin E succinate supplementation on 5-lipoxygenase activity and BL6 cell growth.

Thus 5-LOX activity was inversely related to BL6 and LLCMK growth and was enhanced by vitamin E succinate supplementation. As previously mentioned both 5-LOX and COX activity are thought to contribute to free radical and lipid peroxidation levels within a cell (235). Since vitamin E succinate supplementation significantly decreased COX activity and increased 5-LOX activity in LLCMK cells, it is reasonable to assume that the increase in free radical and lipid peroxidation levels in LLCMK cells following vitamin E succinate supplementation were due to an increase in 5-LOX activity. In BL6 cells on the other hand, increased free radical and lipid peroxidation levels correlated with an increase in both 5-LOX and COX activity.

An additional factor which needed to be considered in these studies was the effect of vitamin E succinate supplementation on intracellular Ca^{2+} levels, since Ca^{2+} has been implicated as a key regulator of cell proliferation through its effects on the cell cycle (278,280,281). Furthermore $Ca^{2+}$is believed to play a critical role in regulating the activity of the COX enzyme and LOX enzymes (276). Supplementation of LLCMK cells with vitamin E succinate did not result in any trend when comparing cell growth and intracellular Ca^{2+}levels. The only exceptions were at vitamin E succinate concentrations of 7 and 10μg/ml, where a marked decrease in both intracellular Ca^{2+}levels and LLCMK cell growth occurred.

5. Secondary messengers and growth.

With respect to BL6 cells (figure 20), vitamin E succinate supplementation resulted in an overall increase in Ca^{2+}levels, with the exception of 10μg/ml supplementation cultures where a non-significant decrease occurred. These changes in intracellular Ca^{2+} levels were inversely related to BL6 cell growth.

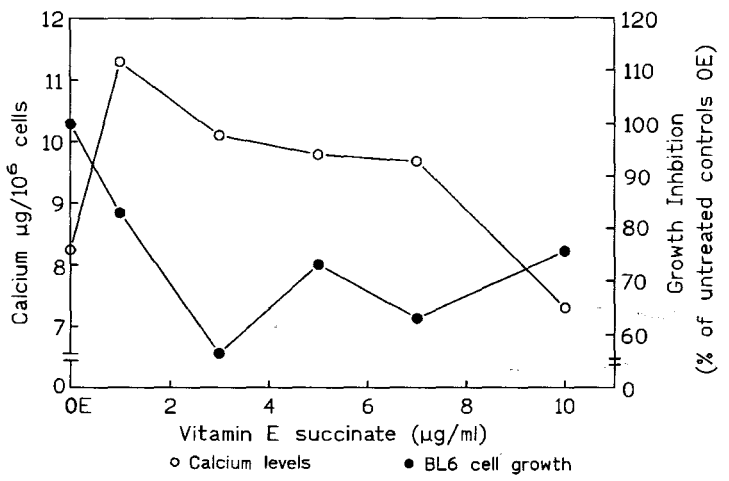


Figure 20: Effect of vitamin E succinate supplementation on calcium levels and BL6 cell growth.

Although intracellular Ca^{2+} levels are believed to regulate the activity of the COX and LOX enzymes (276), it may also affect the activation of the PLA_2 enzyme with consequent AA release, an important rate-limiting precursor of COX activity and eicosanoid synthesis (165,166,273,291-297,299,304). Hence an additional factor which needed to be considered, was the effect of vitamin E succinate supplementation on PLA_2 activity. With the exception of the 1μg/ml supplemented cultures, supplementation of vitamin E resulted in a significant decrease in LLCMK growth, while PLA_2 activity was neither significantly increased nor decreased.

Vitamin E succinate supplementation significantly decreased BL6 cell growth, while PLA_2 activity (figure 21) was generally increased in BL6 cells, significantly so at 1, 3 and 5 μg/ml vitamin E succinate. Thus an inverse relationship again existed between PLA_2 activity and BL6 cell growth.

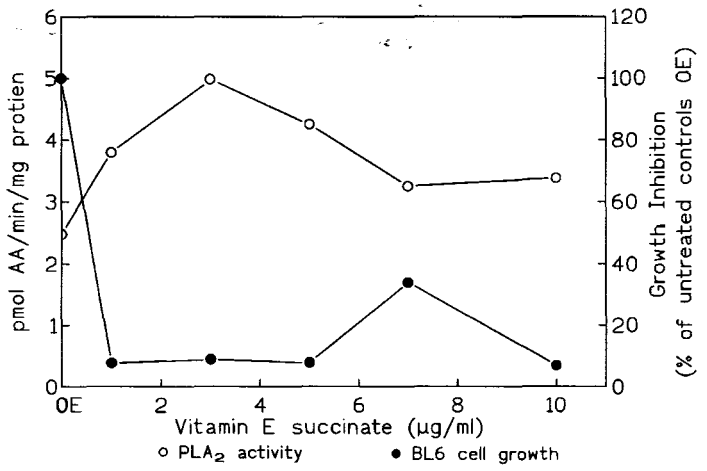


Figure 21: Effect of vitamin E succinate supplementation on phospholipase A_2 activity and BL6 cell growth.

Summarising the results obtained up to this point, it is clear that supplementation of vitamin E succinate significantly inhibits BL6 cell growth and furthermore that this decreases in growth was inversely related to free radical, lipid peroxidation and intracellular Ca^{2+}levels, as well the activities of the 5-LOX, COX and PLA_2 enzymes.

PG synthesis is regulated acutely via the activation of phospholipases and release of AA, while net PG production is dependant on COX activity (166). Since marked changes in the activities of the PLA_2 and COX enzymes have been shown to occur in BL6 and LLCMK cells supplemented with varying levels of vitamin E succinate, it was important to determine the effects of vitamin E succinate supplementation on PG levels in these cells. LLCMK cells showed no significant increase or decrease in growth upon supplementation of vitamin E succinate, while PGE_2 levels, with the exception of the 1μg/ml supplemented cultures, were generally decreased at all vitamin concentrations tested. Although the precise role of PGE_2 in tumour metastasis is unclear (232), studies with BL6 cells (209,232,250,252,255) have suggested that the metastatic potential of these cells is inversely related to the ability of BL6 cells to synthesis PGE_2, and furthermore that the highly metastatic BL6F10 population synthesise far less PGE_2 than the lower metastatic F1 population (233,255).

In the experiment concerning the effect of vitamin E succinate supplementation on PGE_2 levels in BL6 cells (figure 22), supplementation of 1-10μg/ml vitamin succinate resulted in a significant decrease

in growth. The general trend that was apparent was that as BL6 growth decreased, PGE_2 levels increased and *visa versa*. The only exception was that recorded at 3μg/ml supplementation, where both cell growth and PGE_2 levels decrease.

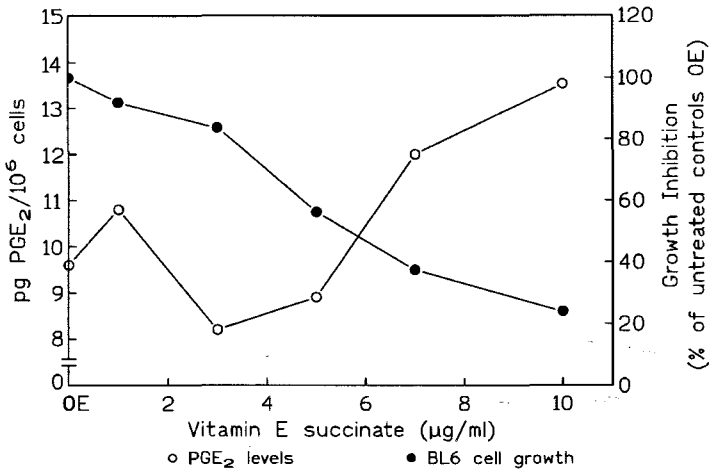


Figure 22: Effect of vitamin E succinate supplementation on prostaglandin E_2 levels and BL6 cell growth.

Since the quantity and type of prostaglandin produced by a given cell is vital to the metabolic effect it has (213), and the fact that BL6 cells are unusual in terms of eicosanoid biology in that they synthesise more than one type of PG (232,256,257), it was of interest to this study to determine whether vitamin E succinate supplementation affected the levels of various other PGs in BL6 cells.

Vitamin E succinate supplementation of BL6 cells resulted in an overall decrease in PGD_2 (figure 23), with a significant decrease occurring at 3 to 10μg/ml supplementation. This decrease in PGD_2 levels at 7 and 10μg/ml respectively, correlated with a non-significant decrease in BL6 growth. Relevant to the $PGF_{2\alpha}$ experiment (figure 24), supplementation of vitamin E succinate resulted in a significant decrease in $PGF_{2\alpha}$ levels, at all vitamin E succinate concentrations tested. Comparing BL6 growth and $PGF_{2\alpha}$ levels, the trend was that of increased growth with decreasing $PGF_{2\alpha}$ levels at 1, 3 and 5μg/ml vitamin E succinate supplementation, while at 7 and 10μg/ml respectively, a decrease in $PGF_{2\alpha}$ levels correlated with a decrease in cell growth.

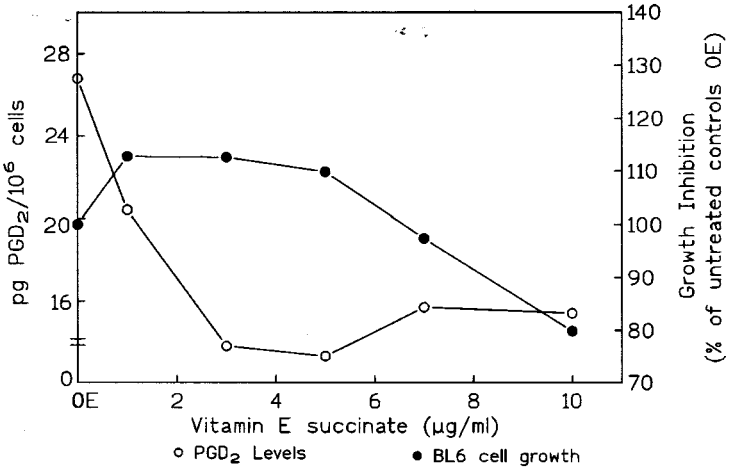


Figure 23: Effect of vitamin E succinate supplementation on prostaglandin D_2 levels and BL6 cell growth.

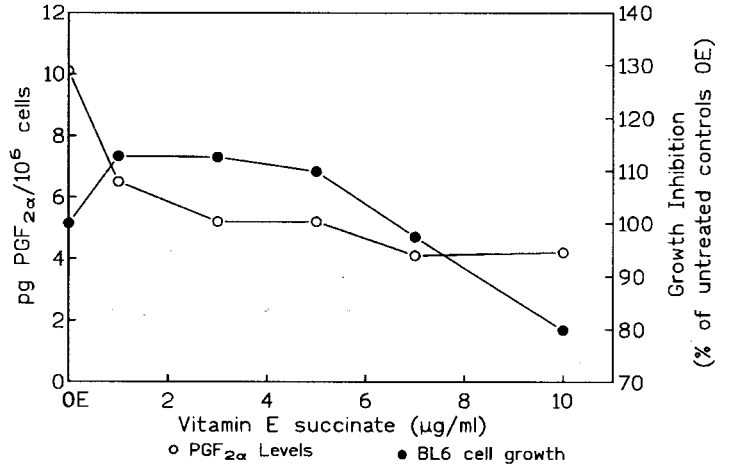


Figure 24: Effect of vitamin E succinate supplementation on prostaglandin $F_{2\alpha}$ levels and BL6 cell growth.

This suggests that at lower concentrations of vitamin E succinate, an inverse relationship exists between BL6 growth and the levels of PGD_2 and $PGF_{2\alpha}$, while at higher concentrations of vitamin E succinate, the inhibitory effects on BL6 cell growth were positively correlated with a decrease in PGD_2 and $PGF_{2\alpha}$ levels.

5. Secondary messengers and growth.

In the experiment examining the effect of vitamin E succinate supplementation on PGI_2 levels in BL6 cells (Figure 25), 3-10μg/ml vitamin E succinate supplementation decreased the growth of these cells to a certain extent, significantly so at 10μg/ml. With respect to PGI_2 levels in the cells, supplementation of 1-7μg/ml vitamin E succinate resulted in no significant increase or decrease in PGI_2 levels, while at 10μg/ml a significant increase in PGI_2 levels occurred. Thus, the growth inhibitory effects observed in BL6 cells at high concentrations of vitamin E succinate were correlated in part by increasing PGE_2 and I_2 levels, and decreasing PGD_2 and $F_{2\alpha}$ levels in these cells.

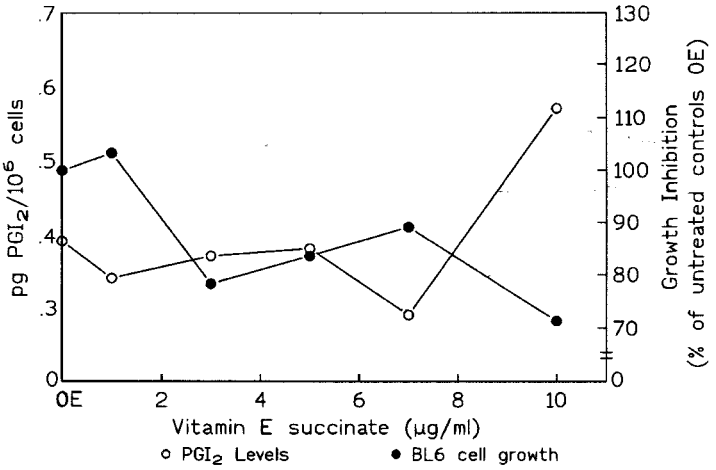


Figure 25: Effect of vitamin E succinate supplementation on prostaglandin I_2 levels and BL6 cell growth.

In general PG effects within a cell are believed to be mediated through activation of AC enzyme and cAMP synthesis (154,227,343,344). PGs are believed to mediate their effects on growth through changes in the intracellular levels of cAMP (205,248,334,347,364,367). PGs of the D, E and I series are capable of increasing cAMP levels in various systems (345). Since vitamin E succinate supplementation modulates intracellular levels of PGE_2, D_2 and I_2 in BL6 cells (485), and the fact that the AC enzyme is responsible for the synthesis of cAMP, it was relevant to this study to determine the effect of vitamin E succinate supplementation on both AC activity and cAMP levels. In LLCMK cells, supplementation of vitamin E succinate resulted in a significant increase in cell growth, with AC activity following a similar trend. The only exception was that recorded at 7 and 10μg/ml vitamin E succinate, where an inverse relationship existed between cell growth and AC activity. With respect to cAMP levels, supplementation of 3-10μg/ml vitamin E succinate resulted in an overall decrease in cAMP levels in LLCMK cells, together with a significant increase in cell growth. This

suggests that intracellular levels of cAMP are negative regulators of LLCMK growth and furthermore that intracellular levels of cAMP in LLCMK cells at 7 and 10μg/ml vitamin E succinate correlate positively with AC activity.

Supplementation of BL6 cells with 1-10μg/ml vitamin E succinate resulted in a significant increase in AC activity (figure 26) at 7 and 10μg/ml respectively. With respect to BL6 cell growth, supplementation of 1 and 3μg/ml vitamin E succinate significantly increased growth, while at 5, 7 and 10μg/ml respectively, a significant decrease in growth occurred. With the exception of 1μg/ml supplemented cultures, the general trend that was apparent was that as AC activity increased, BL6 growth decreased and *visa versa*. Hence an inverse relationship exists between AC activity and BL6 cell growth.

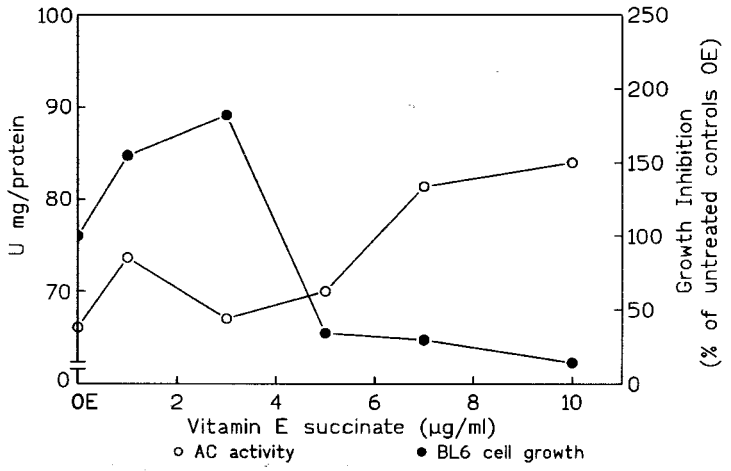


Figure 26: Effect of vitamin E succinate supplementation on adenylate cyclase activity and BL6 cell growth.

When comparing cAMP levels and BL6 cell growth (figure 27), cAMP levels were also inversely related to BL6 cell growth at all vitamin concentrations tested. Thus, supplementation of vitamin E succinate, significantly increases the levels of PGE_2, AC activity and cAMP in BL6 cells, and furthermore these increases were inversely related to BL6 cell growth (486).

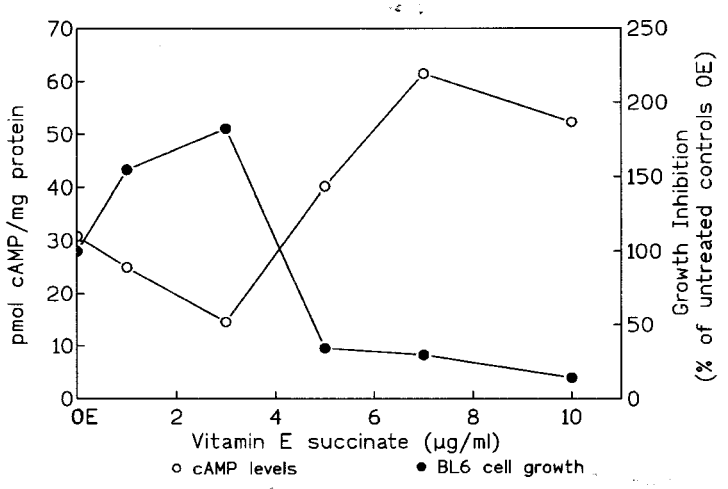


Figure 27: Effect of vitamin E succinate supplementation on cyclic adenosine monophosphate levels and BL6 growth.

Apart from the AC enzyme, intracellular levels of cAMP are regulated in part by the activity of the PDE enzymes. These enzymes catalyse the hydrolysis of cAMP, and are the major physiological pathway for terminating the intracellular effects of cAMP (326). As a result, an investigation of the relationship between total PDE activity and cell growth was undertaken.

Vitamin E succinate supplementation of LLCMK and BL6 cells resulted in an overall decrease in growth, significantly so at 10μg/ml supplementation. PDE activity was generally increased in LLCMK cells, with a significant increase occurring at 7 and 10μg/ml vitamin E succinate supplementation. This increase in PDE activity following vitamin E succinate supplementation could account for the significant decrease in cAMP levels observed in earlier studies with LLCMK cells. In the experiment concerning the effect of vitamin E succinate supplementation on PDE activity in BL6 cells (figure 28), the general trend was that as BL6 growth increased, PDE activity increased and *visa versa*. An exception was at 10μg/ml supplementation where a significant increase in PDE activity was associated with a decrease in growth.

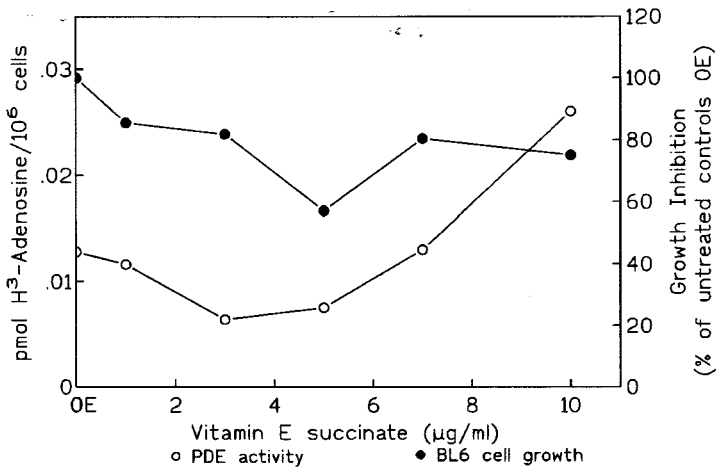


Figure 28: Effect of vitamin E succinate supplementation on phosphodiesterase activity and BL6 cell growth.

In conclusion, although some studies suggest an inverse relationship between LLCMK cell growth and the levels of various enzymes and secondary messengers shown in figure 4, no conclusion can be made since the magnitude of this relationship varied considerably from one experiment to another, while other experiments showed no such inverse relationship. In contrast, the inverse relationship between growth and secondary metabolites in BL6 cells was consistent with the same general trend being observed in the pathway studied, even though the magnitude of this relationship did vary marginally between experiments.

Numerous *in vitro* studies (42,66,125-127) have shown that vitamin E in its esterified form acts as a potent inhibitor of tumour cell growth and development, although the mechanism(s) by which its inhibits tumour cell growth and development is at present unclear. One possible mechanism suggested was through its antioxidant properties associated with the vitamin E component of the vitamin E succinate molecule (66,127). In this study however, supplementation of vitamin E succinate resulted in a significant increase in free radical and lipid peroxidation levels. Since free radical formation (235) and lipid peroxidation (159,235) can be initiated enzymatically through the catalytic action of the COX and LOX enzymes, and the fact that vitamin E succinate supplementation results in an inverse relationship between BL6 growth and the levels of PGE_2, AC activity and cAMP (486), it is proposed that vitamin E succinate inhibitory effects on BL6 growth were mediated via a PGE_2, AC, and cAMP linked system.

5. Secondary messengers and growth.

In an attempt to demonstrate a relationship between the levels of free radicals, lipid peroxidation and the activity of the COX enzyme in BL6 cells, as well as provide further evidence that vitamin E succinate mediates its growth inhibitory effects through a PGE_2, AC and cAMP linked system, BL6 cells were treated with COX inhibitor indomethacin. It must be noted that since supplementation of vitamin E succinate to LLCMK cells resulted in variable and less significant effects on cell growth and secondary messenger metabolism, with no real correlation between the two, these indomethacin studies were carried out on the BL6 cell line only.

6

INHIBITOR TREATMENT AND BL6 CELL GROWTH

6.1 EFFECT OF VITAMIN E SUCCINATE AND INDOMETHACIN SUPPLEMENTATION ON BL6 CELL GROWTH

6.1.1 INTRODUCTION

Numerous PG-synthesising inhibitors or non-steroidal anti-inflammatory drugs have been discovered, which markedly inhibit COX activity. These include aspirin, ibuprofen, piroxicam, sulindac and indomethacin (INDO) (487,488). The best known of these inhibitors and the one used in this study is INDO (figure 29).

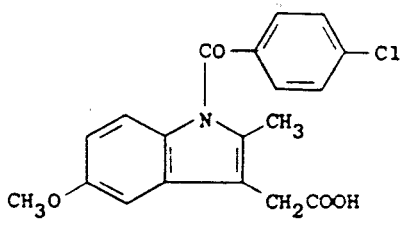


Figure 29: Structure of indomethacin (43).

The mechanism by which INDO inhibits COX activity is at present unknown, although it has been suggested that an initial reversible binding between COX enzyme and INDO occurs, followed by a selective conformational change in PGH synthase which eventually leads to irreversible inactivation of the enzyme and tighter binding of the INDO (165,489).

The inhibition of PG synthesis by indomethacin (INDO) results in growth inhibition of tumours both in experimental animals (205,233,490) and humans (491). These antitumour properties of INDO are believed to be due to its ability to enhance immune responses by inhibiting the production of immune suppressive PGs, notably PGE_2 (232,237,487,488). However, these beneficial effects of INDO must be interpreted with caution, since INDO supplementation has been shown to be ineffective (237) or

even stimulatory (232,256,488) to various tumour cell lines. The reason for this variable effect is at present unclear, although studies by Furuta *et al* (237) suggest that different malignant tumours synthesise specific PGs, and furthermore that their response to INDO treatment was dependant on the expression of PGH synthase activity and the type of PG produced. The aim of this part of the present study was to determine the effect of INDO supplementation on the *in vitro* growth of malignant BL6 cells.

6.1.2 MATERIALS AND METHODS

MATERIALS

Indomethacin was purchased from Sigma Chemical Co., USA. Refer to section 2.2 for the remainder of materials used.

METHODS

6.1.2.1 Preparation of vitamin E succinate and indomethacin stock solutions

Stock solutions of vitamin E succinate (2-20mg/ml) and indomethacin (0.3mM) were freshly prepared in absolute ethanol and diluted 0.5:1 000 in medium containing 10% (v/v) FCS to give a final concentration of 1, 3, 5, 7, 10μg/ml vitamin E succinate and 0.15μM INDO respectively in 0.1% final volume of ethanol. The concentration of INDO required to inhibit COX activity in BL6 cells at all vitamin concentrations tested was determined by preliminary range finding experiments, and was shown to be most effective at 0.15μM INDO.

