ESSENTIAL FATTY ACIDS AND ASCORBIC ACID - INTERACTIONS AND EFFECTS ON MELANOMA GROWTH.

by

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ABSTRACT

The present study was carried out to determine the effects and possible mechanisms of action of the essential fatty acids (EFAs) (linoleic acid (LA), gamma-linolenic acid (GLA) and arachidonic acid (AA)) and ascorbic acid (Asc) on BL6 murine melanoma growth in cell culture and in mice. Interactions between the nutrients in influencing melanoma growth as well as possible mechanisms of the interactions were also examined in the above systems.

Cell culture studies revealed that all three EFAs (0-50μg/ml) and Asc (0-200μg/ml) significantly inhibited melanoma growth at the concentrations used. The EFAs were also found to significantly inhibit growth, although to a lesser extent than BL6 cells, of monkey kidney (LLCMK) cells which were used as a non-malignant control cell line. Asc in contrast was found not to inhibit growth of these cells. Supplementation of Asc (100μg/ml) to EFA containing (0-50μg/ml) medium was found to significantly increase inhibition of cell growth in both cell lines, and in the BL6 cells in particular, after taking into account the growth inhibitory effects of Asc in the absence of EFAs.

The mechanism of cell growth inhibition by the EFAs appeared to involve lipid peroxidation but not enhanced prostaglandin (PG) or leukotriene (LT) synthesis. While Asc was found to increase both lipid peroxidation and PG synthesis in the cells, these mechanisms and enhanced LT synthesis did not appear to have played a role in the inhibition of cell growth by Asc or in the growth inhibitory interaction between Asc and the EFAs.

In vivo studies revealed that diets containing essential or polyunsaturated fatty acids (EFAs/PUFAs) in the form of vegetable oils, and in particular GLA in the form of evening primrose oil, significantly promoted melanoma growth in mice when compared with an EFA/PUFA free diet containing predominantly saturated fats (SF). Supplementary dietary Asc in contrast was found to significantly inhibit melanoma growth in mice fed EFA/PUFA, and in particular GLA, containing diets but not in mice fed SF containing diets. This result appears to indicate the occurrence of an interaction between the two nutrients.

The mechanism of tumour promotion by the EFAs/PUFAs did not appear to have involved enhanced PG or LT synthesis or lipid peroxidation. Since dietary EFA/PUFA manipulation was found to significantly alter the EFA content of tissues, including the melanomas, the mechanism of tumour promotion may have involved changes in the EFA composition of the tumour cells. While supplementary Asc was found to significantly increase the Asc content of certain tissues, including the melanomas, which may have played a role in tumour growth inhibition by Asc, it was found not to affect the EFA content of tissues. Enhanced PG or LT synthesis and lipid peroxidation did not appear to have been involved in the tumour growth inhibitory interaction between Asc and the EFAs/PUFAs.

THe activity of the enzyme delta-6-desaturase, a key enzyme in EFA metabolism which catalyses the desaturation of LA to GLA, and the influence of Asc on activity of the enzyme were also examined. The cultured cells, and BL6 cells in particular, were found to contain significant activity of the enzyme. Whereas murine liver microsomal fractions were found to contain delta-6-desaturase activity, microsomes from melanomas grown in mice were found to lack activity of the enzyme. The significant tumour promoting effects of the GLA containing EPO diet may have been the result of the lack of delta-6-desaturase activity in tumour cells grown in mice. Asc was found to stimulate activity of the enzyme in cultured BL6 cells but not in LLCMK cells, while dietary Asc and EFA/PUFA manipulation did not influence activity of the enzyme in microsomal fractions.

This study has confirmed previous reports of the in vivo tumour promoting effects of dietary EFAs/PUFAs and the tumour growth inhibitory effects of Asc. The in vitro cell growth inhibitory effects of Asc and the EFAs also confirm the results of previous reports. Previous studies investigating possible interactions between Asc and EFAs/PUFAs in influencing tumour cell growth could not be located in the relevant literature. This study may therefore be one of the first investigations of any such interaction between these nutrients in tumour cells. While this study was not able to identify the mechanisms involved in the different tumour promoting or tumour growth inhibitory effects of the two nutrients in the two systems, it did eliminate a number of potential mechanisms. The results of this study also emphasise the difficulty of attempting to compare the results of in vitro and in vivo studies.

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PREFACE

The role played by both the essential fatty acids (EFAs) and ascorbic acid (Asc) in cancer remains controversial. A number of conflicting reports appear in the literature, some claiming therapeutic benefit and others reporting enhanced tumour growth as a result of dietary EFA or Asc manipulation. Although a number of mechanisms whereby these nutrients exert their effects on tumour growth have been proposed on the basis of experimental evidence, the precise nature of these mechanisms remains unclear at the present time. Recently certain studies have reported possible interactions between EFAs and Asc.

This study is concerned primarily with the influence of the n-6 EFAs and Asc on murine melanoma growth. Possible interactions, their influence on tumour growth and the mechanisms of these effects and possible interactions will also be examined.

The terminology used in the literature could give rise to a certain measure of confusion. In this study the term essential fatty acid will be used when referring specifically to particular n-6 EFAs, while the term essential/polyunsaturated fatty acid (EFA/PUFA) will be used in all instances in which a mixture of both essential and non-essential PUFAs might be involved, even though the observed effects are in many instances due to the EFAs. The possible contribution of PUFAs to the observed effects cannot however be discounted, hence the use of the term EFA/PUFA. The terms ascorbic acid and vitamin C are used interchangeably in the literature. In this study only ascorbic acid will be used for clarity.

Chapter 1

LITERATURE REVIEW - ESSENTIAL FATTY ACIDS

1.1 Essential fatty acid metabolism

The naturally occurring polyunsaturated fatty acids (PUFAs) are of four groups, each arising from a particular precursor, namely linoleic acid (LA), α-linolenic acid, stearic acid or palmitic acid. LA and α-linolenic acid cannot be synthesised by man or animals and must be provided in the diet. The PUFAs arising from these precursors are thus known as essential fatty acids (EFAs) (1). This study is primarily concerned with the n-6 EFAs which are derived from LA by desaturation and chain elongation. (figure 1.1) (1).

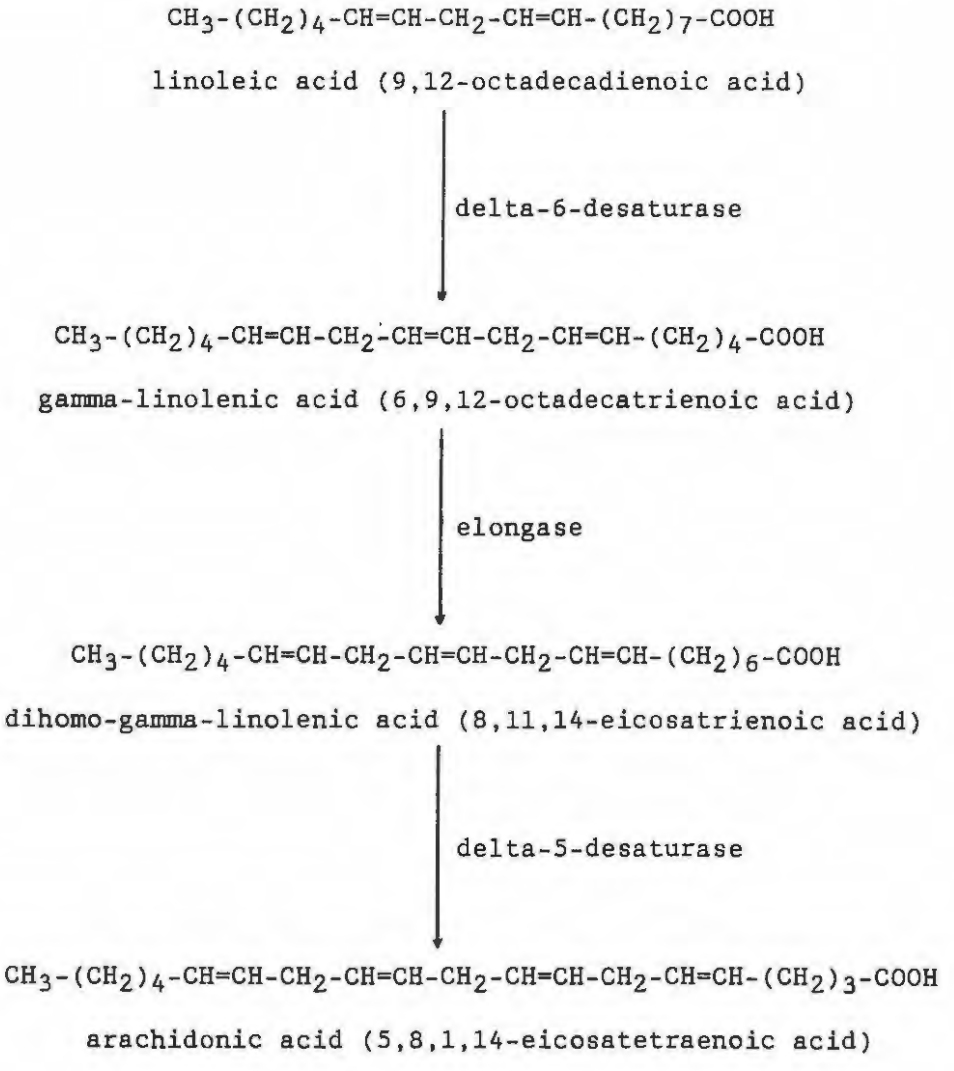

Figure 1.1. Essential fatty acid transformations (1).

All the EFAs arising from LA have been reported to possess EFA activity i.e. they can all cure EFA deficiency symptoms (1).

1.2 Early history

The essential nature of the n-6 fatty acids was first reported in 1929 by Burr and Burr (2). Deficiency symptoms induced by the rigid exclusion of fat from the diet of rats included scaliness and lesions of the tail and skin, hair loss, retarded growth, increased transepidermal water loss and renal and reproductive disorders (2). LA was found to cure the symptoms (3). In 1938 Turpeinen (4) reported that arachidonic acid (AA) was also able to cure EFA deficiency symptoms and suggested that it might be derived from LA. In 1953 Thomasson (5) developed an assay to determine the EFA status of animals which was based on disturbances in water metabolism. Using this assay he found that both gamma-linolenic acid (GLA) and dihomo-gamma-linolenic acid (DGLA) possessed EFA activity and suggested that they might be intermediates in the synthesis of AA from LA. By utilising radiolabelled precursors Mead, Howton and co-workers (6,7,8) were able to confirm the role of LA, GLA and DGLA as precursors or intermediates in AA synthesis.

Early studies also reported that the PUFA/EFA content of tissues could be altered markedly by dietary EFA manipulation. Animals reared on totally fat-deficient diets exhibited a marked decrease in tissue EFA content, while a striking increase in 5,8,11-eicosatrienoic acid tissue content was found (9,10). EFA supplementation resulted in a rapid increase in tissue EFA status and a decrease in trienoic acid levels. The trienoic acid was found to be derived from oleic acid, an endogenous PUFA, via the same pathway utilised in EFA metabolism (11). The ratio of 5,8,11-eicosatrienoic acid:arachidonic acid has subsequently been used as a measure of animal and human EFA status, a ratio of greater than 0,4 being indicative of an EFA deficiency (12,13,14).

EFA deficiency in humans was first described in 1963 by Hansen and colleagues (15) in infants fed a milk diet from which EFAs were virtually absent. Symptoms included severe skin disorders which were corrected by EFAs. Both adults and infants maintained on fat free parenteral nutrition have subsequently been reported to develope various skin disorders, the symptoms of which can be corrected by EFAs (13,14).

1.3. Dietary sources

Sufficient dietary EFAs are important in the prevention of EFA deficiency. USA recommended daily allowances suggest that 1-2% of the total energy intake should be in the form of EFAs (16). There is evidence however, particularly since most Western diets are high in non-essential PUFAs and

saturated fats (SF) which compete with EFAs in their metabolism since they utilise similar metabolic pathways, that the EFA requirement may be as high as 4% of the total energy intake (17). The major sources of EFAs in the diet are summarised in table 1.1 (16,18).

Table 1.1. Dietary sources of essential fatty acids (16,18).

EFA	source
LA	vegetable seed oils (sunflower, safflower, corn, soy), dairy products, organ meats (liver), human milk.
GLA	human milk, certain seed oils (evening primrose, borage, blackcurrant).
DGLA	organ meats (liver, kidneys), human milk.
AA	meat, egg yolks, human milk. Also found in shrimps and seaweed.

1.4 Functions

Despite the fact that the symptoms of EFA deficiency have been thoroughly delineated, the precise functions of the EFAs are still not fully defined. Consideration of deficiency symptoms has shown that many are related to failure of membrane synthesis or maintenance. One of the functions of EFAs thus appears to be a role in membrane structure and function. (19,20).

i) Membrane structure and function

Animal cell membranes appear to represent a composite of many microenviroments with either gel (rigid) or liquid crystalline (fluid) characteristics. The liquid crystalline domains are essential for normal functioning of the membrane (21). The inclusion of PUFAs in the membrane lipid bilayer decreases the temperature at which transition from gel to liquid crystalline phase occurs, thus ensuring that the membrane is in the liquid crystalline phase at physiological temperature (21,22). Since both endogenous PUFAs and EFAs are found within membranes, an absolute requirement for EFAs in maintaining membrane fluidity has not yet been shown. However a large amount of AA and a smaller amount of LA, as well as more highly unsaturated EFA metabolites, are found in biological membranes and must therefore make a contribution to the maintenance of membrane fluidity (21). This concept is supported by the retention of EFAs in membrane phospholipids even during severe EFA deficiency and the rapid replacement of any lost membrane EFAs in EFA deficient animals fed on an EFA sufficient diet (19,22). Dietary EFA manipulation has also been shown to influence

membrane fluidity (23-27). A recent study has provided evidence that platelet membrane fluidity is increased by increasing the level of dietary EFAs in humans and rats (28).

The fluid state of the membrane is important for normal functioning of membrane proteins which include enzymes, hormone receptors, transport proteins and structural proteins among others (21). Changes in membrane unsaturation, and therefore fluidity, have been reported to influence the activity of a number of these proteins including the enzymes acetylcholinesterase, adenyl cyclase, various desaturases and ATPases, the cytochrome P_{450} oxygenase system and cytochrome b_5 reductase to mention but a few (20,21,25,27,29,30). A number of transmembrane transport processes, including passive and facilitated diffusion as well as active transport, appear to be dependant on membrane phospholipid PUFA content. Membrane lipid modification has been shown to influence transport of choline, isobutyric acid, glutamate and glycine among others (21,30). Transport of water and small cations and non-electrolytes, which are transported through specific channels made up of transmembrane proteins, is also thought to be influenced by membrane lipid composition, as is transport of ions dependant on various ATPases (20).In addition membrane lipid modification has been reported to influence the binding properties of various receptors including prostaglandin, opiate, serotonin and insulin receptors (21,30). Besides their importance in membrane structure and function, the EFAs are also precursors for the prostaglandins (PGs), thromboxanes (TXs) and leukotrienes (LTs) collectively known as the eicosanoids.

ii) Prostaglandin and thromboxane biosynthesis

PGs are synthesised from EFA precursors in nearly all tissues where they act as local hormones with highly diverse activities depending on the type of PG or TX synthesised. This is in turn dependant on substrate availability and the activity of the particular PG synthesising enzymes in that tissue (16). The 2-series PGs and TXs are synthesised from AA while the 1-series PGs and TXs are synthesised from DGLA. The biosynthesis of certain 2-series PGs and TXs from AA shown in figure 1.2 (31) can be used to illustrate the pathway involved in PG and TX synthesis. PGs are rapidly degraded in the lungs and about 60% will be removed from the blood during one passage through the pulmonary circulation. They therefore have a role as local regulators of inter- and intra-cellular function in the tissues in which they are synthesised (16).

The different PGs and TXs have widely diverse and in some cases antagonistic effects. These include a role in inhibition or stimulation of platelet aggregation, vasodilation or vasoconstriction, modulation of the immune response, bronchodilation or bronchoconstriction, reproduction, smooth muscle contraction or relaxation and as mediators of the inflammatory response among others (16,32,33). It is thought that most, if not all, of the actions of PGs in biological systems are mediated via changes in the levels of cyclic nucleotides which act as second messengers for the PGs (16,32). In addition to the

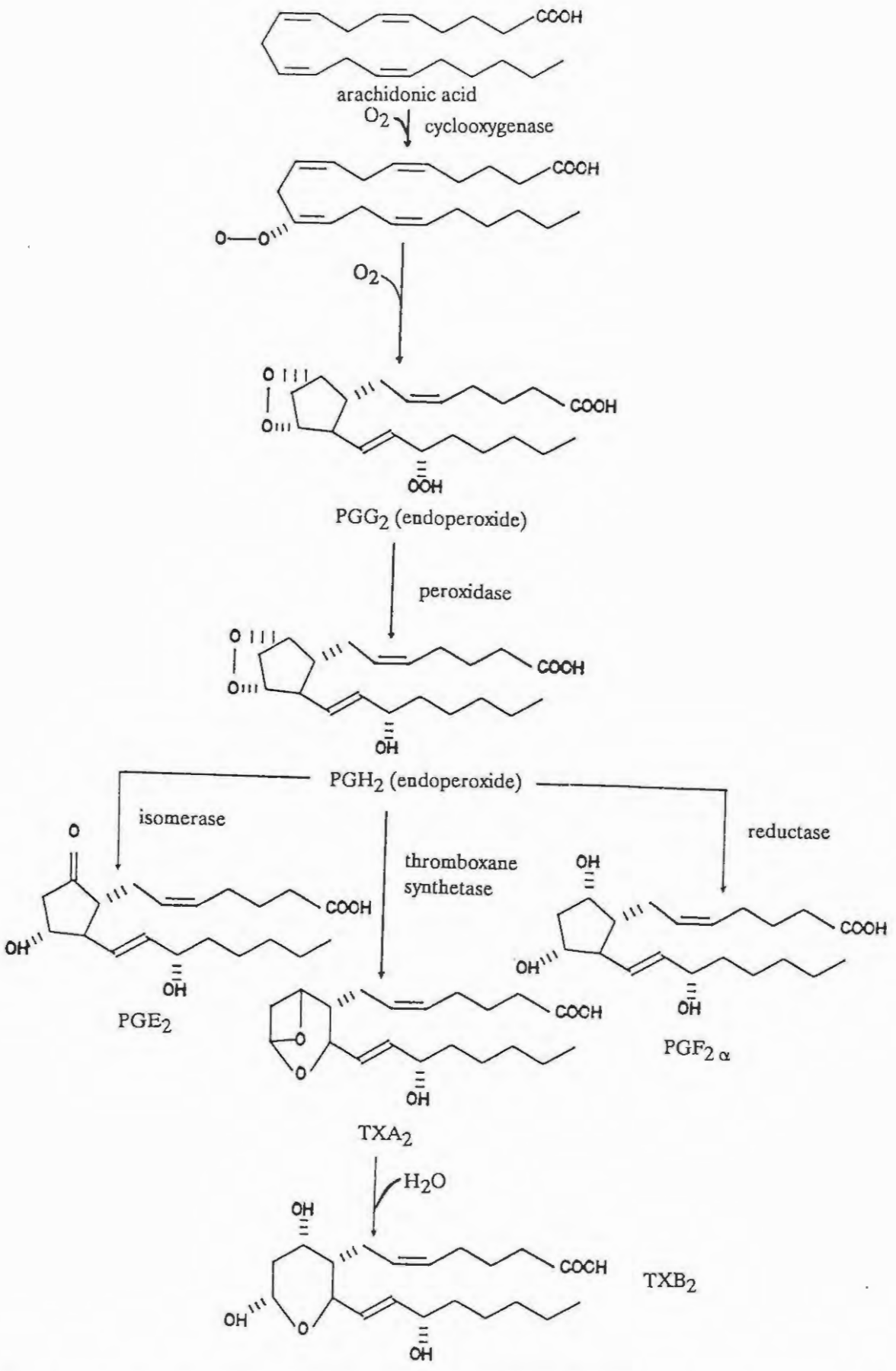

Figure 1.2. Biosynthesis of some 2-series prostaglandins and thromboxanes from arachidonic acid (31). Abbreviations : PG = prostaglandin.

abovementioned effects, PGs are also thought to be mediators of cell growth, a role which will be discussed in more detail in section 1.5. Since PG and TX synthesis can be influenced by dietary EFA manipulation (17,18,26,34-37), the EFAs could play an important role in influencing many of the effects mediated by PGs and TXs.

iii) Leukotriene and hydroxy fatty acid biosynthesis

In the same manner as the PGs, the LTs and hydroxy fatty acids synthesised from EFA precursors by tissues are dependant on substrate availability and the activity of the particular LT synthesising enzymes present in that tissue (38). The biosynthesis of certain LTs and hydroxy fatty acids via the 5-lipoxygenase pathway is illustrated in figure 1.3 (39). The LTs and hydroxy fatty acids appear to have localised effects as they are rapidly degraded in the bloodstream (38). Some of these effects include a role in smooth muscle contraction, bronchoconstriction, vasoconstriction, chemotaxis and as mediators of the immune response and anaphylaxis (31,39-41).

The EFAs thus play an important role as the precursors of the biologically active PGs, TXs and LTs. The biological mechanisms determining which pathway of EFA metabolism is activated have not yet been determined (42).

iv) Other functions.

Besides their role in membrane structure and function and as eicosanoid precursors, the EFAs also play a role in the maintenance of the epidermal water permeability barrier which prevents excessive water loss by animals, increased trans-epidermal water-loss being a well documented symptom of EFA deficiency (43-46).