6.1.2.2 Cell culture procedure

In the experiments relevant to 7.2.2 and 7.2.3, for which further analytical procedures and data are described in chapter 7 (the numbering denoting the relevant experiments), $3x10^6$ BL6 cells were seeded into 6 sets of 5 25cm^2 flasks. To five sets of these flasks, 10ml of MEM basal medium containing 10% (v/v) FCS, 0.15μM INDO and varying levels of vitamin E succinate (1-10μg/ml) was added. The sixth set of flasks (control cultures 0E) received 10ml of 10% (v/v) FCS medium containing 0.1% final volume of ethanol and 0.15μM INDO. In experiments 7.3 and 7.4, $5x10^5$ BL6

cells were seeded into 6 sets of 3 75cm^2 flasks. To 5 of the 6 sets of flasks was added 30ml of medium containing 10% FCS (v/v) containing 0.15μM INDO and varying levels of vitamin E succinate (1-10μg/ml). The sixth set of flasks received 30ml of medium containing 0.1% ethanol and 0.15μM INDO and were referred to as control cultures 0E. All flasks were incubated at 37^{0}C for the duration of the experiment with one medium change during this period.

6.1.2.3 Harvesting of experiments

Refer to section 2.2.3.2 for the method used.

6.1.2.4 Statistical analysis

The results obtained were analysed using a One Way Analysis of Variance (ANOVA) followed by the Student Newmans-Kuels Multiple Range Test. Data in all subsequent sections of this chapter were similarly analyzed.

6.1.3 RESULTS

The results of BL6 growth obtained in the relevant experiments, in which 1-10μg/ml vitamin E succinate and 0.15μM INDO were supplemented, are shown in table 18. Relevant to the discussion in this chapter is the mean percentage growth inhibition of all the experiments. Growth inhibition results for each individual experiment will be discussed relative to cell metabolism in chapter 7.

BL6 cells supplemented with 1 to 7μg/ml vitamin E succinate and 0.15μM INDO showed a general increase in growth compared with control cultures 0E, with a significant ($p \leq 0.05$) increase occurring at 5μg/ml supplementation. At 10μg/ml vitamin E succinate supplementation, a non-significant decrease in growth occurred.

In individual experiments, combined vitamin E succinate and INDO supplementation resulted in similar trends to overall mean growth results, however the differences compared to controls were significant in certain experiments.

Table 18: The effect of vitamin E succinate and indomethacin supplementation on BL6 cell growth. (Values recorded in the table are the mean of 3# or 5* cultures ± SEM).

Vitamin E succinate (μg/ml)	Growth Inhibition (% of untreated controls 0E)				
	Exp 7.2.1*	Exp 7.2.2*	Exp 7.3#	Exp 7.4#	MEAN
0E	100 ±6.1	100 ±12.9	100 ±12.4	100 ±4.4	100 ±4.0
1	182.7[a] ±7.20	133.3 ±7.1	113.5 ±6.0	97.3 ±3.7	131.8 ±8.3
3	191.3[a] ±17.8	110.2 ±5.9	70.7 ±3.8	±102.0 ±6.2	122.8 ±11.2
5	210.5[a] ±13.6	125.7 ±10.4	108.4 ±8.5	100.2 ±5.0	137.2[c] ±11.5
7	199.4[a] ±10.8	115.7 ±8.7	106.93 ±8.9	101.2 ±0.6	130.7 ±10.7
10	126.1 ±11.2	86.2 ±3.1	85.9 ±7.2	76.9[b] ±3.6	93.7 ±5.6

a= p≤0.001:)
b= p≤0.01:) Relative to control cultures 0E.
c= p≤0.05:)

6.1.4 DISCUSSION

In vitro supplementation of BL6 cells with vitamin E succinate results in a significant decrease in growth at 5, 7 and 10μg/ml respectively (486). Other studies (42,127) with BL6 cells have shown similar decreases in growth when supplemented with 1-10μg/ml vitamin E succinate. The supplementation of BL6 cells with 0.15μM INDO and 1, 3, 5 and 7μg/ml vitamin E succinate resulted in a general increase in growth compared with control cultures 0E, with a significant increase occurring at 3μg/ml. The only exception was that recorded at 10μg/ml vitamin E succinate supplementation, where a non-significant decrease in growth occurred.

Researchers (256,492) working with BL6 cells have shown that pretreatment of these cells with INDO also results in a significant increase in growth. This suggests that the growth inhibitory effects of vitamin E succinate supplementation on BL6 cells, was mediated in part by the activation of COX enzyme and increased PG synthesis. However one factor to consider is that inhibition of enzymatic

reactions through one pathway may enhance the activity of other pathways (488). For example, agents which block the COX pathway are believed to enhance the activity of the LOX pathway (232,488). This pathway is believed to synthesise metabolites which affect cell proliferation (232). Hence uncertainty exists as to whether the stimulatory effects of INDO supplementation on BL6 growth in these studies were as a result of decreased COX activity and PG synthesis, or as a result of increased 5-LOX activity and leukotriene synthesis.

In an attempt to demonstrate that the stimulatory effects of INDO supplementation on BL6 cell growth were as a result of decreased COX activity rather than increased 5-LOX activity, the effects of combined nordihydroguaidaretic acid (NDGA) (5-LOX inhibitor) and INDO (COX inhibitor) supplementation on BL6 cell growth was determined.

6.2 EFFECT OF VITAMIN E SUCCINATE, INDOMETHACIN AND NORDIHYDROGUAIDARETIC ACID SUPPLEMENTATION ON BL6 GROWTH

6.2.1 INTRODUCTION

Studies by Lee *et al* (cited in 488), have shown that the inhibition of the COX enzyme with INDO stimulates the synthesis of leukotrienes in TMT-081 rat mammary tumour cells. At least two mechanisms could account for this, namely; a) inhibition of COX enzyme could shunt AA into the LOX pathway resulting in an increase in leukotrienes synthesis, or b) these drugs could directly stimulate LOX enzyme activity and leukotriene synthesis. Since the LOX enzymes synthesise products which affect cell proliferation, and the fact that inhibiting COX activity increases LOX activity (232,488), it was important to this study to determine whether the stimulatory effects of INDO supplementation on BL6 cell growth observed in chapter 6, were due to a decrease in COX activity or increase in LOX activity. Hence an investigation of the effect of combined INDO (COX inhibitor) and NDGA (5-LOX inhibitor) supplementation on BL6 cell growth was determined. The antioxidant NDGA (figure 30) is widely used in the inhibition of 5-LOX activity (449,451), and is of principle concern in this study.

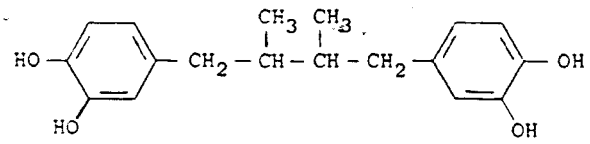


Figure 30: Structure of nordihydroguaidaretic acid (43).

6.2.2 MATERIALS AND METHODS

MATERIALS

Nordihydroguaidaretic acid was purchased from ICN Biomedical Inc., South Africa.

6.2.2.1 Preparation of vitamin E succinate, indomethacin and nordighydroguaidaretic acid stock solutions

Stock solutions of vitamin E succinate (1-10mg/ml) containing INDO (0.15mM) and NDGA (5mM) were prepared freshly in absolute ethanol and diluted 1:1 000 in medium containing 10% (v/v) FCS to give a final concentration of 1, 3, 5, 7 and 10μg/ml vitamin E succinate, 0.15μM INDO and 5μM NDGA. (5μM concentrations of NDGA were found to be effective inhibitors of 5-LOX activity in various other studies (449,451)).

6.2.2.2 Cell culture procedure

$3x10^5$ BL6 cells were seeded into 6 sets of 5 25cm^2 flasks. To five sets of these flasks, 10ml of MEM basal medium containing 10% (v/v) FCS, 0.15μM INDO, 5μM NDGA and varying levels of vitamin E succinate (1-10μg/ml) was added. The sixth set of flasks received 10ml of 10% (v/v) FCS supplemented medium containing 0.1% (v/v) final volume of ethanol and 0.15μM INDO, and were referred to as control cultures 0E. All flasks were incubated at 37^{0}C for the duration of the experiment with one medium change during this period.

6.2.2.3 Harvesting of experiments

The method used is described in section 2.2.3.2.

6.2.3 RESULTS

Supplementation of BL6 cells with varying levels of vitamin E succinate (1-10μg/ml), 0.15μM INDO and 0.5μM NDGA, resulted in no significant increase or decrease in growth compared with control cultures 0E (table 19).

Table 19: The effect of indomethacin and nordihydroguaidaretic acid supplementation on BL6 cell growth. (Each value in the table represents the mean of 5 determinations ± SEM).

Vitamin E succinate (μg/ml)	Growth Inhibition (% of untreated controls 0E)
0E	100 ±4.6
1	119.4 ±4.4
3	98.0 ±3.7
5	103.2 ±5.1
7	96.3 ±7.1
10	102.0 ±6.1

6.2.4 DISCUSSION

In this study, inhibition of 5-LOX activity resulted in no significant increase or decrease in BL6 cell growth. More importantly however, when comparing these results with mean growth results obtained following INDO supplementation alone (section 6.1), the trend was that of marginally decreased growth in combined NDGA and INDO treated cultures, with the exception of the 10μg/ml, where a non-significant increase in growth occurred. This suggests that the observed increase in growth

following INDO supplementation was primarily due to inhibition of COX activity rather than changes in 5-LOX activity.

Since COX activity is believed to contribute to free radical formation (235), lipid peroxidation levels (159,235) and net PG synthesis within a cell (166), the effect of INDO supplementation on free radical formation, lipid peroxidation levels and secondary messengers was determined in BL6 cells.

7

EFFECT OF INDOMETHACIN AND NUTRIENT SUPPLEMENTATION ON CELL OXIDATION STATE AND SECONDARY MESSENGER METABOLISM

7.1 EFFECT OF VITAMIN E SUCCINATE AND INDOMETHACIN SUPPLEMENTATION ON FREE RADICAL FORMATION AND LIPID PEROXIDATION LEVELS IN BL6 CELLS

7.1.1 INTRODUCTION

The mechanism(s) by which vitamin E succinate inhibits tumour cell proliferation is at present unclear, although it has been suggested that the anticarcinogenic effects of vitamin E may reside in their ability to scavenge free radicals and prevent lipid peroxidation (15,22,24,46,54,58,112,117-122,127). Earlier studies described in section 3.2 have shown significant increases in free radical and lipid peroxidation levels in BL6 cells following vitamin E succinate supplementation.

Since lipid peroxidation can be initiated enzymatically through the catalytic action of COX enzyme (159,235), and significant increases in COX activity have been shown to occur in BL6 cells supplemented with vitamin E succinate (see section 4.4), it is possible that the observed increases in free radical and lipid peroxidation levels in BL6 cells were as a result of increased COX activity in these cells.

In an attempt to demonstrate a relationship between free radical formation, lipid peroxidation levels and COX activity, BL6 cells were supplemented with a COX inhibitor INDO.

7.2 MATERIALS

Refer to sections 3.2.1 and 6.1.2 for the materials used.

7.2.1 EFFECT OF VITAMIN E SUCCINATE AND INDOMETHACIN SUPPLEMENTATION ON FREE RADICAL FORMATION IN BL6 CELLS.

METHODS

7.2.1.1 Cell culture procedure

Refer to section 6.1.2.2 for the method used.

7.2.1.2 Harvesting of cells

The method described in section 2.2.3.2 was repeated, except that cell pellets were resuspended in 1.0ml PBS (pH 7.4).

7.2.1.3 Nitroblue tetrazolium assay

Refer to section 3.2.2.2 for the method used.

7.2.2 EFFECT OF VITAMIN E SUCCINATE AND INDOMETHACIN SUPPLEMENTATION ON LIPID PEROXIDATION LEVELS IN BL6 CELLS

METHODS

7.2.2.1 Cell culture procedure

Refer to section 6.1.2.2 for the method used.

7.2.2.2 Harvesting of cells

The method described in section 2.2.3.2 was repeated, except that cell pellets were resuspended in 1.0ml 0.9% saline solution (pH 7.6).

7.2.2.3 Thiobarbaturic acid assay

Refer to section 3.2.3.2 for the method used.

7.2.3 STATISTICAL ANALYSIS

The results obtained were analysed using a One Way Analysis of Variance (ANOVA) followed by the Student Newmans-Kuels Multiple Range Test. Data in all subsequent sections of this chapter were analysed by this method.

7.2.4 RESULTS

Supplementation of BL6 cells (table 20) with 0.15μM INDO and varying levels of vitamin E succinate (1-10μg/ml) resulted in a significant ($p \leq 0.001$) decrease in lipid peroxidation levels, compared with control cultures 0E. The free radical levels on the other hand showed no significant increase or decrease when compared to control cultures.

Table 20: The effect of indomethacin and vitamin E succinate supplementation on free radical formation and lipid peroxidation levels in BL6 cells. (Each value in the table represents the mean of 5 determinations ± SEM)

Vitamin E succinate (μg /ml)	Free radical formation nmol diformazan/10^6 cells	Lipid peroxidation nmol MDA/10^6 cells
0E	2.40 ±0.24	0.75 ±0.07
1	2.14 ±0.06	0.16[a] ±0.05
3	1.99 ±0.014	0.10[a] ±0.03
5	2.18 ±0.16	0.08[a] ±0.02
7	2.16 ±0.11	0.06[a] ±0.01
10	2.35 ±0.16	0.07[a] ±0.02

a= $p \leq 0.001$: Relative to lipid peroxidation levels in control cultures 0E.

7.2.5 DISCUSSION

Analysis of COX catalytic mechanism has revealed that the activity of this enzyme is dependant on the presence of lipid hydroperoxides and that these lipid hydroperoxides are continuously required

during catalysis (168,169,175,181,182). In addition to this, it was found that a wide variety of lipid hydroperoxides could stimulate COX activity, including the product of COX enzyme itself, PGG_2 (168,169,182). These lipid hydroperoxides can decompose into TBA reactive materials such as MDA (186,409,493,494). This suggests that any agent capable of increasing COX activity could indirectly increase MDA levels within the cells through the generation of lipid peroxides.

The PG hydroperoxidase (PG-peroxidase) component of the PGH synthase enzyme complex, is believed to produce numerous oxygen-containing free radicals when catalyzing the conversion of PGG_2 to PGH_2 (168,176,186,235), hence any component capable of increasing COX activity would result in an increase in PGH synthesis activity and indirectly increase free radicals levels. In earlier experiments (section 4.4), vitamin E succinate supplementation of BL6 cells was in fact shown to stimulate COX activity and to increase the levels of free radicals and lipid peroxidation (section 3.2) in these cells. In the present experiment, supplementation of BL6 cells (table 20) with INDO and vitamin E succinate resulted in a significant decrease in lipid peroxidation levels compared with control cultures 0E. The reason for this decrease is unclear, although one factor to consider is that inhibiting the COX enzyme may stimulate other enzymes within the cell which function to remove the MDA precursor lipid hydroperoxides. Free radical levels on the other hand showed no significant increase or decrease following INDO supplementation. More importantly, however, when comparing the effect of vitamin E succinate supplementation on free radical and lipid peroxidation levels in INDO treated cells versus untreated cells (see sections 3.2), a marked decrease in both free radical and lipid peroxidation levels occurred in the INDO treated cells. This suggests that the observed increase in free radical and lipid peroxidation levels in BL6 cells supplemented with vitamin E succinate (484), was due to an increase in COX activity in these cells.

7.3 EFFECT OF VITAMIN E SUCCINATE AND INDOMETHACIN SUPPLEMENTATION ON PROSTAGLANDIN E_2 LEVELS IN BL6 CELLS

7.3.1 INTRODUCTION

The COX inhibitor INDO, functions to irreversibly inhibit the COX activity of the PGH synthase enzyme (165,173,489,495), and has been widely used in the pharmacological intervention of PG biosynthesis (487,488,496). As discussed earlier, although reports have been conflicting regarding the exact role of PGs in tumour development and progression, evidence over the past few years has

suggested a possible link between cancer development, cancer progression, invasiveness and the levels of PGs. The use of various COX inhibitors has contributed greatly to improving the understanding of the role of PGs in tumour growth and development (487,488). In this part of the study, the effect of INDO supplementation on PGE_2 levels in BL6 cells supplemented with varying levels of vitamin E succinate was determined, since PGE_2 has been shown to inhibit tumour cell growth *in vitro* (247-251) and earlier studies in section 4.5 have shown marked increases in PGE_2 levels in BL6 cells following supplementation with vitamin E succinate.

7.3.2 MATERIALS AND METHODS

MATERIALS

Refer to sections 4.5.2 and 6.1.2 for the materials used.

METHODS

7.3.2.1 Cell culture procedure

Methods described in 6.1.2.2 and 2.2.3.2 were repeated, except that cell pellets were resuspended in 2.0ml of PBS buffer pH 7.0.

7.3.2.2 Homogenisation and preparation of samples

Refer to section 4.5.2.2 for the method used.

7.3.2.3 Extraction of prostaglandin E_2

Refer to sections 4.5.2.3 for the procedure used.

7.3.2.4 Determination of prostaglandin E_2 levels

PGE_2 levels were determined using PGE_2-I^{125} assay kit as described in section 4.5.2.4.

7.3.3 RESULTS

The levels of PGE_2 in BL6 cells supplemented with 0.15μM INDO and 1-10μg/ml vitamin E succinate are shown in table 21. Supplementation with 1-10μg/ml vitamin E succinate resulted in no significant increase or decrease in PGE_2 levels in BL6 cells compared with control cultures 0E.

Table 21: The effect of indomethacin and vitamin E succinate supplementation on prostaglandin E_2 levels in BL6 cells. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	PGE_2 levels (pg/10^6 cells)
0E	4.90 ±1.03
1	3.72 ±0.25
3	3.69 ±1.42
5	4.65 ±0.55
7	2.93 ±0.26
10	5.88 ±1.53

7.3.4 DISCUSSION

Various experimental and therapeutic studies have consistently shown that INDO significantly inhibits PG synthesis by inhibiting the enzymes which synthesise them (487). Le Duc *et al* (496) studying the effects of COX inhibition on eicosanoid synthesis in rats showed that INDO supplementation inhibited PGE_2 synthesis by greater than 90% when compared to control animals. In this study supplementation of 1-10μg/ml vitamin E succinate and 0.15μM INDO to BL6 cells resulted in no significant increase or decrease in PGE_2 when compared with control cultures 0E. However, when comparing results obtained from vitamin E succinate treatment alone (section 4.5) to INDO treated cells, a marked decrease in PGE_2 levels was observed in INDO treated cells. INDO supplementation therefore appears to inhibit the stimulatory effects of vitamin E succinate on PGE_2 levels in BL6 cells as observed in

earlier studies (section 4.5).

7.4 EFFECT OF VITAMIN E SUCCINATE AND INDOMETHACIN SUPPLEMENTATION ON ADENYLATE CYCLASE ACTIVITY AND CYCLIC ADENOSINE MONOPHOSPHATE FORMATION

7.4.1 INTRODUCTION

As has been mentioned in earlier studies of this thesis, PGs are believed to exert their effects on tumour growth through a cAMP-mediated pathway (334,347,364,367). Both metastatic and non-metastatic tumour cells in response to PGE_2 synthesis generate elevate levels of cAMP. These effects are believed to be mediated through a PGE_2-receptor-AC-cAMP linked system (154,343,344). Hence any change in PGE_2 levels would result in similar changes in AC activity and cAMP levels. Since in vitamin E succinate supplemented BL6 cells, a decreased PGE_2 synthesis was shown to occur as a result of treatment with INDO, this study was undertaken to determine what effect INDO treatment had on AC activity and intracellular levels of cAMP in BL6 cells.

7.4.2 MATERIALS AND METHODS

MATERIALS

Refer to sections 4.7.2 and 6.1.2 for the materials used.

METHODS

7.4.2.1 Cell culture procedure

The method described in 6.1.2.2 and 2.3.3.2 was repeated, except that cell pellet was resuspended in 1.0ml of Tris-HCl buffer pH 7.5 containing 4mM EDTA.

7.4.2.2 Homogenisation and separation into membrane and stroma fractions

The procedure used is described in section 3.3.1.2.

7.4.2.3 Protein determination

Refer to section 4.7.2.3 for the method used.

7.4.2.4 Adenylate cyclase assay

The method described in 4.7.2.4 was used.

7.4.2.5 Cyclic adenosine monophosphate assay

The method described in 4.7.2.5 was used.

7.4.3 RESULTS

Treatment of BL6 cells (table 22) with 1-10μg/ml vitamin E succinate and 0.15 μM INDO resulted in no significant increase or decrease in AC activity or in the intracellular levels of cAMP, when compared with control cultures 0E.

Table 22: The effect of indomethacin and vitamin E succinate supplementation on adenylate cyclase activity and cyclic adenosine monophosphate levels in BL6 cells. (Each value in the table represents the mean of 3 determinations ± SEM).

Vitamin E succinate (μg/ml)	Adenylate cyclase activity (U/mg Protein)	cAMP levels pmol/mg protein
0E	31.80 ±6.45	3.32 ±0.13
1	25.60 ±1.44	3.28 ±0.075
3	19.67 ±1.04	3.69 ±0.26
5	29.71 ±2.69	2.89 ±0.06
7	23.05 ±1.72	3.74 ±0.84
10	25.33 ±1.98	3.50 ±0.15

7.4.4 DISCUSSION

BL6 cells supplemented with vitamin E succinate and 0.15μM INDO showed no significant increase or decrease in AC activity or cAMP levels when compared with control cultures 0E. However, when comparing the levels of AC activity and cAMP in BL6 cells treated with vitamin E succinate alone (section 4.7) to the INDO treated cells, AC activity and cAMP levels were markedly lower in the INDO treated cells. This suggests that this observed decrease in AC activity and cAMP levels in BL6 cells following INDO supplementation was probably due to a decrease in PGE_2 synthesis, since AC activity is known to be functionally linked to PG synthesis (154,343,344). In summary, results from this study provide further evidence that vitamin E succinate mediates its effects on malignant BL6 cells via a PGE_2-AC-cAMP linked system.

7.5 DISCUSSION OF THE RELATIONSHIP BETWEEN PROSTAGLANDIN E_2, ADENYLATE CYCLASE AND CYCLIC ADENOSINE MONOPHOSPHATE AND CELL GROWTH.

The following discussion will examine the effect of INDO supplementation on the interrelationship between the levels of PGE_2, cAMP and AC activity described in this chapter, and their possible influence on cell growth and metabolism.

Results from chapter 4 on the effects of vitamin E succinate supplementation on secondary messengers in BL6 cells showed that vitamin E succinate supplementation significantly increased the levels of PGE_2, AC activity and cAMP in these cells. Relating these effects to BL6 cell growth, it was evident that an inverse relationship exists between the levels of COX activity, AC activity, PGE_2, cAMP and BL6 cell growth. In terms of PG synthesis inhibitors, although no general rule can be drawn regarding their role in tumour cell growth, it is clear that they greatly affect tumour cell proliferation both *in vitro* (237,492) and *in vivo* (207,235,490).

Studies by Thomas *et al* (249) have shown that the mean basal levels of E series PGs are inversely related to HeLa and Hep2 growth, and furthermore that supplementation of these cells with INDO inhibits PGE synthesis and stimulates cell proliferation. Combined vitamin E succinate and INDO supplementation of BL6 cells in this study resulted in no significant increase or decrease in PGE_2 levels or cell growth (figure 31).

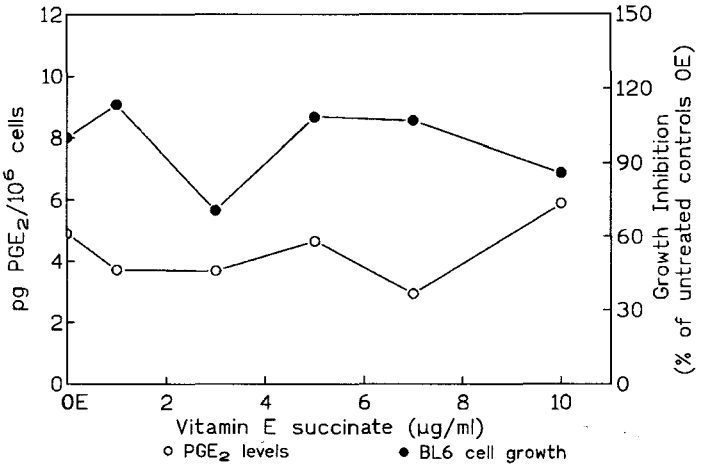


Figure 31: Effect of vitamin E succinate and indomethacin supplementation on prostaglandin E_2 levels and BL6 cell growth.

The relationship between AC activity and BL6 cell growth following combined vitamin E succinate and INDO supplementation is shown in figure 32. Again, BL6 cells showed no significant change in AC activity or cell growth when compared to control cultures 0E. The only exception was that recorded at 10µg/ml vitamin E succinate, where cell growth was significantly decreased.

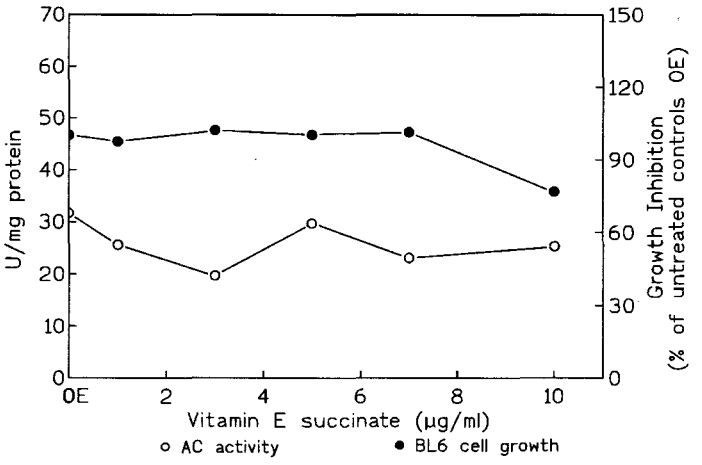


Figure 32: Effect of vitamin E succinate and indomethacin supplementation on adenylate cyclase activity and BL6 growth.

Similarly combined INDO and vitamin E succinate supplementation of BL6 cells (figure 33) resulted in no significant increase or decrease in cAMP levels or cell growth when compared to control cultures 0E, with the exception of the 10μg/ml supplemented cultures where a significant decrease in growth occurred.

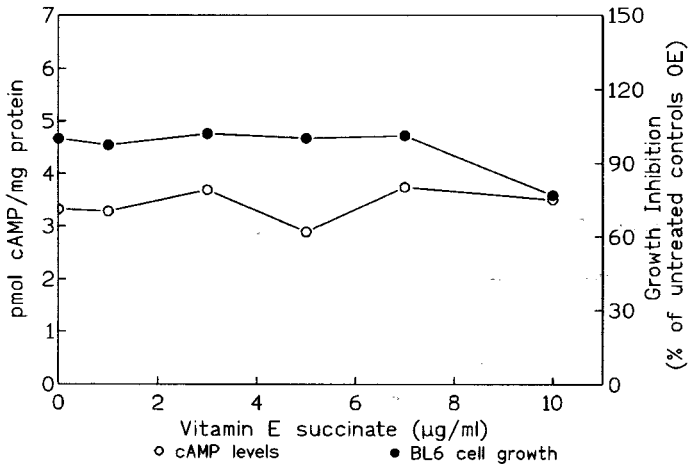


Figure 33: Effect of vitamin E succinate and indomethacin supplementation on cyclic adenosine monophosphate levels and BL6 cell growth.

Consequent use of the COX inhibitor INDO, provides further evidence that the inhibitory effects of vitamin E succinate on BL6 cell growth, were a direct result of the vitamin's ability to increase endogenous levels of PGE_2 and activate the AC-cAMP linked system (497).

8

GENERAL DISCUSSION

Cancer, or more precisely malignant growth, is defined as a disorder in which some cell type in the organism begins to grow in an apparently unchecked fashion. During this proliferation, the cell type, irrespective of its origin, tends to lose or alter some of its normal biochemical characteristics (498). Examples of these fundamental differences between malignant and non-malignant cells with respect to cellular functions and composition, are discussed in chapter one. In general, normal and tumour cells referred to in those reports originate from the same tissue type. In this study however, comparisons were made between malignant and non-malignant cells derived from murine melanoma and monkey kidney cells respectively. Although originating from different tissue types, these cells were used to show differences in the activities of certain enzymes and levels of various secondary metabolites determined in this study, and their response to vitamin E succinate supplementation. One characteristic property which differentiates tumour cells from normal cells is that of cell growth. Both maintenance and control cultures of BL6 cells have consistently shown higher rates of cell proliferation than non-malignant LLCMK control and maintenance cultures.

Although our aerobic lifestyles are advantageous in many ways, the utilization of oxygen by cells for many of their biochemical reactions results in the formation of highly reactive free radical products (60,69). Numerous studies over the past decade have led researchers to suggest that free radicals and products of free radical reactions may be involved in the aetiology of cancer (121,499). Substantial experimental evidence has implicated free radicals in both tumour initiation (8,136) and promotion (5,7-9,136,137). These findings have led to widespread interest in antioxidant vitamins as a means of preventing these processes. Attention has focused in particular on vitamin E, a major lipid-soluble antioxidant present in all membranes (60,69), which functions to scavenge free radicals and prevent lipid peroxidation both *in vitro* and *in vivo* (39,77). Since tumour incidence can be affected by nutritional manipulation (50,51), and an inverse relationship is believed to exist between serum vitamin E and cancer incidence (52,54,60,119,121), this study was directed towards determining the effect of vitamin E supplementation on the growth of a malignant BL6 melanoma and non-malignant monkey kidney (LLCMK) cell line. In addition to this, certain metabolic responses to vitamin E supplementation were studied.

With reference to the overall mean growth studies, supplementation of 5 to 10μg/ml vitamin E succinate resulted in a significant decrease in BL6 cell growth, while LLCMK cells showed no significant increase or decrease in growth. This selective inhibition of vitamin E succinate in BL6 cells may therefore be indicative of its value in the treatment of cancer. These growth inhibitory effects of vitamin E succinate have also been found in various other cell lines (123,126,127), although the mechanism by which the vitamin inhibits growth is at present unclear. In general non-malignant LLCMK cells exhibited no significant trends or relationships with respect to vitamin E succinate supplementation effects on cell growth, and between various metabolites and secondary messengers in the AA pathway (figure 4). Furthermore, with respect to those experiments showing an inverse relationship between LLCMK growth and the levels of secondary 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 tumour cell line (BL6 melanoma cells) used in this study on the other hand exhibited consistent and significant trends or relationships when supplemented with vitamin E succinate, and as a result this discussion will concern itself with the BL6 cells.

This study considered the possibility that the toxic effect of vitamin E succinate on tumour cell growth in BL6 cells was mediated by the antioxidant properties associated with the vitamin E component of the vitamin E succinate molecule. BL6 cells supplemented with 7 and 10μg/ml vitamin E succinate, showed a marked increase in free radical levels, with a significant increase occurring at 10μg/ml supplementation. Lipid peroxidation levels on the other hand were significantly increased at 5,7 and 10μg/ml vitamin E succinate. This increase in free radical and lipid peroxidation levels was inversely related to BL6 cell growth (484). These findings support reports that tumour cell growth is inversely related to lipid peroxidation levels (483,500), and that any agent capable of increasing lipid peroxidation levels within a cell could inhibit tumour cell growth (478,501).

Studies by Cillard *et al* (502,503) have suggested that vitamin E's antioxidant properties can be reversed to a prooxidant effect especially when the concentration of the vitamin is increased. While vitamin E is a naturally-occurring antioxidant, vitamin E esters are non-physiological antioxidants which require esterase activity to liberate the vitamin E component with antioxidant properties (126,403,404). Since no species-specific hydrolysis of vitamin E succinate has been shown to occur in BL6 cells to date, an attempt was made to demonstrate vitamin E succinate cleavage in these cells. Supplementation of BL6 cells with 3 to 10μg/ml vitamin E succinate resulted in a significant increase

in vitamin E succinate uptake compared to untreated control cultures 0E, while vitamin E levels showed no significant increasing or decreasing trend. This suggests that the growth inhibitory effect of vitamin E succinate on BL6 cells was due to the vitamin E succinate molecule as a whole, rather than the antioxidant properties associated with the vitamin E component.

Apart from its possible role as an antioxidant, vitamin E succinate may have numerous other functions within a cell, such as altering membrane-bound enzyme activities (124,415,416). These effects are believed to be mediated through direct interactions with PUFAs and polar phospholipid regions of the membranes (412,413). In an attempt to determine whether vitamin E succinate mediated its effects on BL6 cells through interactions with membranes, BL6 cells were supplemented with ^{3}H-vitamin E succinate and the relative uptake and cellular distribution of the vitamin was determined. Supplementation with ^{3}H-vitamin E succinate resulted in a significant increase in ^{3}H-vitamin E succinate uptake in BL6 cells. With respect to the relative distribution of ^{3}H-vitamin E succinate, generally higher levels of the vitamin occurred in the membrane fractions compared to stroma fractions. The only exceptions were those recorded at 5 and 7μg/ml supplementation, where similar levels of vitamin E succinate were found in membrane and stroma fractions. From the results obtained from free radical, lipid peroxidation, cleavage and uptake studies, it would therefore appear that vitamin E succinate rather than vitamin E mediated its effects on BL6 cell growth, and furthermore that these effects did not involve an antioxidant role, but possibly a modulatory effect on membrane functions. This study then considered the possibility that the inhibitory effect of vitamin E succinate on BL6 cell growth was mediated to a certain extent through an effect on various metabolites, secondary messengers and enzymes in the AA cascade and eicosanoid pathway (figure 4).

Supplementation with vitamin E succinate resulted in a general increase in Ca^{2+} levels in BL6 cells. The availability of Ca^{2+} within a cell is believed to be a key regulator of cell proliferation. In general malignant cells are believed to lose the ability to maintain Ca^{2+} levels below a critical point (283). However, in these studies an increase in Ca^{2+} levels resulted in a decrease in BL6 growth. A factor to consider is that the passage of cells through the G_1 and G_2 phases of the cell cycle is not only cAMP-dependant, but also Ca^{2+}-dependant (362). This cycle can become blocked in either phase under certain conditions and can be altered in tumour cells (289).

The role of Ca^{2+} in modulating the activity of the PLA_2 enzymes also needed to be examined (267-274). These enzymes function to release AA (272,293,307-318,423), an important precursor of PG

and leukotriene biosynthesis (426-428). As regards its possible role in modulating 5-LOX and COX activity, and the inverse relationship that exists between the activity of these enzymes and BL6 growth described in chapter 4, the effect of vitamin E succinate supplementation on PLA_2 activity was determined. Increased PLA_2 activity was observed in BL6 cells at all vitamin E succinate concentrations tested. When comparing PLA_2 activity and Ca^{2+} levels it was clear that PLA_2 activity was positively correlated with changes in intracellular Ca^{2+} levels, and that PLA_2 activity in BL6 cells was Ca^{2+}-dependant. The only exception was that recorded at 10μg/ml supplementation, where Ca^{2+} levels decrease and PLA_2 activity increases. This suggests that lower concentrations of vitamin E succinate may modulate PLA_2 activity in BL6 cells through changes in intracellular Ca^{2+} levels, while at higher concentrations of vitamin E succinate (10μg/ml), these effects were independent of intracellular Ca^{2+} levels. Since vitamin E succinate is believed to stabilise membranes (412-414) and alter membrane-bound enzyme activities (124,415,416), the possibility exists that high concentrations of vitamin E succinate may modulate PLA_2 activity through changes in membrane fluidity. As indicated, uptake studies showed markedly higher levels of vitamin E succinate in membrane fractions of BL6 cells supplemented with 10μg/ml vitamin E succinate, compared to stroma fractions.

Although 5-LOX and COX activities were similar in control cultures of BL6 cells, supplementation of vitamin E succinate resulted in markedly higher levels of COX activity in comparison with 5-LOX activity, at all concentrations of vitamin E succinate tested. An exception however did occur in the 3μg/ml supplemented cultures where 5-LOX activity was higher than COX activity. This suggests that vitamin E succinate's growth inhibitory effects on BL6 cells were more likely to be mediated through changes in COX activity than 5-LOX and that products of COX activity may play an important role in modulating tumour cell growth. This hypothesis was further strengthened by findings that supplementation of BL6 cells with the COX inhibitor indomethacin, abolishes the growth inhibitory effects of vitamin E succinate supplementation on BL6 cells. Since net PG production is regulated by COX activity (166), and marked changes in COX activity have been shown to occur in BL6 cells supplemented with varying levels of vitamin E succinate, it was important to determine the effects of vitamin E succinate supplementation on PG levels in these cells.

As previously mentioned, most PG-producing cells predominantly synthesis one type of PG due to the existence of a single PGH_2-metabolising enzyme (165). When comparing the levels of PGs in BL6 control cultures, it was clear that BL6 cells convert AA to PGD_2, $F_{2\alpha}$, E_2 and I_2 in descending order of magnitude (485). Other studies with BL6 cells have shown that PGD_2 is the major AA metabolite

produced by these cells (255,256), while PGE_2 and $PGF_{2\alpha}$ were produced in smaller amounts (cited in 232,255). PGI_2 levels in BL6 cells on the other hand were found to be markedly lower than PGD_2, E_2 and $F_{2\alpha}$ levels. Upon supplementation with vitamin E succinate in this study, a marked increase in PGE_2 levels occurred in the 7 and 10μg/ml supplemented cultures, while PGI_2 levels were also significantly increased at 10μg/ml supplementation (PGI_2 levels were however markedly lower than PGE_2 levels in BL6 cells). It has been suggested that AA metabolism can be diverted towards PGE_2 synthesis when elevated levels of lipid hydroperoxides (known inhibitors of PGI_2 synthase activity) occur (215). Since COX enzymes catalyse the conversion of AA to various lipid hydroperoxides, and vitamin E succinate supplementation has been shown to significantly increase COX activity in BL6 cells, it is reasonable to assume that the growth inhibitory effects of vitamin E succinate supplementation on BL6 cells were mediated largely through an increase in PGE_2 synthesis rather than an increase in PGI_2 synthesis.

One major intracellular signalling system identified thus far is that of the AC enzyme, which functions to generate cAMP from ATP and is regulated by G-proteins (422). PGE_2 is believed to stimulate cAMP formation through a PGE_2-receptor-cAMP-linked system (366), with the cAMP acting as a secondary messenger for PGE_2 mediated modulation of biological activity within the cell (368). In this study, supplementation with vitamin E succinate significantly increased both AC activity and cAMP levels in BL6 cells, while cell growth was significantly decreased. Since PGE_2 levels were inversely related to BL6 cell growth, and an increase in PGE_2 was positively correlated with increases in AC activity and cAMP levels, it is reasonable to conclude that the increase in AC activity and cAMP levels observed in BL6 cells supplemented with vitamin E succinate was due to an increase in PGE_2 synthesis (486). Supplementation of E-series PGs to various malignant and non-malignant cell lines have been shown to inhibit the growth of these cells, and furthermore this inhibition of growth correlated with changes in cAMP levels (205,248,347).

With respect to another possible effector of cAMP levels in cells, and the consequent effects of cAMP on BL6 cell growth already described, the effect of vitamin E succinate supplementation on PDE activity in BL6 cells was determined. Total PDE activity in BL6 cells was neither significantly increased nor decreased upon vitamin E succinate supplementation, suggesting that PDE activity plays no role in altering intracellular cAMP levels. However, caution should be exercised when drawing such conclusions, since two distinct forms of PDE are known to occur, namely a sPDE and pPDE enzyme (326,371). Of interest to this study was the pPDE enzyme, which controls basal levels of

cAMP and whose activity is dependant on the levels of intracellular activators such as cAMP (326,370,372).

As indicated, in BL6 cells, supplementation of vitamin E succinate resulted in a significant increase in cAMP levels (486). In contrast, a non-significant decrease in pPDE activity was observed in BL6 cells following vitamin E succinate supplementation. The only exception was that recorded at 10μg/ml supplementation, where a non-significant increase in pPDE activity occurs. This effect was contrary to the expected result. If cAMP was responsible for modulating pPDE activity as suggested (326,370,372), one would have expected pPDE activity to increase as the levels of cAMP were increased. One important consequence of this decreased PDE activity, however, is that lower pPDE would result in an increase in cAMP levels within the cell, which was found in these cells. Since pPDE activity is a membrane-bound enzyme (369,370), and vitamin E succinate is known to modulate the activity of membrane-bound enzymes (124,415,416), we propose that the observed decrease in pPDE activity may therefore be a direct result of the vitamin E succinate supplementation.

One question that may arise, is how cAMP levels in BL6 cells increase at 10μg/ml vitamin E succinate supplementation, when some increase in pPDE and sPDE activity occurred. This may be explained by the fact that intracellular cAMP levels are determined in large part by AC and cPDE activity (325,378,379). Supplementation of BL6 cells with 10μg/ml vitamin E succinate resulted in a significant increase in AC activity and this increase in AC activity was greater than the small rise noted in pPDE and sPDE activity, resulting in a net production of cAMP rather than net removal. Thus, the increase in cAMP levels in BL6 cells following vitamin E succinate supplementation was largely due to changes in AC activity rather than PDE activity.

In an attempt to provide further evidence that vitamin E succinate mediated its growth inhibitory effects on BL6 cells through a PGE_2-AC-cAMP linked system and that a direct link exists between free radical formation, lipid peroxidation levels and COX activity, BL6 cells were supplemented with COX inhibitor INDO. With reference to overall mean growth studies, supplementation of BL6 cells with 0.15μM INDO and 1-10μg/ml vitamin E succinate resulted in no significant increase or decrease in growth when compared to control cultures. Since vitamin E succinate is believed to inhibit BL6 growth through increases in the levels of PGE_2, cAMP and AC activity (486), the possibility that INDO stimulatory effects on BL6 growth were due to decreased levels of PGE_2, cAMP and AC activity was considered. Supplementation of BL6 cells with INDO and vitamin E succinate revealed

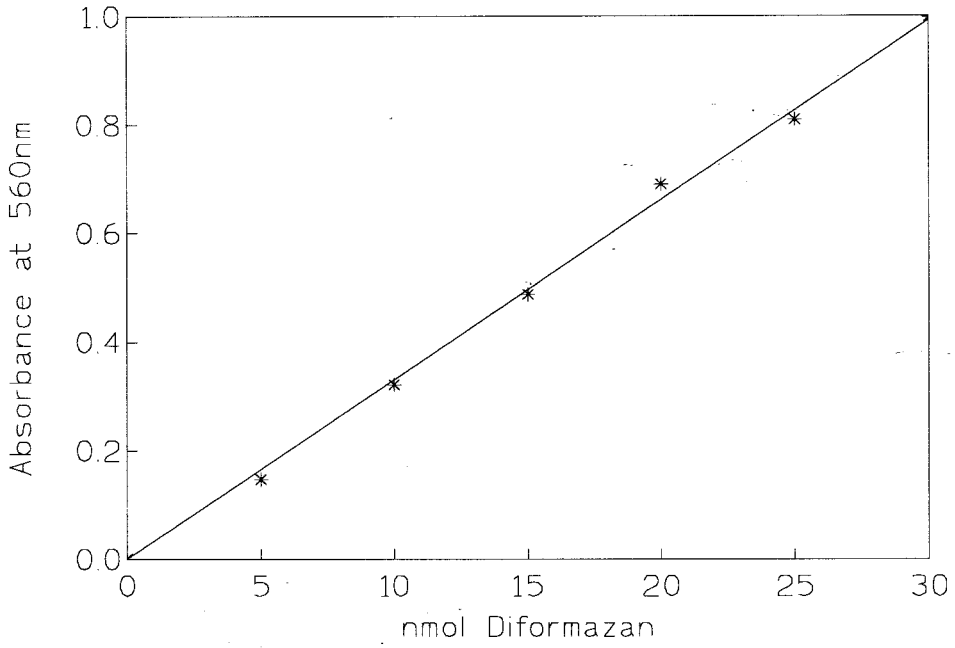
that no significant increase or decrease in PGE_2 and cAMP levels or AC activity occurred when compared to control cultures (498). These results provide further evidence that vitamin E succinate's growth inhibitory effects on BL6 cells were mediated through a PGE_2-AC-cAMP linked system.

As regards free radical and lipid peroxidation levels in BL6 cells. The metabolism of AA by COX and LOX enzymes are believed to contribute to free radical formation (235) and lipid peroxidation levels within a cell (159,235), and since vitamin E succinate supplementation significantly increased free radical formation and lipid peroxidation levels in BL6 cells (484), this study considered the effect of vitamin E succinate supplementation on the activity of these enzymes. Supplementation with 7 and 10μg/ml vitamin E succinate resulted in a significant increase in COX activity, while 5-LOX activity was significantly increased at 10μg/ml. Furthermore, it was established in chapter 4 that an inverse relationship existed between the activities of these two enzymes and BL6 cell growth. Although vitamin E succinate is believed to stabilise membranes (412-414) and modulate the activities of membrane-bound enzymes (124,415,416), the effects of vitamin E succinate supplementation on BL6 cells may be more complex than simply stabilising membranes, since 5-LOX enzymes are located in the cytosol and not in the membranes (438,443,446). Another important factor to also consider is the intracellular Ca^{2+} found in vitamin supplemented cells, since Ca^{2+} is believed to influence the pattern of AA metabolites formed, by regulating the activities of both the COX and 5-LOX enzymes (276). INDO supplementation resulted in a significant decrease in lipid peroxidation levels, while free radicals returned to levels similar to those found in control cultures. It is therefore reasonable to conclude that the observed increase in free radical and lipid peroxidation levels in BL6 cells following supplementation with vitamin E succinate was an indirect result of the vitamin's ability to increase COX activity in these cells.

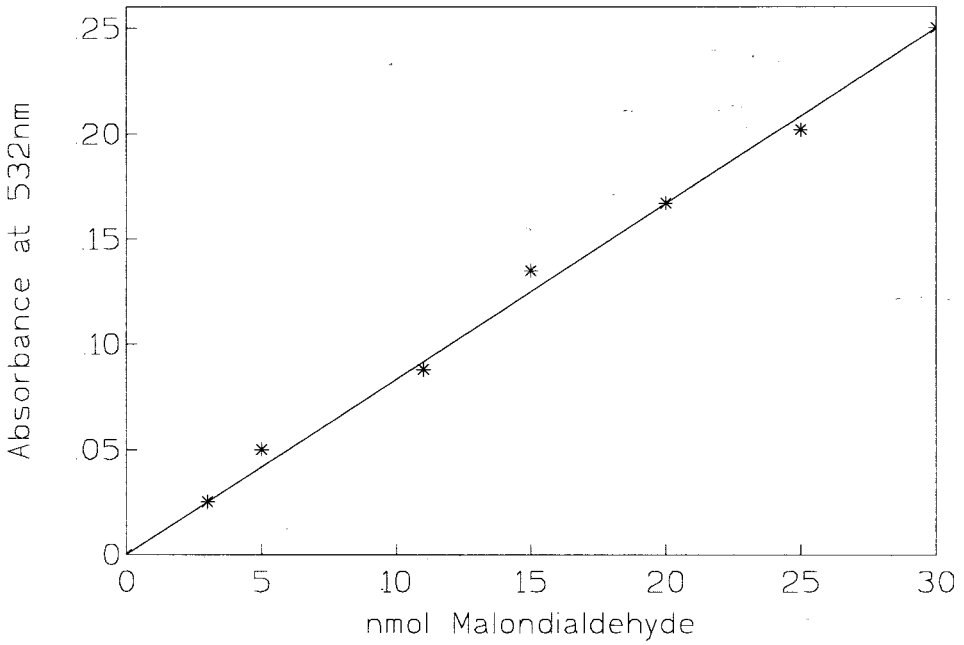
Results from these studies suggest that vitamin E in the form of vitamin E succinate inhibits BL6 growth, and furthermore that this inhibitory effect on cell growth did not involve antioxidant properties, but rather changes in AA and eicosanoid metabolism. This alteration in AA metabolism was brought about by a cascade effect, initiated by vitamin E succinate increasing PLA_2 activity possibly via an alteration in intracellular Ca^{2+} levels and/or membrane-stabilising properties of the vitamin. This in turn results in an increase in PGE_2 levels in BL6 cells via the action of the COX enzyme. These increases in PGE_2 stimulate AC activity and increase cAMP levels in BL6 cells. Since cAMP functions as a negative regulator of the cell cycle (362), elevated levels of cAMP may inhibit BL6 cell proliferation through cell cycle inhibition.

In conclusion, it is clear that vitamin E succinate has a profound effect on malignant cell growth in *in vitro* cultured cells and that this effect can be explained by the interaction of the vitamin ester with a number of biochemical pathways. The extension of these findings to chemoprevention of cancer will however require further investigation.