Since EFAs and their metabolites play a role in many cellular processes, alterations or imbalances in EFA metabolism could have serious consequences for the health of an individual. Imbalances in the levels of EFAs or their metabolites have been recognised in several diseases and are thought to play a role in the disease process. Dietary EFA manipulation has been proposed as a form of treatment for certain of these diseases which include cardiovascular and renal diseases (16,47,48), atheroschlerosis (14), diabetes mellitus (14,16,49,50), hypothyroidism (16), arthritis (16), cystic fibrosis (14,51), dermatitis (14,16), psoriasis (16), eczema (16) and multiple schlerosis (14). Several hypotheses have proposed that imbalances of EFAs or their metabolites might play a role in acrodermatitis enteropathica (50), hyperactivity in children (52), acquired immunodeficiency syndrome (53), cirrhosis

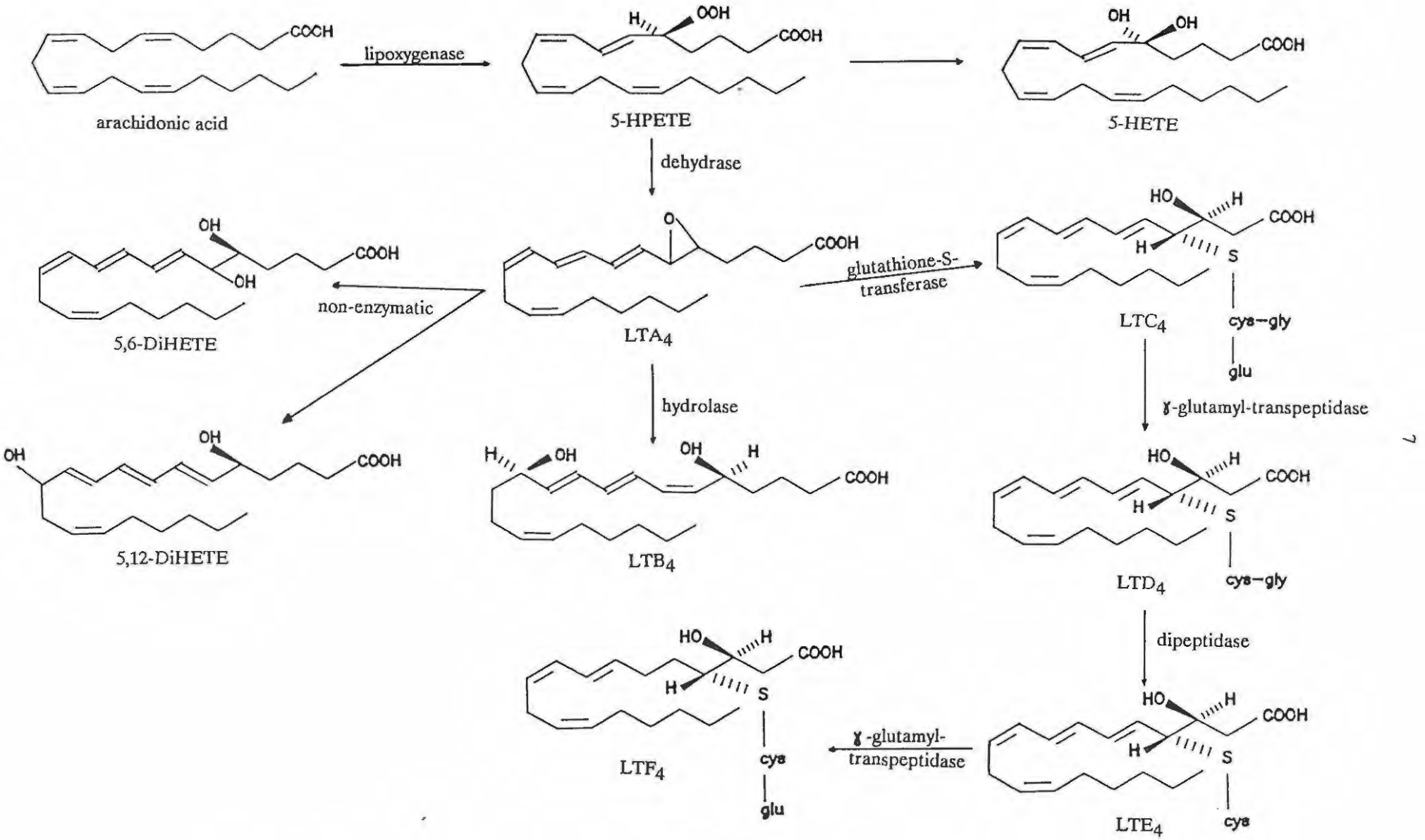

Figure 1.3. Biosynthesis of leukotrienes and hydroxy fatty acids from arachidonic acid via the 5-lipoxygenase pathway (39). Abbreviations : HETE = hydroxyeicosatrienoic acid; HPETE = hydroperoxyeicosatrienoic acid; LT = leukotriene.

of the liver (54), sickle cell anaemia (16) and alchoholism (55). Of particular interest to this study is the role played by EFAs and their metabolites in cancer which will be discussed in more detail.

1.5 ESSENTIAL/POLYUNSATURATED FATTY ACIDS AND CANCER

Much of the evidence relating to dietary fat and cancer has been reviewed by the USA National Research Council Committee on Diet, Nutrition and Cancer (56), who concluded that experimental and epidemiological evidence was most suggestive of a causative relationship between dietary fat and cancer. Although PUFAs, including EFAs, do not cause cancer, a relationship between increased cancer incidence and elevated dietary fat has been demonstrated by several studies (57,58). Both the amount and/or type of fat have been shown to influence carcinogenesis (59) although the influence, if any, is also dependant on the type of cancer (57).

1.5.1. Influence of polyunsaturated/essential fatty acids on in vivo tumour growth.

i) Animal studies

The relationship between dietary fat and cancer has been known for some time. In 1930 Watson and Mellanby (60) demonstrated increased growth of tar induced tumours in rats fed high fat diets. In 1939 Bauman and Rausch (61) reported that dietary fat enhanced the incidence of skin cancer in rats after exposure to UV light. In a number of studies in the 1940s, Tannenbaum (62,63) reported enhanced incidence and growth of spontaneous mammary tumours and benzpyrene induced skin tumours in rats as a result of a high fat diet. Subsequent studies have demonstrated a relationship between increased dietary fat and a number of types of cancer, and in particular mammary gland cancer and cancer of the skin, colon and prostate (56). A more comprehensive list of evidence indicating a possible relationship between dietary PUFAs/EFAs and cancer in animals is summarised in table 1.2.

In general it can be seen from table 1.2 that diets high in PUFAs/EFAs, and in particular LA which is a major component of many oils such as corn, sunflower and safflower oil, promote tumour growth in experimental animals. It can also be seen however that the effect, if any, is dependant on the type of tumour. More recently diets containing high levels of SF have been shown by Carroll and colleagues to promote tumour growth, but only when some PUFAs in the form of EFAs are present in the diet (84,87,93). From these results it has been proposed that once the PUFA/EFA requirement of the tumour is met, further enhancement of tumourigenesis is dependant on the level and not the type of dietary fat (81). It is interesting to note that recent studies have reported decreased tumour growth in animals fed high fish oil diets, the main constituent of which are n-3 EFAs (83,94,95).

Table 1.2. Experimental evidence relating dietary polyunsaturated fatty acids to cancer in experimental animals.

cancer type	species	PUFA source	effect	references
i) spontaneous tumours				
mammary	mouse	COO	↑	65
	rat	COO + SNO	↑	66
reticuloendothelial	rat	COO + SNO	↓	66
adrenocortical	rat	COO + SNO	↓	66
ii) transplantable tumours				
mammary	mouse	COO	↑	64,69-72
adenocarcinoma	mouse	SNO	↑	73
	mouse	pure LA	↑	70
	rat	COO	↑	64
	rat	COO + SNO	NE	67
	rat	EPO	↓	68
human mammary	mouse	COO	↑	74
murine melanoma	mouse	SFO	↑	57
human hepatoma	rat	EPO	NE	75
human melanoma	mouse	CO	NE	74
ascites	mouse	pure LA	↓	77
	rat	pure LA or AA	↓	78
leukemia	mouse	SNO	↑	79
	rat	COO + SNO	NE	67
carcinoma	rat	COO + SNO	NE	67
iii) carcinogen induced tumours				
DMBA mammary	rat	COO	↑	80,81,83
	rat	SFO	↑	82
	rat	EPO	↑	82
	rat	SNO	↑	83,84,87
	rat	OO	↑	85
	rat	EPO	NE	86
	rat	ethyl linoleate	↑	87
DMBA skin	mouse	COO	↑	88
BP skin	rat	CSO	↑	62
AAF mammary	rat	lard	↑	89
DMH colon	rat	COO	↑	90
AOM colon	rat	SFO	↑	91
	rat	COO	↑	91
DES mammary	rat	COO	↑	92
MCA skin	mouse	pure LA	↑	76

Abbreviations used: COO = corn oil; SNO = sunflower oil; EPO = evening primrose oil; SFO = safflower oil; OO = olive oil, CSO = cottonseed oil; DMBA = 7,12-dimethylbenzanthracene; AAF = 2-acetylaminofluorine; DMH = 1,2-dimethylhydrazine; AOM = azoxymethane; BP = benzpyrene; DES = diethylstilbesterol; MCA = methylcholanthracene. ↑ = increased growth; ↓ = decreased growth; NE = no effect.

ii) Epidemiological studies

Epidemiological data on human populations has indicated a strong positive correlation between high dietary fat intake and breast cancer mortality in particular (56,81,96,97). Further examination of the data shows a strong positive correlation with total fat and animal fat intake, but little or no apparent correlation with vegetable fat intake. Since vegetable fats generally contain higher levels of PUFAs/EFAs than animal fat, this data does not appear to be in agreement with data from animal experiments. An explanation of this apparently contradictory data may be provided by the previously mentioned studies of Carroll and colleagues (84,87,93). The small amount of PUFAs/EFAs required for enhancement of tumour promotion would probably be provided by the normal diet of most populations, particularly since in countries with a low fat intake, a larger proportion is derived from plant sources (81,96). Any additional fat whether animal or vegetable could thus lead to promotion of tumour growth.

The epidemiological studies linking dietary fat and cancer have been critisised by some researchers. They have cited other dietary factors such as trans fatty acids, increased caloric intake and low fibre diets, rather than increased dietary fat intake as causes of the increased tumour incidence found in some populations (98,99).

1.5.2. Mechanisms of in vivo tumour growth promotion

A number of mechanisms have been proposed to account for the tumour promoting effects of dietary PUFAs/EFAs. These include altered PG synthesis, enhanced formation of lipid peroxides, changes in membrane lipid composition and fluidity, alterations in intercellular communication, hormonal effects, caloric effects and suppression of the host immune system (100). PUFAs/EFAs may also play an indirect role in initiation of carcinogenesis by triggering cooxygenation of various carcinogens, thus activating them, by cyclooxygenase or lipoxygenase action (101-104).

i) Prostaglandin synthesis

Although it is generally accepted that PGs have an active role in cell proliferative processes in normal tissues, their precise role in the growth and development of tumours remains unclear. Two themes are found in the literature, the first proposing that the increased PG production observed in tumour bearing animals and humans is involved in initiation and enhancement of tumour growth, and the second proposing that increased PG production is part of a homeostatic response directed toward limitation of tumour growth (105).

Several studies have provided evidence linking PGs to enhanced growth and development of tumours.

Synthesis of PGs, and in particular PGE_2, has been reported to be greatly elevated in humans and animals bearing a wide variety of tumours (105-114). In addition many tumour promoters, such as the phorbol esters, stimulate PG synthesis (101,102,105-108,114),while PGs have also been reported to reduce the latent period of tumour induction by carcinogens (118). Increased PG synthesis is also thought to play a role in the hypercalcaemia, osteolysis and metastatic spread of cancer (101,105,107,114). Since increased levels of dietary EFAs have been reported to enhance synthesis of PGs (17,18,26,34-37), the effects of EFAs on tumour growth might be mediated by increased PG synthesis. Inhibitors of PG synthesis, such as indomethacin and ibuprofen, have in fact been reported to reverse the tumour promoting effects of high fat diets (101,105,106,112,115-117).

The mechanism whereby PGs enhance tumour growth is thought to involve immunosuppression. While PGs appear to play a role in the normal homeostatic control of the immune response (119), increased PG production in tumour bearing animals is thought to play a role in the immunosuppression observed in these animals thus providing a mechanism whereby the tumour could escape the immune surveillance mechanisms of the host (108,114). PGs have been reported to inhibit lymphocyte proliferation and lymphocyte mediated tumour cell cytolysis, to inhibit lymphokine and antibody production, and to inhibit macrophage and natural killer cell cytotoxicity toward tumour cells (107,112,119-123). PG synthesis inhibitors have also been found to enhance both cellular and humoral immune responses (107,110,122,123), while immunization of animals against PGE_2 has been reported to enhance immunity in tumour bearing mice (115,124). In addition to their effects on the host immune response, PGs have been reported to promote tumour angiogenesis (125).

The existence of evidence supporting a role for PGs in the inhibition of tumour growth must also be noted however. PG mediated tumour growth inhibition as well as anti-metastatic activity of various PGs and PG analogues have been reported (107,126-132). Treatment of animals with PG synthesis inhibitors has also been shown to increase growth of transplantable tumours in mice (132,133). Proponents of the anti-cancer role of PGs propose that the elevated PG levels observed in tumour bearing animals are produced as part of a homeostatic mechanism on the part of the host animal to reduce tumour growth. (105).

ii) Membrane lipid modification and lipid peroxidation

Tumour cell membranes are reported to contain less PUFAs/EFAs than normal cell membranes and will thus be less fluid than those of normal cells (93,134-136). These changes in membrane lipid composition are thought to play a role in the tumourigenic process, since changes in membrane fluidity are known to influence activity of certain membrane enzymes, hormone receptor availability and transmembrane transport processes. Tumour cell antigen availability could also be affected, allowing the tumour cell to escape immune surveillance by the host (93,137). The fatty acid composition of

tumour cells and their membranes can be modified by dietary PUFA/EFA manipulation, as tumour cell membranes are reportedly enriched in PUFAs/EFAs by feeding animals diets high in PUFAs/EFAs (93,138). The physical and physiological functions of the tumour cell membrane could thus be altered by dietary PUFA/EFA manipulation leading to changes in the growth of tumour cells. A related effect is lipid peroxidation which involves free radical attack on biological membranes leading to oxidative destruction of PUFAs/EFAs. Peroxidation of PUFAs/EFAs is a feature of many types of cell injury in which free radicals are produced in excess of local defence mechanisms (139). The PUFA/EFA substrates do not have to be in the free carboxylate form but can also undergo peroxidation while esterified as in membrane phospholipids (139). All PUFAs/EFAs share a common structure (figure 1.4) which facilitates the initiation of lipid peroxidation (144). The presence of an adjacent double bond weakens the carbon-allylic hydrogen bonds. These allylic hydrogens, especially those on the carbon atom between two double bonds, are thereby partially activated towards abstraction by reactive chemical species containing one or more unpaired electrons (free radicals) (144). Susceptibility to peroxidation is thus increased by increasing unsaturation.

The reactions of lipid peroxidation (schematically represented in figure 1.5) may be categorised as initiation, propagation and termination (144). Initiation is the reaction of the PUFA/EFA side chain of a lipid with a reactive free radical to form a lipid radical, which then reacts with molecular oxygen forming a peroxylipid radical. Propagation is the reaction of a peroxylipid radical with another PUFA/EFA side chain of a lipid to form a lipid hydroperoxide and lipid radical. The number of free radicals in the reaction sequence, which can then react with further PUFAs/EFAs, is thus conserved. Termination is the removal of free radicals, where two radicals combine to yield a non-radical product, thus ending the chain reaction. The lipid hydroperoxide formed during propagation can decompose, especially in the presence of transition metals, to yield a number of breakdown products including alkoxy radicals, peroxyl radicals, malondialdehyde and hydroxy alkenals to mention but a few (139,144). These reactions thus generate more reactive free radicals. It has been suggested that PUFA/EFA derivatives, and in particular lipid peroxides and their breakdown products, might play a role in the promotion of tumourigenesis by high fat diets (93,100,139).

One of the characteristic biochemical changes known to occur in tumours is a significant reduction in lipid peroxidation when compared with normal tissue of the same origin (135,136,140,141). This is particularly surprising considering that the actvity of enzymes which protect against oxygen free radical attack, namely superoxide dismutase and glutathione peroxidase, has been reported to be reduced in tumour cells (134). A number of mechanisms have been proposed to explain this phenomenon. One mechanism is based on the reduced PUFA/EFA content of tumour cell membranes which would result in reduced lipid peroxidation due to decreased substrate availability (134,135,140,142,143). High PUFA/EFA diets, by increasing membrane PUFA/EFA content, would thus increase substrate availability for lipid peroxidation. A second mechanism is based on the observed reduction in the

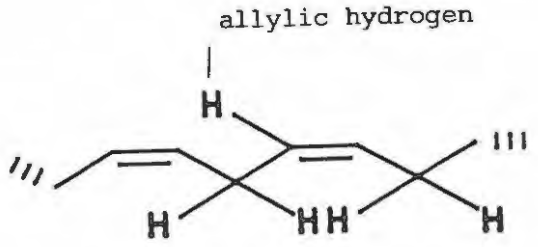

Figure 1.4. A common structural feature of polyunsaturated fatty acids which facilitates the initiation of lipid peroxidation. The presence of two adjacent double bonds activates the allylic hydrogen for abstraction by free radicals (144).

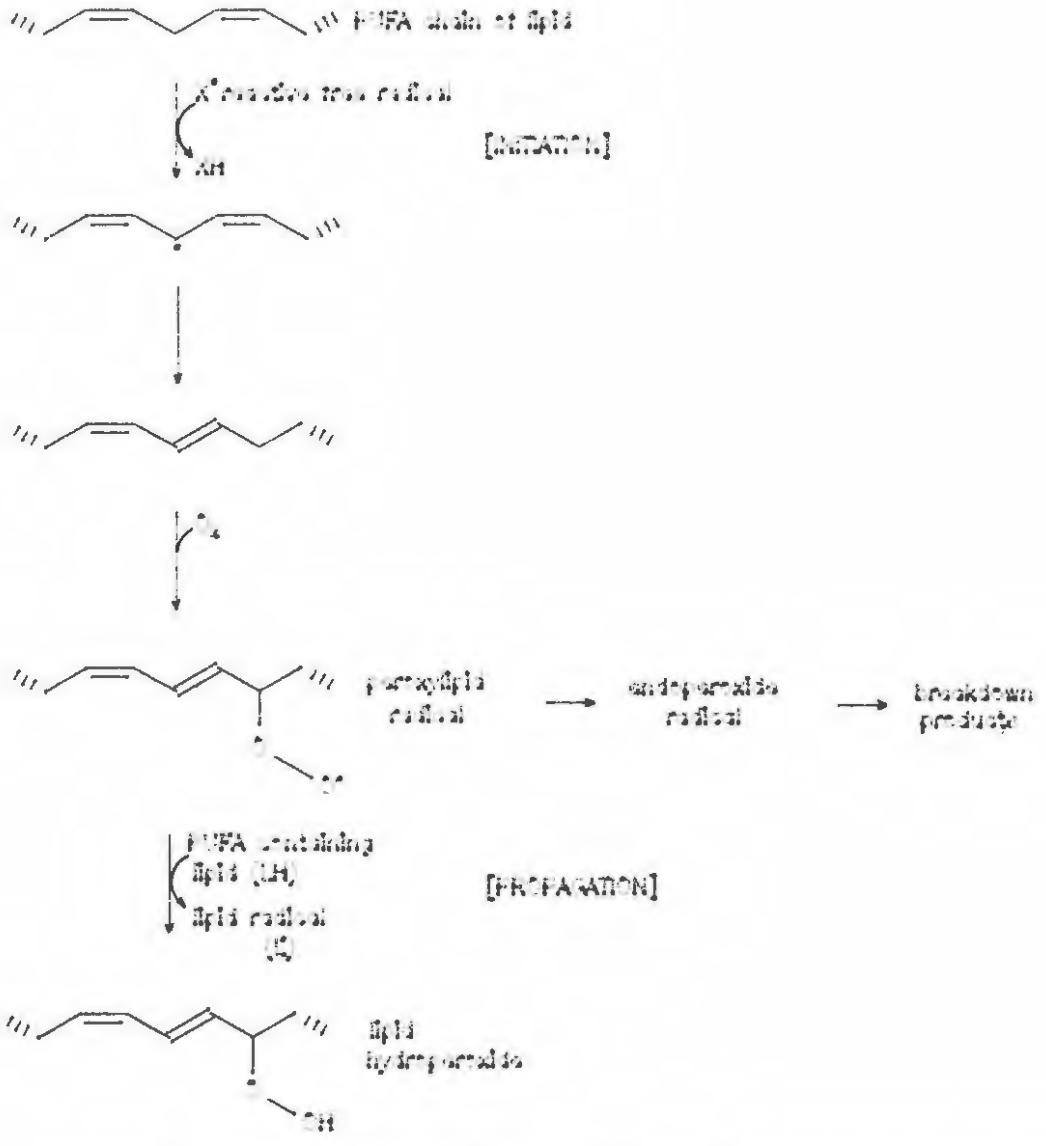

Figure 1.5. The formation of lipid radicals, peroxylipid radicals and lipid hydroperoxides in the sequence of reactions that constitute lipid peroxidation. Most of the peroxylipid radical will abstract a hydrogen atom from another PUFA giving rise to a hydroperoxide and another lipid radical. Some peroxylipid radicals will form endoperoxides which then break down to short chain products generating more free radicals (144).

activity of certain enzyme systems in tumour cells, including the NADPH-cytochrome P_{450} and NADPH-cytochrome c reductase electron transport chains, which are known to be involved in initiation and propagation of lipid peroxidation (136,142,143). A third mechanism is based on reports that tumour cell membranes contain increased levels of the lipid soluble anti-oxidant α-tocopherol, which is an effective scavenger of lipid peroxy and oxygen radicals (136,140,142). Lipid peroxidation has been implicated in promotion of tumourigenesis since anti-oxidants such as α-tocopherol and butylated hydroxytoluene have been reported to inhibit 7,12-dimethylbenzanthracene (DMBA) induced mammary carcinogenesis in animals fed high PUFA/EFA diets (100). More recently the hydroperoxy and hydroxy auto-oxidation breakdown products of LA and AA have been shown to stimulate DNA synthesis in cells (100). The mechanism whereby lipid peroxidation influences tumour growth is at this stage unclear. In membranes undergoing lipid peroxidation, free radical chain termination reactions can result in lipid-lipid, lipid-protein and protein-protein crosslinking leading to decreased membrane fluidity and associated effects (see section 1.4) which may influence the metabolism and growth of tumours (144). Certain of the breakdown products of lipid peroxides, such as the 4-hydroxyalkanals which cause similar effects to lipid peroxides, can reportedly diffuse from the cell membrane thus transmitting the effects of lipid peroxidation throughout the cell (139,144). In addition to the plasma membrane, membranes of subcellular organelles such as mitochondrial, lysosomal, endoplasmic reticulum and nuclear membranes can also undergo lipid peroxidation thereby influencing the functioning of these organelles (142). These effects of lipid peroxidation are thought to play a role in the promotional stage of carcinogenesis by some as yet unknown mechanism. (145).

The validity of this concept has been questioned by several researchers however. It has been suggested that the observed effects of anti-oxidants on carcinogen-induced tumourigenesis might have been the result of modification of carcinogen metabolism rather than of lipid peroxidation, since butylated hydroxytoluene has been reported not to influence methylnitrosourea induced tumour growth (100) and methylnitrosourea, unlike DMBA, does not require metabolic activation. Hydroxypentanal, a breakdown product of lipid peroxidation, has also been reported to inhibit growth of certain murine tumours (139). In addition diets containing menhaden (fish) oil, which is rich in long chain PUFAs/EFAs (eicosapentaenoic and docosahexaenoic acids) which are very susceptible to lipid peroxidation, have been reported to inhibit DMBA induced mammary tumour growth in rats (100).

iii) Caloric consumption

High caloric intake has been identified as a risk factor in certain types of cancer and since high fat diets are also high in calories, this may provide a mechanism whereby these diets promote tumourigenesis. Human epidemiological studies have provided a positive correlation between breast, colon and prostrate cancer mortality and both increased dietary fat and caloric intake (100,146,147). However it is difficult to distinguish between the effects of dietary fat or caloric consumption in human

populations since the diet cannot be manipulated as readily as with experimental animals (146). High fat, high calorie diets have been found to promote carcinogen induced tumourigenesis in experimental animals (89,146,147), while reduced tumour growth has been reported in animals whose food intake was restricted when compared with animals fed unrestricted diets (92,147). However the animals fed the restricted diets also had reduced body weights which might have contributed to the observed effects. Restrictions of other dietary components could also have influenced tumour growth (147). Studies in which animals were fed identical, isocaloric diets containing either high levels of PUFAs/EFAs or SF, reported that the PUFA/EFA containing diets promoted tumourigenesis when compared with the SF containing diets. These results suggest that a specific effect of PUFAs/EFAs rather than a general caloric effect is responsible for the increased tumour growth observed in animals fed high PUFA/EFA diets (57,100,146,147).

iv) Immunoinhibition

A number of studies have provided evidence that the concentration and degree of saturation of dietary fat may exert an influence on the immune response and on lymphocyte function in particular. The immune response has been reported to be suppressed in EFA deficient mice, while supplementation of PUFAs/EFAs to the diet resulted in immunostimulation. Further PUFA/EFA or SF supplementation resulted in immunosuppression (57,148,149). It would therefore appear that while PUFAs, and in particular EFAs, are required for normal functioning of the immune system, high levels of dietary fat inhibit the immune system. Reported effects of high levels of dietary PUFAs/EFAs on the immune response include inhibition of lymphocyte transformation, proliferation and cytotoxicity and inhibition of the reticuloendothelial system (which is thought to be associated with decreased immunoglobin G synthesis) among others (57,93,100,119,148-152).