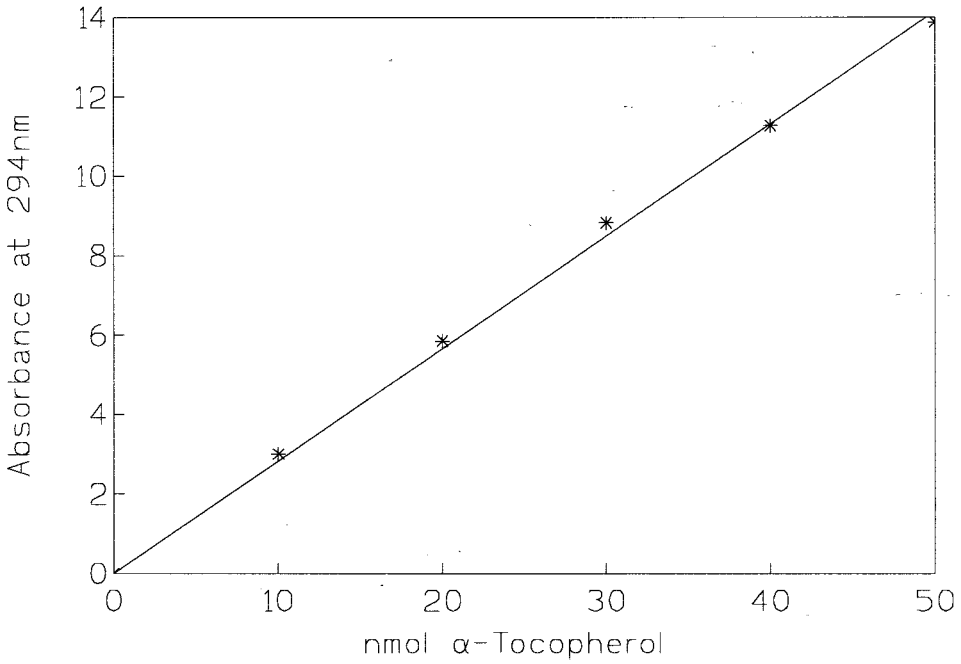
APPENDICES



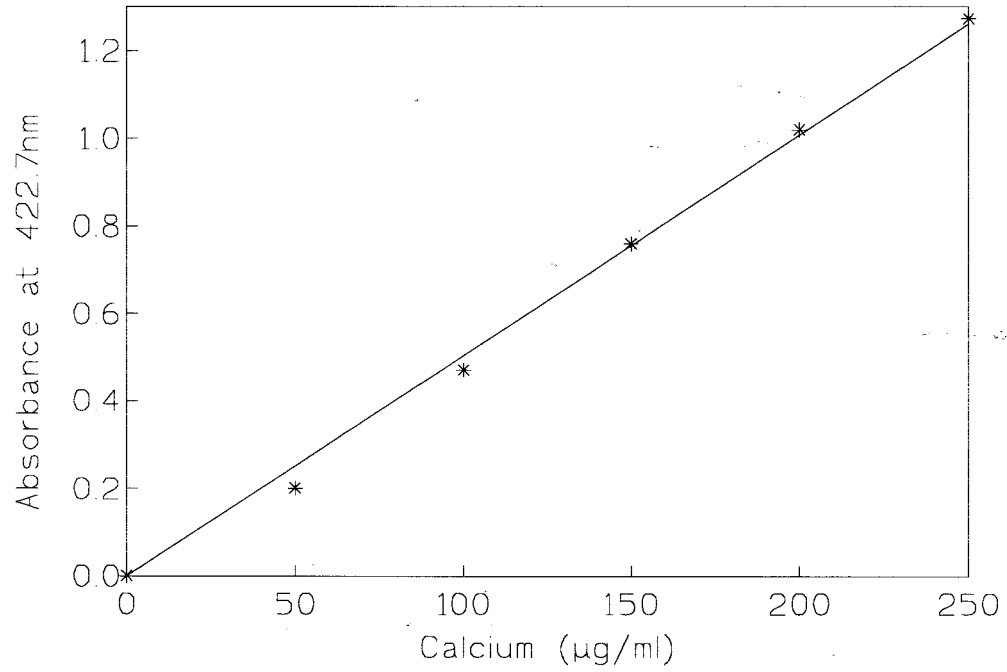
APPENDIX 1: Diformazan standard curve



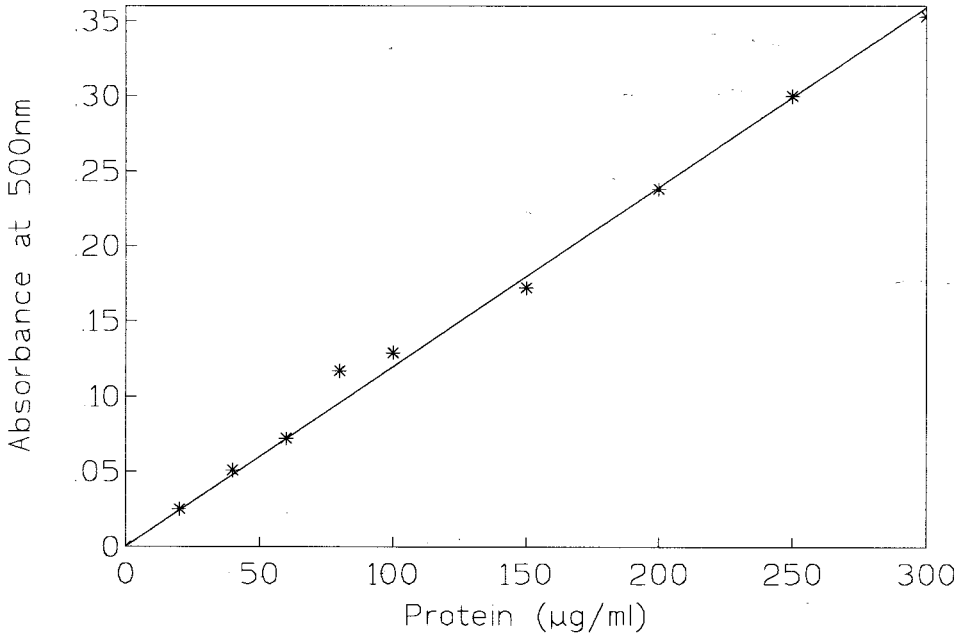
APPENDIX 2: Malondialdehyde standard curve



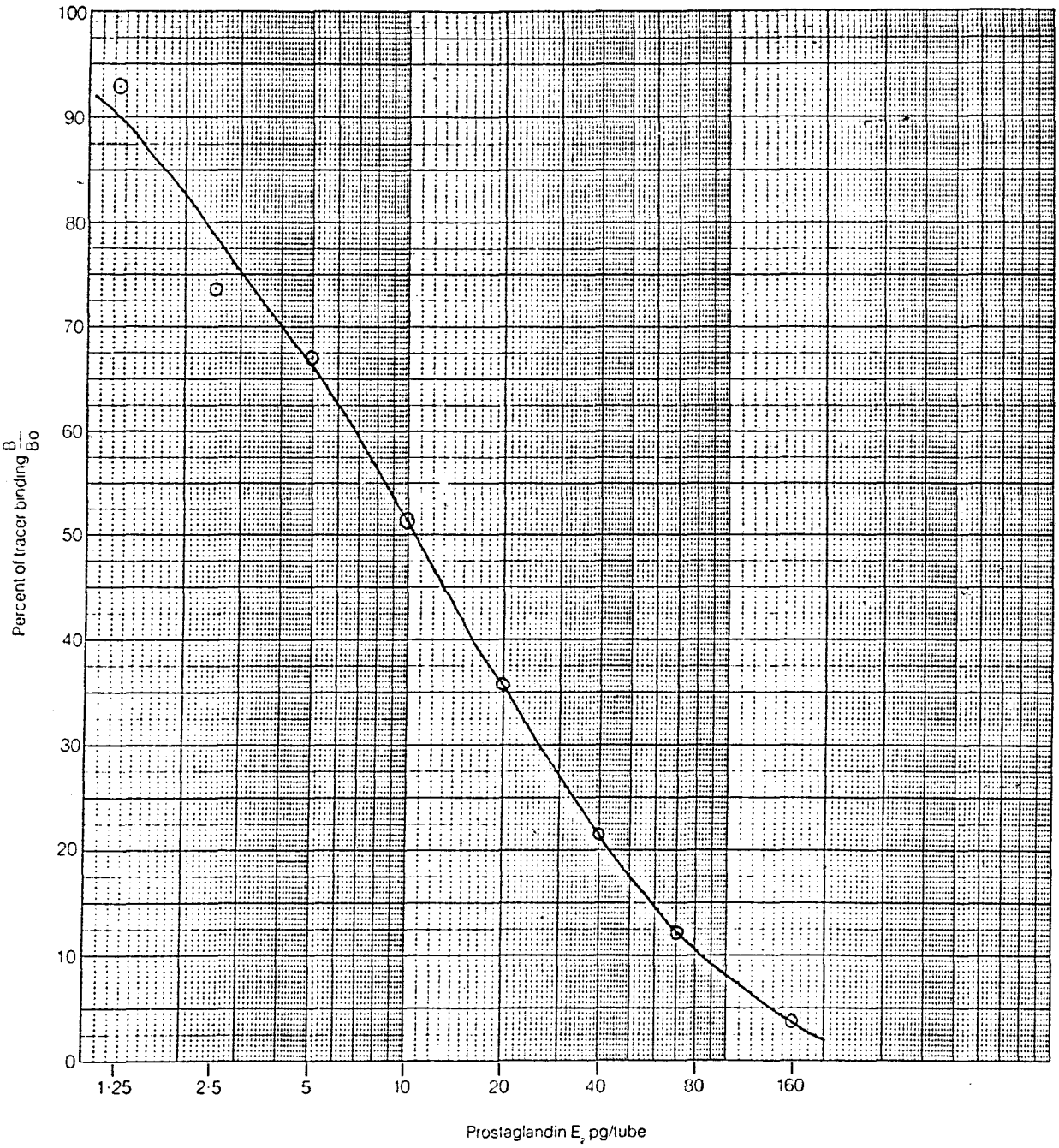
APPENDIX 3: α-Tocopherol standard curve



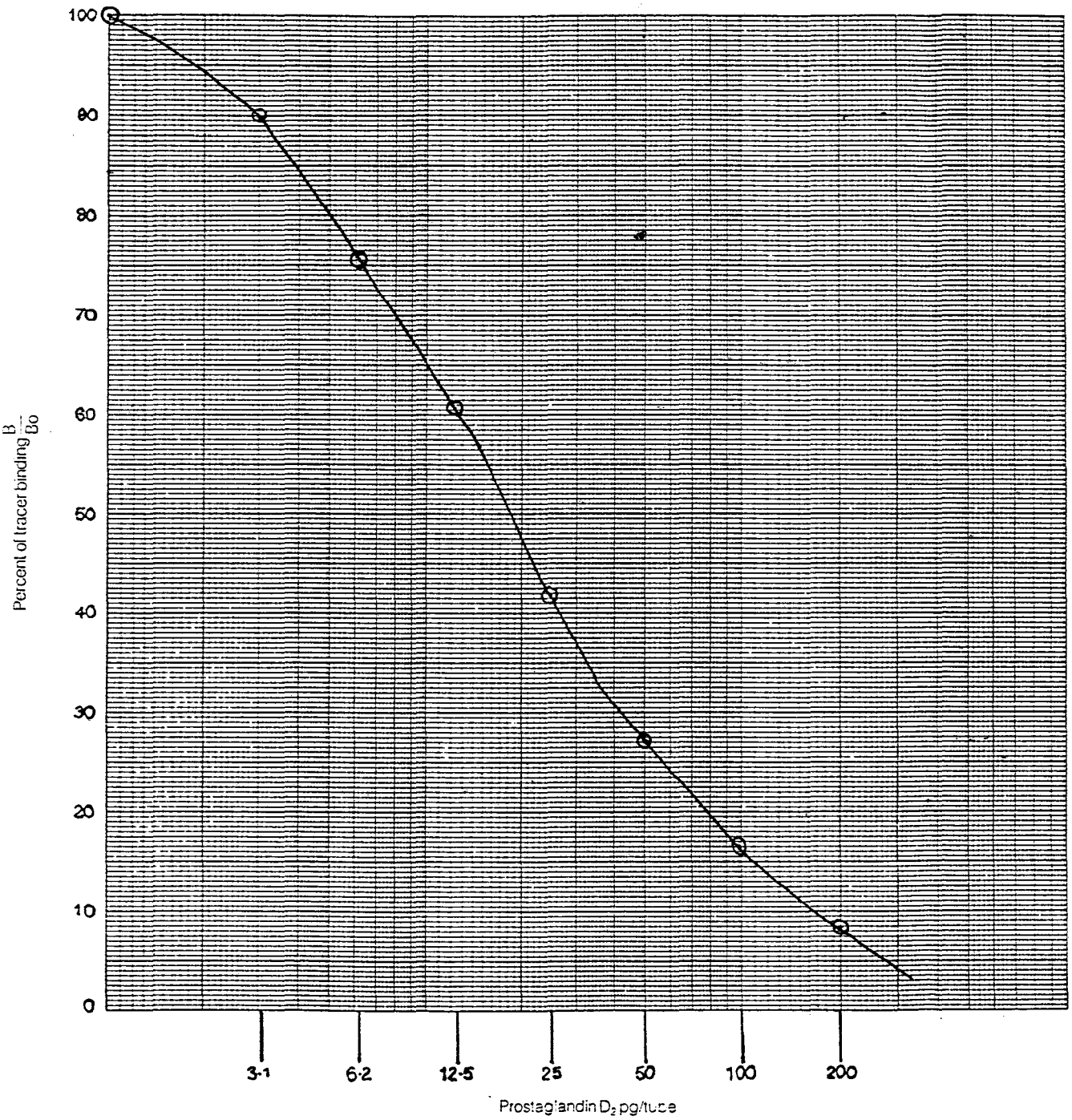
APPENDIX 4: Calcium standard curve



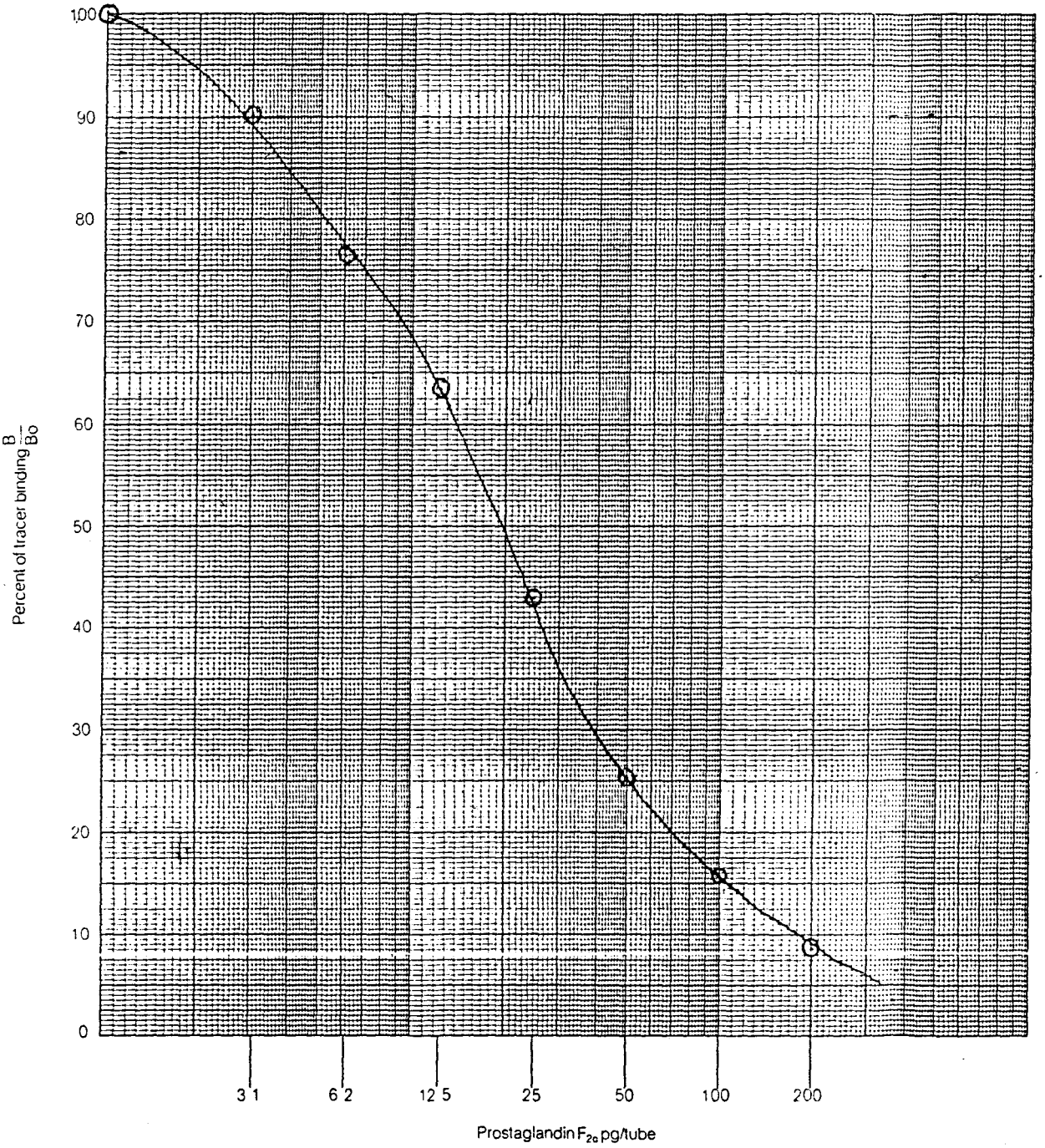
APPENDIX 5: Protein standard curve



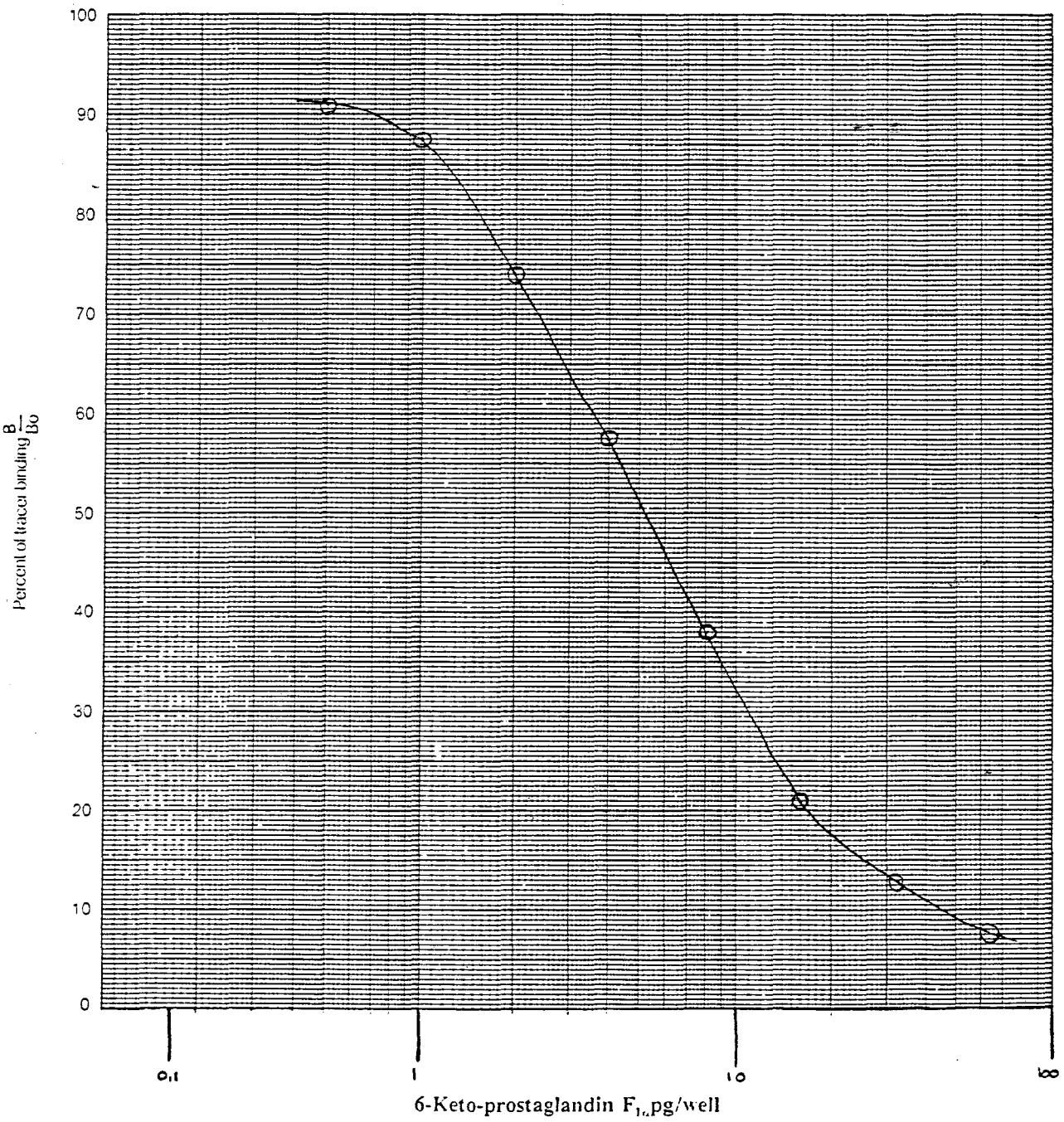
APPENDIX 6: Prostaglandin E_2 standard curve



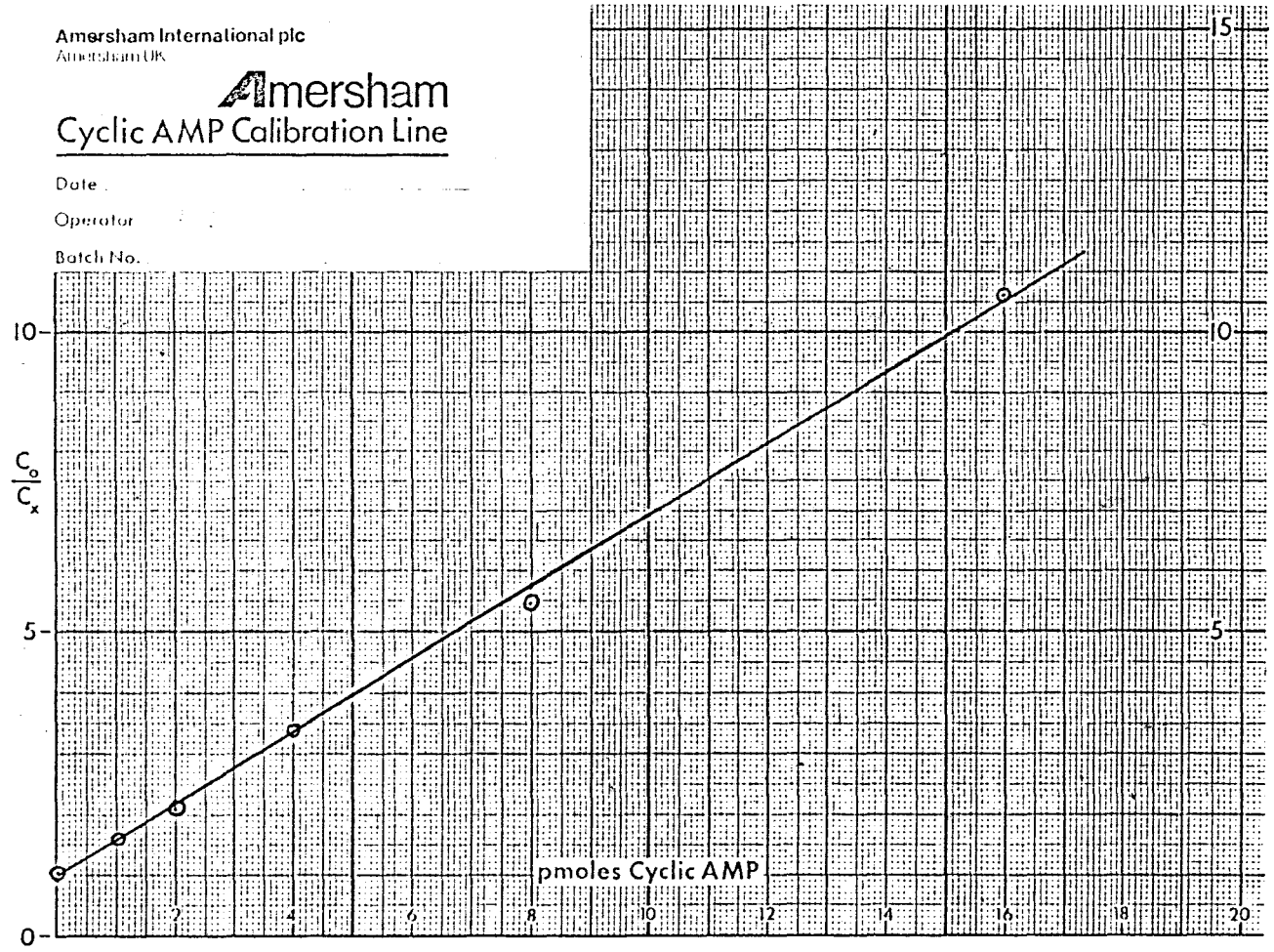
APPENDIX 7: Prostaglandin D_2 standard curve



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



APPENDIX 9: Prostaglandin I_2 standard curve



APPENDIX 10: Cyclic adenosine monophosphate standard curve

References

1. Pitot HC and Dragan YP. Facts and theories concerning the mechanisms of carcinogenesis. FASEB J. **5**: 2280-2286, 1991
2. Cairns J. The cancer problem. Nature **255**: 197-200, 1975.
3. Brock TD and Madigan MT. Biology of Microorganisms. Prentice Hall, New Jersey 1991.
4. Hennekens CH. Micronutrients and cancer prevention. New Eng. J. Med. **315**: 1288-1289, 1986.
5. Witz G. Active oxygen species as factors in multistage carcinogenesis $_{(43306)}$. Proc. Soc. Exp. Biol. Med. **198**: 675-682, 1991.
6. Sporn MB and Harris ED. Proliferative diseases. Am. J. Med. **70**: 1231-1236, 1981.
7. Troll W and Wiesner R. The role of oxygen radicals as possible mechanism of tumour promotion. Ann. Rev. Pharmacol. Toxicol. **25**: 509-528, 1985.
8. Sun Y. Free radicals, antioxidant enzymes, and carcinogenesis. Free Radical Biol. Med. **8**: 583-599, 1990.
9. Kensler TW and Taffe BG. Free radicals in tumor promotion. Adv. Free Radical Biol. Med. **2**: 347-387, 1986.
10. Slaga TJ. Multistage skin carcinogenesis and specificity of inhibitors. p 10-23 in Modulation and Mediation of Cancer by Vitamins. (FL Meyskens, KN Prasad eds) Krager Basel, London, 1983.
11. Bertram JS, Kolonel LN and Meyskens FL. Rationale and strategies for the chemoprevention of cancer in humans. Cancer Res. **47**: 3012-3031, 1987.
12. Flavin DF and Kolbye AC Jr. Nutritional factors with the potential to inhibit critical pathways of tumor promotion. p24-38 in Modulation and Mediation of Cancer by Vitamins. (FL Meyskens, KN Prasad eds) Krager Basel, London, 1983.
13. Weinberg RA. Finding the anti-oncogene. Sci. Amer. **259**: 34-41, 1988.
14. Travali S, Koniecki J, Petralia S and Baserga R. Oncogenes in growth and development. FASEB J. **4**: 3209-3214, 1990.
15. Hunter T. Oncogenes and growth control. TIBS **10**: 275-280, 1985.
16. Hellström KE and Hellström I. Oncogene-associated tumor antigens as targets for immunotherapy. FASEB J. **3**: 1715-1722, 1989.
17. Roos E and Dingemans KP. Mechanisms of metastasis. Biochim. Biophys. Acta **560**: 135-166, 1979.

18. Sugarbaker EV and Ketcham AS. Mechanisms and prevention of cancer dissemination: an overview. Semin. Oncol. **4**: 19-32, 1977.

19. Liotta LA. Cancer cell invasion and metastasis. Sci. Amer. **266**: 34-41, 1992.

20. Lippman SM, Benner SE and Ki Hong W. Chemoprevention. Cancer **72**: 984-990, 1993.

21. Meyskens FL. Coming of age-The chemoprevention of cancer. New Eng. J. Med. **323**: 825-827: 1990.

22. Stavric B. Role of chemopreventers in human diet. Clin. Biochem. **27**: 319-332, 1994.

23. Huber MH, Lee JS and Hong WK. Chemoprevention of lung cancer. Semin. Oncol. **20**: 128-141, 1993.

24. Knekt P. Role of vitamin E in the prophylaxis of cancer. Ann. Med. **23**: 3-12, 1991.

25. Gould MN, Haag JD, Kennan WS, Tanner MA and Elson CE. A comparison of tocopherol and tocotrienol for the chemoprevention of chemically induced rat mammary tumors. Amer. J. Clin. Nutr. **53**: 1068s-1070s, 1991.

26. Scott ML. Vitamin E. p 133-210 in Handbook of Lipid Research; The fat soluble vitamins. (HF Deluca ed) Plenum Press, New York, 1987.

27. Burton GW. Vitamin E: molecular and biological function. Proc. Nutr. Soc. **53**: 251-262, 1994.

28. Pehr K and Forsey R. Why don't we use vitamin E in dermatology? Can. Med. Ass. J. **149**: 1247-1253, 1993.

29. Kasparek S. Chemistry of tocopherols and tocotrienols. p7-53 in Vitamin E: A Comprehensive Treatise. (LJ Machlin ed) Marcel Dekker Inc., New York, 1980.

30. Mason KE. The first two decades of vitamin E. Fed. Proc. **36**: 1906-1910, 1977.

31. Osaki FA. Metabolism and physiologic roles of vitamin E. Hosp. Practice **12**: 79-85, 1977.

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

33. Behrens WA and Madere R. Mechanism of absorption, transport and tissue uptake of RRR-α-Tocopherol and d-γ-Tocopherol in the white rat. J. Nutr. **117**: 1562-1569, 1987.

34. Drevon CA. Absorption, transport and metabolism of vitamin E. Free Radical Res. Commun. **14**: 229-246, 1991.

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

36. Liebler DC. The role of metabolism in the antioxidant function of vitamin E. CRC Crit. Rev. Toxicol. **23**, 147-149, 1993.

37. Fritsma GA. Vitamin E and autoxidation. Am. J. Med. Technol. **49**: 453-456, 1983.

38. Bendich A and Machlin LJ. Safety of oral intake of vitamin E. Am. J. Clin. Nutr. **48**: 612-619, 1988.

39. Burton GW and Ingold KU. Vitamin E as an *in vitro* and *in vivo* antioxidant. Ann. New York Acad. Sci. **570**: 7-22, 1989.

40. Cheeseman KH, Holley AE, Kelly FL, Wasil M, Hughes L and Burton G. Biokinetics in humans of RRR-α-Tocopherol: The free phenol, acetate ester, and succinate ester forms of vitamin E. Free Radical. Biol. Med. **19**: 591-598, 1995.

41. Bieri JG, Corash L and Hubbard Van S. Medical uses of vitamin E. New Eng. J. Med. **308**: 1063-1070, 1983.

42. Prasad KN and Rama BN. Modification of the effect of pharmacological agents on tumor cells in culture by vitamin C and vitamin E. p244-257 in Modulation and Mediation of Cancer by Vitamins. (Meyskens FL and Prasad KN eds) Krager Basel, Switzerland, 1983.

43 Windholz M. The Merck index. Merck and Co., 1983.

44. Bauernfeind J. Tocopherols in foods. p100-160 in Vitamin E: A Coprehensive Treatise. (LJ Machlin ed) Marcel Dekker, New York, 1980.

45. Hoffman FA. Micronutrient requirements of cancer patients. Cancer **55**: 295-300, 1985.

46. Watson RR and Leonard TK. Selenium and vitamins A, E, and C: Nutrients with cancer prevention properties. J. Am. Diet. Ass. **86**: 505-510, 1986.

47. Horwitt MK. Therapeutic uses of vitamin E in medicine. Nutr. Rev. **38**: 105-113, 1980.

48. Gallo-Torres HE. Absorption. p170-192 in Vitamin E: A Coprehensive Treatise (LJ Machlin ed) Marcel Dekker, New York, 1980.

49. Farrell PM. Deficiency states, pharmacological effects, and nutrient requirements. p520-604 in Vitamin E: A Comprehensive Treatise (LJ Machlin ed) Marcel Dekker, New York, 1980.

50. Willet WC and MacMahon B. Diet and cancer-an overview. New Eng. J. Med. **310**: 633-638, 1984.

51. Bertino JR. Nutrients, vitamins and minerals as therapy. Cancer **43**: 2137-2142, 1979.

52. Menkes MS, Comstock GW, Vuilleumier JP, Helsing KJ, Rider AA and Brookmeyer R. Serum beta-carotene, vitamins A and E, selenium, and the risk of lung cancer. New Eng. J. Med. **315**: 1250-1254, 1986.

53. Gey KF, Brubacher GB and Stähelin HB. Plasma levels of antioxidant vitamins in relation to ischemic heart disease and cancer. Am. J. Clin. Nutr. **45**: 1368-1377, 1987.

54. Knekt P. Serum vitamin E level and risk of female cancers. Int. J. Epidemiol. **17**: 281-288, 1988.

55. Stähelin HB, Rösel F, Buess E and Brubacher G. Cancer, vitamins, and plasma lipids: Prospective basel study. J. Natl. Cancer Inst. **73**: 1463-1468, 1984.

56. Garewal HS and Schantz S. Emerging role of β-Carotene and antioxidant nutrients in prevention of oral cancer. Arch. Otolaryngol Head Neck Surg. **121**: 141-144, 1995.

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

58. Odeleye OE, Eskelson CD, Mufti SI and Watson RR. Vitamin E protection against nitrosamine-induced oesophageal tumor incidence in mice immunocompromised by retroviral infection. Carcinogenesis **13**: 1811-1816, 1992.

59. Palamanda JR and Kehrer JP. Involvement of vitamin E and protein thiols in the inhibition of microsomal lipid peroxidation by glutathione. Lipids **28**: 427-431, 1993.

60. Packer L. Protective role of vitamin E in biological systems. Am. J. Clin. Nutr. **53**: 1050s-1055s, 1991.

61. Aruoma OI. Nutrition and health aspects of free radicals and antioxidants. Fd. Chem. Toxic. **32**: 671-683, 1994.

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

63. Thurnham DI. Anti-oxidant vitamins and cancer prevention. J. Micronutr. Anal. **7**: 279-299, 1990.

64. Maxwell SRJ. Prospects for the use of antioxidant therapies. Drugs **49**: 345-361, 1995.

65. Cheesman KH and Slater TF. An introduction to free radical biochemistry. Br. Med. Bull. **49**: 481-493, 1993.

66. Borek C. The role of free radicals and antioxidants in cellular and molecular carcinogenesis *in vitro*. p1461-1469 in Medical, Biochemical and Chemical Aspects of Free Radicals. (O Hayaishi, E Niki, M Kondo and T Yoshikawa eds) Elsevier Science Publishers, Amsterdam, 1989.

67. Lambelet P, Saucy F and Löliger J. Chemical evidence for interactions between vitamins E and C. Experientia **41**: 1384-1388, 1985.

68. Palozza P and Krinsky NI. The inhibition of radical-initiated peroxidation of microsomal lipids by both α-Tocopoherol and β-carotene. Free Radical Biol. Med. **11**: 407-414, 1991.

69. Di Macsio P, Murphy ME and Sies H. Antioxidant defence systems: the role of carotenoids, tocopherols, and thiols. Am. J. Clin. Nutr. **53**: 194s-200s, 1991.

70. Goldring CEP, Rice-Evans CA, Burdon RH, Rao R, Haq I and Diplock RH. α-Tocopherol uptake and its influence on cell proliferation and lipid peroxidation in transfromed and nontransfromed baby hamster kidney cells. Arch. Biochem. Biophys. 303: 429-435, 1993.

71. Balasubramaniyan N, Subramanian S and Govindasamy S. Status of antioxidant systems in human carcinoma of uterine cervix. Cancer Lett. **87**: 187-192, 1994.

72. Krinsky NI. Mechanism of action of biological antioxidants. Proc. Soc. Exp. Biol. Med. **200**: 248-254, 1992.

73. Borek C. Vitamin E as an anticarcinogen. Ann. New York Acad. Sci. **570**: 417-420, 1990.

74. Bowry VW, Ingold KU. Extraordinary kinetic behaviour of the α-Tocopheroxyl (vitamin E) radical. J. Org. Chem. **60**: 5456-5467, 1995.

75. Buechter DD. Free radicals and oxygen toxicity. Pharm. Res. **5**: 253-260, 1988.

76. Anderson R and Theron AJ. Physiological potential of ascorbate, β-carotene and α-Tocopherol individually and in combination in the prevention of tissue damage, carcinogenesis and immune dysfunction mediated by phagocyte-derived reactive oxidants. World Rev. Nutr. Diet **62**: 27-58, 1990.

77. Murphy ME, Scholich H, Wefers H and Sies H. Alpha-Tocopherol in microsomal lipid peroxidation. Ann. New York Acad. Sci. **570**: 480-486, 1989.

78. McCay PB. VITAMIN E: Interactions with free radicals and ascorbate. Annu. Rev. Nutr. **5**: 323-340, 1985.

79. Serbinova EA, Packer L. Antioxidant properties of α-Tochopherol and α-Tocotrienol. p354-366 in Methods in Enzymology. (Packer L ed) Academic Press, New York. **234**, 1994.

80. Wefers H and Sies H. The protection by ascorbate and glutathione against microsomal lipid peroxidation is dependant on vitamin E. Eur. J. Bioch. **174**: 353-357, 1988.

81. Kaiser S, Di Mascio P, Murphy ME and Sies H. Physical and chemical scavenging of singlet molecular oxygen by tocopherols. Arch. Biochem. Biophys. **277**: 101-108, 1990.

82. Burton GW and Ingold KU. Autoxidation of biological molecules. 1. The antioxidant activity of vitamin E and related chain-breaking phenolic antioxidants in vitro. J. Amer. Chem. Soc. **103**: 6472-6477, 1981.

83. Burton GW, Joyce A and Ingold KU. Is vitamin E the only lipid soluble, chain breaking antioxidant in human blood plasma and erythrocyte membranes. Arch. Biochem. Biophys. **221**: 281-290, 1983.

84. Burton GW, Le Page Y, Gabe EJ and Ingold KU. Antioxidant activity of vitamin E and related phenols. Importance of stereoelectronic factors. J. Amer. Chem. Soc. **102**: 7791-7792, 1980.

85. Niki E, Kawakami A, Saito M, Yamamoto Y, Tsuchiya J and Kamiya Y. Effect of phytyl side chain of vitamin E on its antioxidant activity. J. Biol. Chem. **260**: 2191-2196, 1985.

86. Diplock AT and Lucy JA. The biochemical modes of action of vitamin E and selenium a hypothesis. FEBS Lett. **29**: 205-210, 1973.

87. Horvath PM and Clement Ip. Synergistic effect of vitamin E and selenium in the chemoprevention of mammary carcinogenesis in rats. Cancer Res. **43**: 5335-5341, 1983.

88. Tappel AL. Vitamin E and selenium protection from *in vivo* lipid peroxidation. Ann. New York Acad. Sci. **355**: 18-29, 1980.

89. Locker GY, Doroshow JH, Baldinger JC and Myers CE. The relationship between dietary tocopherol and glutathione peroxidase acitvity in murine cardiac tissue. Nutr. Rep. Int. **19**: 671-678, 1979.

90. Buettner GR. The pecking order of free radicals and antioxidants: lipid peroxidation, α-Tocopherol, and ascorbate. Arch. Biochem. Biophys. **300**: 535-543, 1993.

91. Niki E, Kawakami A, Yamamoto Y, Kamiya K. Oxidation of lipids. VIII. Synergistic inhibition of oxidation of phosphatidylcholine liposomes in aqueous dispersion by vitamin E and vitamin C. Bull. Chem. Soc. Jap. **58**: 1971-1975, 1985.

92. Rifici VA and Khachadurian AK. Dietary supplementation with vitamins C and E inhibits *in vitro* oxidation of lipoproteins. J. Am. College Nutr. **12**: 631-637, 1993.

93. Scarpa M, Rigo A, Maiorano M, Ursini F and Gregolin C. Formation of α-Tocopherol by ascorbate during peroxidation of phosphatidylcholine liposomes. Biochim. Biophys. Acta **801**: 215-219, 1984.

94. Doba T, Burton GW and Ingold KU. Antioxidant and co-oxidant 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, 1985.

95. Chan AC. Partners in defense, vitamin E and vitamin C. Can. J. Physiol. Pharm. **71**: 725-731, 1993.

96. Niki E, Saito T, Kawakami A and Kamiya Y. Inhibition of oxidation of methyl linoleate in solution by vitamin E and vitamin C. J. Biol. Chem. **259**: 4177-4182, 1984.

97. Palozza P and Krinsky NI. β-Carotene and α-Tocopherol are synergistic antioxidants. Arch. Biochem. Biophys. **297**: 184-187, 1992.

98. Molennaar I, Hulstaert CE and Hardonk MJ. Role in function and ultrastructure of cellular membranes. p372-389 in Vitamin E: A Coprehensive Treatise. (LJ Machlin ed) Marcel Dekker, New York, 1980.

99. Packer L and Landvik S. Vitamin E: Introduction to biochemistry and health benefits. Ann. New York Acad. Sci. **570**: 1-6, 1989.

100. Urano S, Yano K and Matsuo M. Membrane-stabilizing effect of vitamin E: effect of α-Tocopherol and its model compounds on fluidity of lecithin liposomes. Biochem. Biophys. Res. Comms. **150**: 469-475, 1988.

101. Linder MC. Nutritional biochemistry and metabolism-With clinical applications. p94-99 Elsevier Science Publishing Co., New York, 1985.

102. Greenwald P and Schreiber G. Perspectives on chemoprevention in man. p40-46 in Modulation and Mediation of Cancer by Vitamins. (FL Meyskens and KN Prasad eds) Krager Basel, London, 1983.

103. Bright-See E and Newmark HL. Potential and probable role of vitamin C and E in the prevention of carcinogenesis. p95-103 in Modulation and Mediation of Cancer by Vitamins. (FL Meyskens and KN Prasad eds) Krager Basel, London, 1983.

104. Birt DF. Update on the effects of vitamins A, C, and E and Selenium on carcinogenesis (42424). Proc. Soc. Exp. Biol. Med. **183**: 311-320, 1986.

105. Byers T and Perry G. Dietary carotenes, vitamin C, and vitamin E as protective antioxidants in human cancers. Annu. Rev. Nutr. **12**: 139-159, 1992.

106. Meydani SN, Meydani M, Verdon CP, Shapiro AA, Blumberg JB and Hayes KC. Vitamin E supplementation suppresses prostaglandin E^1_2 synthesis and enhances the immune response of aged mice. Mech. Ageing Dev. **34**: 191-201, 1986.

107. Schmidt K. Antioxidant vitamins and β-carotene: effects on immunocompetance[1,2]. Amer. J. Clin. Nutr. **53**: 383s-385s, 1991.

108. Tengerdy RP. Effect of vitamin E on immune responses. p429-443 in Vitamin E: A Comprehensive Treatise. (LJ Machlin ed) Marcel Deker, New York, 1980.

109. Eskelson CD. Odeleye OE, Watson RR, Earnest DL and Mufti SI. Modulation of cancer growth by vitamin E and alcohol. Alcoh. Alcoh. **28**: 117-125,1993.

110. El Attar TMA, and Lin HS. Inhibition of human oral squamous carcinoma cell (SCC-25) proliferation by prostaglandin E_2 and vitamin E succinate. J. Oral Pathol. Med. **22**: 425-427, 1993.

111. Sakamoto W. Fujie K, Nishihira J and Handa H. Effect of vitamin E on arachidonic acid-release in rat peritoneal macrophages. Biochim. Biophys. Acta **1170**: 296-300, 1993.

112. Schwartz J, Shklar G and Trickler D. p53 in the anticancer mechanism of vitamin E. Oral Oncol, Eur. J. Cancer **29B**: 313-318, 1993.

113. Douglas K. Making friends with death-wish genes. New Sci. **143**:30-34, 1994.

114. Cullota E and Koshland DE. p53 sweeps through cancer research. SCI. **262**: 1958-1961, 1993.

115. Friend S. p53: A glimpse at the puppet behind the shadow play. SCI. **265**: 334-335, 1994.

116. Yamada Y, Yoshida T, Hayasmhi K, Sekiya T, Yokota J, Hirohashi S, Nakatani K, Nakano H, Sugimura T and Terada M. p53 gene mutations in gastric cancer metastases and in gastric cancer cell lines derived from metastases. Cancer Res. **51**: 5800-5805, 1991.

117. Shklar G. Oral mucosal carcinogenesis in hamsters: Inhibition by vitamin E. J. Natl. Cancer Inst. **68**: 791-797, 1982.

118. Slaga TJ and Bracken WM. The effects of antioxidants on skin tumor initiation and aryl hydrocarbon hydroxylase. Cancer Res. **37**: 1631-1635, 1977.

119. Diplock AT. Antioxidant nutrients and disease prevention: an overview. Amer. J. Clin. Nutr. **53**: 189s-193s, 1991.

120. Knekt P, Aromaa A, Maatela J, Aaran R-V, Nikkari T, Hakama M, Hakulinen T, Peto R and Teppo L. Vitamin E and cancer prevention. Amer. J. Clin. Nutr. **53**: 283s-286s, 1991.

121. Gey KF. Prospects for the prevention of free radical disease, regarding cancer and cardiovascular disease. Br. Med. Bull. **49**: 679-699, 1993.

122. Cook MG and McNamara P. Effect of dietary vitamin E on dimethylhydrazine-induced colonic tumours in mice. Cancer Res. **40**: 1329-1331, 1980.

123. Elattar TMA and Lin HS. Vitamin E succinate potentiates the inhibitory effect of prostaglandins on oral squamous carcinoma cell proliferation. Prostaglandins Leukot. EFAs. **52**: 69-73, 1995.

124. Torelli S, Masoudi F and Prasad KN. Effect of alpha Tocopheryl acid succinate on cAMP dependant protein kinase activity in murine B-16 melanoma cells in culture. Cancer Lett. **39**: 129-136, 1988.

125. Schwartz J and Shklar G. The selective cytotoxic effect of carotenoids and α-Tocopherol on human cancer cell lines *in vitro*. J. Oral Maxillofac Surg. **50**: 367-373, 1992.

126. Kline K, Cochran GS and Sanders BG. Growth-inhibitory effects of vitamin E succinate on retrovirus-transformed tumour cells *in vitro*. Nutr. Cancer **14**: 27-41, 1990.

127. Rama BN, Prasad KN. 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, 1983.

128. Prasad KN, Edwards-Prasad J, Ramanujam S and Sakamoto A. Vitamin E increases the growth inhibitory and differentiating effects of tumour therapeutic agents on neuroblastoma and glioma cells in culture (40840). Proc. Soc. Exp. Biol. Med. **164**: 158-163, 1980.

129. Garland M, Willet WC, Manson JE and Hunter DJ. Antioxidant micronutrients and breast cancer. J. Am. Coll. Nutr. **12**: 400-411, 1993.

130. Sakamoto A, Chougule PB and Prasad KN. Retrospective analysis of the effect of vitamin A, C, and E in human neoplasms. p330-333 in Modulation and Mediation of Cancer by Vitamins. (FL Meyskens and KN Prasad eds) Krager Basel, London, 1983.

131. Bierke P and Svedenstål B-M. The influence of selenium, vitamin E, and oestrogen on the development of tumours in mice exposed to ^{90}Sr. Acta Ocol. **33**: 963-967, 1994.

132. Dean RT, Gieseg S and Davies MJ. Reactive species and their accumulation on radical-damaged proteins. TIBS **18**: 437-441, 1993.

133. McCay PB and Margret King M. Vitamin E: Its role as a biological free radical scavenger and its relationship to the microsomal mixed-function oxidase system. p289-317 in Vitamin E: A Comprehensive Treatise. (LJ Machlin ed) Marcel Dekker, New York, 1980.

134. Porter NA, Caldwell SE and Mills KA. Mechanisms of free radical oxidation of unsaturated lipids. Lipids **30**: 277-290, 1995.

135. Slater TF. Free radical studies in relation to cancer and liver regeneration. South Afr. J. Sci. **87**: 588-590, 1991.

136. Thompson HJ. Effect of deficiencies of selenium and vitamin E alone or in combination on the induction of mammary carcinogenesis by 1-methyl-1-nitrosourea. Carcinogenesis **12**: 2175-2179, 1991.

137. Salim AS. Removing oxygen-derived free radicals delays hepatic metastases and prolongs survival in colonic cancer. Oncology **49**: 58-62, 1992.

138. Zhang J-R and Sevanian A. The genotoxic effects of arachidonic acid in V79 cells are mediated by peroxidation products. Tox. Appl. Ph. **121**: 193-202, 1993.

139. Hendrickse CW, Kelly RW, Radley S, Donovan IA, Keighley MRB and Neoptolemos JP. Lipid peroxidation and prostaglandins in colorectal cancer. Br. J. Surg. **81**: 1219-1223, 1994.

140. Wang M-Y and Liehr JG. Induction by estrogens 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, 1995.

141. Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson JD. Membrane structure. p477-487 in Molecular Biology of the Cell. Garland Publishing, New York, 1994.

142. Mathews CK and Van Holde KE. Lipids, membranes and cellular transport. p298-332 in Biochemistry. Benjamin Cummings Publishing Company Inc., New York, 1990.