The mechanisms proposed for the immunoinhibitory effects of PUFAs/EFAs include enhanced PG production (already discussed) and changes in membrane lipid composition and fluidity and the effects associated with these changes (149,151,152). Lymphocyte mediated cytolysis requires direct contact between tumour antigens and antigen receptors located on the lymphocyte membrane, the number and distribution of which could be influenced by changes in membrane fluidity (119,151). Recent studies have provided evidence that changes in lymphocyte membrane lipid composition rather than in tumour membrane lipids appear to be responsible for the decreased lymphocyte cytotoxicity observed in animals fed PUFA/EFA rich diets (149,151,152).

In contrast to these reports, certain studies using experimental animals have reported that diets high in PUFAs/EFAs do not influence the immune response, including lymphocyte proliferation and complement mediated tumour cell cytolysis (153,154).

v) Hormonal mechanism

A number of studies have provided evidence which suggests that high levels of dietary PUFAs/EFAs promote mammary tumourigenesis in particular, by stimulating the endocrine system. Secretion of prolactin by the pituitary gland and of oestrogens by the ovaries are reportedly stimulated in rats fed high PUFA/EFA diets (100,138,155). Increased serum prolactin and oestrogen levels have been reported in carcinogen treated animals (87), while anti-oestrogen and anti-prolactin drugs have been shown to diminish tumour incidence in animals fed high PUFA/EFA diets (96).

Contradictory evidence has also been provided by a number of studies however. Serum prolactin and oestrogen levels were shown to be unchanged in normal and carcinogen treated animals fed either high PUFA/EFA or low PUFA/EFA diets (87,100,138). High PUFA/EFA diets have also been found to increase promotion of DMBA induced tumours when circulating levels of prolactin and oestrogen were controlled by endocrine and drug manipulations (138). Growth of tumours known to be non-responsive to hormones has also been found to be stimulated by high PUFA/EFA diets (100,138). It would thus appear that at this stage insufficient evidence exists to definitely implicate a hormonal mechanism in promotion of tumourigenesis by high PUFA/EFA diets.

A related effect is the possible influence of dietary PUFAs/EFAs on hormone receptors. Some studies have reported increased numbers of prolactin and oestrogen receptors in animals fed high PUFA/EFA diets (100,138). In contrast, other studies have reported that high PUFA/EFA diets do not influence hormone receptor levels (100,138).

vi) Intercellular communication

Cell to cell communication is thought to play an important role in the control of differentiation of both normal and neoplastic tissues. Recently interruption of this process has been proposed as a possible mechanism of tumour promotion (100,138). Many tumour promoters have been reported to block a specific type of intercellular communication known as metabolic cooperation, in which regulatory molecules and/or ions are transported between adjacent cells via membrane structures called gap junctions (100,138). Observations that PUFAs/EFAs inhibit intercellular communication, possibly by membrane lipid modification, has led to the hypothesis that the effects of dietary PUFAs/EFAs on tumour growth might be mediated via this mechanism (100,138). However since proliferating cells communicate less than non-proliferating cells, these findings may be the result of cell growth stimulation by PUFAs/EFAs involving some other mechanism (100).

A number of mechanisms have thus been proposed to account for the tumour promoting effects of dietary PUFAs/EFAs. As yet evidence conclusively proving the role of one or more of these mechanisms to the exclusion of others is lacking. It is also possible that the mechanism of tumour promotion by PUFAs/EFAS might be due to a combination of a number of the proposed mechanisms.

1.5.3. Influence of essential fatty acids on growth of cultured cells

Numerous studies have examined the influence of EFAs on the growth of various cell types in culture. Results from several of these studies are listed in table 1.3. These studies have utilised transformed cells, from both malignant and normal tissues, with an indefinite lifespan in culture, as well as untransformed cells from normal tissues which have a limited lifespan in culture. Despite the large number of different cell types used, growth of most cultured cells was inhibited by supplementary EFAs. Although growth of both malignant and normal, or benign, cells is inhibited, the effects appear to be more pronounced in malignant cells (158,162,171). In co-cultures of malignant and normal cells, exogenous EFAs have been reported to selectively kill malignant cells while lowering the rate of cell division of normal cells (158,171).

The growth inhibitory effects of EFAs appear to be concentration dependant as low levels of EFAs have been reported to enhance cell growth (58,167,170) with higher levels inhibiting cell growth (156-166,168,169). A large number of different types of cultured cells, both malignant and normal, do not appear to have a growth requirement for EFAs, some containing no detectable EFAs in their cellular lipids (58,72). Other cell types however exhibit a definite growth requirement for EFAs (167,170,172-174). Supplementation with low levels of EFAs could thus satisfy the EFA requirements of the latter group resulting in cell growth stimulation, while higher levels of supplementary EFAs, as used in most studies listed in table 4, in excess of the EFA requirement would result in cell growth inhibition regardless of whether or not the cells require EFAs for growth.

1.5.4. Mechanisms of in vitro cell growth inhibition

A number of mechanisms have been proposed for the growth inhibitory effects of EFAs on cells in culture. These include effects on PG synthesis, membrane lipid modification and lipid peroxide formation.

i) Prostaglandin synthesis

Both endogenous (177,181,187) and exogenous (156,164,169,175-182) PGs and PG analogues have been reported to inhibit proliferation of cells in culture, while PG synthesis inhibitors have been reported to stimulate cell growth (177,181). The effects of exogenous PGs on cell growth appear to be

Table 1.3. Influence of essential fatty acids on in vitro cell growth

cell type	EFA	effect	references
i)Malignant cells			
human sarcoma	LA	↓	156
	GLA	↓	156,157
	AA	↓	156
human carcinoma	LA	↓	158-160,171
	GLA	↓	157-160,161,163,171
	DGLA	↓	158,159,171
	AA	↓	158,159,171
human hepatoma	LA	NE	162
	GLA	↓	162
human adenocarcinoma	LA	↓	158,171
	GLA	↓	158,171
	DGLA	↓	158,171
	AA	↓	158,171
human leukaemic lymphocytes	LA	↓	77
human neuroblastoma	GLA	↓	164,165
murine melanoma	GLA	↓	166
murine leukaemic thymocytes	GLA	↓	77
DMBA induced murine mammary	LA	↑	167
rat ascites	LA	↓	94
	AA	↓	94
ii)Benign cells			
human glioma	AA	↓	168
human foetal brain	AA	↓	168
human lymphocytes	LA	↓	77
human fibroblasts	LA	NE	158,171
	GLA	NE	158,171
	AA	↓	158,171
monkey kidney	LA	NE	158,162,171
	GLA	NE	158,162,171
	AA	↓	158,171
canine kidney	LA	NE	158,171
	AA	↓	158,171
guinea pig smooth muscle	LA	NE	169
	GLA	↓	169
	AA	↓	169
murine epithelial	LA	↑	167
bovine kidney	GLA	↓	157
	GLA	NE	166

Abbreviations used: DMBA = 7,12-dimethylbenzanthracene; LA = linoleic acid; GLA = gamma-linolenic acid; DGLA = dihomo-gamma-linolenic acid; AA = arachidonic acid; ↓ = increased growth; ↑ = decreased growth; NE = no effect.

dependant on both PG concentration and cell type. Certain PGs have been reported to stimulate cell growth at low concentrations (169,183,184) while higher levels of these exogenous PGs have been reported to inhibit growth of the same cell types (184,185). Since exogenous EFAs have been reported to stimulate PG synthesis in cultured cells (178,186-188,202), the growth inhibitory effects of EFAs might be mediated via stimulation of PG synthesis. Recent studies have however provided evidence that a mechanism other than enhanced PG synthesis appears to be responsible for the growth inhibitory effects of EFAs. PG synthesis inhibitors have been reported not to reduce the growth inhibitory effects of EFAs (158,164,190), while PUFAs which are not PG precursors, some of which have been reported to inhibit PG synthesis, have also been shown to inhibit cell growth (169,186). Additionally compounds which have no influence on PG synthesis, including certain anti-oxidants, have been reported to reverse the growth inhibitory effects of EFAs (169).

ii) Membrane lipid composition and lipid peroxidation

Membranes of cultured tumour cells have been reported to contain less PUFAs/EFAs, and in particular AA, than those of normal cultured cells (137,191). These membrane lipid alterations are thought to play a role in several biological properties of tumour cells. Supplementation of EFAs to cultured cells has been shown to increase the cell and cell membrane PUFA/EFA composition, thus influencing membrane fluidity and the effects associated with changes in membrane fluidity (30,165,192,193). Thus the growth inhibitory effects of EFAs may be mediated via changes in cell membrane composition.

A related effect is the modification of intracellular EFA levels. Morphological observations suggest that while EFAs readily enter cultured cells, not all the EFAs can be effectively metabolised. Consequently extensive morphological changes (196), including lipid accumulation in the form of lipid droplets and triglycerides, occur in the cytoplasm (173,189,194-197). The lipid accumulation is thought to interfere with normal functioning of the cell leading to growth inhibition, although other studies have reported that a moderate increase in the accumulation of lipid droplets and triglycerides does not appear to exert any deleterious effects on cells (408).

While cultured cells have been reported to generate free radicals and lipid peroxides (159,168,186,198,199), tumour cells undergo less lipid peroxidation than normal cells, probably as a result of low substrate availability due to the lower levels of PUFAs/EFAs found in the tumour cells (168,199). Supplementary EFAs have been found, probably by providing substrate, to increase free radical generation and lipid peroxidation in cultured cells including tumour cells (159,168,186,198,199). Since malignant cells in particular have low activity of the free radical scavenging enzymes superoxide dismutase and glutathione peroxidase (199), they would be more susceptible to growth inhibition by

the resultant increase in lipid peroxidation. Reports that both free radicals and lipid peroxides are toxic to cultured tumour cells (198,200) and that the growth inhibitory effects of EFAs can be reversed by anti-oxidants (159,165,168,169,197,201,202), suggest that increased lipid peroxidation might be the mechanism whereby EFAs inhibit growth of cultured cells. The potency of a given EFA in killing malignant cells has also been correlated with the extent of lipid peroxidation of that EFA in cells (159).

While the cyclooxygenase, lipoxygenase and cytochrome P_{450} pathways are known to generate lipid peroxides in cultured cells (202), recent studies have shown that the production of free radicals and lipid peroxides are not influenced by inhibitors of the above pathways (188,202). General peroxidation reactions are thus thought to be involved in the control of cell proliferation.

A number of mechanisms have thus been proposed to explain the growth inhibitory effects of EFAs on cells grown in culture. As found in the in vivo situation, evidence conclusively proving the role of one or more of these mechanisms to the exclusion of others is lacking. Furthermore the mechanisms of the growth inhibitory effects appear to vary among different cell types.

1.5.5. Comparison of the effects of essential fatty acids on cell growth in experimental animals and in cultured cells.

EFAs appear to promote growth of tumours in experimental animals while inhibiting growth of cultured cells particularly at higher EFA concentrations. Many of the mechanisms proposed for the observed effects of the EFAs in the two systems are the same. An explanation for the apparently contradictory results obtained might be provided if the variables in the two systems are examined. While EFAs can be supplied directly to the target cells in high concentration in cell culture, processes such as absorption, transport and uptake by other tissues dilute the level of EFAs reaching the target, or tumour tissue, in experimental animals. The results might thus not be contradictory, as different levels of EFAs might be involved in the in vivo and in vitro situations, particularly since lower levels of EFAs have been reported to promote growth of cultured cells. In experimental animals other factors, such as the immune response, which are not found in cultured cells and are influenced by dietary EFAs, might also play a role in mediating the effects of the EFAs on tumour growth. Furthermore while EFAs are required for normal growth by animals and also presumeably by tumours grown in animals, many cultured cells do not have an EFA requirement for normal growth and developement.

Chapter 2

LITERATURE REVIEW - ASCORBIC ACID

2.1 Ascorbic acid biosynthesis and metabolism

Ascorbic acid (Asc) differs from other vitamins in that it is an essential dietary nutrient in only certain species - man, primates, the guinea pig, certain flying mammals and Passeriform birds and in a certain number of insects. (203,204). Other animals are able to synthesis Asc via the pathway shown in figure 2.1. The inability of man and certain animals to synthesise Asc is thought to be due to loss of activity of the enzyme L-gulonolactone oxidase (203,205). It has been proposed that the loss of activity is due either to deletion of the relevant genetic material (204) or due to defective expression of the genes coding for the enzyme (203).

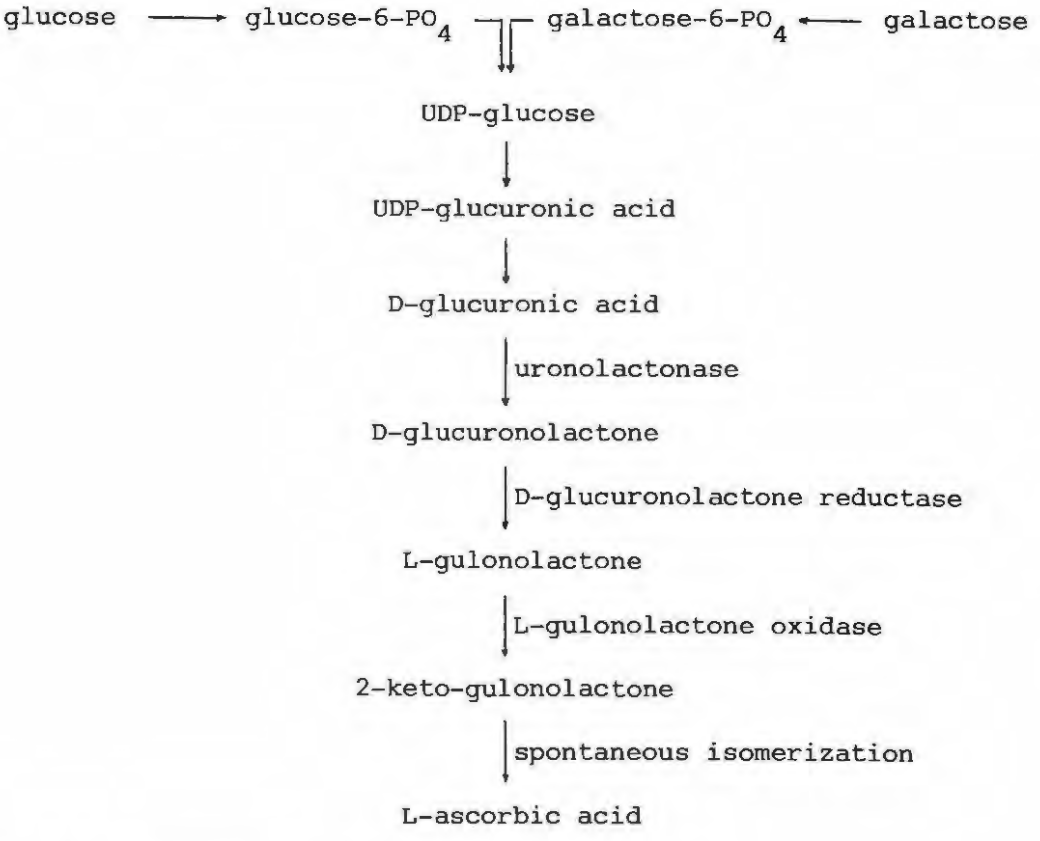

Figure 2.1. Biosynthesis of ascorbic acid (203).

In man dietary Asc is metabolised via the pathway shown in figure 2.2. The principle route for the elimination of metabolic products of Asc in humans is urinary excretion, the major urinary metabolites being unchanged Asc, dehydroascorbic acid (DHA), 2,3-diketogulonic acid and oxalate (205,207,208). Minor urinary metabolites include ascorbate-2-sulphate and saccharoascorbate (205,209,210). In animals 2,3-diketogulonic acid can be further metabolised to predominantly CO_2, the major route for Asc elimination in animals being repiratory exhalation of CO_2 (205,207,211). Urinary excretion of other Asc metabolites constitutes a minor route of elimination in these animals (207).

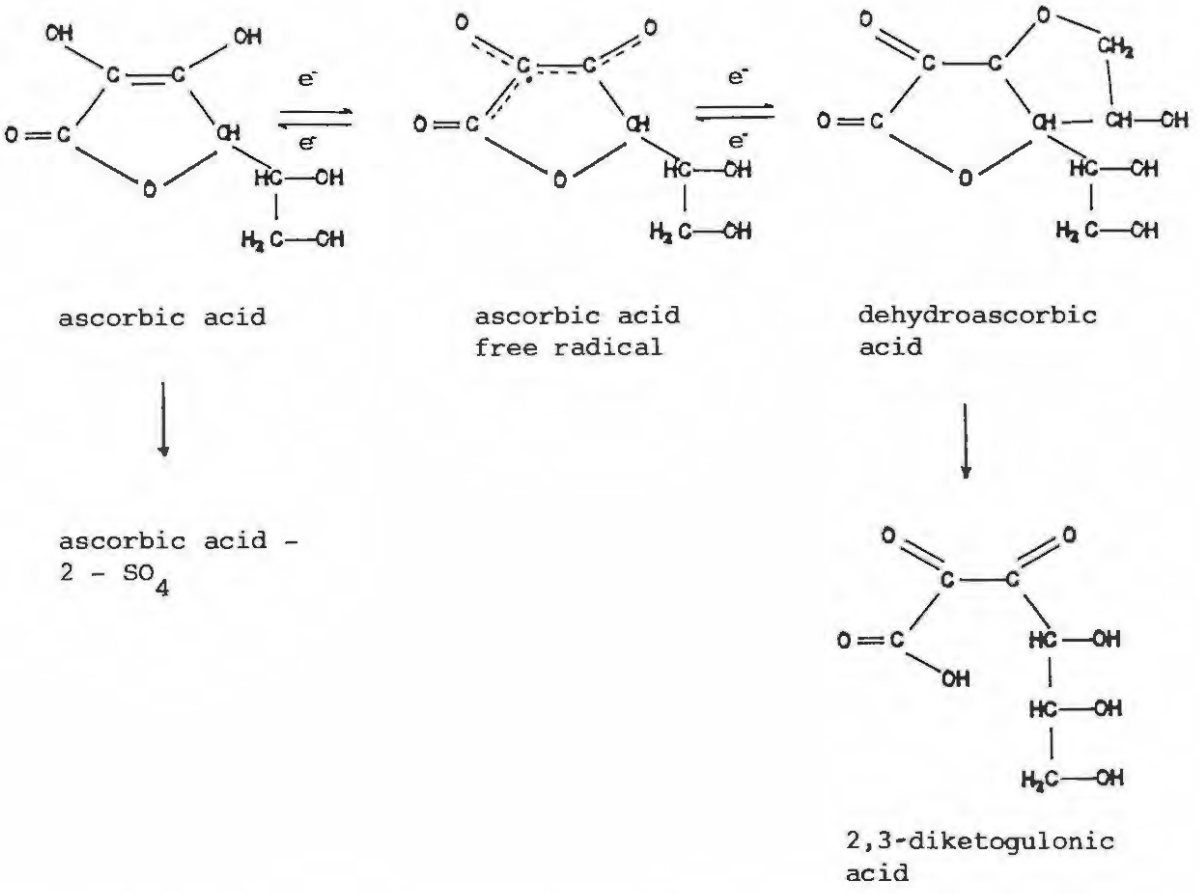

Figure 2.2. Metabolism of ascorbic acid in man (206).

2.2 Early history

A deficiency of Asc gives rise to the disease scurvy, the existence of which was reported in various population groups in a number of early studies (212,213). During the period of empire expansion in the seventeenth and eighteenth centuries, the principle health problem encountered by seamen undertaking long voyages was scurvy. During the eighteenth century Lind (cited in 214), in studies on scorbutic seamen, demonstrated that the juice of fresh citrus fruit could cure scurvy. The active ingredient was named vitamin C, but it was not until 1928 that Svent-Gyorgi isolated the active anti-scorbutic agent and identified it as Asc (212). By the mid-1930s chemical synthesis of Asc led to the availability of large quantities at low cost for experimental purposes (215). Subsequent studies utilising radiolabelled precursors by Burns, Isherwood, King and colleagues, led to the identification of the biosynthetic pathway of Asc represented in figure 2.1 (216-220). Studies by the same scientists utilising radiolabelled Asc led to the determination of the metabolic fate of the vitamin in man (figure 2.2) and animals (207,211). A number of early studies in the 1930s and 1940s also attempted to determine the Asc requirement of man by determining the level of intake which saturated plasma and tissues with Asc, excess Asc being excreted unchanged in the urine (221-225). A requirement of between 60-75mg per day was proposed. Recent studies have confirmed that the body pool of Asc is well regulated, with any excess being excreted (226,227). During the 1940s and 1950s a number of the functions of Asc, including its role in collagen synthesis, tyrosine metabolism and steroid hormone biosynthesis were also delineated (216,225,228,229). These and other functions will be discussed more fully in section 2.4.

2.3. Scurvy, dietary requirements and turnover.

A deficiency of Asc is characterised by the disease scurvy, symptoms of which include haemorrhages throughout the body due to increased capillary fragility, swollen and bleeding gums, anaemia and behavorial changes (230-333). During the initial stages of a deficiency, a decline in body weight is noted along with fatigue, bone fragility, painful and swollen joints and poor wound healing (230-233). If left untreated scurvy can be fatal.

The Asc requirement of man is a subject of some controversy. The recommended daily allowance (RDA) in the UK is 30mg (230) while in the USA it has recently been increased from 45mg to 60mg (235). A committee recently appointed by the US Academy of Sciences to review RDAs proposed that the RDA for Asc be reduced (234). Their proposal is based on reports that intakes of Asc as low as 10mg per day are sufficient to prevent symptoms of scurvy (209). In contrast to this view however, some researchers have proposed that the RDA for Asc is too low. An intake of 45mg per day is reported to be insufficient to maintain serum and body Asc at optimal levels (236). Since scurvy is a

clinical manifestation of prolonged and extreme Asc deficiency, subclinical deficiency of the vitamin could persist in individuals before onset of symptoms (203,237). It has been proposed that this condition may influence the health of individuals, particularly since intakes above the minimum required to prevent scurvy have been shown to improve the health and growth of guinea pigs (237). Additionally increased Asc turnover and excretion of Asc metabolites, probably reflecting increased Asc requirement, have been reported under extreme physiological and pathological conditions including pregnancy, disease, strenuous physical exercise, stress or drug intake and smoking (203,209,237-239). Studies have estimated that the daily turnover of Asc in the adult man is approximately 45mg (236) and since absorption of Asc from the gastrointestinal tract has been reported to be significantly less than 100% (209,240), it has been suggested that the RDA for Asc is too low and should be increased to 120-150mg to ensure optimal levels of Asc in the body in health and disease (203,238).

A third view is presented by scientists who propose that pharmacological doses of Asc (up to 10g per day) may be beneficial in the prevention and treatment of certain diseases including cancer, influenza, the common cold and atheroschlerosis among others (203,241,242). The role of pharmacological doses of Asc in cancer therapy will be discussed in more detail in section 2.6.