143. Singer SJ and Nicolson GL. The fluid mosaic model of the structure of cell membranes. SCI. **175**: 720-731, 1972.

144. Needlands PJ and Clandinin MT. Diet fat influences liver plasma-membrane lipid composition and glucagon-stimulated adenylate cyclase activity. Biochem. J. **212**: 573-583, 1983.

145. Houslay MD and Gordon LM. The activity of adenylate cyclase is regulated by the nature of its lipid environment. p179-231 in Current Topics in Membranes and Transport. Academic Press, New York, 1983.

146. Spector AA and Yorek MA. Membrane lipid composition and cellular function. J. Lipid Res. **26**: 1015-1035, 1985.

147. Ahmad SN, Alam SQ, Alam BS. Fatty acid incorporation into membranes of dispersed rat submandibular salivary gland cells and their effect on adenylate cyclase activity. Archs. Oral Biol. **35**: 879-883, 1990.

148. Murphy MG. Dietary fatty acids and membrane protein function. J. Nutr. Biochem. **1**: 68-79, 1990.

149. Alam SQ and Alam BS. 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, 1986.

150. Kaplán P, Račay P, Lehotský J and Mézešová V. Change in fluidity of brain endoplasmic reticulum membranes by oxygen free radicals: a protective effect of stobadine, α-tocopherol acetate, and butylated hydroxytoluene. Neurochem. Res. **20**: 815-820, 1995.

151. Cutris MT, Gilfor D and Farber JL. Lipid peroxidation increases the molecular order of microsomal membranes. Arch. Biochem. Biophys. **235**: 644-649, 1984.

152. Dinis TCP, Almeida LM and Madeira VMC. Lipid peroxidation in sarcoplasmic reticulum membranes: Effect on functional and biophysical properties. Arch. Biochem. Biophys. **301**: 256-264, 1993.

153. Vajreswari A and Narayanareddy K. 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, 1992.

154. Alam SQ, Alam BS and Ren Y-F. Adenylate cyclase activity, membrane fluidity and fatty acid composition of rat heart in essential fatty acid deficiency. J. Mol. Cell Cardiol. **19**: 465-475, 1987.

155. Farías RN, Bloj B, Morero RD, Siñeriz F and Trucco RE. Regulation of allosteric membrane-bound enzymes through changes in membrane lipid composition. Biochim. Biophys. Acta **415**: 231-251, 1975.

156. Dobretsov GE, Borshevskaya TA, Petrov VA and Vladimirov Y-A. The increase of phospholipid bilayer rigidity after lipid peroxidation. FEBS Lett. **84**: 125-128, 1977.

157. Housley MD. Regulation of adenylate cyclase (EC 4.6.1.1) activity by its lipid environment. Proc. Nutr. Soc. **44**: 157-165, 1985.

158. Sandermann H. Regulation of membrane enzymes by lipids. Biochem. Biophys. Acta **515**: 209-237, 1978.

159. Galeotti T, Borello S, Minotti G, and Masotti L. Membrane alterations in cancer cells: The role of oxy radicals. Ann. New York Acad. Sci. **488**: 468-480, 1986.

160. Inbar M, Shinitzky M and Sachs L. Rotational relaxation time of concanavalin A bound to the surface membrane of normal and malignant transformed cells. J. Mol. Biol. **81:** 245-253, 1973.

161. Galeotti T, Borrello S, Palombini G, Masotti L, Ferrari MB, Cavatorta P, Arcioni A, Stremmenos C and Zannoni C. Lipid peroxidation and fluidity of plasma membranes from rat liver and morris hepatoma 3924A. FEBS **169**: 169-173, 1984.

162. Coman DR and Anderson TF. A structural difference between the surface of normal and of carcinomatous epithelial cells. Cancer Res. **15**: 541-543, 1955.

163. Sawada M, Sester U and Carlson JC. 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, 1992.

164. Choi J-H and Yu BP. Brain synaptosomal aging; free radicals and membrane fluidity. Free Radical Biol. Med. **18**: 133-139, 1995.

165. Smith WL and Marnett LJ. Prostaglandin endoperoxide synthase: structure and catalysis. Biochim Biophys. Acta **1083**: 1-17, 1991.

166. DeWitt DL. Prostaglandin endoperoxide synthase: regulation of enzyme expression. Biochim. Biophys. Acta **1083**: 121-134, 1991.

167. Marnett LJ. Polycyclic aromatic hydrocarbon oxidation during prostaglandin biosynthesis. Life Sci. **29**: 531-546, 1981.

168. Lands WEM and Hanel AM. Inhibitors and activators of prostaglandin biosynthesis. p203-223 in Prostaglandins and related substances. (C Pace-Asciak and E Granström eds) Elsevier science Publishers Co., Amsterdam, 1983.

169. Yamamoto S. Enzymes in the arachidonic acid cascade. p171-202 in Prostaglandins and related substances. (C Pace-Asciak and E Granström eds) Elsevier science Publishers, Amsterdam, 1983.

170. Regier MK, DeWitt DL, Schindler MS and Smith WL. Subcellular localization of prostaglandin endoperoxide synthase-2 in murine 3T3 cells. Arch. Biochem. Biophys. **301**: 439-444, 1993.

171. Egan RW, Gale PH, Baptista EM, Kennicott KL, Vandenheuvel WJA, Walker RW, Fagerness PE and Kuehl FA. Oxidation reactions by prostaglandin cyclooxygenase-hydroperoxidase. J. Biol. Chem. **256**: 7352-7361, 1981.

172. Kulmacz RJ. Topography of prostaglandin H synthase. J. Biol. Chem. **264**: 14136-14144, 1989.

173. Sigal E. The molecular biology of mammalian arachidonic acid metabolism. Am. J. Physiol. **260**: L13-L28, 1991.

174. Otto JC, DeWitt DL and Smith WL. N-glycosylation of prostaglandin endoperoxide synthase-1 and -2 and their orientations in the endoplasmic reticulum. J. Biol. Chem. **268**: 18234-18242, 1993.

175. Kulmacz RJ, Pendleton RB and Lands WEM. Interaction between peroxidase and cyclooxygenase activities in prostaglandin-endoperoxide synthase. J. Biol. Chem. **269**: 5527-5536, 1994.

176. Needleman P, Turk J, Jaschik BA, Morrison AR and Lefkowith JB. Arachidonic acid metabolism. Ann. Rev. Biochem. **55**: 69-102, 1986.

177. Kukreja RC, Kontos HA, Hess ML and Ellis EF. PGH synthase and lipoxygenase generate superoxide in the presence of NADH or NADPH. Circ. Res. **59**: 612-619, 1986.

178. Ogino N, Ohki S, Yamamoto S and Hayaishi O. Prostaglandin endoperoxide sythetase from bovine vesicular gland microsomes. J. Biol. Chem. **253**: 5061-5068,1978.

179. Ohki S, Ogino N, Yamamoto S, and Hayaishi O. Prostaglandin hydroperoxidase, an integral part of prostaglandin endoperoxide sythetase from bovine vesicular gland microsomes. J. Biol. Chem. **254**: 829-836, 1979.

180. Van Dorp DA. Aspects of the biosynthesis of prostaglandins. Prog. Biochem. Pharmacol. **3**: 71-82, 1967.

181, Hemler ME and Lands WEM. Evidence for a peroxide-initiated free radical mechanism of prostaglandin biosynthesis. J. Biol. Chem. **255**: 6253-6261, 1980.

182. Hemler ME and Cook HW and Lands WEM. Prostaglandin biosynthesis can be triggered by lipid peroxides. Arch. Biochem. Biophys. **193**: 340-345, 1979.

183. Marshall PJ. Kulmacz RJ and Lands WEM. Constraints on prostaglandin biosynthesis in tissues. J. Biol. Chem. **262**: 3510-3517, 1987.

184. Egan RW, Gale PH, VandenHeuvel WJA, Baptista EM and Kuehl FA. Mechanism of oxygen transfer by prostaglandin hydroperoxidase. J. Biol. Chem. **255**: 323-326, 1980.

185. Markey CM, Alward A, Weller PE and Marnett LJ. Quantitative studies of hydroperoxide reduction by prostaglandin H synthase. J. Biol. Chem. **262**: 6266-6279, 1987.

186. Egan RW, Paxton J and Kuehl FA. Mechanism for irreversible self-deactivation of prostaglandin synthetase. J. Biol. Chem. **251**: 7329-7335, 1976.

187. Egan RW, Gale PH and Kuehl FA. Reduction of hydroperoxides in the prostaglandin biosynthetic pathway by a microsomal peroxidase. J. Biol. Chem. **254**: 3295-3302, 1979.

188. Nanayama T, Hara S, Inoue H, Yokoyama C and Tanabe T. Regulation of two isozymes of prostaglandin endoperoxide synthase and thromboxane synthase in human monoblastoid cell line U937. Prostagland **49**: 371-382, 1995.

189. Pilbeam CC, Raisz LG, Voznesensky O, Alander CB, Delman BN and Kawaguchi H. Autoregulation of inducible prostaglandin G/H synthase in osteoblastic cells by prostaglandins. J. Bone Mineral Res. **10**: 406-414, 1995.

190. Sano H, Kawahito Y, Wilder RL, Hashiramoto A, Mukai S, Asai K, Kimura S, Kato H, Kondo M and Hla T. Expression of cyclooxygenase-1 and -2 in human colorectal cancer. Cancer Res. **55**: 3785-3789, 1995.

191. Mitchell MD, Edwin SS, Lundin-Schiller S, Silver RM, Smotkin D and Trautman MS. Mechanism of interleukin-1β stimulation of human amnion prostaglandin biosynthesis: mediation via a novel inducible cyclooxygenase. Placenta **14**: 615-625, 1993.

192. Tordjman C, Coge F, Andre N, Rique H, Spedding M and Bonnet J. Characterisation of cyclooxygenase 1 and 2 expression in mouse resident peritoneal macrophages *in vitro*; interactions of non steroidal anti-inflammatory drugs with COX2. Biochim. Biophys. Acta 1256: 249-256, 1995.

193. Goppelt-Struebe M. Regulation of prostaglandin endoperoxide synthase (cyclooxygenase) Prostaglandin Leukot. EFAs **52**: 213-222, 1995.

194. Bennett PR, Slater D, Sullivan M, Elder MG and Moore GE. Changes in amniotic arachidonic acid metabolism associated with increased cyclooxygenase gene expression. Br. J. Obstet. Gynaecol. **100**: 1037-1042, 1993.

195. Rollins TE and Smith WL. Subcellular localization of prostaglandin-forming cyclooxygenase in swiss mouse 3T3 fibroblasts by electron microscopic immunocytochemistry. J. Biol. Chem. **255**: 4872-4875, 1980.

196. Smith WL, De Witt DL, Bimodal distribution of the prostaglandin I_2 synthase antigen in smooth muscle cells. J. Biol. Chem. 258: 5922-5926, 1983.

197. Dvořàk AM, Morgan EL, Tzizik DM and Weller PF. Prostaglandin endoperoxide synthase (cyclooxygenase): Ultrastructural localization to non-membrane-bound cytoplasmic lipid bodies in human eosinophils and 3T3 fibroblasts. Int. Arch. Allergy Immunol. **105**: 245-250, 1994.

198. Merlie JP, Fagan D, Mudd J and Needleman P. Isolation and characterisation of the complementary DNA for sheep seminal vesicle prostaglandin endoperoxide synthase (Cyclooxygenase). J. Biol. Chem. **263**: 3550-3553, 1988.

199. Feng L, Xia Y, Garcia GE, Hwang D and Wilson CB. Involvement of reactive oxygen intermediates in cyclooxygenase-2 expression induced by interleukin-1, tumor necrosis factor-α, and lipopolysaccharide. J. Clin. Invest. **95**: 1669-1675, 1995.

200. Ramwell PW, Foegh M, Loeb R and Leovey EMK. Synthesis and metabolism of prostaglandins, prostacyclin, and thromboxanes: The arachidonic acid cascade. Semin Perinatology **4**: 3-13, 1980.

201. Samuelsson B. Biosynthesis of prostaglandins. Fed. Proc. **31**: 1442-1450,1972.

202. Miyamoto T, Ogino N, Yamamoto S and Hayaishi O. Purification of prostaglandin endoperoxide synthetase from bovine vesicular gland microsomes. J. Biol. Chem. **251**: 2629-2636, 1976.

203. Pike JE. Prostaglandins. Sci. Amer. **225**: 84-92, 1971.

204. Oliw E. Granström E and Änggård E. The prostaglandins and essential fatty acids. p1-44 in Prostaglandins and related substances. (C Pace-Asciak and E Granström eds) Elsevier Science Publishers, Amsterdam, 1983.

205. Honn KV, Bockman RS and Marnett LJ. Prostaglandins and cancer: a review of tumor initiation through tumor metastasis. Prostagland. **21**: 833-864, 1981.

206. Turini ME, Basu TK and Clandinin MT. Prostaglandins-diet-cancer: A review. Nutr. Res. **10**: 819-827, 1990.

207. Karmali RA. Review: Prostaglandins and cancer. Prostaglandins and Med. **5**: 11-28, 1980.

208. Hansen HS. Dietary essential fatty acids and *in vivo* prostaglandin production in mammals. World. Rev. Nutr. Diet **42**: 102-134, 1983.

209. Levine L. Arachidonic acid transformation and tumour production. Adv. Cancer Res. **35**: 49-79, 1981.

210. Smith C, Otto AM and Jimenez de Asua L. Prostaglandins and the proliferation of cultured animal cells. p132-160 in Growth and Maturation Factors. (G Guroff ed) John Wiley and Sons, New York. 2, 1984.

211. Bennett A. The production of prostanoids in human cancers, and their implications for tumor progression. Prog. Lipid Res. **25**: 539-542, 1986.

212. Hoffmann P and Mest H.-J. What about the effects of dietary lipids on endogenous prostanoid synthesis? A state-of-the-art review. Biomed. Biochim. Acta **46**: 639-650, 1987.

213. Polgar P, Douglas WHJ, Terracio L and Taylor L. Release of archidonic acid and its conversion to prostaglandins in various diploid cell types in culture. Advances in Prostaglandin and Thromboxane Research. (B Samuelsson, PW Ramwell and R Paoletti R eds) Raven Press, New York. **6**, 1980.

214. Hayashi H, Fujii Y, Watanabe K, Urade Y and Hayaishi O. Enzymatic conversion of prostaglandin H_2 to prostaglandin $F_{2\alpha}$ by aldehyde reductase from human liver: Comparison to the prostaglandin F synthetase from bovine lung. J. Biol. Chem. **264**: 1036-1040, 1989.

215. Powell WS. Formation of 6-Oxoprostaglandin $F_{1\alpha}$, 6,15-dioxoprostaglandin $F_{1\alpha}$ and monohydroxyiconsatetrenoic acids from arachidonic acid by fetal calf aorta and ductus arteriosus. J. Biol. Chem. **257**: 9457-9464, 1982.

216. Salmon JA, Smith DR, Flower RJ, Moncada S and Vane JR. Further studies on the enzymatic conversion of prostaglandin endoperoxide into prostacyclin by porcine aorta microsomes. Biochim. Biophys. Acta **523**: 250-262, 1978.

217. Moncada S, Gryglewski RJ, Bunting S and Vane JR. A lipid peroxide inhibits the enzyme in blood vessel microsomes that generates from prostaglandin endoperoxides the substance (prostaglandin X) which prevents platelet aggregation. Prostagland. **12**: 715-737, 1976.

218. Peters HD, Peskar BA and Schönhöfer PS. Influence of prostaglandins on connective tissue cell growth and function. Naunyn-Schmiedeberg's Arch. Pharmakol. **297**: s89-s93, 1977.

219. Taylor L and Polgar P. Cell growth and the regulation of prostaglandin synthesis. Prostagland. **22**: 723-728, 1981.

220. Uribe A, Alam M and Midtvedt T. E_2 prostaglandins modulate cell proliferation in the small intestinal epithelium of the rat. DIGEB **52**: 157-164, 1992.

221. Wickremasinghe RG. The role of prostaglandins in the regulation of cell proliferation. Prostaglandins Leukot. EFAs. **31**: 171-179, 1988.

222. Huttner JJ, Gwebu ET, Panganamala RV, Milo GE, Cornwell DG, Shamra HM and Geer JC. Fatty acids and their prostaglandin derivatives: inhibitors of proliferation in aortic smooth muscle cells. SCI. **197**: 289-291, 1977.

223. Grenier FC, Allen ML and Smith WL. Interrelationships among prostaglandins, vasopressin and cAMP in renal papillary collecting tubule cells in culture. Prostagland. **24**: 547-565, 1982.

224. Van Valen F, Jürgens H, Winkelmann W and Keck E. β-adrenergic agonist- and prostaglandin-mediated regulation of cAMP levels in ewings sarcoma cells in culture. Biochem. Biophys. Res. Comms. **146**: 685-691, 1987.

225. Sakai T, Yamaguchi N, Shiroko Y, Sekiguchi M, Fujii G and Nishino H. Prostaglnadin D_2 inhibits the proliferation of human malignant tumor cells. Prostagland. **27**: 17-26, 1984.

226. Kawamura M and Koshihara Y. Prostaglandin D_2 strongly inhibits growth of murine mastocytoma cells. Prostaglandins Leukotrienes Med. **12**: 85-93, 1983.

227. Bhuyan BK, Adams EG, Badiner GJ, Li LH and Barden K. Cell cycle effects of prostaglandins A_1, A_2, and D_2 in human murine melanoma cells in culture. Cancer Res. **46**: 1688-1693, 1986.

228. Keyaki A, Handa H, Yamashita J, Tokuriki Y, Otsuka S-I, Yamasaki T and Gi H. Growth-inhibitory effect of prostaglandin D_2 on mouse glioma cells. J. Neurosurg. **61**: 912-917, 1984.

229. Simmet T and Jaffe BM. Inhibition of B-16 Melanoma growth *in vitro* by prostaglandin D_2. Prostagland. **25**: 47-54, 1983.

230. Fukushima M, Kato T, Ueda R, Ota K, Narumiya S and Hayaishi O. Prostaglandin D_2, a potential antineoplastic agent. Biochem. Biophys. Res. Commun. **105**: 956-964, 1982.

231. Bennett A. Prostaglandins and tumour metastasis. p223-228 in Icosanoids and Cancer. (H Thaler-Dao ed) Raven Press, New York, 1984.

232. Fulton AM. The role of eicosanoids in tumor metastasis. Prostaglandins Leukot. EFAs. **34**: 229-237, 1988.

233. Fulton AM. Interactions of natural effector cells and prostaglandins in the control of metastasis. J. Natl. Cancer Inst. **78**: 735-741, 1987.

234. Hubbard WC, Alley MC, McLemore TL and Boyd MR. Profiles of prostaglandin biosynthesis in sixteen established cell lines derived from human lung, colon, prostate and ovarian tumours. Cancer Res. **48**: 4770-4775, 1988.

235. De Vries CEE and Van Noorden CJF. Effects of dietary fatty acid composition on tumor growth and metastasis. Anticancer Res. **12**: 1513-1522, 1992.

236. Lupulescu A. Enhancement of carcinogenesis by prostaglandins. Nature **272**: 634-636, 1978.

237. Furuta Y, Hall ER, Sanduja S, Barkley T and Milas L. Prostaglandin production by murine tumours as a predictor for therapeutic response to indomethacin. Cancer Res. **48**: 3002-3007, 1988.

238. Bennett A and Del Tacca M. Prostaglandins in human colonic carcinoma. GuT **16**: 409, 1975.

239. Ohno K, Sakai T, Fukushima M, Narumiya S and Fujiwara M. Site and mechanism of growth inhibition by prostaglandins.IV. Effect of cyclopentenone prostaglandins on cell cycle progression of G_1-enriched HeLa S3 cells. J. Pharmacol. Exp. Ther. **245**: 294-298, 1988.

240. Whatley RE, Satoh K, Zimmerman GA, McIntyre TM and Prescott SM. Proliferation-dependant changes in release of arachidonic acid from endothelial cells. J. Clin. Invest. **94**: 1889-1900, 1994.

241. Jaffe BM. Prostaglandins and cancer: An update. Prostagland. **6**: 453-461, 1974.

242. Horrobin DF. The reversibility of cancer: The relevance of cyclic AMP, calcium, essential fatty acids and prostaglandin E_1. Med. Hypotheses **6**: 469-486, 1980.

243. Tashjian AH, Voelkel EF and Levine L. Prostaglandins, tumor cells and bone metabolism. p513-523 in Prostaglandins and Related Lipids (AR Liss ed) New York, 1982.

244. Stamford IF, Carroll MA, Civier A, Hensby CN and Bennett A. Identification of arachidonic metabolites in normal, benign and malignant human mammary tissues. J. Pharm. Pharmacol. **35**: 48-49, 1983.

245. Bockman R, Bellin A and Hickok N. Disordered prostaglandin production and cell differentiation/ proliferation in cancer. p169-182 in Icosanoids and Cancer. (H Thaler-Dao ed) Raven Press, New York, 1984.

246. Young MR and Hoover CS. Inhibition of spleen cell cytotoxic capacity toward tumour by elevated prostaglandin E_2 levels in mice bearing lewis lung carcinoma. J.Natl. Cancer Inst. **77**: 425-429, 1986.

247. Stoll KE, Ottino P and Duncan JR. Interrelationship of ascorbate, arachidonic acid and prostaglandin E_2 in B16 melanoma cells. Prostaglandins Leukot. EFAs. **50**: 123-131, 1994.

248. Nakamura A, Chiba T, Yamatani T, Yamaguchi A, Inui T, Morishita T, Kadowaki S and Fujita T. Prostaglandin E_2 and $F_{2\alpha}$ inhibit growth of human gastric carcinoma cell line KATO lll with simultaneous stimulation of cyclic AMP production. Life Sci. **44**: 75-80, 1988.

249. Thomas DR, Philpott GW and Jaffe BM. The relationship between concentration of prostaglandin E and rates of cell replication. Exp. Cell Res. **84**: 40-46, 1974.

250. Santoro MG, Benedetto A and Jaffe BM. Effect of endogenous and exogenous prostaglandin E on friend erythroleukaemia cell growth and differentiation. Br. J. Canc. **39**: 259-267, 1979.

251. Karmali RA, Horrobin DF, Menezes J and Patel P. The relationship between concentrations of prostaglandin A1, E1, E2 and E2α and rates of cell proliferation. Pharmacol. Res. Commun. **11**: 69-75, 1979.

252. Santoro MG and Jaffe BM. Inhibition of friend erythroleikaemia-cell tumours *in vivo* by a synthetic analogue of prostaglandin E_2. Br. J. Cancer **39**: 408-413, 1979.

253. Foecking MK, Kibbey WE, Abou-Issa H, Matthews RH and Minton JP. Hormone dependence of 7,12-dimethylbenz[a]anthracene-induced mammary tumor growth: correlation with prostaglandin E_2 content. J. Natl. Cancer Inst. **69**: 443-446, 1982.

254. Honn KV, Cicone B and Skoff A. Prostacyclin: A potent antimetastatic agent. SCI. **212**: 1270-1272, 1981.

255. Fitzpatrick FA and Stringfellow DA. Prostaglandin D_2 formation by malignant melanoma cells correlates inversely with cellular metastatic potential. Proc. Natl. Acad. Sci. **76**: 1765-1769, 1979.

256. Stringfellow DA and Fitzpatrick FA. Prostaglandin D_2 controls pulmonary metastasis of malignant melanoma cells. Nature **282**: 76-78, 1979.

257. Fitzpatrick FA and Stringfellow DA. Prostaglandin formation by murine malignant melanoma cells: inverse correlation with metastasis. p705-708 in Essential Fatty Acids and Prostaglandins. (Holman RT ed) Pergamon Press, New York. **20**, 1981.

258. Santoro MG, Crisari A, Benedetto A and Amici C. Modulation of the growth of a human erythroleukemic cell line (K562) by prostaglandins: Anti-proliferative action of prostaglandin A^1. Cancer Res. **46**: 6073-6077, 1986.

259. Tanaka H, Yamamuro T, Matsumoto M, Kotoura Y and Tanaka C. The effects of PGD_2 and 9-deoxy-Δ^9-PGD_2 on colony formation of murine osteosarcoma cells. Prostagland. **30**: 167-174, 1985.

260. Bregman MD and Meyskens FL. *In vitro* modulation of human and murine melanoma growth by prostaglandin analogues. Prostagland. **26**: 449-456, 1983.

261. Honn KV and Meyer J. Tromboxanes and prostacyclin: positive and negative modulators of tumor growth. Biochem. Biophys. Res. Commun. **102**: 1112-1129, 1981.

262. Pozzan T, Rizzuto R, Volpe P and Meldolesi J. Molecular and cellular physiology of intracellular calcium stores. Physiol. Rev. **74**: 595-636, 1994.

263. Whitfield JF, Boynton AL, MacManus JP, Rixon RH, Sikorska M, Tsang B and Walker PR. The roles of calcium and cyclic AMP in cell proliferation. Ann. New York Acad. Sci. **339**: 216-240, 1980.

264. Dawson AP. Regulation of intracellular Ca^{2+}. Essays Biochem. **25**: 1-37, 1990.

265. Ross EM and Gilman AG. Biochemical properties of hormone-sensitive adenylate cyclase. Annu. Rev. Biochem. **49**: 533-564, 1980.

266. Mac Neil S, Walker SW, Senior HJ, Pollock A, Brown BL, Bleehen SS, Munro DS and Tomlinson S. Calmodulin activation of adenylate cyclase in the mouse B16 melanoma. Biochem. J. **224**: 453-460, 1984.

267. Xing M and Mattera R. Phosphorylation-dependant regulation of phospholipase A_2 by G-proteins and Ca^{2+} in HL60 Granulocytes. J. Biol. Chem. **267**: 25966-25975, 1992.

268. Channon JY and Leslie CC. A calcium-dependant mechanism for associating a soluble arachidonyl-hydrolysing phospholipase A_2 with membrane in the macrophage cell line RAW 264.7. J. Biol. Chem. **265**: 5409-5413, 1990.

269. Brooks RC, McCarthy KD, Lapetina EG and Morell P. Receptor-stimulated phopholipase 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, 1989.

270. Murthy M, Rao GH, Robinson P and Reddy S. Influx of extracellular calcium and agonist-coupling appear essential for the activation of thromboxane A_2-dependant phospholipase A_2 in human platelets. Protaglandins Leukot. EFAs 53: 31-39, 1995.

271. Wijkander J and Sundler R. Macrophage arachidonate-mobilizing phopholipase A_2: role of Ca^{2+} for membrane binding but not for catalytic activity. Biochem. Biophys. Res. Commun. **184**: 118-124, 1992.

272. Kramer RM, Roberts EF, Manetta J and Putnam JE. The Ca^{2+}-sensitive cytosolic phospholipase A_2 is 100-kDa protein in human monoblast U937 cells. J. Biol. Chem. **266**: 5268-5272, 1991.

273. De Winter JM, Korpancova J and Van Den Bosch H. Regulatory aspects of rat liver mitochondrial phopholipase A_2: Effects of calcium ions and calmodulin. Arch. Biochem. Biophys. **234**: 243-252, 1984.

274. Peplow PV. Modification to dietary intake of sodium, potassium, calcium, magnesium and trace elements can influence archidonic acid metabolism and eicosanoid production. Prostaglandins Leukot. EFAs **45**: 1-19, 1992.

275. Watterson DM, Van Eldik LJ, Smith RE and Vanaman TC. Calcium-dependant regulatory protien of cyclic nucleotide metabolism in normal and transformed chicken embryo fibroblasts. Proc. Natl. Acad. Sci. **73**: 2711-2715, 1976.

276. Volterra A, Trotti D, Bezzi P, Civenni G and Racagni G. [Ca^{2+}] modulates the ratio between cyclooxygenase and lipoxygenase metabolism of arachidonic acid in homogenates of hippocampal astroglial cultures. Neurosci. Lett. **183**: 160-163, 1995.

277. Cooper DMF, Mons N and Karpen JW. Adenyl cyclases and the interaction between calcium and cAMP signalling. Nature **374**: 421-424, 1995.

278. Durham ACH and Walton JM. Calcium ions and the control of proliferation on normal and cancer cells. Biosci. Rep. **2**: 15-30, 1982.

279. Batra S, Popper LD and Hartley-Asp B. Effect of calcium and calcium antagonists on ^{45}Ca influx and cellular growth of human prostatic tumour cells. The Prostate **19**: 299-311, 1991.

280. Berridge MJ. Calcium signalling and cell proliferation. BioEssays **17:** 491-500, 1995.

281. Whitfield JF, Bird RP, Chakravarthy BR, Isaacs RJ and Morley P. Calcium-Cell cycle regulator, differentiator, killer, chemopreventor, and maybe, tumor promoter. J. Cell. Biochem. Suppl. **22**: 74-91, 1995.

282. Sasaki Y and Hidaka H. Calmodulin and cell proliferation. Biochem. Biophys. Res. Commun. **104**: 451-456, 1982.

283. Horrobin DF. The reversibility of cancer: The relevance of cyclic AMP, calcium, essential fatty acids and prostaglandin E1. Med. Hypotheses **6**: 469-486, 1980.

284. Martin TJ and Grill V. Hypercalcaemia in cancer. J. Steroid Biochem. Molec. Biol. **43**: 123-129, 1992.

285. Hickie RA and Kalant H. Calcium and magnesium content of rat liver and morris hepatoma 5123 tc. Cancer Res. **27**: 1053-1057, 1967.

286. Yoneda T, Kitamura M, Ogawa T, Aya S-i and Sakuda M. Control of VX_2 carcinoma cell growth in culture by calcium, calmodulin, and prostaglandins. Cancer Res. **45**: 398-405, 1985.

287. Anghileri LJ, Miller ES, Robinette J, Prasad KN and Lagerborg VA. Calcium metabolism in tumours. Its relationship with chromium complex accumulation II. Calcium, magnesium and phosphorus in human and animal tumours. Oncology **25**: 193-209, 1971.

288. Boynton AL and Whitfield JF. Different calcium requirements for proliferation of conditionally and unconditionally tumorigenic mouse cells. Proc. Natl. Acad. Sci. **73**: 1651-1654, 1976.

289. Parsons PG, Musk P, Goss PD and Leah J. 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, 1983.

290. Van Den Bosch H. Intracellular phospholipases A. Biochim. Biophys. Acta **604**: 191-246, 1980.

291. Pfeilschifter J, Schalkwijk C, Briner VA and Van Den Bosch H, Cytokine-stimulated secretion of group II phospholipase A_2 in rat mesangial cells. J. Clin. Invest. **92**: 2516-2523, 1993.

292. Leslie CC. Kinetic properties of a high molecular mass arachidonoyl-hydrolyzing phopholipase A_2 that exhibits lysophospholipase activity. J. Biol. Chem. **266**: 11366-11371, 1991.

293. Mayer RJ and Marshall LA. New insights on mammalian phospholipase A_2(s); comparison of arachidonoyl-selective and -nonselective enzymes. FASEB J. **7**: 339-348, 1993.

294. Hara S, Kudo I, Komatani T, Takahashi K, Nakatani Y, Natori Y, Ohshima M and Inoue K. Detection and purification of two 14kDa phospholipase A_2 isoforms in rat kidney: their role in eicosanoid synthesis. Biochim. Biophys. Acta **1257**: 11-17, 1995.

295. Steiner MR, Bomalaski JS and Clark MA. Responses of purified phospholipase A_2 activating protein (PLAP) and melittin. Biochem. Biophys. Acta **1166**: 124-130, 1993.

296. Angel J, Berenbaum F, Le Denmat C, Nevalainen T, Masliah J and Fournier C. Interleukin-1-induced prostaglandin E_2 biosynthesis in human synovial cells involves the activation of cytosolic phospholipase A_2 and cyclooxygenase-2. Eur. J. Biochem. **226**: 125-131, 1994.

297. Ross BM, Kim DK, Bonventre JV and Kish SJ. Characterization of a novel phospholipase A_2 activity in human brain. J. Neurochem. **64**: 2213-2221, 1995.

298. Ohara O, Ishizaki J and Arita H. Structure and function of phopholipase A_2 receptor. Prog. Lipid Res. **34**: 117-138, 1995.

299. Rosenthal MD, Rzigalinski BA, Blackmore PF and Franson RC. Cellular regulation of arachidonic mobilisation and metabolism. Prostaglandins Leukot. EFAs **52**: 93-98, 1995.

300 Saris N-EL, Steinbrückner B, Thurén T, Lalla MLT, Van Den Bosch H, Kinnunen PKJ and Hoffman GE. Characterization of cellular and elevated serum phopholipase A_2 activities with a comparison of two methods. Scand. J. Clin. Lab. Invest. **52**: 19-25, 1992.

301. Ransac S, Aarsman AJ, Van Den Bosch H, Gancet C, De Haas GH and Verger R. Rat platelet phospholipase A_2. Kinetic characteristics using the monomolecular film technique. Eur. J. Biochem. **204**: 793-797, 1992.

302. Ballou LR and Cheung WY. Inhibition of human platelet phospholipase A_2 activity by unsaturated fatty acids. Proc. Natl. Acad. Sci. **82**: 371-375, 1985.

303. Ballou LR and Cheung WY. Marked increase of human platelet phospholipase A_2 activity *in vitro* and demonstration of an endogenous inhibitor. Proc. Natl. Acad. Sci. **80**: 5203-5207, 1983.

304. Lister MD, Deems RA, Watanabe Y, Ulevitch RJ and Dennis EA. Kinetic analysis of the Ca^{2+}-dependant, membrane-bound, macrophage phopholipase A_2 and the effects of arachidonic acid. J. Biol. Chem. **263**: 7506-7513, 1988.

305. Clark MA, Conway TM, Shorr RGL and Crooke ST. Identification and isolation of a mammalian protein which is antigenically and functionally related to the phospholipase A_2 stimulatory peptide melittin. J. Biol. Chem. **262**: 4402-4406, 1987.

306. Sevanian A, Muakkassah-Kelly SF and Montestruque S. The influence of phopholipase A_2 and glutathione peroxidase on the elimination of membrane lipid peroxides. Arch. Biochem. Biophys. **223**: 441-452, 1983.

307. Axelrod J. Receptor-mediated activation of phopholipase A_2 and arachidonic acid release in signal transduction. Biochem. Soc. T. **18**: 503-507, 1990.

308. Axelrod J, Burch RM and Jelsema CL. Receptor-mediated activation of phospholipase A_2 via GTP-binding proteins: archidonic acid and its metabolites as second messengers. TINS **11**: 117-123, 1988.

309. Burgoyne RD and Morgan A. The control of free arachidonic acid levels. TIBS **15**: 365-366, 1990.

310. Buckley BJ, Barchowsky A, Dolor RJ and Whorton AR. Regulation of arachidonic acid release in vascular endothelium. Biochem. J. **280**: 281-287, 1991.

311. Lin L-L, Lin AY and Knopf JL. Cytosolic phopholipase A_2 is coupled to hormonally regulated release of arachidonic acid. Proc. Natl. Acad. Sci. **89**: 6147-6151, 1992.

312. Gronich JH, Bonventre JV and Nemenoff RA. Identification and characterisation of a hormonally regulated form of phospholipase A_2 in rat renal mesangial cells. J. Biol. Chem. **263**: 16645-16651, 1988.

313. Ando M, Furui H, Suzuki K, Taki F and Takagi K. Direct activation of phospholipase A_2 by GTP-binding protein in human peripheral polymorphonuclear leucocytes. Biochem. Biophys. Res. Commun. **183**: 708-713, 1992.

314. Burch RM and Axelrod J. Dissociation of bradykinin-induce prostaglandin formation from phosphotidylinositol turnover in swiss 3T3 fibroblasts: evidence for G protein regulation of phospholipase A_2. Proc. Nat. Acad. Sci. **84**: 6374-6378, 1987.

315. Burch RM, Luini A and Axelrod J. Phospholipase A_2 and phospholipase C are activated by distinct GTP-binding proteins in response to α_1-adrenergic stimulation in FRTL5 thyroid cells. Proc. Nat. Acd. Sci. **83**: 7201-7205, 1986.

316. Burch RM, Luini A, Mais DE, Corda D, Vanerhoek JY, Kohn LD and Axelrod J. α_1-Adrenergic stimulation of arachidonic acid release and metabolism in a rat thyroid cell line. J. Biol. Chem. **261**: 11236-11241, 1986.

317. Jelsema CL and Axelrod J. Stimulation of phopholipase A_2 activity in bovine rod outer segments by the $\beta\gamma$ subunits of transducin and its inhibition by the α subunit. Proc. Nat. Acad. Sci. (USA) **84**: 3623-3627, 1987.

318. Kim D, Lewis DL, Graziadei L, Neer EJ, Bar-Sagi D and Clapham DE. G-protein $\beta\gamma$-subunits activate the cardiac muscarinic K^+-channel via phopholipase A_2. Nature **337**: 557-560, 1989.

319. Simon MI, Strathmann MP and Gautam N. Diversity of G proteins in signal transduction. SCI. **252**: 802-808, 1991.

320. Hepler JR and Gilman AG. G proteins. TIBS: **17**, 383-387, 1992.

321. Schultz G and Jakobs KH. Adenylate cyclase. p369-378 in Methods of Enzymatic Analysis. (J Bergmeyer and M Graßl eds) Verlag Chemie, Weinheim, 1974.

322. Cole OF, Elder MG and Sullivan MHF. Decidual adenylate cyclase and prostaglandin production *in vitro*. Prostagland. **49**: 167-174, 1995.

323. Emmelot P and Bos CJ. Studies on plasma membranes. XIV. Adenylate cyclase in plasma membranes isolated from rat and mouse livers and hepatomas, and its hormone sensitivity. Biochim. Biophys. Acta **249**: 285-292, 1971.

324. Monn E and Christiansen RO. Adenosine 3',5'-monophosphate phosphodiesterase: multiple molecular forms. SCI. **173**: 540-542, 1971.

325. D'Armiento M, Johnson GS and Pastan I. Regulation of adenosine 3':5'-cyclic monophosphate phosphodiesterase activity in fibroblasts by intracellular concentrations of cyclic adenosine monophosphate. Proc. Natl. Acad. Sci. **69**: 459-462, 1972.

326. Amer MS and Kreighbaum WE. Cyclic nucleotide phosphodiesterases: properties, activators, inhibitors, structure-activity relationships, and possible role in drug development. J. Pharm. Sci. **64**: 1-36, 1975.

327. Gao B and Gilman AG. Cloning and expression of a widely distributed (type 1V) adenyl cyclase. Proc. Nat. Acad. Sci. 88: 10178-10182, 1991.

328. Steer ML and Wood A. Regulation of human platelet adenylate cyclase by epinephrine, prostaglandin E_1, and guanine nucleotides. J. Biol. Chem. **254**: 10791-10797, 1979.

329. Lefkowitz RJ, Stadel JM and Caron MG. Adenylate cyclase-coupled beta-adrenergic receptors: structure and mechanisms of activation and desensitization. Ann. Rev. Biochem. **52**: 159-186, 1983.

330. Sheppard JR, Lester B, Doll J, Buscarino C, Gonzales E, Corwin S, Greig R and Poste G. Biochemical regulation of adenylate cyclase in murine melanoma clones with different metastatic properties. Int. J. Cancer **37**: 713-722, 1986.

331. Engelhard VH, Esko JD, Storm DR and Glaser M. Modification of adenylate cyclase activity in LM cells by manipulation of the membrane phospholipid composition *in vivo*. Proc. Natl. Acd. Sci. **73**: 4482-4486, 1976.

332. Thierauch K-H, Dinter H and Stock G. Prostaglandins and their receptors: II. Receptor structure and signal transduction. J. Hypertens. **12**: 1-5, 1994.

333. Cutler LS, Christian CP and Feinstein MB. 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**: 403-410, 1985.

334. Yatsunami K, Ichikawa A and Tomita K. Accumulation of adenosine 3',5'-monophosphate induced by prostaglandin E_1 binding to mastocyctoma P-815 cells. Biochem. Pharmacol. **30**: 1325-1332, 1981.

335. Michael AE, Abayasekara DRE and Webley GE. 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, 1993.

336. Tsai BS and Lefkowitz RJ. Multiple effects of guanine nucleotides on human platelet adenylate cyclase. Biochim. Biophys. Acta **587**: 28-41, 1979.

337. Negishi M, Ito S and Hayaishi O. Prostaglandin E receptors in bovine adrenal medulla are coupled to adenylate cyclase via Gi and to phosphoinositide metabolism in a pertussis toxin-insensitive manner. J. Biol. Chem. **264**: 3916-3923, 1989.

338. Hildebrant JD, Sekura RD, Codina J, Iyengar R, Manclark CR and Birnbaumer L. Stimulation and inhibition of adenylate cyclases mediated by distinct regulatory proteins. Nature **302**: 706-708, 1983.

339. Murphy MG, Moak CM and Rao BG. Effects of membrane polyunsaturated fatty acids on opiate peptide inhibition of basal and prostaglandin E_1-stimulated cyclic AMP formation in intact N1E-115 neurobalstoma cells. Biochem. Pharmacol. **36**: 4079-4084, 1987.

340. Stryer L. Biochemistry. W.H. Freeman and company, New York, 1988.

341. Canuto RA, Paradisi L, Muzio G, Maggiora M, Mengozzi G, Garramone A and Dianzani MU. 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, 1995.

342. Keirns JJ, Kreiner PW, Brock WA, Freeman J and Bitensky MW. Prostaglandins and the adenyl cyclase of the cloudman melanoma. p181-198 in The Role of Cyclic Nucleotides in Carcinogenesis. (J Schultz and HG Gratzner eds) Academic Press, New York, 1973.

343. Torikai S and Kurokawa K. Distribution of Prostaglandin E_2-sensitive adenylate cyclase along the rat nephron. Prostagland. **21**: 427-438, 1981.

344. Yamaguchi DT, Hahn TJ, Beeker TG, Kleeman CR and Muallem S. Relationship of cAMP and calcium messenger systems in prostaglandin-stimulated UMR-106 cells. J. Biol. Chem. **263**: 10745-10753, 1988.

345. Sonnenburg WK and Smith WL. Regulation of cyclic AMP metabolism in rabbit cortical collecting tubule cells by prostaglandins. J. Biol. Chem. **263**: 6155-6160, 1988.

346. Toriyama K, Morita I and Murota S-i. The existence of distinct classes of prostaglandin E_2 receptors mediating adenylate cyclase and phospholipase C pathways in osteblastic clone MC3T3-E1. Prostaglandins Leukot. EFAs **46**: 15-20, 1992.

347. Nakamura A, Yamatani T, Fujita T and Chiba T. Mechanism of inhibitory action of prostaglandins on the growth of human gastric carcinoma cell line KATO III. Gastroent. **101**: 910-918, 1991.

348. Yamashita L and Sweat FW. The stimulation of rat liver adenylate cyclase by prostaglandin E_1 and E_2. Biochem. Biophys. Res. Commun. **70**: 438-444, 1976.

349. Wisner A, Bommelaer-Bayet M-C, Tiberghien C, Renard C-A, David F and Dray F. Hypothalamic prostaglandin E_2 receptors coupled to an adenyl cyclase. Eur. J. Pharmacol. **162**: 89-100, 1989.

350. Dominguez JH, Shuler F, Olszowy MW, Brown T and Puschett JB. Prostaglandin E_2 is an inhibitor of adenylate cyclase in rabbit proximal tubule. Am. J. Physiol. **254**: C304-C309, 1988.

351. Lapetina EG, Schmitges CJ, Chandrabose K and Cuatrecasas P. Regulation of phospholipase activity in platelets. p127-135 in Advances in Prostaglandin and Thromboxane Research. (C Galli ed) Raven Press, New York. **3**, 1978.

352. Gottesman MM and Fleischmann RD. The role of cAMP in regulating tumour cell growth. Cancer Surveys **5**: 291-308, 1986.

353. Chlapowski FJ, Kelly LA and Butcher RW. Cyclic nucleotides in cultured cells. p246-337 in Advances in Cyclic Nucleotide Research. (P Greengard and GA Robinson eds) Raven Press, New York. **6**, 1975.

354. Hunt NH and Martin TJ. Hormone receptors and cyclic nucleotides; significance for growth and function of tumours. Mol. Aspects Med. **3**: 59-118, 1980.

355. Pastan I and Johnson GS. Cyclic AMP and the transformation of fibroblasts. Adv. Cancer Res. **19**: 303-329, 1974.

356. Johnson GS. Regulation of cell functions in fibroblasts by cyclic AMP. p39-45 in The Role of Cyclic Nucleotides in Carcinogenesis. (J Schultz and HG Gratzner eds) Academic Press, New York, 1973.

357. Anderson WB, Johnson GS and Pastan I. 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, 1973.

358. Gericke D and Chandra P. Inhibition of tumour growth by nucleoside cyclic 3',5'-monophosphates. Hoppe-Seyler's Z. Physiol. Chem. **Bd.350**: s1469-1471, 1969.

359. Negishi M, Ichikawa AS, Oshio N, Yatsunami K and Tomha K. Cell cycle specific fluctuations of adenosine 3', 5'-monophosphate and prostaglandin binding in synchronized mastocytoma P-815 cells. Biochem. Proc. **31**: 173-179, 1982.

360. Friedman DL. Role of cyclic nucleotides in cell growth and differentiation. Phsiol. Rev. **56**: 652-708, 1976.

361. Ryan WL and Heidrick ML. Role of cyclic nucleotides in cancer. p8-116 in Advances in Cyclic Nucleotide Research. (P Greengard and GA Robinson eds) Raven Press, New York. **4**, 1974.

362. Boynton AL and Whitfield JF. The role of cyclic AMP in cell proliferation: A critical assessment of the evidence. p195-293 in Advances in Cyclic Nucleotide Research. (P Greengard and GA Robinson) Raven Press, New York. **15**, 1983.

363. Revillon F, Vandewalle B, Lassalle B and Lefebvre J. cAMP effect on extracellular matrix synthesis in human breast cancer cells. Cell Prolif. **25**: 633-642, 1992.

364. Johnson GS and Pastan I. Change in growth and morphology of fibroblasts by prostaglandins. J. Natl. Cancer Inst. **47**: 1357-1364, 1971.

365. Manganiello V and Vaughan M. Prostaglandin E_1 effects on adenosine 3':5'-cyclic monophosphate concentration and phophodiesterase activity in fibroblasts. Proc. Nat. Acad. Sci. **69**: 269-273, 1972.

366. Ballard TA and Elmer WA. Effect of prostaglandin E_2 on cyclic AMP levels in limb cells of mouse mutant brachypodism. Developm. Biol. **142**: 489-492, 1990.

367. Anderson WB, Gallo M, Wilson J, Lovelace E and Pastan I. Effect of epidermal growth factor on prostaglalndin E_1-stimulated accumulation of cyclic AMP in fibroblastic cells. FEBS Lett. **102**: 329-332, 1979.

368. Ishihara K, Ono S, Takahama Y, Hirayama F, Hirano H, Itoh K, Dobashi K, Murakami S, Katoh Y, Yamaguchi M and Hamaoka T. 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. Immun. **68**: 154-162, 1989.

369. Russell TR, Thompson WJ, Schneider FW and Appleman MM. 3':5'-cyclic adenosine monophosphate phosphodiesterase: negative cooperativity. Proc. Nat. Acad. Sci. **69**: 1791-1795, 1972.

370. Appleman MM, Thompson WJ and Russel TR. Cyclic nucleotide phosphodiesterases. p66-98 in Advances in Cyclic Nucleotide Research. (P Greengard and GA Robinson eds) Raven Press, New York. **3**, 1973.

371. Weiss B and Hait WN. Selective cyclic nucleotide phosphodiesterse inhibitors as potential therapeutic agents. Ann. Rev. Pharmacol. Toxicol. **17**: 441-477, 1977.

372. Wells JN and Hardman JG. Cyclic nucleotide phosphodiesterases. p119-143 in Advances in Cyclic Nucleotide Research. (P Greengard and GA Robinson eds) Raven Press, New York. **8**, 1977.

373. Sheppard JR. Difference in the cyclic adenosine 3',5'-Monophosphate levels in normal and transformed cells. Nat. New Biol. **236**: 14-16, 1972.

374. Pastan I, Willingham M, Carchman R and Anderson WB. Cyclic AMP metabolism in normal and transformed fibroblasts. p47-52 in The Role of Cyclic Nucleotides in Carcinogenesis. (J Schultz and HG Gratzner eds) Academic Press, New York, 1973.

375. Otten J, Johnson GS and Pastan I. Cyclic AMP levels in fibroblasts: relationship to growth rate and contact inhibition of growth. Biochem. Biophys. Res. Commun. **44**: 1192-1198, 1971.

376. Heidrick ML and Ryan WL. Adenosine 3':5'-cyclic monophosphate and contact inhibition. Cancer Res. **31**: 1313-1315, 1971.

377. Bürk RR. Reduced adenyl cyclase activity in a polyoma virus transformed cell line. Nature **219**: 1272-1275, 1968.

378. Anderson WB, Russell TR, Carchman RA and Pastan I. Interrelationship between adenylate cyclase activity, adenosine 3':5' cyclic monophosphate phosphodiesterase activity, adenosine 3':5' cyclic monophosphate levels, and growth of cells in culture. Proc. Nat. Acad. Sci. **70**: 3802-3805, 1973.

379. Hait WN and Weiss B. Increased cyclic nucleotide phosphodiesterase activity in leukaemic lymphocytes. Nature **259**: 321-323, 1976.