The body pool of Asc in man is approximately 1500mg (226,227). The first symptoms of scurvy become apparent when the pool size is depleted to 300-400mg and upon repletion of Asc the last symptoms do not disappear until a pool size of about 1000mg is attained (226,231). The body pool of Asc is thought to be maintained at about 1500mg by plasma Asc levels of 0,8-0,9mg/100ml (238). The first symptoms of scurvy become apparent at plasma Asc levels of 0,2-0,3mg/100ml (238). The upper limit of the Asc body pool appears to be closely regulated by a number of mechanisms.In man Asc is absorbed in the gastrointestinal tract by an energy requiring Na^+-dependant transport system which is a saturable process exhibiting decreased relative absorption with increasing Asc intake (209,240). For example at Asc intakes of up to 180mg an average absorption of 70% has been reported (240), while in another study it was shown that increasing the Asc intake from 1,5g to 12g resulted in a decrease in relative absorption of ingested Asc from 50% to 16% (240). Some measure of control thus appears to be exerted at the level of absorption.

Transport of Asc from the blood into a number of tissues has also been reported to occur via a saturable active transport system (205,208,243). Less efficient Asc uptake would thus be found at higher blood Asc levels. The upper level of Asc in the blood is limited by glomerular reabsorption in the kidneys, which is a saturable Na^+-dependant process (244). The large increase in renal turnover of unmetabolised Asc observed at plasma Asc concentrations above 0,8-0,9mg/100ml is reported to be a consequence of the saturability of the reabsorption process, this plasma level being thought to

represent the plasma threshold level of Asc (244). Thus with increasing intake of Asc a decrease in the relative absorption and distribution to tissues as well as increased excretion of Asc is found.

The regulation of the Asc body pool size has been used as an argument against the proposed beneficial effects of large doses of Asc. Regular daily intake of Asc at levels as low as 180mg has however been reported to increase both plasma and tissue Asc levels in man (226,227,246,247). Upon reduction of the Asc intake both the plasma Asc levels and body pool size rapidly return to normal (241). Thus at this stage there appears to be no clear indication of the exact Asc requirement by man.

Dietary sources of Asc, excluding vitamin supplements, include most foods of plant origin. While fresh citrus fruit and leafy vegetables are the usual sources, appreciable amounts are also found in tomatoes, potatoes, berries and other fruit and vegetables. Some Asc is also found in the organ meats of animals (245).

2.4. Functions

The known functions of Asc have been delineated by using two major approaches. Firstly by inducing a deficiency in dependant animals and relating symptoms to functions and secondly by identifying the biochemical reactions in which Asc is involved and determining the role of Asc in these enzyme catalysed reactions.

Many of the functions of Asc can be attributed to its ability to undergo reversible oxidation and reduction to dehydroascorbic acid (figure 2.2). A two step process, each involving the transfer of a single electron, via the Asc free radical is involved (203,248). A requirement for Asc as a cofactor in a number of enzymatic hydroxylation reactions has been reported. These include reactions involved in collagen, proteoglycan, carnitine, steroid hormone and catecholamine biosynthesis, tyrosine degradation, peptidyl amidation and drug metabolism (203,206,249-263). Asc has also been implicated in histamine, folate and iron metabolism and in regeneration of α-tocopherol (203,264-271). As the present study is concerned with the influence and inter-relationships between Asc and EFAs on tumour growth, only those functions thought to be involved in these processes, namely collagen and proteoglycan synthesis and α-tocopherol regeneration, will be discussed in more detail.

i) Collagen synthesis

Hydroxylation of lysine and proline is essential for the synthesis of functional and stable collagen macromolecules (206,254). Studies utilising purified prolyl and lysyl hydroxylases have indicated a specific requirement for Asc in these reactions (206,251,254). Evidence from these studies suggests that Asc acts by maintaining enzyme bound iron in a loosely bound ferrous form preventing its

oxidation to the more tightly bound ferric form which would render the enzymes inactive. In addition Asc is thought to maintain certain essential thiol groups of the enzymes in the reduced form (206). In vivo studies utilising scorbutic guinea pigs and in vitro studies using tissue homogenates and cultured cells have also demonstrated an Asc requirement for efficient hydroxylation of lysine and proline (206,249,250,252,254,272,273). In the absence of Asc a polypeptide termed procollagen, which is similar to collagen but is deficient in hydroxylated lysine and proline, is synthesised (252). Disturbances in collagen metabolism are thought to result in the poor wound healing and increased capillary fragility which are characteristic of scurvy (254).

ii)Proteoglycan biosynthesis

Asc is thought to play a role in the synthesis and maintenance of proteoglycans which, together with collagen, make up a large part of the gel-like extracellular matrix (253). In vitro studies using cell and organ cultures derived from human articular cartilage have shown that the presence of Asc stimulates synthesis of sulphated proteoglycans and increases deposition of these macromolecules into the extracellular matrix (253). The mechanism whereby Asc exerts these effects has not yet been established, but Asc is known to increase the ratio of cysteine:cystine residues in proteins as a result of its reducing activity (203,253) and cysteine residues are known components of the protein core of proteoglycans (253). In addition Asc, or its metabolite Asc-2-sulphate, has been reported to play a role in the sulphation of certain macromolecules which may include proteoglycans (274). Asc has also been reported to inhibit the lysosmal enzymes arylsulfatase A and B which are involved in the degradation of sulphated macromolecules including proteoglycans (253). Asc thus plays a role in the synthesis and maintenance of the extracellular matrix which is thought to play a major role in influencing cellular function and behaviour (275).

iii)α-Tocopherol regeneration

Both α-tocopherol and Asc react rapidly with free radicals and it is widely accepted that the anti-oxidant properties of these compounds are responsible in part for their biological properties (271). Although tissue Asc levels are often considerably greater than those of α-tocopherol, the latter is considerably more lipophilic than Asc and in biomembranes has been found to be a more effective anti-oxidant, particularly with respect to lipid peroxidation (271). It has been proposed that the two vitamins act synergistically,α-tocopherol acting as the primary anti-oxidant and the resulting α-tocopheroxyl radical then reacting with Asc to regenerate α-tocopherol (figure 2.3).

The Asc free radical formed in this reaction is stable and generally unreactive toward biological molecules, reacting preferentially with other free radicals (276). This mechanism would permit a single α-tocopherol molecule to scavenge many free radicals and also links Asc to the protection of

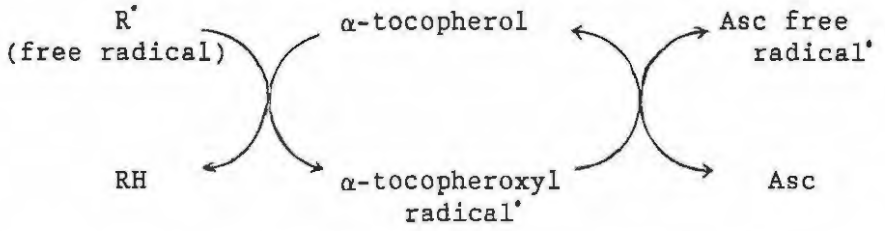

Figure 2.3. Synergistic interaction between α-tocopherol and ascorbic acid (271).

membrane lipids against free radical damage. A number of studies utilising cell-free chemical systems or tissue homogenates and subcellular fractions thereof, have confirmed the role of Asc in regeneration of the α-tocopheroxyl radical to yield α-tocopherol (270,271,276). The relevance of this interaction in vivo has been questioned however since lipid soluble α-tocopherol is membrane bound while Asc is confined to the aqueous fluids within and surrounding cells (270,276). Recent studies employing liposomes as model biomembranes have however confirmed the ability of Asc to interact with membrane bound α-tocopherol (270,276), while a number of in vivo studies have also reported an interaction between the two nutrients. Increasing the level of dietary Asc has been reported to increase tissue α-tocopherol levels in guinea pigs (cited in 276) while Asc deficient diets were shown to reduce tissue α-tocopherol levels (cited in 276). In a human study, supplementary α-tocopherol and Asc were reported to reduce the level of serum lipid peroxides, the cumulative effects of both supplements being greater than for either vitamin administered alone (276).

2.5. Therapeutic benefit of large ascorbic acid intakes

A number of scientists have proposed that large pharmacological doses of Asc (up to 10g per day) would be of therapeutic value (203,241,242,277). These recommendations were based on the amount of Asc found in diets consumed by primates (242) and also by determining the amount of Asc synthesised in other animals in relation to body weight (277) and extrapolating these results to humans. As a result of these recommendations, supplementary intakes of Asc far in excess of the RDA are ingested by many individuals (203). Much research has subsequently been carried out to determine the effects and proposed therapeutic benefits of large intakes of Asc.

The medical value of large intakes of Asc in humans is a controversial issue. Whereas therapeutic benefit has been claimed by some scientists, a number of other studies have been unable to confirm these reports (241,242,242,278,279). Large intakes of Asc have also been reported to exert undesirable side effects which include increased urinary oxalate excretion which may lead to the formation of kidney stones (203,244), ulcer development (280), diarrhea (203), destruction of vitamin B12 in food

(244), decreased copper absorption (280), increased clotting time of blood (203) and mutagenic effects (281,182). The relevanee of a number of these studies to the in vivo situation is unclear however since many of them were performed in vitro. In addition there is no evidence of any of these side effects, with the exception of diarrhea, occuring in humans and animals ingesting large doses of Asc (241,244,283). Of possible concern are the studies which report that large doses of Asc lead to induction of Asc metabolising enzymes (241,284,285). Upon cessation of large intakes the activities of these enzymes were found to remain elevated for a short period leading to a reduction in body Asc levels. A number of other studies using guinea pigs were unable to confirm the existence of this effect which has been called the rebound effect. (244,286)

The diseases against which Asc is claimed to yield therapeutic benefit include atherosclerosis (203,246), the common cold and influenza (203,242) and cancer (203,241,287-293). Since the present study is primarily concerned with the effect of Asc on cell and tumour growth, only the latter aspect will be discussed in detail.

2.6 ASCORBIC ACID AND CANCER

The first report of the USA National Research Council Committee on Diet, Nutrition and Cancer (56) identified Asc as one of the nutrients which might prove beneficial in the treatment and prevention of cancer. This suggestion was made on the basis of results from many studies of animal model and cell culture systems and from epidemiological studies of dietary Asc intake in human populations either with cancer or at risk of developing particular types of cancer. A number of clinical trials have also provided evidence that Asc might prove beneficial in the treatment of cancer (241,287-293).

2.6.1. Influence of ascorbic acid on in vivo tumour growth

i)Human studies

A possible role for Asc in cancer was first recognised in the 1930s and 1940s as a result of a number of studies which reported accumulation of Asc in both human and animal tumours (294,295). Patients suffering from various types of cancer were also found to have depleted tissue and blood Asc levels, presumably as a result of Asc accumulation by the tumours as well as utilisation of Asc by the defence mechanisms of the body, including the immune system, in response to the presence of the tumours (241,247,296,299-304). Increased utilisation of Asc by tumour bearing patients is illustrated by a recent study in which patients treated with 3g Asc daily had decreased urinary Asc excretion in comparison to healthy controls on the same Asc regimen (247). In the same study supplementary Asc was found to increase plasma Asc levels more rapidly in healthy controls than in tumour bearing patients, probably reflecting increased utilisation of Asc in the tumour bearing individuals.

Although large doses of Asc had been utilised to a limited extent, with some success, in the treatment of cancer as early as in 1940 by Deucher (cited in 241), it was not until 1971 that extensive studies on the influence and role of large doses of Asc in cancer began. These were stimulated by the proposals of Cameron and Pauling (241,287-293) that large doses of Asc might prove beneficial in the treatment of cancer since Asc was known to play an important role in the synthesis and maintenance of collagen and proteoglycans making up the extracellular matrix and to stimulate the immune response. The inadequate state of conventional therapy for most solid tumours at that time and the non-toxic nature of Asc motivated Cameron to perform clinical trials of high dose Asc (10g per day) in the treatment of terminal cancer patients at the Vale of Leven Hospital, Loch Lomondside, Scotland in the 1970s (241,288-291). Significantly prolonged survival times when compared with those of a control group selected from hospital case files as well as increased general health were reported. In certain individuals tumour regression was also observed. While the primary tumours originated from a number of sites, therapeutic benefit was observed irrespective of the origin of the tumours. The results of these studies have been confirmed in patients with apparently terminal cancer admitted to Fukuoka Torikai Hospital, Fukuoka, Japan (cited in 241). Asc in doses of between 5g and 10g daily was reported to significantly increase life expectancy when compared with patients treated with less than 5g daily.

The studies of Cameron and Pauling have however been critisised on the basis that they involved a retrospective comparison between selected study patients and historical control patients thus giving rise to the possibility of potential bias (278,279,305,306). These considerations motivated Moertel and colleagues at the Mayo Clinic, Rochester, Minnesota, USA to conduct randomised, placebo controlled, double blind studies to determine the influence of 10g Asc administered daily on the survival times of terminally ill cancer patients (278,279). In contrast to the results of Pauling and Cameron no therapeutic benefit was derived from Asc treatment when compared with placebo treated patients. Cameron and Pauling (307, cited in 308) have suggested that these studies were inconclusive since the patients used in the first study of Moertel and colleagues had undergone prior chemotherapy or radiotherapy and would thus have had considerably depressed immune systems. In the second study of Moertel and colleagues patients supposedly on the placebo regimen were reported to have urinary Asc levels of up to 100 times greater than those expected in people not ingesting Asc supplements, which suggests that these patients may in fact have been ingesting large doses of Asc (308). At the present time the controversy surrounding the use of Asc in the treatment of cancer appears to be no closer to resolution than when it began.

ii) Epidemiological studies

The epidemiological data pertaining to the effect of Asc on the occurrence of cancer is not extensive. Furthemore the studies provide mostly indirect evidence since they are based on the consumption of foods known to contain high levels of Asc rather than on actual measurements of Asc intake (309).

Possible influences of other dietary nutrients can thus not be discounted. Bearing in mind the above limitations, an inverse correlation between Asc consumption and the incidence of a number of types of cancer, including laryngeal, gastric and oesophagal cancer, has been reported. (56,309,310). In contrast other studies have reported no apparent influence of dietary Asc on mouth, colon and gastric cancer. (309).

iii) Experimental animal studies

As already mentioned, a role for Asc in tumour growth was first recognised due to observations that tumours sequestered Asc and that the tissue and blood Asc levels of tumour bearing animals were significantly reduced (294,295). Early studies examined the growth of tumours in scorbutic guinea pigs in which tumour growth was found to be reduced, presumeably due to a requirement for Asc by the tumours (311). Growth and health of the animals was also severely affected however with symptoms of scurvy appearing quite rapidly (311). Since the reports by Cameron and Pauling of the therapeutic value of large doses of Asc in cancer treatment (241,287-293), many investigators have examined the effect of large pharmacological doses of Asc on the growth and development of transplantable and induced tumours in experimental animals. The results of a number of these studies are summarised in table 2.1.

As can be seen from table 2.1 the majority of studies, with some exceptions, have shown that Asc reduces the growth of transplantable tumours in experimental animals. Studies involving carcinogen induced tumours have reported variable results, although in the majority of studies tumour growth appears to have been inhibited by Asc. Studies utilising the carcinogenic nitrosamines have reported little protective effect of Asc against carcinogenesis induced by preformed nitrosamines (304,318,319), while significant protection was afforded animals treated with nitrosamine forming compounds (304,310,318,319). Asc thus appears to prevent the formation of nitrosamines rather than to exert a direct effect on the initiation of carcinogenesis by nitrosamines. This effect has been confirmed in a number of vitro investigations (321,322).

2.6.2. Mechanisms of tumour growth inhibition by ascorbic acid

A number of mechanisms have been proposed to account for the tumour growth inhibitory effects of Asc observed with most transplantable tumours and certain carcinogen induced tumours. These include immunostimulation, maintenance of collagen and proteoglycan integrity, inhibition of the enzyme hyaluronidase and antioxidant action.

Table 2.1. Influence of large doses of ascorbic acid on tumour growth in experimental animals.

tumour type	species	effect	references
i) transplantable tumours			
sarcoma	mouse	↓	299,312
	rat	↑	309
leukaemia	mouse	↓	299,312-314
carcinoma	mouse	↓	312,313
	rat	NE	315
fibrosarcoma	mouse	↓	299,312
lymphoma	mouse	↓	299
ii) spontaneous tumours			
mammary	mouse	↓	316
iii) carcinogen induced tumours			
UV squamous cell carcinoma	mouse	↓	317
DMBA mammary	mouse	NE	318
DMBA skin	mouse	↓	315
DEN tracheal	mouse	↓	318
DMH colon	mouse	↓	318
	rat	↓	318
HTA bladder	mouse	↓	318
DMN lung	mouse	↓	318
DMH renal	rat	↓	318
DEN tracheal	hamster	↓	318
MNU colon	rat	NE	318
MNU bladder	rat	↓	318
BP	rat	↓	315
AP	rat	↓	304
MCA	rat	↑	310
	guinea pig	NE	318
NNM	rat	NE	318
MNP	mouse	↑	319
$NaNO_2$ + methylurea lung	mouse	↓	319
$NaNO_2$ + morpholine lung	mouse	↓	319
$NaNO_2$ + morpholine liver	rat	↓	318

Abbreviations used: DMBA = 7,12-dimethylbenzanthracene; DEN = N-nitrosodiethylamine; MNP = mononitrosopiperazine; MNU = methylnitrosourea; DMH = 1,2-dimethylhydrazine; HTA = 3=hydroxyanthranillic acid; DMN = dimethylnitrosourea; NNM = N nitrosomorpholine; BP = benzpyrene; AP = aminopyrene; MCA = methylcholanthracene; ↑ = increased growth; ↓ = decreased growth; NE = no effect.

i) Immunostimulation

A number of studies involving both humans and experimental animals have provided evidence that Asc might play an important role in stimulation of the immune response. Reduced immunocompetence, associated with reduced Asc levels, in both human and animals suffering from a number of diseases including cancer has been reported (203,304), while Asc supplementation in both humans and animals has been shown to enhance immunocompetence (304,323). The exact role of Asc in immunostimulation is still unclear although a number of reports have provided evidence that the immunostimulation may involve several components of the immune response including both humoral and cell mediated responses.

Increased production of certain immunoglobins (Ig), including IgG, IgA and IgM, have been reported in healthy individuals ingesting supplementary Asc (324,325). Lymphocyte concentrations are reportedly depressed in scorbutic individuals (241,304) while Asc supplementation has been found to increase lymphocyte blastogenesis and reactivity (323,326,327). Enhanced macrophage motility and phagocytosis (326,328,329), enhanced production of complement in humans (304,330) and of inteferon in mice and cultured cells (331,332) have also been found as a result of Asc supplementation. Asc thus appears to play a role in the recognition and subsequent destruction of tumour cells by the immune system.

ii) Collagen and proteoglycan synthesis and maintenance

In normal cells the tendency to proliferate is thought to be inhibited by the surrounding extracellular matrix (241,304,333). Maintenance of matrix integrity could thus play an important role in prevention of tumour cell invasiveness. Since Asc plays an important role in the synthesis of normal, functional collagen and proteoglycans it could play an important role in maintaining matrix integrity.

Tumour cell invasiveness is thought to depend on the release of lysosomal glycosidases (for example hyaluronidase) and perhaps other lysosomal degradative enzymes (collagenase and other proteases) by the tumour cells leading to depolymerisation of the extracellular matrix (293,304,333). Supplementary Asc has been reported to decrease collagen degradation in cancer patients presumeably as a result of its role in the synthesis of collagen (303,304). In addition Asc is suggested to play a role in the synthesis of physiological hyaluronidase inhibitor, a glycoprotein thought to be derived from proteoglycan degradation in which one or more Asc residues have been substituted for glucuronic acid residues (304,333). Since hyaluronidase catalyses proteoglycan degradation, supplementary Asc could afford protection to the proteoglycans by stimulating physiological hyaluronidase inhibitor synthesis.

iii) Lipid peroxidation

The reactive nature and possible role of lipid peroxides in tumour growth as well as the tumour growth inhibitory effects of certain anti-oxidants have been discussed previously (section 1.5.2). A number of in vitro studies involving cell free systems, liposomes and subcellular organelles have reported decreased peroxidation of lipids in the presence of Asc (334-336). The relevance of these studies to in vivo tumour growth are at present unclear, although a few studies have reported inhibition of lipid peroxidation by Asc in both humans and animals (276,337-339).

The protective properties of Asc against free radical induced lipid peroxidation are most likely due to the ability of Asc to regenerate α-tocopherol, the fat soluble anti-oxidant found in cell membranes (271). Asc itself can act as a free radical scavenger and may protect membrane PUFAs/EFAs from free radical attack by intercepting free radicals generated in the aqueous phase (276). It must be noted however that a number of in vitro studies have reported that Asc can also undergo auto-oxidative destruction, especially in the presence of some transition metal ions, which could lead to the promotion of PUFA/EFA oxidation (276,340-342).

iv) Ascorbic acid metabolites

It has been proposed that the growth inhibitory effects of Asc might be mediated by one or more of its metabolites. Both DHA and 2,3-diketogulonic acid have been reported to inhibit tumour growth in mice by a mechanism thought to involve interaction with DNA (304). DHA has also been shown to be a mitotic inhibitor (343).

A number of other mechanisms for the anti-tumour activity of Asc have been proposed on the basis of in vitro studies. These include inhibition of catalase leading to H_2O_2 accumulation, DNA strand breakage and a direct effect on tumour cell metabolism. The relevance of these mechanisms to tumour cell growth in vivo is at present unclear. These mechanisms will be discussed in more detail in the next section.

2.6.3. Influence of ascorbic acid on growth of cultured cells

A number of studies have examined the effect of Asc on the in vitro growth of cells, the results of a number of these studies being listed in table 2.2. In general it can be seen that growth of cultured cells is inhibited by supplementary Asc. While there appears to be no Asc requirement by cells in culture (358), a number of studies have reported that certain cells grow very poorly in the absence of Asc (346,355,358). Inhibition of cell growth appears to be more pronounced for malignant cells than for normal or benign cells. For example in simultaneous cultures of leukaemic and normal marrow cells, the leukaemic cells were preferentially inhibited at physiological Asc concentrations while higher concentrations inhibited growth of both cell types (351).

2.6.4. Mechanisms of in vitro cell growth inhibition

A number of mechanisms have been proposed for the growth inhibitory effects exerted by Asc in cultured cells. These include enhanced production of H_2O_2, DNA strand breakage and alterations in cellullar metabolic pathways. It has also been suggested that metabolites of Asc, rather than Asc itself, might exert the growth inhibitory effects. This possibility has previously been discussed.

i) Hydrogen peroxide formation

Studies utilising both cell free systems and cultured cells have reported the formation of H_2O_2 in cells supplemented with Asc, either as a result of the auto-oxidation of Asc or by a cell mediated reaction (248,315,354,356). Reversal of the growth inhibitory effects of Asc by exogenous catalase, which catalyses the degradation of H_2O_2 to O_2, led to the proposal that the growth inhibitory effects of Asc might be mediated via formation of H_2O_2 (315,354,356,357). In addition Asc has been found to inhibit the activity of catalase which leads to accumulation of H_2O_2 in cells (359,360). H_2O_2 can react with various metal ions including Cu^{2+} to produce hydroxyl radicals which are known to be highly reactive with a number of biological molecules including membrane PUFAs/EFAs, proteins and DNA (315). Since Asc and certain of its metabolites including DHA and diketogulonic acid have been demonstrated to enhance lipid peroxidation in the presence of metal ions, including Cu^{2+} and Fe^{2+}, in certain cell free systems and subcellular fractions (340-342,361),it is possible that the growth inhibitory effects of Asc could be mediated via this mechanism.

Cancer cells have been reported to be more sensitive than normal cells to the growth inhibitory effects of H_2O_2 since they have been found to possess lower catalase activity than normal cells (349). The more pronounced growth inhibition as a result of Asc supplementation observed in cancer cells when compared with normal cells might thus be explained by this proposed mechanism.

Table 2.2. Effect of ascorbic acid on in vitro cell growth

cell type	effect	references
i) malignant cells		
murine melanoma	↓	344
murine neuroblastoma	↓	345
murine sarcoma	↓	312
	↑	346
murine leukaemia	↓	312
murine carcinoma	↓	312
human melanoma	↓	344,347
human carcinoma	↓	348,349
human leukaemia	↓	350,351
	NE	350
	↑	350
human neuroblastoma	↓	315
HeLa	↓	344
Ehrlich ascites carcinoma	↓	352,353
ii) benign cells		
murine fibroblasts	↓	344,354
	NE	312
murine plasmacytoma	↑	355
human fibroblasts	↓	344,354
	NE	347
human amniotic	↓	344
human marrow	↓	350,351
	NE	350
rat glioma	↓	345
chicken embryo fibroblasts	↓	354
Chinese hamster lung	↓	344,356
Chinese hamster ovary	↓	344,357

Abbreviations used: ↑ = increased growth; ↓ = decreased growth; NE = no effect.

ii) DNA scission

Asc has been reported to possess DNA strand breaking activity particularly in the presence of Cu^{2+} ions in both cell free systems and in cell culture (315,362-365). The mechanism is thought to involve formation of oxygen radicals from H_2O_2 since catalase has been shown to inhibit the DNA scission activity of Asc (363,364). The relevance of these studies to the in vivo situation are at present unclear although it must be noted that certain anti-tumour drugs also possess DNA scission activity (363).

iii) Cancer cell metabolism

A number of metabolic changes are known to occur during the process of cell transformation. These include an increase in anaerobic glycolysis coupled with a decrease in oxidative phosphorylation and increased gluconeogenesis among others (304). Since the activity of a number of enzymes and cytochromes are known to be influenced by Asc, it has been proposed that Asc might play a role in normalising certain metabolic pathways in cancer cells (304). Asc supplementation to various types of cancer cells in culture has been found to increase oxygen consumption, decrease lactate production and to inhibit anaerobic glycolysis (304,349). The relevance of these studies to in vivo tumour development is at present unknown although oxidative energy production is reportedly reduced in scorbutic guinea pigs, suggesting that Asc might play a role in stimulation of the relevant pathways involved (304).

2.6.5. Comparison of the effects of ascorbic acid on tumour cell growth in experimental animals and in cell culture

Asc has been reported to inhibit both in vivo tumour growth and in vitro cell growth. A number of the mechanisms proposed for the growth inhibitory effects in the two systems are the same. These include a role in H_2O_2 synthesis and lipid peroxidation, DNA strand scission and in altering cancer cell metabolism. The possibility that metabolites of Asc may be responsible for the growth inhibitory effects has also been mentioned. As yet evidence conclusively proving the role of one or more of the suggested mechanisms to the exclusion of others is lacking.

Chapter 3

LITERATURE REVIEW - INTERACTIONS BETWEEN ESSENTIAL FATTY ACIDS AND ASCORBIC ACID

Although extensive studies have been carried out relating to the effects and functions of both EFAs and Asc, very few reports on possible interactions between the two nutrients are found. Such interactions may be of particular relevance to the role of these two nutrients in tumour cell metabolism since both EFAs and Asc have been found to influence the rate of tumour cell growth as has been discussed. Possible interactions between EFAs and Asc may be mediated at the level of PG synthesis since recent studies have reported that Asc influences PG synthesis from precursor EFAs (366-372). A second possible site of interaction is at the level of lipid peroxidation (276,334-342).

i) Prostaglandin synthesis

Asc has been reported to both inhibit and stimulate synthesis of certain PGs in a number of in vitro studies utilising animal tissues, tissue homogenates and cultured cells (366-372). The effects appear to be concentration dependant and to depend on the tissue or cell type used. At physiological concentrations Asc has been shown to increase synthesis of PGE_1 and TXB_1 from DGLA in human platelets while exerting no effect on synthesis of PGE_2 and TXB_2 from AA (366). In another study using human platelets Asc was reported to increase synthesis of PGE_1 and $PGF_{1\alpha}$ from DGLA and to lesser degree increased $PGF_{2\alpha}$, PGE_2 and TXB_2 synthesis from AA (368). In experiments using guinea pig lung parenchymal slices, physiological concentrations of Asc increased synthesis of TXB_2 and 6-keto-$PGF_{1\alpha}$ while higher concentrations increased TXB_2, PGE_2 and $PGF_{2\alpha}$ synthesis (367). Production of prostacyclin in rat aortic rings (368) and of PGE_2 in murine macrophages (370) was also stimulated by physiological concentrations of Asc. High concentrations of Asc were also found to stimulate PG production by human lung fibroblasts and rabbit pulmonary smooth muscle cells while inhibiting PG synthesis by calf pulmonary endothelial cells and rat prostrate cells (371). The influence on synthesis of individual PGs was not determined in this study. At physiological concentrations Asc has also been shown to inhibit synthesis of $PGF_{2\alpha}$ in guinea pig uterine homogenates (369).

The mechanism whereby Asc influences PG synthesis is at present unclear but it is suggested to involve either stimulation of the enzyme cyclooxygenase or increased release of EFA substrate from tissue stores (366-368). Studies using cultured lung fibroblasts have provided evidence that the effects of Asc on these processes might be mediated by increased H_20_2 levels induced by Asc since addition of catalase to Asc treated cultures was found to abolish the PG stimulatory effects of Asc (372). Further support for this mechanism is provided by the fact that H_2O_2 can decompose to form oxygen radicals

(315) which are known to play a role in the reaction catalysed by cyclooxygenase during PG synthesis (373,374).

The relevance of enhanced or decreased PG production, as a result of Asc supplementation, to cancer growth inhibition is at present unclear since PGs have been reported to both enhance and inhibit tumour growth as previously discussed (section 1.5).

ii) Lipid peroxidation

As already mentioned Asc has been found to both inhibit and promote lipid peroxidation (276,334-342). The anti-oxidant effects of Asc resulting in decreased lipid peroxidation appear to be more relevant to in vivo studies since the pro-oxidant effects have been demonstrated only in studies using in vitro systems in the presence of exogenous metal ions (276,340-342), while a number of in vivo and in vitro studies have reported that Asc reduces lipid peroxidation (267,334-339). The role played by lipid peroxidation in in vivo tumour growth promotion and in inhibition of growth of cultured cells has been discussed previously (section 1.5). It is thus possible that the effects of Asc on tumour growth may be mediated by its influence on lipid peroxidation, although no studies relating directly to Asc and lipid peroxidation in tumour cells could be found in the relevant literature.

Recent reports indicating a possible relationship between EFAs and Asc in the treatment of cancer, involved the treatment of terminally ill patients suffering from primary liver cancer with both GLA and large doses of Asc (156,375,376). Increased survival times were found in comparison to the average survival times of patients at the Medical University of South Africa suffering from primary liver cancer not treated in this manner. Some clinical improvement as well as increased well being and a decrease in pain was also reported in a number of patients.

While extensive data exists in the literature about the effects of EFAs and Asc on tumour and cell growth, the precise mechanism of these effects remains unclear. The possibility that lipid peroxidation might be a mechanism whereby these effects are mediated has only been examined relatively recently and hence limited information about the role played by lipid peroxidation in tumour and tumour cell growth is available in the relevant literature. While the role of PG synthesis in tumour and cell growth has been well documented, controversy still exists regarding the role of PGs in EFA/PUFA mediated effects on cell or tumour growth growth. Furthermore no accounts relating to the possible role of PG synthesis in the interactions between Asc and EFAs in tumour cell growth and metabolism could be found in the relevant literature. In this dissertation the effect of EFAs and Asc on the growth of BL6

melanoma cells, in vivo and in vitro, and on LLCMK monkey kidney (normal) cells in vitro were examined, as well as possible interactions between the two nutrients in influencing cell and tumour growth. The role played by lipid peroxidation and PG synthesis in the mechanism, and as a site of interaction between the two nutrients, whereby these nutrients affected cell growth was also investigated.

Since much of the relevant literature has been discussed in the literature review, the introduction to the various chapters will be relatively brief. A detailed comparison of the literature and results obtained in this dissertation will however be provided in the discussion of each chapter.

Chapter 4

CELL CULTURE METHODS

4.1. Introduction

Cell culture systems provide greatly simplified models for studies on the metabolism of nutrients, including EFAs and Asc, since the effects of these nutrients or their metabolites, on processes such as cell proliferation,can be related directly to the target cells (58).

Since the aim of any cancer therapy would be to destroy tumour cells while minimising damage to normal host cells, it is useful to compare the influence of various compounds on the growth of both tumour and normal cells. Cultured, transplantable BL6 murine melanoma and normal or benign monkey kidney cells were used in this study to investigate the effects and mechanisms of EFAs and Asc on cell growth. In this chapter routine maintenance of the cells as well as the methods used in setting up experimental cultures and the reagents and media required will be described.

4.2 Materials and Methods.

Reagents

Trypsin subculturing solution contained in a final volume of 1000ml : 8,0g NaCl; 0,4g KCl; 1,0g D-glucose; 0,58g $NaHCO_3$; 0,2g EDTA; 0,02g phenol red; 10^5IU penicillin; 10^5µg streptomycin and 500 units/ml trypsin.

Media

Eagle's Basal Medium (modified) (MEM) was purchased from Flow Laboratories, Irvine, Scotland and contained Hank's salts and glutamine but no $NaHCO_3$. Each sachet was made up to a final volume of 10 litres with milli-Q water to which 0,5g Asc; 0,1g serine; 0,06g glycine; 7,5g $NaHCO_3$; 500IU/ml penicillin and 500µg/ml streptomycin were added. The media was filtered through a Millipore filtration unit (Millipore, Bedford, Massachusets, USA) with a Sartorius pre-filter and 0,45µm and 0,22µm pore size filters (Millipore).

Foetal Calf Serum (FCS) (filtered and ultraviolet irradiated) was purchased from the State Vaccine Institute, Cape Town, R.S.A. and was sterile filtered into the culture medium through a 0,45µm Millipore filter using a Swinnex-25 holder (Millipore).

Experimental medium consisted of MEM containing 10% FCS.

Maintenance medium consisted of MEM to which 5% FCS was added and was used to maintain cells at a slightly lower growth rate between experiments.

Freezing medium consisting of MEM containing 20% FCS and 10% dimethylsulphoxide was used to freeze cells in liquid nitrogen.

Phosphate buffered saline (PBS) in a final volume of 1000ml contained: 0,2g KCL; 8,0g NaCl; 1,15g Na_2HPO_4; 0,2g KH_2PO_4.

Cells

Benign monkey kidney cells (LLCMK) were obtained from the Department of Physiology, Medunsa, R.S.A. These cells were used as the non-cancerous control as they were the only normal cell line available to this laboratory at the time.

Transplantable BL6 murine melanoma cells were obtained from Dr. C. Albrecht, Department of Pharmacology, University of Stellenbosch Medical School, Tygerberg, R.S.A.

The BL6 and LLCMK cells were used for all cell culture studies.

Methods

Cell maintenance Cells were maintained at 37°C in a Labcon forced circulation incubator Type FSIE. Procedures were carried out on a laminar flow bench using aseptic technique. Cells were grown in 80cm^2 Nunc tissue culture flasks (Weil Organisation, Durban, R.S.A.). Passaging of confluent cells was carried out once or twice weekly, depending on the rate of cell proliferation, using the trypsin sub-culturing solution. Cell culture medium was decanted and 10ml of trypsin solution was sterile filtered into the flask. After 2 minutes of incubation at 37°C most of the trypsin solution was decanted leaving a thin film covering the cells. The flask was incubated at 37°C until all the cells had detached from the flask surface. The cell population was reduced by decanting off excess cells but leaving sufficient cells to allow propagation of the culture. After addition of 30ml maintenance medium to the flasks, cells were again incubated at 37°C. Cells were maintained in this manner for about 6 weeks before being discarded and replaced by fresh frozen cells. Cells were discarded after 6 weeks of passaging since the BL6 cells were found to change characteristics and could not be transplanted successfully in mice after passage for longer periods.

Experimental Cell culture. Cells were trypsinised by adding 5ml of the trypsin solution to the flasks and incubating at 37°C until all the cells had lifted off from the flask surface. The cells were then decanted into a sterile Nunc centrifuge tube (Weil Organisation) to which was added 5ml experimental medium to dilute the trypsin solution thus diminishing any possible action of the trypsin on the cell walls which might have led to rupture of the cells.Cells were centrifuged at 1000g in a Hettich Universal K2S centrifuge for 10 minutes, the supernatant decanted and the cell pellet resuspended in experimental medium by gentle passage through a sterile pasteur pipette. Cells were seeded at a density of $3x10^5$ cells per $25cm^2$ Nunc culture flask to which had been added 10ml experimental medium. Appropriate concentrations of the test compounds were sterile filtered into the flasks which were then incubated at 37°C until the most rapidly growing cultures reached confluency. Control cultures contained appropriate concentrations of the solvent in which test compounds were dissolved. Five replicates were prepared at each concentration of the test compounds. When the most rapidly growing cultures reached confluency (4 days) cells were trypsinised, pelleted by centrifugation and resuspended in 5ml PBS for enumeration. This method was used throughout the study unless otherwise stated.

Cell enumeration. Two methods were used to determine cell number. a) Haemocytometry. A Neubauer haemocytometer (Neubauer, West Germany) was used to count cells prior to and at the end of each experiment. b) A counting plate was used to establish growth curves. Cell number was determined daily using a counting plate made by a colleague (377). The plate was made to the dimensions of the undersurface of the $25cm^2$ culture flasks. Lines were etched 1mm and 0,5mm apart on the plate as indicated in figure 4.1. Six blocks, either of size 0,5mm x 0,5mm ($0,25mm^2$) or 1mm x 1mm ($1mm^2$), were counted using a set pattern (indicated in figure 4.1.) throughout to ensure uniformity. Since the total surface area of the bottom of the flasks used is $2500mm^2$, counting six blocks of $0,25mm^2$ required a multiplication factor of 1666,67 while counting six blocks of $1mm^2$ required a multiplication factor of 416,67 to determine cell number. The advantage of this method is that a growth curve can be determined by counting the same flask(s) daily without trypsinisation. The accuracy of this method is attested to by the low standard error of the mean (SEM values) obtained and the close correlation of cell numbers with those obtained by haemocytometry (377).

Freezing of Cells. Samples of both cell lines were frozen and stored in the manner described below for later use. Actively growing, nearly confluent cells were trypsinised and centrifuged and the cell pellet resuspended in 2ml freezing medium. The cell suspension was transferred to a 2ml freezing vial (Sterilin, Feltham, England) which was tightly capped and placed in the vapour phase of a Dewar flask, containing liquid nitrogen, for 60 minutes before being submerged in the liquid phase. Cells remained viable for several months if stored in this manner. New cultures were started from frozen cells by placing a vial from the liquid nitrogen in a 37°C waterbath to thaw. The outside of the vial was washed with ethanol to reduce the risk of contamination and the cell suspension was transferred to a $80cm^2$ culture flask containing 60ml of culture medium, to which 15% FCS had been added, and

incubated at 37°C. After 24 hours this medium was replaced by maintenance medium and the cells incubated as before.

As already mentioned these methods were used throughout the study for maintenance of cells and for experimental purposes.

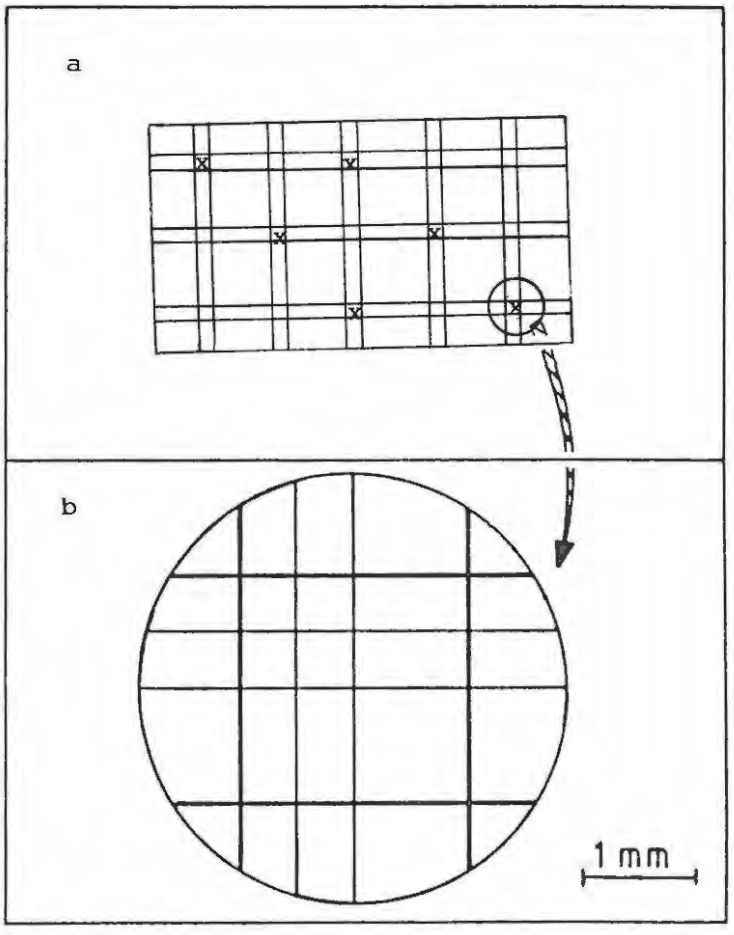

Figure 4.1. (a) Diagrammatic representation of the cell counting plate illustrating the pattern followed for enumeration of cells. (b) Enlargement of the counting area to illustrate the squares of area $1mm^2$ and $0,25mm^2$ within which cells were counted.

Chapter 5

INFLUENCE OF ESSENTIAL FATTY ACIDS ON CELL GROWTH

5.1 Introduction

EFA metabolism varies widely in different cell lines, some requiring EFAs for growth (58,167,170,172,173) and others not (58,172). Despite this variation in EFA requirement, cell culture data is remarkably consistent regarding the effects of EFAs on cell growth. Low concentrations of exogenous EFAs appear to stimulate growth of many cells while higher concentrations inhibit cell growth (58). The growth inhibitory effects are more pronounced in tumour cells than in normal or benign cells (158,162,166,171).

Although the precise mechanism(s) whereby EFAs inhibit growth of cultured cells have not been established, a number of mechanisms have been proposed for the growth inhibitory effects. These include enhanced PG synthesis (156,175,178), enhanced lipid peroxidation (159,168,199,201,202), altered membrane lipid composition (164,165,192) and intracellular accumulation of EFAs (194). As previously mentioned this study will examine the possible role of enhanced PG or LK synthesis and enhanced lipid peroxidation in EFA mediated cell growth inhibition. Inhibitors of the pathways involved in the synthesis of PGs and LKs and in lipid peroxidation have been widely used in examining the possible role of these pathways in EFA mediated cell growth inhibition. In this study the PG synthesis inhibitor indomethacin, the LK synthesis inhibitor caffeic acid and the anti-oxidant α-tocopherol will be used to examine the possible involvement of these pathways in EFA mediated cell growth inhibition.

5.2 Effect of essential fatty acids on in vitro cell growth.

5.2.1. Materials and Methods

Materials

Essential fatty acid stock solutions. LA, GLA and AA (Sigma, St Louis, USA) were dissolved in a minimum volume of 95% ethanol and diluted to a final concentration of 10mg/ml with distilled and deionised water. These were stored at -20^{o}C under nitrogen and protected from light until use.

Experimental medium and cells were described in section 4.2. While the foetal calf serum added to this medium contains trace amounts of EFAs, this medium is essentially EFA deficient (B.Davidson - personal communication).

Radiochemicals (Radiochemical Centre, Amersham, UK). [1-^{14}C]-LA (specific activity 58,7mCi/mmol) and [1-^{14}C]-AA (specific activity 59,6 mCi/mmol) were supplied dissolved in toluene which is not miscible with aqueous culture medium. They were thus evaporated to dryness at 90^{o}C under a stream of nitrogen and reconstituted in propyleneglycol (377) at a final concentration of 0,02 µg/ul. They were stored under nitrogen at -20^{o}C and protected from light until use.

Methods

Effect of essential fatty acids on cell growth. Cells were set up as described in section 4.2. LA, GLA and AA were added to flasks at final concentrations of 0 (control), 10, 25 or 50µg/ml. These concentrations of EFAs have been used in many of the studies listed in table 1.3 which examined the influence of EFAs on the growth of cultured cells. All flasks including control flasks contained an equal volume of ethanol (0,1%). Cells were incubated and enumerated as described in section 4.2. In a separate study to determine whether or not the effects of the EFAs on cell growth were reversible, the EFA supplemented medium in cultures was replaced with fresh experimental medium, containing no supplementary EFAs, after 24 hours of incubation at 37^{o}C and the cells incubated and enumerated as before.

Uptake of radiolabelled essential fatty acids by cells. Cells were set up as before and incubated at 37^{o}C until they were nearing confluency (3 days). The medium was decanted and replaced with fresh experimental medium. Either 0,5µCi ^{14}C-LA or 0,5µCi ^{14}C-AA was then added to the flasks which were incubated for a further 24 hours before the cells were harvested. Cells were then homogenised using a Dounce homogeniser (30 strokes with the tight plunger). Total uptake of EFAs by cells was determined by counting the radioactivity of the samples in 10ml scintillation fluid (EP ready solv, Beckman, Galway, Ireland) in a Beckman LS 3801 scintillation counter.

Statistical analysis. One way analysis of variance (ANOVA) and Scheffe's multiple range test were used for statistical analysis. In several instances a comparison of data from different tables or figures was carried out. The statistical significance of these comparisons will be referred to in the relevant text and was not included in the figures or tables to avoid complicating the tables and figures.

5.2.2 Results

Effect of essential fatty acids on cell growth. The three EFAs used, namely LA, GLA, and AA all significantly inhibited growth of both BL6 and LLCMK cells in a dose dependant manner (figures 5.1-5.3). Inhibition was significant ($p < 0,001$) at the lowest concentration (10µg/ml) of each EFA used in both cell lines.

Whereas the growth inhibition was more pronounced in BL6 cells than in LLCMK cells at all concentrations of the three EFAs (figures 5.1-5.3), this difference was not significant in LA treated cultures but was significant ($p < 0,05$) at 50µg/ml for GLA treated cultures and at 25 and 50µg/ml for AA treated cultures. The growth inhibitory effects of the three EFAs were also evident in the growth curves obtained by daily enumeration of cells (figures 5.4-5.9). From these curves it was also evident that the growth inhibitory effects required the presence of the EFAs in the medium and were reversible upon removal of the EFA supplemented medium.

Cultures treated with 50µg/ml EFA for 24 hours and subsequently grown in experimental medium containing no supplementary EFAs, exhibited an increased rate of growth compared with cells grown in medium containing 50µg/ml EFA throughout the experimental period. While a difference in the number of BL6 cells treated in the two methods mentioned above was evident at 48 hours, this difference only became significant ($p < 0,001$) after 72 hours. In LLCMK cells a difference in growth rates was evident at 72 hours but only became significant ($p < 0,001$) after 96 hours. From the growth curves it was also evident that the BL6 cells divided more rapidly than the LLCMK cells. Whereas cultures from both cell lines were seeded with $0,3 \times 10^6$ cells, at confluency (4 days) the control BL6 cultures contained approximately $1,8 \times 10^6$ cells while the control LLCMK cultures contained approximately $1,3 \times 10^6$ cells.

The degree of cell growth inhibition by the three EFAs increased with increasing unsaturation (AA > GLA > LA). A significant difference ($p < 0,001$) was however only evident between AA and LA or GLA treated cultures at the highest concentration used, namely 50µg/ml, in LLCMK and BL6 cells.