380. Peery CV, Johnson GS and Pastan I. Adenyl cyclase in normal and transformed fibroblasts in tissue culture. J. Biol. Chem. **246**: 5785-5790, 1971.

381. Allen DO, Munshower J, Morris HP and Weber G. Regulation of adenyl cyclase in hepatomas of different growth rates. Cancer Res. **31**: 557-560, 1971.

382. Murphy MG. Studies of the regulation of basal adenylate cyclase activity by membrane polyunsaturated fatty acids in cultured neroblastoma. J. Neurochem. **47**: 245-253, 1986.

383. Murphy MG. Membrane fatty acids, lipid peroxidation and adenylate cyclase activity in cultured neural cells. Biochem. Biophys. Res. Comms. **132**: 757-763, 1985.

384. Heidrick ML and Ryan WL. Cyclic nucleotides on cell growth *in vitro*. Cancer Res. **30**: 376-378, 1970.

385. Krieder JW, Wade DR, Rosenthal M and Densley T. Maturation and differentiation of BL6 melanoma cells induced by theophylline treatment. J. Natl. Cancer Inst. **54**: 1457-1467, 1975.

386. Krieder JW, Rosenthal M and Lengle N. Brief communication: Cyclic adenosine 3':5' monophosphate in the control of melanoma cell replication and differentiation. J. Natl. Cancer Inst. **50**: 555-558, 1973.

387. Sheppard JR. Restoration of contact-inhibited growth to transformed cells by dibutyryl adenosine 3':5' cyclic monophosphate. Proc. Nat. Acad. Sci. **68**: 1316-1320, 1971.

388. Minton JP, Wisenbaugh T and Mathews RH. Breif Communication: Elevated cyclic AMP levels in human breast-cancer tissue. J. Natl. Cancer Inst. **53**: 283-284, 1974.

389. Perkins JP, Macintyre EH, Riley WD and Clark RB. Adenyl cyclase, phosphodiesterase and *cAMP* dependant protein kinase of malignant glial cells in culture. Life Sci. **10**: 1069-1080, 1971.

390. Minton JP, Matthews RH and Wisenbaugh TW. Elevated adenosine 3':5'-cyclic monophosphate levels in human and animal tumours *in vivo*. J. Natl. Cancer Inst. **57**: 39-41, 1976.

391. Guerinot F, Delarue J-C, Contesso G and Bohuon C. Adenosine 3':5'-cyclic monophosphate and guanosine 3':5'-cyclic monophosphate levels in human breast cancer tissue. Oncology **34**: 261-263, 1977.

392. Chayoth R, Epstein S and Field JB. Increased cyclic AMP levels in malignant hepatic nodules of ethionine treated rats. Biochem. Biophys. Res. Commun. **49**: 1663-1670. 1972.

393. Sarria A, Prasad KN. dl-α-Tocopheryl succinate enhances the effect of γ-irradiation on neuroblastoma cells in culture (41772). Proc. Soc. Exp. Biol. Med. **175**: 88-92, 1984.

394. Prasad KN, Ramanujam S and Gaudreau D. Vitamin E induces morphological differentiation and increases the effect of ionizing radiation on neuroblastoma cells in culture (40598)[1]. Proc. Soc. Exp. Biol. Med. **161**: 570-573, 1979.

395. Sakagami H, Asaka K, Abe E, Miyaura C, Suda T and Konno K. Effect of DL-α-Tocopherol (Vitamin E) on the differentiation of mouse myeloid leukaemia cell. J. Nutr. Sci. Vitaminol **27**: 291-300, 1981.

396. Freshney RI. Introduction: Principles of sterile technique and cell propagation. p1-11 in Animal cell culture. A practical approach. (RI Freshney ed) IRL Press, Washington DC, 1986.

397. Helson L, Verma M and Helson C. Vitamin E and human neuroblastoma. p258-265 in Modulation and Mediation of Cancer by Vitamins. (FL Meyskens and KN Prasad eds) Krager Basel, London, 1983.

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

399. Prasad KN and Edwards-Prasad J. Effects of Tocopherol (Vitamin E) acid succinate on morphological alterations and growth inhibition in melanoma cells in culture. Cancer Res. **42**: 550-555, 1982.

400. Oberley TD, Schultz JL, Li N and Oberley LW. Antioxidant enzyme levels as a function of growth state in cell culture. Free Radical Biol. Med. **19**: 53-65, 1995.

401. Horton AA and Fairhurst S. Lipid peroxidation and mechanisms of toxicity. CRC Crit. Rev. Toxicol. **18**: 27-77, 1987.

402. Tisdale MJ and Mahmoud MB. Activities of free radical metabolising enzymes in tumours. Br. J. Cancer **47**: 809-812, 1983.

403. Carini R, Dianbzani MU, Maddix SP, Slater TF and Cheeseman KH. 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. Pharmacol. **39**: 1597-1601, 1990.

404. Trevithick JR and Mitton KP. Topical application and uptake of vitamin E acetate by the skin and conversion to free vitamin E. Biochem. Molecular Biol. Int. **31**: 869-878, 1993.

405. Pascoe GA and Reed DJ. Vitamin E protection against chemical-induced cell injury. Arch. Biochem. Biophys. **256**: 159-166, 1987.

406. Sagar PS, Das UN, Koratkar R, Ramesh G, Padma M and Sravan Kumar G. Cytotoxic 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, 1992.

407. Das UN, Padma M, Sangeetha Sagar P, Ramesh G and Koratkar R. Stimulation of free radical generation in human leucocytes by various agents including tumor necrosis factor is a calmodulin dependant process. Biochem. Biophys. Res. Commun. **167**: 1030-1036, 1990.

408. Drapper HH and Hardley M. Malondialdehyde determination as an index of lipid peroxidation. p421-431 in Methods in Enzymology. (L Packer and AN Glazer eds) Academic Press, New York. **182**, 1990.

409. Pryor WA, Stanley JP and Blair E. Autoxidation of polyunsaturated fatty acids: II. A suggested mechanism for the formation of TBA-reactive materials from prostaglandin-like endoperoxides. Lipids **11**: 370-379, 1975.

410. Kelly EE, Buettner GR and Burns CP. 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, 1995.

411. Scalia S, Renda A, Ruberto G, Bonina F and Menegatti E. 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, 1995.

412. Fariss MW. Cadmium toxicity: unique cytoprotective properties of alpha Tocopheryl succinate in hepatocytes. Toxicology **69**: 63-77, 1991.

413. Fariss MW. Oxygen toxicity: Unique cytoprotective properties of vitamin E succinate in hepatocytes. Free Radical Biol. Med. **9**: 333-343, 1990.

414. Lai MZ, Duzgunes N, Szoka FC. Effects of replacement of hydroxyl group of cholesterol and tocopherol on the thermotropic behaviour of phospholipid membranes, Biochem. **24**: 1646-1653, 1985.

415. Toivanen JL. Effects of selenium, vitamin E and vitamin C on human prostacyclin and thromboxane synthesis *in vitro*. Prostaglandins Leukot. EFAs **26**: 265-280, 1987.

416. Brase DA, Westfall TC. Stimulation of rat liver phenylalanine hydroxylase activity by derivatives of vitamin E. Biochem. Biophys. Res. Commun. **48**: 1185-1191, 1972.

417. Panganamala RV and Cornwell DG. The effects of vitamin E on arachidonic acid metabolism. Ann. New York Acad. Sci. **393**: 376-391, 1982.

418. Dupont J. Essential fatty acids and prostaglandins. Prev. Med. **16**: 485-492, 1987.

419. Cheung WY. Calmodulin plays a pivotal role in cellular regulation. SCI. **207**: 19-27, 1980.

420. De Carvalho MS, McCormack FX and Leslie CC. The 85-kDa, arachidonic acid-specific phospholipase A_2 is expressed as an activated phosphoprotein in Sf9 cells. Arch. Biochem. Biophys. **306**: 534-540, 1993.

421. Mundy GR. Calcium and cancer. Life Sci. **23**: 1735-1744, 1978.

422. Hill SE, Rees RC and MacNeil S. 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, 1990.

423. Wright S. Essential fatty acids and the skin. Br. J. Dermatol. **125**: 503-515, 1991.

424. Stoll KE and Duncan JR. Phospholipase A_2 activity *in vitro* cultured cells-influence of ascorbate supplementation. J. Lipid Mediators Cell Signalling **11**: 261-266, 1995.

425. Krumhardt B and Dupont J. Rat platelet phospholipase A_2 activity and thromboxane synthesis are concomitantly affected by dietary linoleic acid. J. Nutr. Biochem. **2**: 443-447, 1991.

426. Dennis EA. Regulation of eicosanoid production: role of phospholipases and inhibitors. Biotechnol. **5**: 1294-1300, 1987.

427. Hendrickse CW, Radely S, Donovan IA, Keighley MRB and Neoptolemos JP. Activities of phospholipase A_2 and diacylglycerol lipase are increased in human colorectal cancer. Br. J. Surg. **82**: 475-478, 1995.

428. Guthridge CJ, Stampfer MR, Clark MA and Steiner MR. Phospholipase A_2 in ras-transformed and immortalised human mammary epithelial cells. Cancer Lett. **86**: 11-21, 1994.

429. Flower RJ and Blackwell GJ. The importance of phospholipase-A_2 in prostaglandin biosynthesis. Biochem. Pharmacol. **25**: 285-291, 1976.

430. Fraizer LW and Yorio T. Eicosanoids: Their function in renal epithelia ion transport [43503A]. P.S.E.B.M. **201**: 229-243, 1992.

431. Nemenoff RA, Winitz S, Qian N-X, Van Putten V, Johnson GL and Heasley LE. Phosphorylation and activation of a high molecular weight form of phospholipase A_2 by p42 microtubule-associated protein 2 kinase and protein kinase C. J. Biol. Chem. **268**: 1960-1964, 1993.

432. Rustenbeck I and Lenzen S. Regulation of transmembrane ion transport by reaction products of phospholipase A_2. II. Effects of arachidonic acid and other fatty acids on mitochondrial Ca^{2+} transport. Biochem. Biophys. Acta **982**: 147-155, 1989.

433. Clarke JM and Switzer RL. Photometry. p12-13 in Experimental Biochemistry. WH Freeman and Co., New York, 1977.

434. Borrello S, Minotti G, Palombini G, Grattagliano A and Galeotti T. Superoxide-Dependant lipid peroxidation and vitamin E content of microsomes from hepatomas with different growth rates. Arch. Biochem. Biophys. **238**: 588-595, 1985.

435. Cheeseman KH, Emery S, Maddix SP, Slater TF, Burton GW and Ingold KU. Studies on lipid peroxidation in normal and tumour tissues. Biochem. J. **250**: 247-252, 1988.

436. Boyington JC, Gaffney BJ and Amzel LM. The three-dimensional structure of an arachidonic acid 15-lipoxygenase. SCI. **260**: 1482-1486, 1993.

437. Gao X and Honn KV. Biological properties of 12(s)-HETE in cancer metastasis. p439-444 in Advances in Prostaglandin, Thromboxane, and Leukotriene Research. (B Samuelsson ed) Raven Press, New York, 1995.

438. Yamamoto S. Mammalian lipoxygenases: molecular structures and functions. Biochim. Biophys. Acta **1128**: 117-131, 1992.

439. Maccarrone M, Veldink GA, Vliegenthart JFG and Agrò AF. Inhibition of soybean lipoxygenase-1 by chain-breaking antioxidants. Lipids **30**: 51-54, 1995.

440. Mayatepek E and Hoffman GF. Leukotrienes: Biosynthesis, metabolism, and pathophysiological significance. Pediatric Res. **37**: 1-9, 1995.

441. Soberman RJ. 5- and 15(ω-6)-Lipoxygenases from human polymorphonuclear leucocytes. p344-349 in Methods in Enzymology. (JN Abelson and MI Simon eds) Academic Press, New York. **163**, 1988.

442. Furukawa M, Yoshimoto T, Ochi K and Yamamoto S. Studies on arachidonic acid 5-LOX of rat basophilic leukemia cells. Biochim. Biophys. Acta **795**: 458-465, 1984.

443. Noguchi M, Miyano M, Matsumoto T and Noma M. Human 5-lipoxygenase associates with phosphatidylcholine liposomes and modulates LTA_4 synthetase activity. Biochem. Biophys. Acta **1215**: 300-306, 1994.

444. Radmark O, Zhang Y-Y, Hammarberg T, Lind B, Hamberg M, Brungs M, Steinhilber D and Samuelsson B. 5-Lipoxygenase: structure and stability of recombinant enzyme, regulation in mono mac 6 cells. p1-9 in Advances in Prostaglandin, Thromboxane, and Leukotriene Research. (B Samuelsson ed) Raven Press, New York. **23**, 1995.

445. Rouzer CA and Samuelsson B. The importance of hydroperoxide activation for the detection and assay of mammalian 5-lipoxygenase. FEBS **204**: 293-296, 1986.

446. Rouzer CA and Kargman S. Translocation of 5-lipoxygnesase to the membrane in human leucocytes challenged with ionophore A23187. J. Biol. Chem. **263**: 10980-10988, 1988.

447. Natsue K, Ueda N, Yamamoto S, Komatsu N and Watanabe K. Arachidonate 5-lipoxygenase of porcine pancreas: its localization in acinar cells. Biochim. Biophys. Acta **1085**: 241-247, 1991.

448. Hansson G, Malmsten C and Rådmark O. The Leukotrienes and other lipoxygenase products. p127-167 in Prostaglandins and related substances. (C Pace-Asciak and E Granström eds) Elsevier Science Publishers, Amsterdam, 1983.

449. Jakobsson P-J, Larsson P, Feltenmark S, Odlander B, Runarsson G, Björkholm M and Claesson H-E. The 5-lipoxygenase pathway in normal and malignant human B lymphocytes. p293-298 in Advances in Prostaglandin, Thromboxane, and Leukotriene Research. (B Samuelsson ed) Raven Press, New York, 1995.

450. Goetzl EJ. Vitamin E modulates the lipoxygenation of arachidonic acid in leucocytes. Nature **288**: 183-185, 1980.

451. Smith WL and Lands WEM. Oxygenation of unsaturated fatty acids by soybean lipoxygenase. J. Biol. Chem. **247**: 1038-1047, 1972.

452. Buckman DK, Hubbard NE and Erickson KL. Eicosanoids and linoleate-enhanced growth of mouse mammary tumour cells. Prostaglandins Leukot. EFAs **44**: 177-184, 1991.

453. Fujita T, Fujimoto Y and Tanioka K. Antioxidant effects on prostaglandin synthesis in rabbit kidney medulla slices. Experientia **38**: 1472, 1982.

454. Yamamoto S. Purification and assay of PGH synthase from bovine seminal vesicles. p55-72 in Methods in Enzymology. (WEM Lands and WL Smith eds) Academic Press, New York. **86**, 1982.

455. Vajreswari A and Narayanareddy K. 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, 1992.

456. Powell WS. Rapid extraction of oxygenated metabolites of archidonic acid from biological samples using octadecyclsilyl silica. Prostagland. **20**: 947-957, 1980.

457. Powell WS. High-pressure liquid chromatography in the analysis of arachidonic acid metabolites. p75 in Prostaglandins and related substances. A practical approach. (C Benedetto, S Nigam, RG McDonald-Gibson and TF Slater eds) Irls Press, Oxford, 1987.

458. Nigan S. Extraction of eicosanoids from biological samples. p45-52 in Prostaglandins and related substances. A practical approach. (C Benedetto, S Nigam, RG McDonald-Gibson and TF Slater eds) Irls Press, Oxford, 1987.

459. Prostaglandin E_2 (^{125}I) assay kit. Code RPA 530. Amersham International, plc., UK.

460. Elattar TMA and Lin HS. Effect of vitamin C and vitamin E on prostaglandin synthesis by fibroblasts and squamous carcinoma cells. Prostaglandins Leukot. EFAs **47**: 253-257, 1992.

461. Fulton AM and Heppner GH. Relationship of PGE and natural killer sensitivity to metastatic potential in murine mammary adenocarcinomas. Cancer Res. **45**: 4779-4784, 1985.

462. Prostaglandin D_2 [^3H] assay Kit. Code TRK 890. Amersham International, plc., UK.

463. Prostaglandin F_2 [^3H] assay Kit. Code TRK 900. Amersham International, plc., UK.

464. 6-Keto-Prostaglandin $F_{1\alpha}$ enzymeimmunoassay (EIA) Kit. Code RPN 221. Amersham International, plc., UK.

465. Fitzpatrick FA, Stringfellow DA, Maclouf J and Rigaud M. Analytical methods that reveal prostacyclin synthesis by cell cultures: Glass capillary gas chromatography with electron capture detection. p55-64 in Prostacyclin. (JR Vane and S Bergström eds) Raven Press, New York, 1979.

466. Chan AC and Hamelin S st-J. The effects of vitamin E and corn oil on prostacyclin and TBX B_2 synthesis in rats. Ann. New York Acad. Sci. **393**: 201-202, 1982.

467. Chan AC, Pitchard ET and Choy PC. Differential effects of dietary vitamin E and antioxidants on eicosanoid synthesis in young rabbits. J. Nutr.**113**: 813-819, 1983.

468. Kozawa O, Tokuda H, Suzuki A, Kotoyori J, Ito Y and Oiso Y. Effect of glucocorticoid on prostaglandin $F_{2\alpha}$-induced prostaglandin E_2 synthesis in osteoblast-like cells: inhibition of phosphoinositide hydrolysis by phopholipase C as well as phospholipase A_2. Eur. J. Endocrinol. **131**: 510-515,1994.

469. MacNeil S, Walker SW, Senior HJ, Pollock A, Brown BL, Bleehen SS, Munro DS and Tomlinson S. Calmodulin activation of adenylate cyclase in the mouse B16 melanoma. Biochem. J. **224**: 453-460, 1984.

470. Otten J, Johnson GS and Pastan I. Regulation of cell growth by cyclic adenosine 3',5'-monophosphate. J. Biol. Chem. **247**: 7082-7087, 1972.

471. Salomon Y. Adenylate cyclase assay. p35-55 in Advances in Cyclic Nucleotide Research. (G Brooker, P Greengard and GA Robinson eds) Raven Press, New York. **10**, 1979.

472. cAMP (^{3}H) assay kit. Code TRK 432. Amersham International plc., England.

473. Chatterjee SK and Kim U. Adenosine-3',5'-cyclic monophosphate levels and adenosine-3',5'-cyclic monophosphate phosphodiesterase activity in metastasizing and non metastasizing rat mammary carcinomas. J. Natl. Cancer Inst. **54**: 181-186, 1975.

474. Perkins JP, Macintyre EH, Riley WD and Clark RB. Adenyl cyclase, phosphodiesterase and cyclic AMP dependant protein kinase of malignant glial cells in culture. Life Sci. **10**: 1069-1080, 1971.

475. Elks ML and Manganiello VC. Selective effects of phosphodiesterase inhibitors on different phosphodiesterases, adenosine 3',5'-cyclic monophosphate metabolism, and lipolysis in 3T3-L1 adipocytes. J. Endocrinol. **115**: 1262-1268, 1984.

476. Thompson WJ, Brooker G, Appleman MM. Assay of cyclic nucleotide phosphodiesterase with radioactive substrates. p205-213 in Methods in enzymology. (JG Hardman, and BW O'Malley eds) Academic Press, New York. **38**, 1974.

477. Ames BN. Dietary carcinogens and anticarcinogens. SCI. **221**: 1256-1263, 1983.

478. Bégin ME, Ells G and Horrobin DF. Polyunsaturated fatty acid-induced cytotoxicity against tumour cells and its relationship to lipid peroxidation. J. Natl. Cancer Inst. **80**: 188-194, 1988.

479. Gavino VC, Miller JS, Ikharebha SO, Milo GE and Cornwell DG. Effect of polyunsaturated fatty acids on lipid peroxidation in tissue cultures. J. Lipid Res. **22**: 763-769, 1981.

480. Morisaki N, Sprecher H, Milo GE and Cornwell DG. Fatty acid specificity in the inhibition of cell proliferation and its relationship to lipid peroxidation and prostaglandin biosynthesis. Lipids **17**: 893-899, 1982.

481. Cheeseman KH, Collins M, Proudfoot K, slater TF, Burton GW, Web AC and Ingold KU. Lipid peroxidation in normal and tumour tissues. The novikoff rat liver tumour. Biochem. J. **235**: 507-514, 1986.

482. Slater TF, Cheesman KH, Benedetto C, Collins M, Emery S, Maddix SP, Nodes JT, Proudfood K, Burton GW and Ingold KU. Studies on the hyperplasia (regeneration) of the rat liver, following partial hepatectomy. Changes in lipid peroxidation and general biochemical aspects. Biochem. J. **265**: 51-59, 1990.

483. Yoshikawa T, Kokura S, Tainaka K, Naito Y and Kondo M. A novel cancer therapy based on oxygen radicals. Cancer Res. **55**: 1617-1620, 1995.

484. Ottino P and Duncan JR. (*In press*) Effect of α-Tocopherol succinate on free radical and lipid peroxidation levels in BL6 melanoma cells. Free Radical Biol. Med. 1996.

485. Ottino P and Duncan JR. (*in press*) Prostaglandin levels in BL6 melanoma cells cultured *in vitro*: The effect of vitamin E succinate supplementation. Prostaglanidins Leukot. EFAs, 1996.

486. Ottino P and Duncan JR. The role of adenylate cyclase, cAMP and PGE_2 *in vitro* growth regulation of murine melanoma cells by vitamin E. Prostaglandins Leukot. EFAs **54**: 375-383, 1996.

487. Lupulescu A. Prostaglandins, their inhibitors and cancer. Prostaglandins Leukot. EFAs **54**: 83-94, 1996.

488. Ara G and Teicher BA. Cyclooxygenase and lipoxygenase inhibitors in cancer therapy. Prostaglandins Leukot. EFAs **54**: 3-16, 1996.

489. Kulmacz RJ and Lands WEM. Stoichiometry and kinetics of the interaction of prostaglandin H synthase with anti-inflammatory agents. J. Biol. Chem. **260**: 12572-12578, 1985.

490. Carter CA, Milholland RJ, Shea W and Ip MM. Effect of the prostaglandin synthetase inhibitor indomethacin on 7,12-dimethylbenz(a)anthracene-induced mammary tumourigenesis in rats fed different levels of fat. Cancer Res. **43**: 3559-3562, 1983.

491. Al-Saleem T, Ali ZS and Quassab M. Skin carriers in xeroderma pigmentosum: response to indomethacin and steroids. Lancet 2: 264-265,1980.

492. Santoro MG, Philpott GW and Jaffe BM. Inhibition of tumour growth *in vivo* and *in vitro* by prostaglandin E. Nature 263: 777-779, 1976.

493. Smith JB, Ingerman CM and Silver MJ. Malondialdehyde formation as an indicator of prostaglandin production by human platelets. J. Lab. Clin. Med. **88**: 167-172, 1976.

494. Fujimoto Y and Fujita T. Effects of lipid peroxidation on prostaglandin synthesis in Rabbit kidney medulla slices. Biochim. Biophys. Acta **710**: 82-86, 1982.

495. Mizuno K, Yamamoto S and Lands WEM. Effects of non-steroidal anti-inflammatory drugs on fatty acid cyclooxygenase and prostaglandin hydroperoxidase activities. Prostagland. **23**: 743-757, 1982.

496. LeDuc LE, Su KC, Guth E, Reedy T and Guth PH. Effects of cyclooxygenase and lipoxygenase inhibition on eicosanoids and healing of acetic acid colitis in rats. Dig. Dis. Sci. **38**: 289-294, 1993.

497. Ottino P and Duncan JR. (*in press*) The effect of vitamin E and indomethacin treatment of adenylate cyclase activity, PGE_2 and cAMP levels in murine melanoma cells. Prostaglandins Leukot. EFAs.

498. Lajtha LG. The nature of cancer. p34-54 in What we know about cancer. (RJC Harris ed) George Allen and Unwin LTD, London, 1970.

499. Gutteridge JMC. Invited review: Free radicals in disease processes: a complication of cause and consequence. Free Rad. Res. Commun. **19**: 141-158,1993.

500. Dormandy TL. In praise of lipid peroxidation. Lancet **12**: 1126-1128, 1988.

501. Gardiner NS and Duncan JR. Effect of dietary polyunsaturated fatty acid and α-Tocopherol manipulation on murine melanoma growth. Med. Sci. Res. **18**: 923-925, 1990.

502. Cillard J, Cillard P, Cormier M and Girre L. α-Tocopherol prooxidant effect in aqueous media: increased autoxidation rate of linoleic acid. J. Am. Oil. Chem. Soc. **57**: 252-255, 1980.

503. Cillard J, Cillard P, and Cormier M. Effect of experimental factors on the prooxidant behaviour of α-Tocopherol. J. Am. Oil. Chem. Soc. **57**: 255-261, 1980.