Uptake of radiolabelled essential fatty acids by cells. LLCMK cells were found to take up significantly more ^{14}C-LA ($p < 0,001$) and ^{14}C-AA ($p < 0,001$) than BL6 cells (figure 5.10). Both cell lines absorbed significantly more ($p < 0,001$) ^{14}C-AA than ^{14}C-LA.

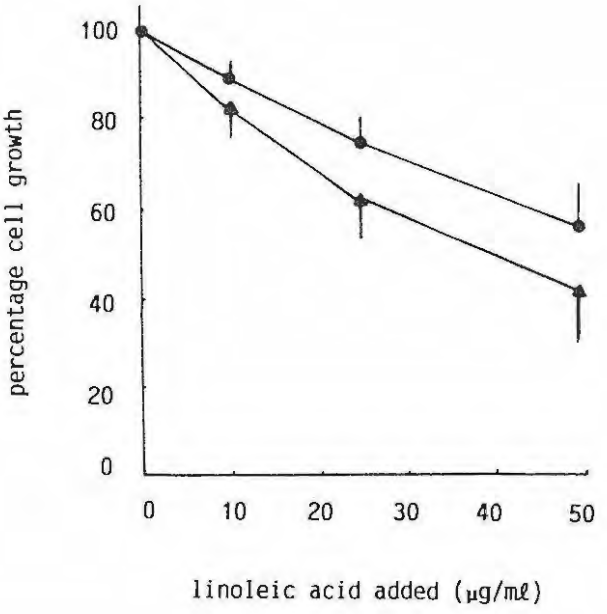

Figure 5.1. Effect of increasing concentrations of supplementary LA on the growth of cultured cells. ● LLCMK cells; ▲ BL6 cells. Results are mean and SEM of 5 cultures. Growth inhibition was significant ($p < 0,01$) at all concentrations for both cell lines. No significant difference between inhibition of the two cell lines was evident.

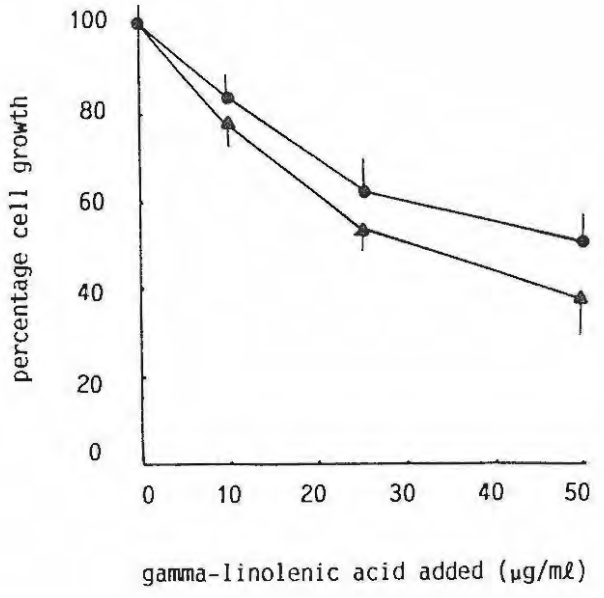

Figure 5.2. Effect of increasing concentrations of supplementary GLA on the growth of cultured cells. ● LLCMK cells; ▲ BL6 cells. Results are mean and SEM of 5 cultures. Growth inhibition was significant ($p < 0,01$) at all concentrations in both cell lines. Inhibition of BL6 cells was significantly ($p < 0,05$) more pronounced than inhibition of LLCMK cells at 50µg/ml supplementary GLA.

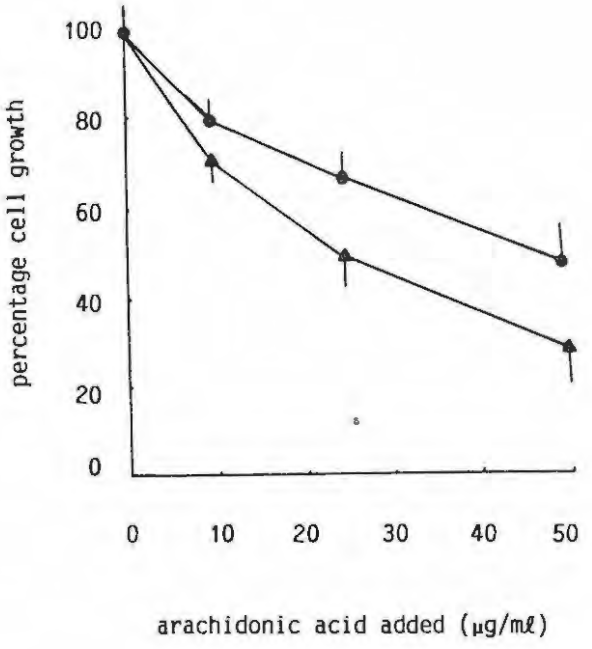

Figure 5.3. Effect of increasing concentrations of supplementary AA on the growth of cultured cells. ● LLCMK cells; ▲BL6 cells. Results are mean and SEM of 5 cultures. Growth inhibition was significant ($p < 0,01$) at all concentrations in both cell lines. Inhibition of BL6 cells was significantly ($p < 0,05$) more pronounced than inhibition of LLCMK cells at 25 and 50µg/ml supplementary AA.

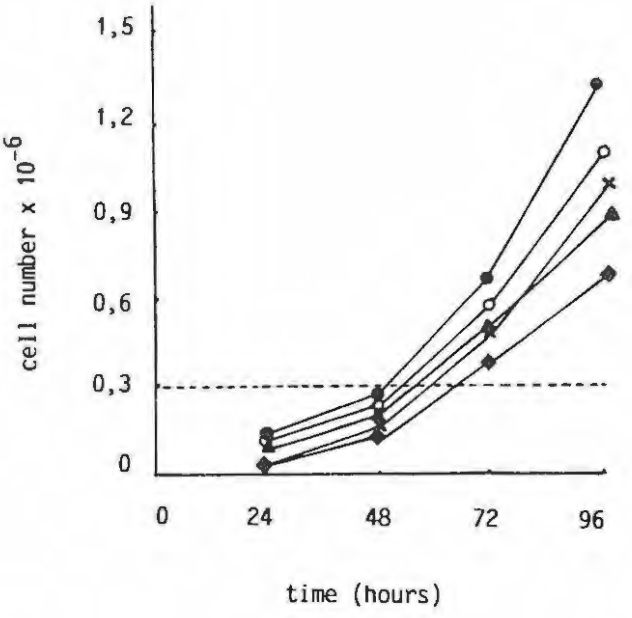

Figure 5.4. Growth curves for LLCMK cells supplemented with different concentrations of LA.● control;○ 10µg/ml LA;▲25µg/ml LA;◆50µg/ml LA;✖ cells grown in medium containing 50µg/ml LA for 24 hours and thereafter in unsupplemented medium. Results are mean and SEM of 5 cultures.

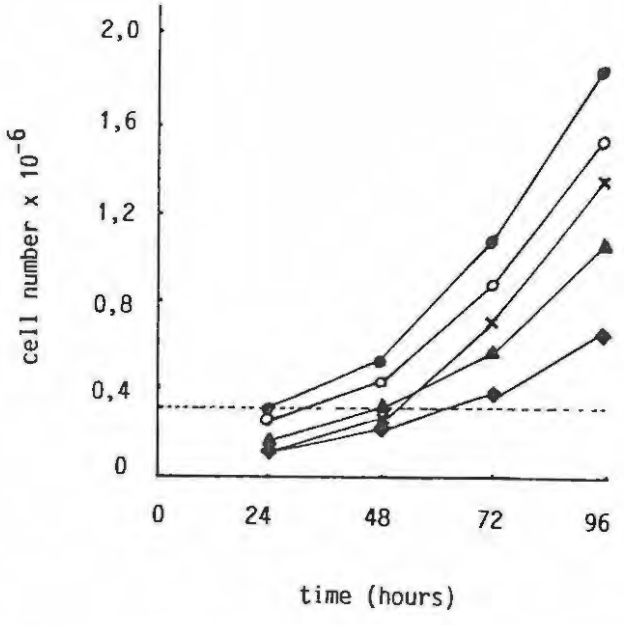

Figure 5.5. Growth curves for BL6 cells supplemented with different concentrations of LA.● control;○ 10µg/ml LA;▲25µg/ml LA;◆50µg/ml LA;✖ cells grown in medium containing 50µg/ml LA for 24 hours and thereafter in unsupplemented medium. Results are mean and SEM of 5 cultures.

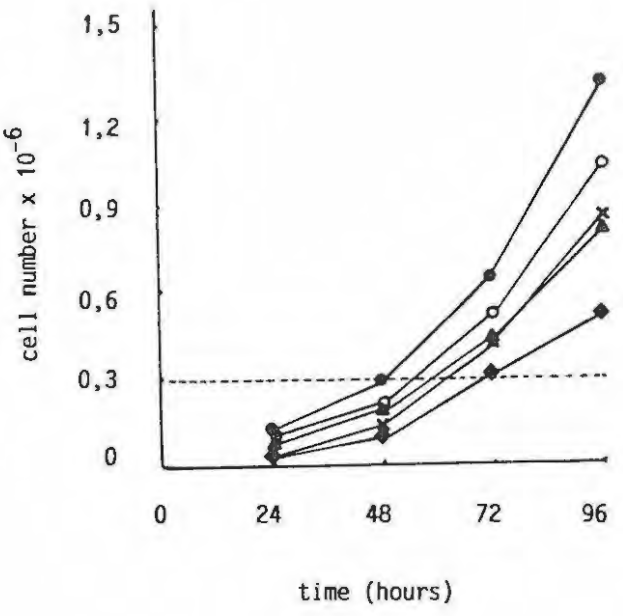

Figure 5.6. Growth curves for LLCMK cells supplemented with different concentrations of GLA. ● control;○ 10µg/mlGLA;▲ 25µg/ml GLA;◆50µg/ml GLA; ✖cells grown in medium containing 50µg/ml GLA for 24 hours and thereafter in unsupplemented medium. Results are mean and SEM of 5 cultures.

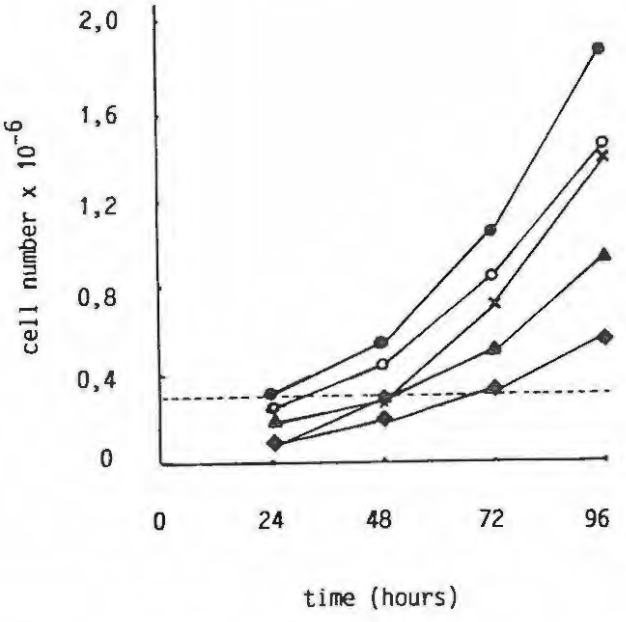

Figure 5.7. Growth curves for BL6 cells supplemented with different concentrations of GLA. ● control;○ 10µg/ml GLA; ▲25µg/ml GLA;◆50µg/ml GLA; ✖cells grown in medium containing 50µg/ml GLA for 24 hours and thereafter in unsupplemented medium. Results are mean and SEM of 5 cultures.

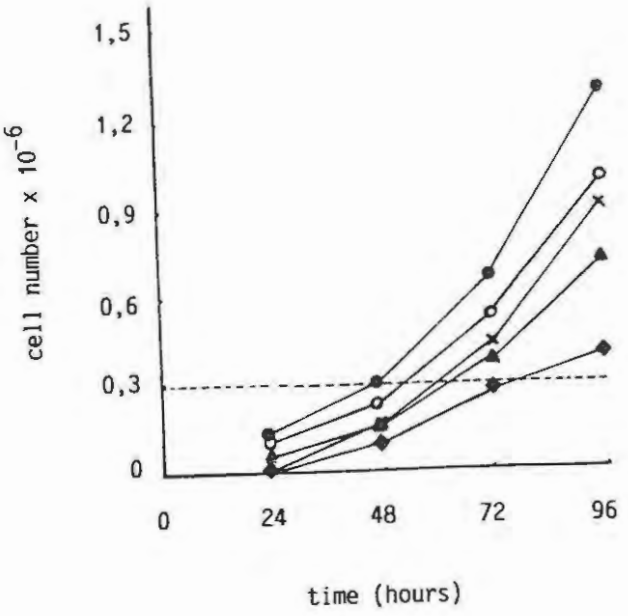

Figure 5.8. Growth curves for LLCMK cells supplemented with different concentrations of AA. ● control; ○ 10µg/ml AA; ▲ 25µg/ml AA; ◆ 50µg/ml AA; ✖ cells grown in medium containing 50µg/ml AA for 24 hours and thereafter in unsupplemented medium. Results are mean and SEM of 5 cultures.

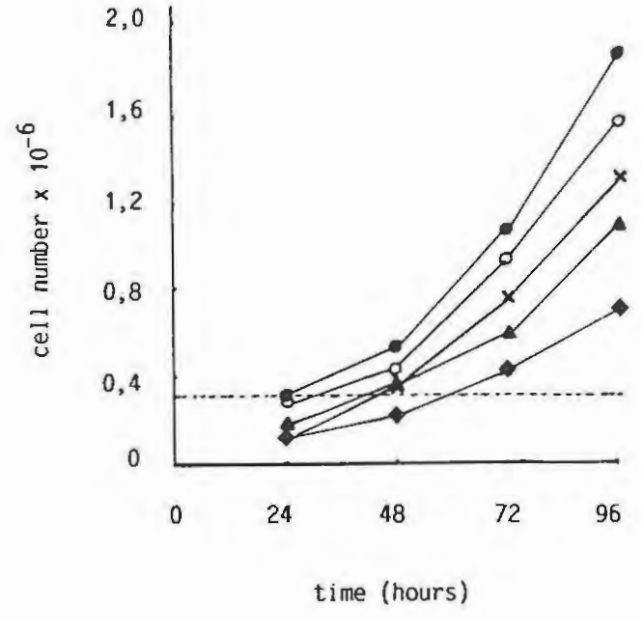

Figure 5.9. Growth curves for BL6 cells supplemented with different concentrations of AA. ● control; ○ 10µg/ml AA; ▲ 25µg/ml AA; ◆ 50µg/ml AA; ✖ cells grown in medium containing 50µg/ml AA for 24 hours and thereafter in unsupplemented medium. Results are mean and SEM of 5 cultures.

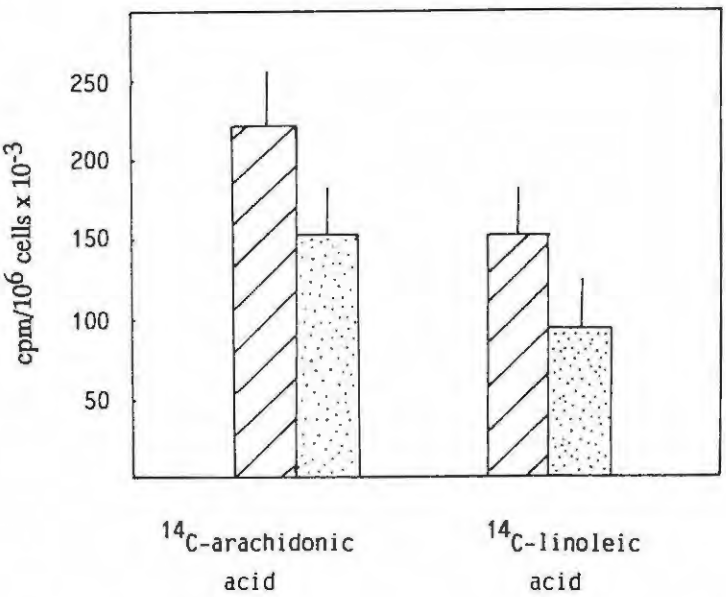

Figure 5.10. Uptake of ^{14}C-LA and ^{14}C-AA by cultured cells. ▨ LLCMK cells; ▩ BL6 cells. Results are mean and SEM of 5 cultures. LLCMK cells absorbed significantly more ($p < 0,01$) ^{14}C-AA and ^{14}C-LA than BL6 cells while both cell lines absorbed significantly more ($p < 0,01$) ^{14}C-AA than ^{14}C-LA.

5.3 Mechanisms of essential fatty acid cell growth inhibition

5.3.1 Materials and Methods

Materials

Essential fatty acids were prepared as described in section 5.2.

Experimental medium and cells were described in section 4.2.

A number of reagents were made up in a minimum volume of 95% ethanol and diluted to their final concentrations with distilled and deionised water. These included:

Prostaglandin E_1 and E_2 stock solutions (PGE_1 and PGE_2) (Sigma) were diluted to 1mg/ml and stored at -20°C under nitrogen and protected from light until use.

Indomethacin (IM), a gift from Lennon's Pharmaceuticals, Port Elizabeth, R.S.A., was diluted to a final concentration of 5mM. Fresh solutions were made up prior to use.

Caffeic acid (CA) (Sigma) was diluted to 5mM. Fresh solutions were made up prior to use.

D-α-tocopherol (TOC) (Sigma) was diluted to 5mM. Fresh solutions were made up prior to use.

Oleic acid (OA) (Sigma) was made up to 10mg/ml and stored at -20°C under nitrogen and protected from light until use.

2-methyl-1,2-di-3-pyridyl-1-propanone (MPP) (Sigma) was diluted to 5mM. Fresh solutions were made up prior to use.

Nitroblue tetrazolium (NBT) (Sigma) was made up to a final volume of 0,2% in PBS.

Trichloroacetic acid (TCA) (Sigma) was made up to a final volume of 20% in distilled and deionised water.

Thiobarbituric acid (TBA) (Sigma) was made up to a final concentration of 0,67% in 10% TCA.

1,1,3,3-tetramethoxypropane (Sigma) was used to generate a standard curve (0-25 µg/ml) for measurement of lipid peroxidation.

Methods

Influence of prostaglandins E_1 and E_2 on cell growth. PGE_1 and PGE_2 were added to cultures at final concentrations of 0 (control); 0,5; 1; 2,5; 5 or 10µg/ml. Control cultures contained an appropriate volume of ethanol. Cultures were incubated and enumerated as described in section 4.2.

Influence of indomethacin, caffeic acid and α-tocopherol on essential fatty acid cell growth inhibition. EFA containing cultures (0-50µg/ml) were prepared as described in section 5.2. To these flasks was added 5µM of either IM, CA or TOC, while a relevant volume of ethanol was added to control flasks containing EFAs only. Cultures were then incubated and enumerated as before. These compounds were added at this concentration, which is sufficient to inhibit the relevant pathways, in a number of previous studies investigating the mechanism(s) of EFA mediated cell growth inhibition (159,164,169). In this study cell growth in cultures containing no supplementary EFAs was not significantly influenced by these compounds at this concentration. It has been reported that the cellular uptake of EFAs is more rapid than uptake of certain compounds including TOC (201). To examine the possibility that the growth inhibitory effects of the EFAs might be mediated before uptake of IM, CA and TOC could occur in sufficiently high levels for these reagents to exert their effects, cells were preincubated for 24 hours with these reagents before supplementation with EFAs.

Effect on oleic acid on cell growth. OA, a fatty acid which is not a PG precursor; was added to cultures at final concentrations of 0 (control); 10; 25 or 50µg/ml. Control cultures contained a relevant volume of ethanol. Cultures were then incubated and enumerated as before.

Measurement of free radical formation. Free radical generation in confluent EFA containing cultures was measured by the ability of free radicals to reduce NBT to insoluble blue formazan (Matsui *et al* cited in 198). Cells were grown to confluency in medium containing no supplementary EFAs whereupon the medium was replaced with fresh experimental medium. EFAs were added to the cultures at final concentrations of 0 (control); 10; 25 or 50 µg/ml. The cultures were then incubated at 37°C for a further 24 hours before measurement of free radical generation.

The culture medium was decanted and cells were rinsed three times with PBS before addition of 4ml of NBT. Cells were then incubated for 4 hours at 37°C before the NBT solution was decanted and the cells again rinsed three times with PBS. Since these cells were not readily detached by trypsinisation, all cells were carefully detached using a rubber policeman and resuspended in 5ml PBS. Cells were then lysed by homogenisation using a Dounce homogeniser (30 strokes with the tight plunger). The

lysate was centrifuged at 1000g (Hettich Universal K2S centrifuge) to pellet the cell debris and the absorbance of the supernatant was then determined at 490nm in a Bausch and Lomb 1001 spectrophotometer. In order to compare free radical production in control cultures of BL6 and LLCMK cells, PBS was used as a blank. However since samples from cells may contain other compounds which might interfere with the assay, for analysis of EFA containing cultures the supernatants from control culture samples were used as blanks to allow a more accurate assessment of the level of free radical formation by the cells.

To investigate the possibility that the measured free radical production might be due to auto-oxidation of the EFAs in the medium, free radical production was also measured in EFA containing medium which had been incubated at 37^{o}C for 24 hours in the absence of cells.

Measurement of lipid peroxidation. Lipid peroxidation was estimated by the TBA assay for malonaldehyde (MDA) (201). MDA in this assay is formed by the thermal or acid catalysed decomposition of lipid peroxides and allows for an estimation of the levels of lipid peroxides generated by the cells (405). Cells were grown to confluency in medium containing no supplementary EFAs before replacement of the medium with fresh experimental medium. EFAs were then added to cultures at final concentrations of 0 (control); 10; 25 or 50 μg/ml. After incubation at 37^{o}C for 24 hours cells and the medium were assayed for production of lipid peroxides.

Cells were disrupted by addition of 2ml of TCA to the medium in the flasks, whereafter 4ml of TBA was added. This mixture was incubated at 97^{o}C for 20 minutes. The flask contents were then decanted and centrifuged at 12000g for 10 minutes (Beckman L8-80M ultracentrifuge). The absorbance of the supernatant was then measured at 532nm in a Bausch and Lomb 1001 spectrophotometer. Since tissue culture media contains the pH indicator phenol red, which contributes a background absorbance at 532nm, experimental medium treated in the same manner as the samples was used as a blank to compare lipid peroxidation in control BL6 and LLCMK cultures. However since TBA is known to react with certain compounds other than MDA (405), the control cultures were used as blanks to allow a more accurate estimation of lipid peroxide production in EFA treated cultures. Absorbance was converted to nmoles MDA from a standard curve generated with 1,1,3,3-tetramethoxypropane (appendix 1).

To investigate the possibility that the measured lipid peroxidation might be due to auto-oxidation of the EFAs in the medium, lipid peroxide formation was also measured in EFA supplemented medium which had been incubated at 37^{o}C for 24 hours in the absence of cells.

Mechanism of free radical and lipid peroxide production. Separate experiments were performed in an attempt to identify the mechanism of free radical generation and lipid peroxidation in the cells. The

cyclooxygenase synthesis inhibitor IM (5μM), the lipoxygenase inhibitor CA (5μM), the cytochrome P_{450} inhibitor MPP (5μM) and the anti-oxidant TOC (5μM) were added together with EFAs (25μg/ml) to confluent cultures. After incubation at 37°C for 24 hours free radical production and lipid peroxidation were measured as previously described in this section. The three enzyme inhibitors were used since the reactions catalysed by the enzymes mentioned above have been reported to generate free radicals and lipid peroxides (202). TOC was used since it is a general inhibitor of lipid peroxidation as a result of its anti-oxidant, free radical scavenging function.

Statistical analysis was performed as before using analysis of variance and Scheffe's multiple range test. As previously mentioned a number of comparisons of data contained in separate tables and figures were carried out, the statistical significance of which is contained for simplicity in the text and not in the tables or figures.

5.3.2. Results

Effect of prostaglandins E_1 and E_2 on cell growth. PGE_1 and PGE_2 were found to significantly ($p < 0,001$) inhibit growth of BL6 and LLCMK cells in a dose dependant manner (figure 5.11).

PGE_1 was found to be a more potent ($p < 0,001$) at 10 μg/ml) inhibitor of cell growth than PGE_2 in both cell lines.

Inhibition of BL6 cell growth by PGE_1 and PGE_2 was significantly ($p < 0,01$ at all concentrations above 1μg/ml) more pronounced than inhibition of LLCMK cells.

Effect of indomethacin on cell growth inhibition by essential fatty acids. A slight but non-significant reduction of the growth inhibitory effects of LA, GLA and AA was observed in both BL6 and LLCMK cells as a result of IM supplementation into the medium (figures 5.12-5.17).

Effect of caffeic acid on cell growth inhibition by essential fatty acids. CA was found not to influence cell growth inhibition of either BL6 or LLCMK cells supplemented with EFAs (figures 5.12-5.17).

Effect of oleic acid on cell growth. OA was found to significantly ($p < 0,001$) inhibit growth of both BL6 and LLCMK cells in a dose dependant manner (figure 5.18.) The inhibition was significantly less than cell growth inhibition by EFAs (for example $p < 0,001$ when comparing inhibition by 50μg/ml OA to growth inhibition by 50μg/ml LA in either cell line).

Effect of α-tocopherol on cell growth inhibition by essential fatty acids. A significant reduction in the growth inhibitory effects of LA, GLA and AA in both BL6 and LLCMK cells was observed as a result of TOC supplementation to the medium (figures 5.12-5.17). The reduction in growth inhibition was significant ($p < 0,001$) for all three EFAs at all concentrations in BL6 cells and at all concentrations except 10µg/ml in LLCMK cells. TOC was also found to non-significantly reduce the cell growth inhibitory effects of OA (figure 5.18).

Preincubation studies. Preincubation of cells with IM, CA or TOC for 24 hours prior to the addition of EFAs was found to have no influence on the effects of these compounds on EFA mediated cell growth inhibition (results not shown).

Free radical production. A dose dependant increase in free radical generation was observed in both LLCMK and BL6 cells upon EFA supplementation (figures 5.19 and 5.20). This increase was found to be significant ($p < 0,001$) at all levels of supplementary EFAs in both cell types.

While free radical production increased with increasing unsaturation (AA > GLA > LA) of the EFAs, this effect was only significant ($p < 0,05$) at 25 and 50µg/ml between all three EFAs in both BL6 and LLCMK cells. At 10µg/ml little difference between the effects of the three EFAs on free radical production was found in the two cell lines .

When comparing results from the two cell lines, the control LLCMK cells were found to produce slightly more (although non-significant) free radicals than the control BL6 cells (figure 5.23). However upon EFA supplementation higher levels of free radicals were measured in BL6 cells. This effect was only significant ($p < 0,05$) in cultures supplemented with 50µg/ml AA however.

EFA containing medium incubated in the absence of cells was found to contain a negligible level of free radicals when compared with the levels measured in EFA supplemented cell cultures. (the level of free radicals measured in AA supplemented medium incubated in the absence of cells in depicted in figure 5.23.)

Lipid peroxidation. A dose dependant increase in lipid peroxidation was measured in EFA treated BL6 and LLCMK cells (figures 5.21 and 5.22). This effect was significant ($p < 0,001$) at all EFA levels in both BL6 and LLCMK cells.

Lipid peroxidation was found to increase with increasing unsaturation (AA > GLA > LA) of the EFAs. This effect was significant between all three EFAs ($p < 0,05$) at 25 and 50µg/ml in both BL6 and LLCMK cells. At 10 µg/ml little difference between the effects of the three EFAs on lipid peroxidation was evident in the two cell lines.

When comparing results from the two cell lines, control LLCMK cells were found to produce significantly more ($p < 0,05$) lipid peroxides than control BL6 cells. While higher levels of lipid peroxidation were measured in BL6 cells than in LLCMK cells upon EFA supplementation, this effect was only significant at 25µg/ml ($p < 0,05$) and 50µg/ml ($p < 0,001$) supplementary AA.

EFA containing medium incubated in the absence of cells was found to contain a negligible level of lipid peroxides when compared with EFA supplemented cell cultures (the level of lipid peroxides measured in AA supplemented medium incubated in the absence of cells is depicted in figure 5.24).

Meehanisms of free radical production and lipid peroxidation. IM, CA and MPP did not influence free radical production (figure 5.23) or lipid peroxidation (figure 5.24) in either of the cell lines treated with any of the three EFAs. TOC was found to significantly ($p < 0,001$) reduce both free radical (figure 5.23) and lipid peroxide (figure 5.24) production in both cell lines, treated with any of the three EFAs, to levels approaching those found in control cultures which were not supplemented with any EFAs.

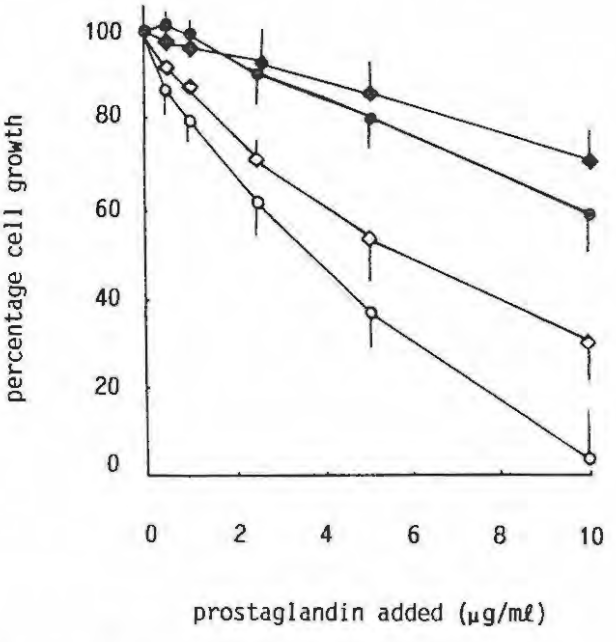

Figure 5.11. Effect of increasing concentrations of supplementary PGE_1 and PGE_2 on growth of cultured cells. ◆ PGE_2 LLCMK cells; ◇ PGE_2 BL6 cells; ● PGE_1 LLCMK cells; ○ PGE_1 BL6 cells. Results are mean and SEM of 5 cultures. Both PGE_1 and PGE_2 were found to significantly ($p < 0,01$) inhibit growth of both cell lines. PGE_1 was found to inhibit cell growth significantly more ($p < 0,001$) than PGE_2 at 10µg/ml in BL6 cells. Inhibition of BL6 cells by both PGE_1 and PGE_2 was found to be significantly ($p < 0,01$ at all concentrations above 1µg/ml) more pronounced than inhibition of LLCMK cells.

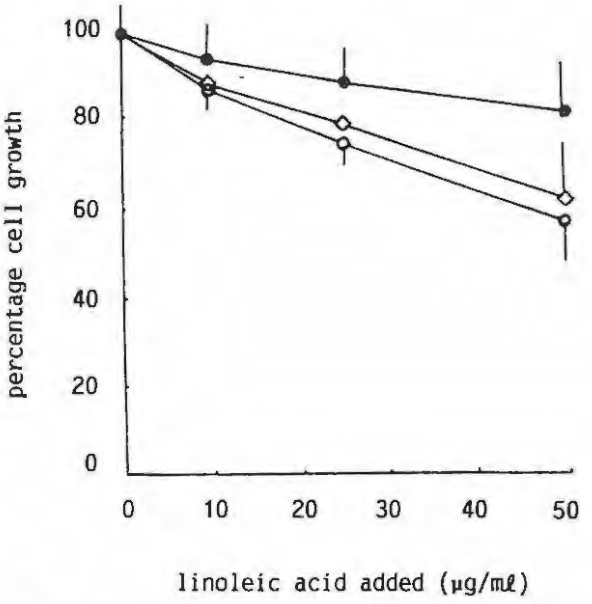

Figure 5.12. Effect of the PG synthesis inhibitor IM and the antioxidant TOC on LA mediated LLCMK cell growth inhibition. o LA; ◇ LA + 5µM IM; ● LA + 5µM TOC. The lipoxygenase inhibitor CA (5µM) did not influence LA mediated cell growth inhibition. These results were ommitted for clarity as they overlap with the curve for cells treated with LA only. Results are mean and SEM of 5 cultures. Neither IM or CA were found to significantly affect LLCMK cell growth inhibition by LA while TOC was found to significantly ($p < 0,01$ at all LA concentrations except 10µg/ml) reduce growth inhibition by LA.

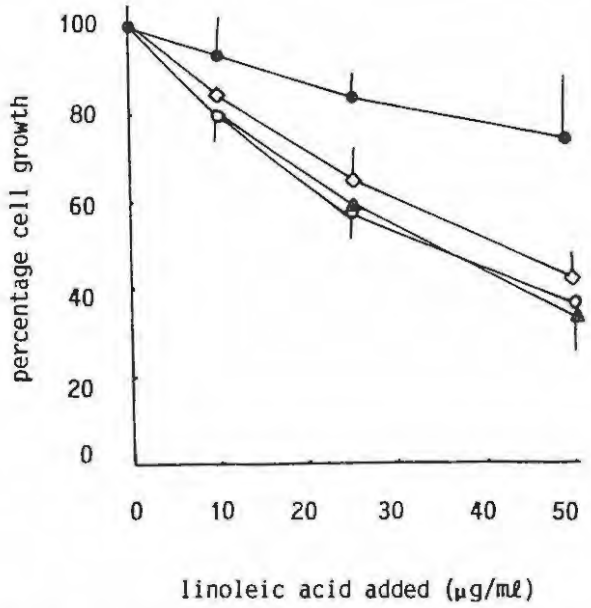

Figure 5.13. Effect of IM, CA and TOC on LA mediated BL6 cell growth inhibition. o LA; ◇ LA + 5µM IM; ▲ LA + 5µM CA; ● LA + 5µM TOC. Results are mean and SEM of 5 cultures. IM and CA were found not to significantly affect growth inhibition of BL6 cells by LA while TOC was found to significantly ($p < 0,01$ at all LA concentrations) reduce the BL6 cell growth inhibitory effects of LA.

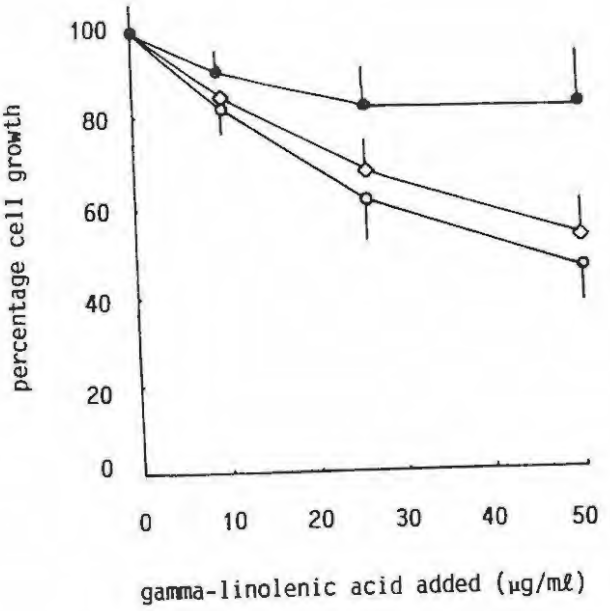

Figure 5.14. Effect of IM and TOC on GLA mediated LLCMK cell growth inhibition. ○ GLA; ◇ GLA + 5µM IM; ● GLA + 5µM TOC. Results for cells treated with GLA and 5µM CA overlap closely with those for cells treated with GLA only and were ommitted for clarity. Results are mean and SEM for 5 cultures. IM and CA did not affect LLCMK cell growth inhibition by GLA while TOC was found to significantly ($p < 0,01$ at all GLA concentrations except 10µg/ml) reduce LLCMK cell growth inhibition by GLA.

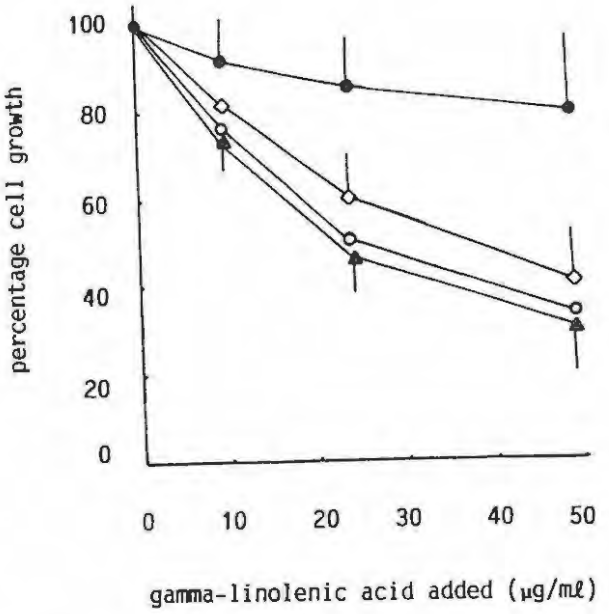

Figure 5.15. Effect of IM, CA and TOC on GLA mediated BL6 cell growth inhibition. ○ GLA; ◇ GLA + 5µM IM; ▲ GLA + 5µM CA; ● GLA + 5µM TOC. Results are mean and SEM for 5 cultures. IM and CA were found not to significantly affect BL6 cell growth inhibition by GLA while TOC was found to significantly ($p < 0,01$ at all GLA concentrations) reverse the BL6 cell growth inhibitory effects of GLA.

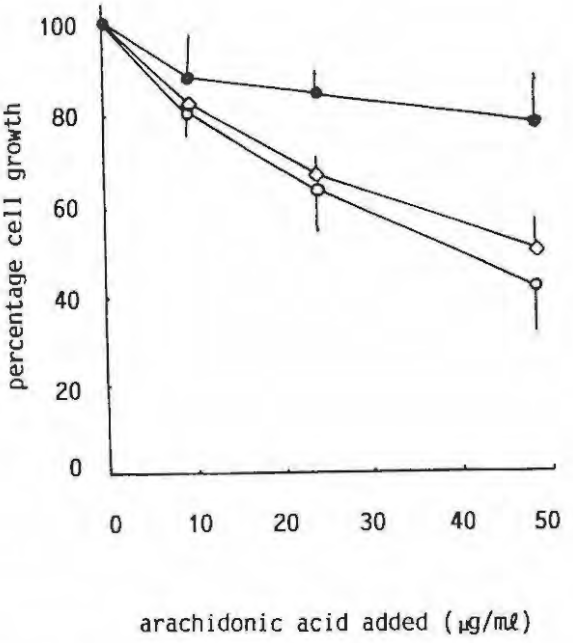

Figure 5.16. Effect of IM and TOC on AA mediated LLCMK cell growth inhibition. o AA; ◇ AA + 5µM IM; ● AA + 5µM TOC. Results for cells treated with AA and 5µM CA overlap closely with those of cells treated with AA only and were ommitted for clarity. Results are mean and SEM of 5 cultures. IM and CA were found not to significantly affect LLCMK cell growth inhibition by AA while TOC was found to significantly ($p < 0,01$ at all AA concentrations except 10µg/ml) reduce the LLCMK cell growth inhibitory effects of AA.

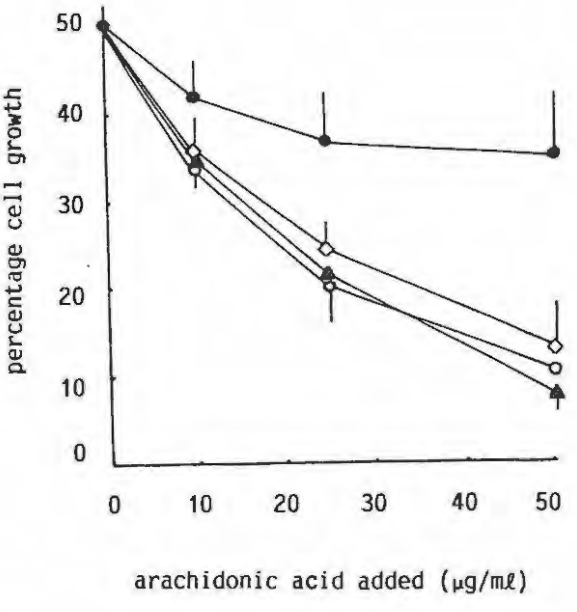

Figure 5.17. Effect of IM, CA and TOC on AA mediated BL6 cell growth inhibition. oAA; ◇AA + 5µM IM; ▲AA + 5µM CA; ● AA + 5µM TOC. Results are mean and SEM of 5 cultures. IM and CA were found not to significantly affect BL6 cell growth inhibition by AA while TOC was found to significantly ($p < 0,01$ at all AA concentrations) reduce the BL6 cell growth inhibitory effects of AA.

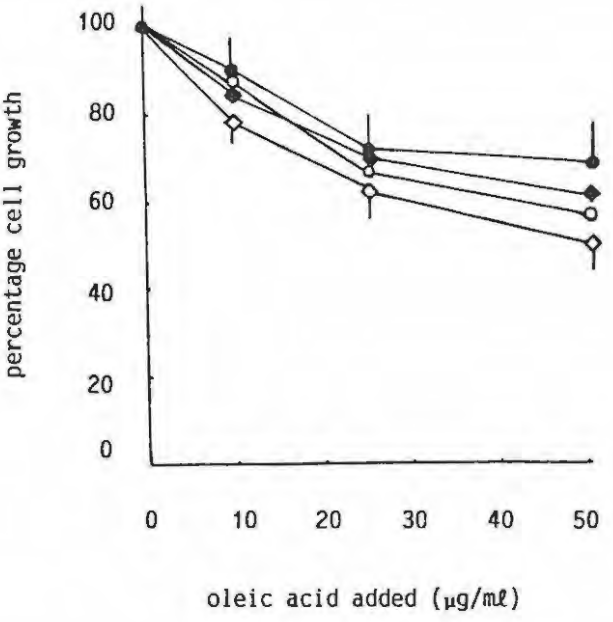

Figure 5.18. Effect of increasing concentrations of supplementary OA on growth of cultured cells and the influence of TOC on these effects. o LLCMK cells; ● LLCMK cells + 5µM TOC;◇BL6 cells; ◆ BL6 cells + 5µM TOC. Results are mean and SEM of 5 cultures. Cell growth inhibition by OA was significant ($p < 0,01$) in both cell lines. Reversal of OA cell growth inhibition by TOC was not significant in either cell line.

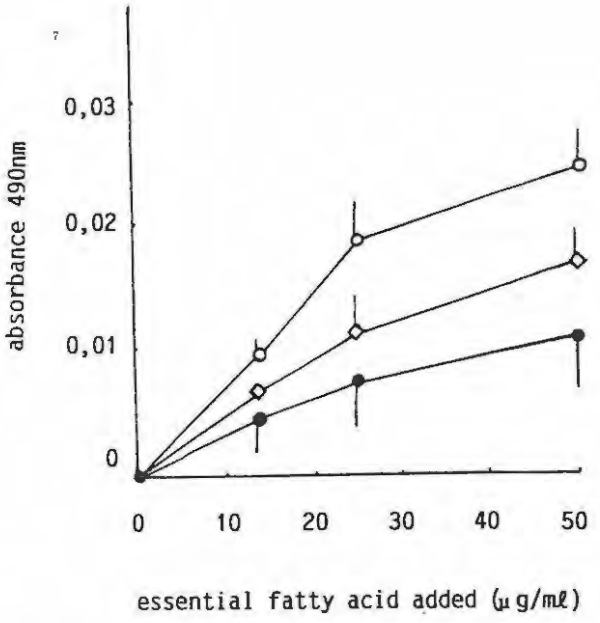

Figure 5.19. Effect of increasing concentrations of supplementary EFAs on free radical formation by LLCMK cells. ● LA; ◇ GLA; o AA. Results are mean and SEM of 5 cultures. The increase in free radical production was significant ($p < 0,01$) at all levels of supplementation of the three EFAs. Free radical production was found to increase with increasing unsaturation of the EFAs, this effect being significant ($p < 0,05$) between all three EFAs at concentrations of 25 and 50µg/ml.

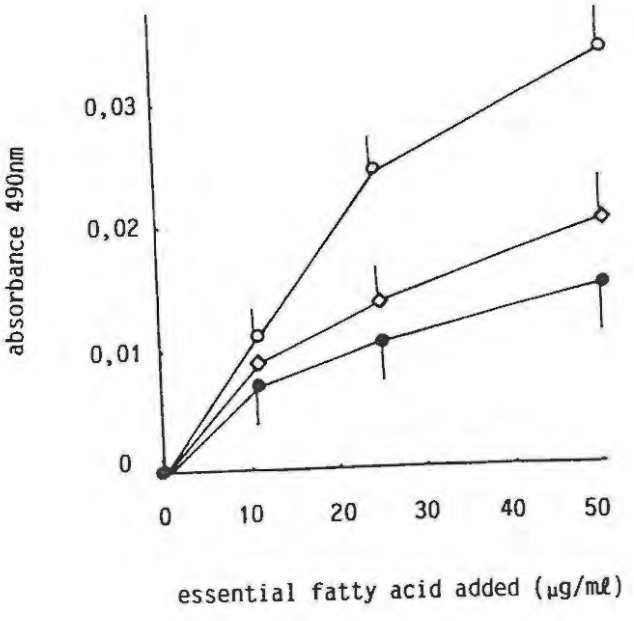

Figure 5.20. Effect of increasing concentrations of supplementary EFAs on free radical formation by BL6 cells. ● LA; ◇ GLA; o AA. Results are mean and SEM of 5 cultures. The increase in free radical production was significant ($p < 0,01$) at all levels of supplementation of the three EFAs. Free radical production was found to increase with increasing unsaturation of the EFAs, this effect being significant ($p < 0,05$) between all three EFAs at concentrations of 25 and 50µg/ml.

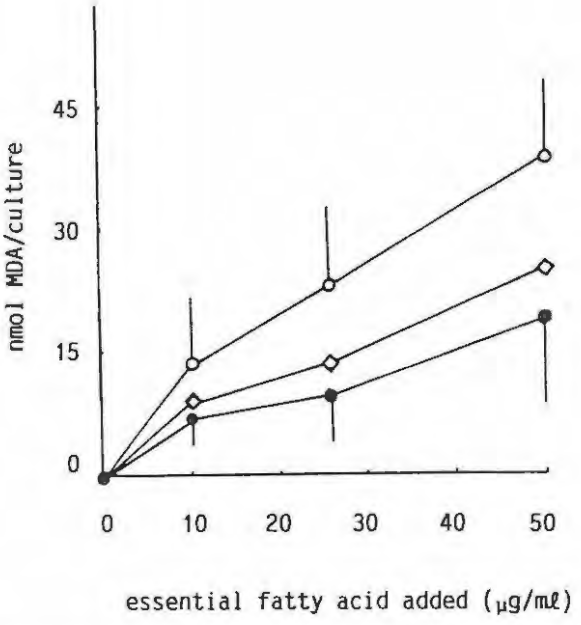

Figure 5.21. Effect of increasing concentrations of supplementary EFAs on lipid peroxidation by LLCMK cells. ● LA; ◇ GLA; ○ AA. Results are mean and SEM of 5 cultures. The increase in lipid peroxidation was significant ($p < 0,01$) at all levels of supplementation of the three EFAs. Lipid peroxidation was found to increase with increasing unsaturation of the EFAs, this effect being significant ($p < 0,05$) between all three EFAs at concentrations of 25 and 50µg/ml.

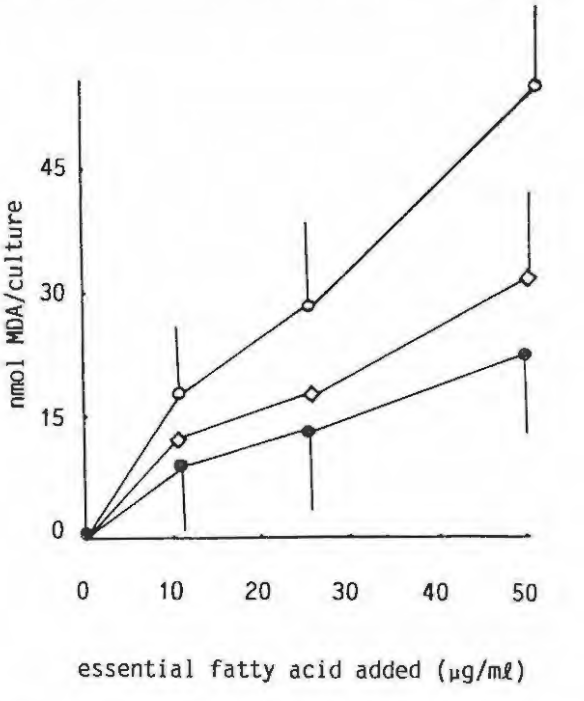

Figure 5.22. Effect of increasing concentrations of supplementary EFAs on lipid peroxidation by BL6 cells. ● LA; ◇ GLA; ○ AA. Results are mean and SEM of 5 cultures. The increase in lipid peroxidation was significant ($p < 0,01$) at all levels of supplementation of the three EFAs. Lipid peroxidation was found to increase with increasing unsaturation of the EFAs, this effect being significant ($p < 0,05$) between all three EFAs at concentrations of 25 and 50µg/ml.

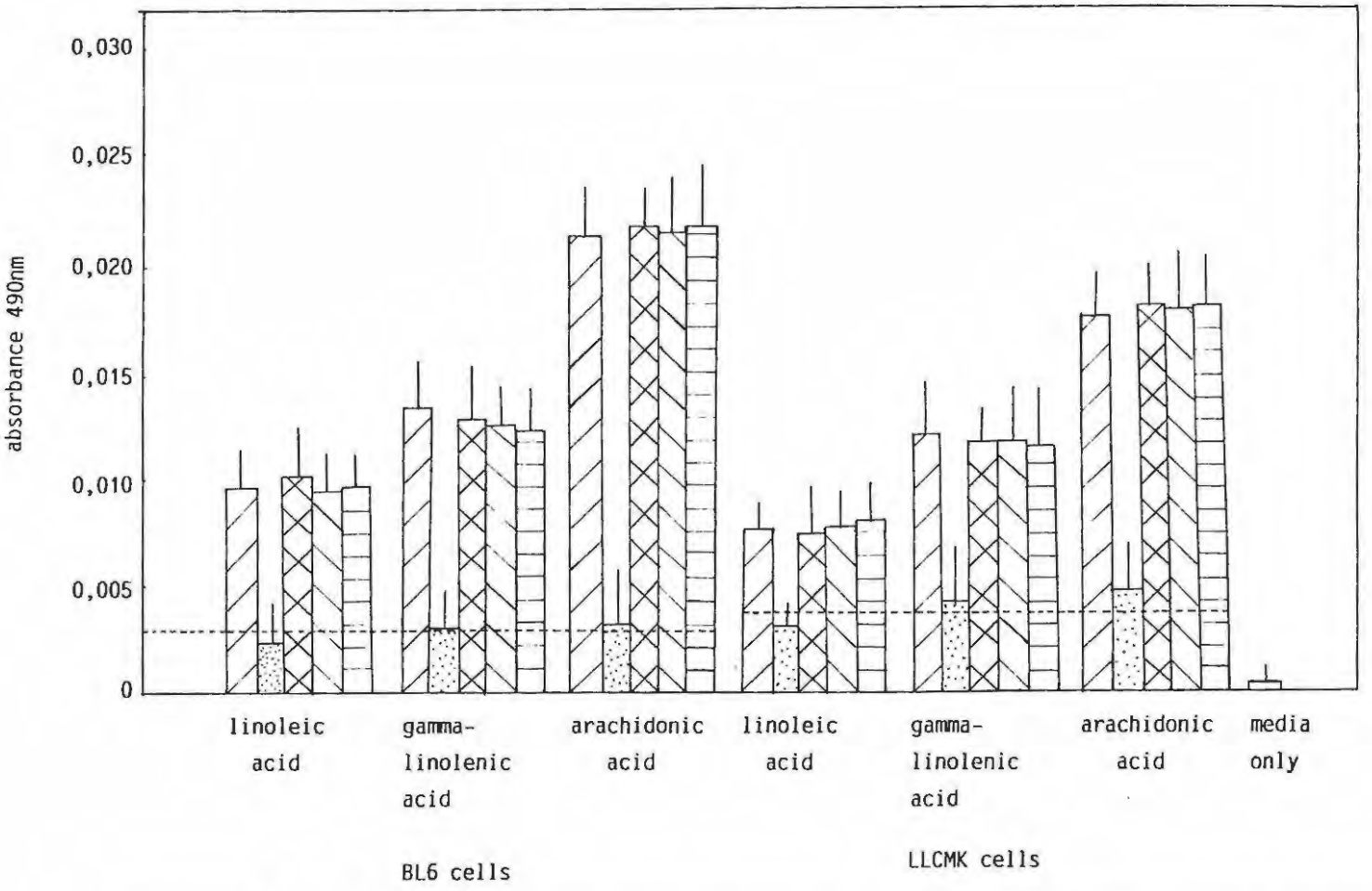

Figure 5.23. Effect of PG synthesis (IM), LT synthesis (CA) and cytochrome P_{450} (MPP) inhibitors and TOC (all 5μM) on free radical formation by cultured cells containing supplementary EFAs (25μg/ml). ▨ control (control cultures were EFA supplemented but contained none of the other chemicals tested); ▩ 5μM TOC; ▩ 5μM IM; ▩ 5μM CA; ▩ 5μM MPP. Results are mean and SEM of 5 cultures. IM, CA and MPP were found not to significantly influence free radical formation in either cell line while TOC was found to significantly ($p < 0,001$) reduce free radical formation in both cell lines treated with any of the three EFAs. The dotted line represents the level of free radical formation measured in cultures with no EFA supplementation. Free radical formation in media only refers to the level of radicals measured in medium containing 25μg/ml AA incubated in the absence of cells.

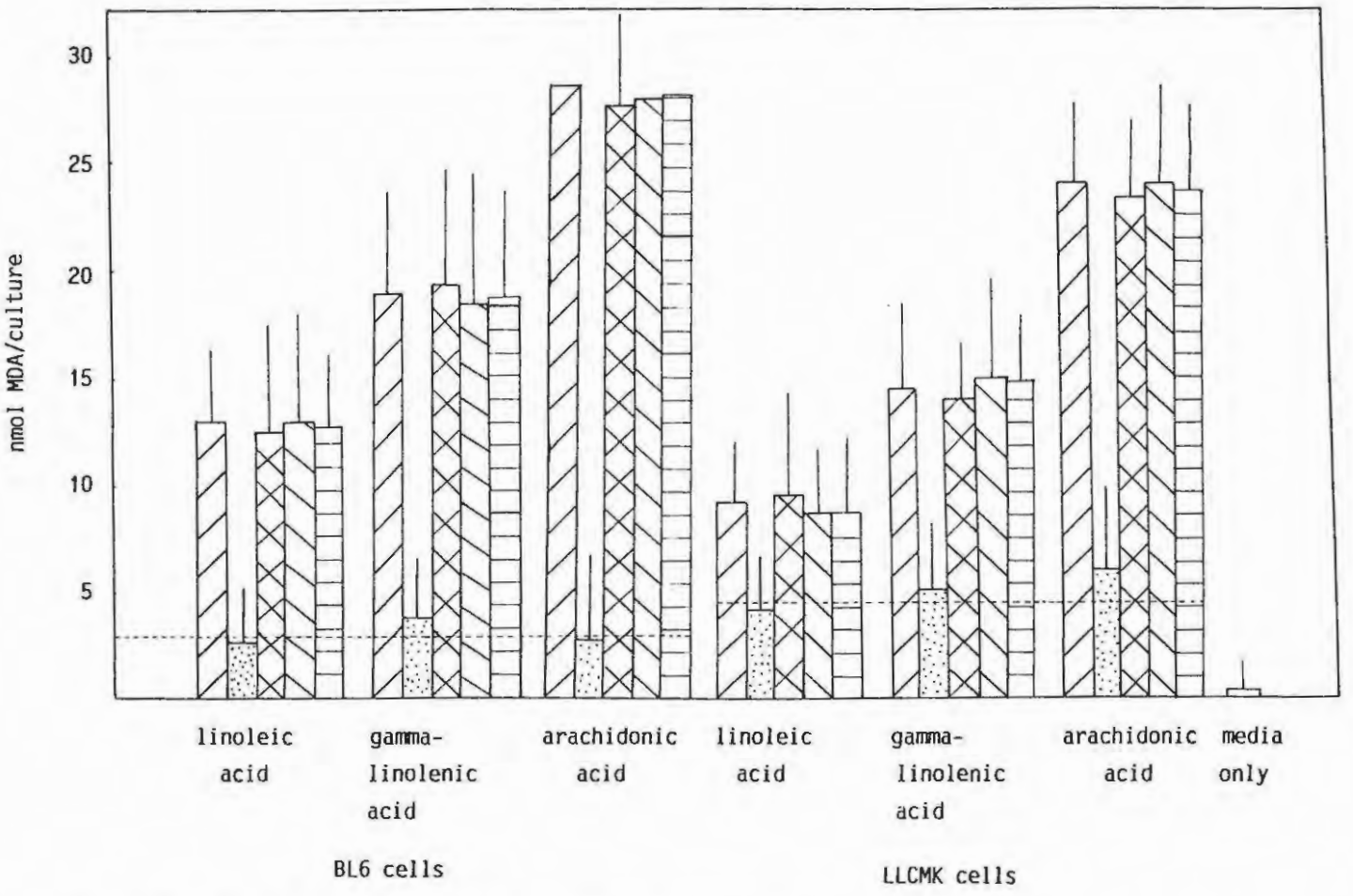

Figure 5.24. Effect of IM, CA, MPP and TOC on lipid peroxidation in cultures containing supplementary EFAs (25µg/ml). ▨ control (control cultures were EFA supplemented but contained none of the other chemicals tested); ⁘ 5µM TOC; ▩ 5µM IM; ▧ 5µM CA; ▤ 5µM MPP. Results are mean and SEM of 5 cultures. IM, CA and MPP were found not to significantly influence lipid peroxidation in either cell line while TOC was found to significantly ($p < 0{,}001$) reduce lipid peroxidation in both cell lines treated with any of the three EFAs. The dotted line represents the level of lipid peroxidation measured in cultures containing no supplementary EFAs. Lipid peroxidation in media only refers to the level of peroxidation measured in medium containing 25µg/ml AA incubated in the absence of cells.

5.4 Discussion

All three EFAs used in this study, namely LA, GLA and AA significantly inhibited growth of both malignant BL6 melanoma and benign LLCMK cells in a dose dependant manner, inhibition being significant at all concentrations (10-50μg/ml). The growth inhibitory effects were more pronounced in the BL6 cells than in the LLCMK cells with this effect being significant at higher concentrations of GLA and AA. Numerous other studies have previously reported similar growth inhibitory effects of EFAs on cultured cells (156-166,168,169,171). Since these studies are too numerous to discuss, a few pertinent examples should suffice. Horrobin and colleagues (158,159) reported that LA, GLA and AA at a concentration of 20μg/ml were more effective in inhibiting growth of three human carcinoma cell lines than of four benign cell lines. In co-cultures of carcinoma cells and normal human fibroblasts, the EFAs were selectively cytotoxic towards the carcinoma cells. Booyens and colleagues have examined the effects of EFAs, and in particular GLA, on the growth of a number of human cancer and benign cell lines. GLA at concentrations ranging from 10-60μg/ml was reported to inhibit growth of human sarcoma and carcinoma cells (157,161,163), while at concentrations ranging from 1-10μg/ml GLA was reported to significantly inhibit BL6 cell growth while not influencing growth of benign MDBK cells (166).

Since LLCMK cells were shown to take up significantly more radiolabelled LA and AA than BL6 cells, the increased growth inhibition observed in BL6 cells was obviously not due to greater uptake of EFAs by BL6 cells. It would thus appear that BL6 cells are more susceptible to the growth inhibitory effects of intracellular EFAs than LLCMK cells as they incorporate less EFAs than the LLCMK cells but are inhibited to a greater extent by supplementary EFAs. These results confirm the observations of Skeef (377) that LLCMK cells take up more ^{14}C-LA than BL6 cells and those of Das et al (198,199) that human breast tumour, human leukaemic and BALB transformed murine fibroblast cells incorporate more LA and AA than normal human fibroblasts. In the latter study the tumour cells were also found to be more susceptible to the growth inhibitory effects of the EFAs than the benign cells. The increased susceptibility of the BL6 cells to the growth inhibitory effects of the EFAs may be the result of the more rapid proliferation of the BL6 cells in comparison to the LLCMK cells. Furthermore tumour cell membranes are reported to contain less PUFAs/EFAs than those of normal cells (137,191), consequently their lipid content would presumeably be more susceptible to manipulation by supplementary EFAs. Since changes in membrane lipid composition, and therefore membrane fluidity, are thought to influence cell growth (100), the tumour cells would obviously be more susceptible to the growth inhibitory effects of supplementary EFAs if the mechanism of cell growth inhibition involved alterations in membrane EFA/PUFA content.

The growth inhibitory effects of the EFAs were found to be to be reversible and to require the presence of the EFAs in the medium. In this study, replacement of EFA containing medium with fresh medium containing no supplementary EFAs, resulted in increased growth of cells when compared with cells grown in medium containing EFAs throughout the experimental period. Booyens *et al* (162), in contrast, reported that the growth inhibitory effects of GLA were not reversible in human carcinoma and LLCMK cells exposed to 10μg/ml GLA for 5 days and subsequently grown in GLA free medium. The longer exposure of cells to GLA in that study in comparison to the present study may have given rise to the apparently contradictory results.

Several studies have however reported that while cells rapidly absorb exogenous EFAs, not all the EFAs can be metabolised and are stored in the cytoplasm of the cells in the form of lipid droplets and triglycerides which are thought to interfere with cellular metabolism leading to cell growth inhibition (160,189,194). Ultrastructural investigations have shown that upon removal of exogenous EFAs, the cells metabolise the excess EFAs in the lipid droplets thus enabling growth of the cells to proceed at an increased rate (194). The results of this study appear to support these observations. The reversal of growth inhibition upon removal of exogenous EFAs became significant after 72 hours in BL6 cells and after 96 hours in LLCMK cells, the cells presumeably having utilised this period to metabolise some of the excess EFAs. This effect presumeably became evident sooner in BL6 cells than in LLCMK cells since the more rapidly dividing BL6 cells would be expected to have a higher rate of metabolism. It must be noted however that some studies have reported that most cultured cells can tolerate a moderate increase in the level of triglycerides and lipid droplets without any apparent deletrious effects (cited in 408). It would therefore appear, particularly since cell growth was inhibited even at lower EFA concentrations in this study, that other mechanisms are also involved in the observed growth inhibitory effects of the EFAs.

A comparison of the growth inhibitory effects of the three EFAs used in this study revealed that the potency of the individual EFAs in inhibiting cell growth corresponded to increasing unsaturation of the EFAs although in both cell lines this was only significant at an EFA supplementation of 50ug/ml. Several previous studies have compared the growth inhibitory effects of individual EFAs on cultured cells. Booyens and colleagues (162) reported that GLA was a more effective inhibitor of human hepatoma cell growth than LA and that AA was more effective than LA or GLA in inhibiting human sarcoma cell growth. Robinson *et al* (160) reported that GLA was a more effective inhibitor of human carcinoma growth than LA, while Cornwell *et al* (169) found AA to be a more effective inhibitor of guinea pig aorta smooth muscle cell growth than GLA. LA was found not to influence the growth of the same cells at concentrations of up to 100μM in the same study.

Since the cells used in the present study were found to take up more radiolabelled AA than LA, it is possible that the different potencies of the EFAs in inhibiting cell growth may be related to different levels of uptake of the individual EFAs by the cells. However since excess EFAs which may be absorbed by cells are stored in the form of cytoplasmic lipid droplets and triglycerides (160,189,194,408), which may (160,189,194) or may not (408) result in cell growth inhibition, it is also possible that a more specific effect of the EFAs might be involved in the growth inhibition. As previously mentioned several other mechanisms have been proposed for the growth inhibitory effects of the EFAs . In this study the possible involvement of increased PG or LT synthesis and lipid peroxidation in the mechanism of cell growth inhibition by the EFAs was examined.

Endogenous synthesis of PGs has been found to be involved in the regulation of cell proliferation in certain cells including human lung fibroblasts (187), HeLa and HEp-2 cells (177) and polyoma virus transformed 3T3 fibroblasts (181). Since supplementary EFAs have been reported to increase endogenous PG synthesis (178,186-188) and since PG synthesis inhibitors, including IM, have been shown to stimulate the growth of certain cells (176,177,181), it has been proposed that inhibition of cell growth by EFAs might be mediated by increased PG synthesis. Furthermore, as found in this study, exogenous PGs including PGE_1 and PGE_2 have been reported to inhibit cell growth (156,164,169,175-182). As found in studies examining the effect of exogenous EFAs on cell growth, inhibition of malignant BL6 cell growth by the PGs used in this study was found to be more pronounced than inhibition of the benign LLCMK cells.

The role played by endogenous PGs in the growth inhibitory effects of the EFAs appears to be dependant on cell type however. In this study IM was found not to significantly influence the growth inhibitory effects of the EFAs in either BL6 or LLCMK cells. This result appears to preclude enhanced PG synthesis as the mechanism whereby EFAs exerted their growth inhibitory effects in these cells. Furthermore in this study OA, which is not a PG precursor, was also found to inhibit cell growth, although this inhibition was not as great as that observed in EFA treated cells. Several other studies have also provided evidence that enhanced PG synthesis may not be the mechanism whereby EFAs exert their growth inhibitory effects. Begin *et al* (171) and Botha *et al* (190) reported enhanced GLA mediated cell growth inhibition in several human carcinoma cell lines upon IM supplementation. Fujiwara *et al* (164) have shown that IM and aspirin (also a PG synthesis inhibitor) did not influence GLA cytotoxicity in four human neuroblastoma cell lines, while Cornwell and associates (169,202) reported that IM did not influence GLA and AA mediated cell growth inhibition in guinea pig aorta smooth muscle cells.

While it has been proposed that lipoxygenase products including the LTs and hydroxy fatty acids might play a role in inhibition of cell growth by EFAs (169), the lack of effect of CA, a lipoxygenase inhibitor,

on the EFA mediated growth inhibition observed in this study would appear to preclude LTs and other lipoxygenase products from involvement in EFA growth inhibition.

Reports that both lipid peroxides and free radicals are toxic to cultured cells (198,200) and that the growth inhibitory effects of the EFAs can be reversed by antioxidants (159,164,168,169,197,201,202) led to the proposal that increased lipid peroxidation might play a role in the growth inhibitory effects of EFAs on cultured cells. Since susceptibility to peroxidation of fatty acids increases with increasing unsaturation (144), this hypothesis was particularly attractive to the present study as cell growth inhibition by the EFAs was found to increase with increasing unsaturation. The antioxidant TOC was found, in this study, to significantly reverse the growth inhibitory effects of the EFAs in both cell lines. Since this result suggested that lipid peroxidation might be involved in the growth inhibitory effects of the EFAs, the production of lipid peroxides in EFA supplemented cultures was determined. Furthermore the level of free radicals which are the initiating agents in lipid peroxidation (144) were determined.

A dose dependant increase in the generation of free radicals and lipid peroxides by both BL6 and LLCMK cells supplemented with EFAs was observed in this study. The level of free radical production and of lipid peroxidation in the cells was found to increase with increasing unsaturation of the EFAs (AA > GLA > LA). This result was not unexpected since susceptibility to lipid peroxidation is known to be associated with increasing unsaturation as previously mentioned. Since lipid peroxides are known to decompose to form free radicals (139,144) the increased free radical production observed was also expected.

In this study LLCMK cells grown in medium containing no supplementary EFAs were found to produce higher levels of free radicals and lipid peroxides than control BL6 cells. Upon supplementation with EFAs however, BL6 cells were found to produce significantly higher levels of free radicals and lipid peroxides than LLCMK cells. These observations suggest that tumour cells do not have a decreased capacity for lipid peroxidation or free radical generation when compared with normal cells, but that these processes may be inhibited by lack of substrate, particularly since tumour cell membranes are known to contain lower levels of PUFAs/EFAs (93,134-136). Since tumour cells have been found to have reduced activity of the free radical scavenging enzymes, superoxide dismutase and glutathione peroxidase (199), they would presumeably be more susceptible to free radical induced lipid peroxidation when provided with substrate in the form of exogenous EFAs. The reduced activity of these enzymes could also provide an explanation for the observation that, upon EFA supplementation, BL6 cells generate more lipid peroxides than LLCMK cells despite the fact that the LLCMK cells were found in this study to take up more radiolabelled LA and AA than BL6 cells. These results confirm those of Das *et al* (198,199) who reported that human breast cancer cells

produced significantly higher levels of free radicals than normal human fibroblasts when supplemented with EFAs.

The results of this study suggest that the mechanism of EFA mediated cell growth inhibition may have involved lipid peroxidation since a correlation appears to exist between the level of growth inhibition by the EFAs and their ability to induce lipid peroxidation. The most potent inhibitor of cell growth, namely AA, was found to induce the highest level of lipid peroxidation while GLA which was a more potent inhibitor of cell growth than LA also induced a higher level of lipid peroxidation than LA. Furthermore the growth inhibitory effects of the EFAs were more pronounced in the BL6 cells which were found to generate more lipid peroxides than the LLCMK cells. Several studies have arrived at a similar conclusion using different cell lines. Begin *et al* (159) in a study using human breast cancer cells reported a correlation between the effectiveness of a given EFA in inhibiting tumour cell growth and the extent of lipid peroxidation induced by that EFA. Liepkalns *et al* (168) in a study using human glioblastoma and foetal brain cells and Gavino *et al* (201) in a study utilising guinea pig smooth muscle cells also provided support for the involvement of lipid peroxidation in the mechanism of EFA cell growth inhibition. In contrast Morisaki *et al* (186) found that while exogenous PUFAs, including EFAs, induced lipid peroxidation in guinea pig smooth muscle cells, no correlation could be found between the level of lipid peroxidation and cell growth inhibition by the PUFAs/EFAs.

Fujiwara *et al* (164) in a study using four human neuroblastoma cell lines could find no correlation between the level of reversal of GLA mediated cell growth inhibition by TOC and the reduction in the level of lipid peroxidation by TOC in these cells. While the results of this study suggest that lipid peroxidation might be involved in the mechanism of cell growth inhibition by EFAs, a closer examination of the data reveals, as in the study of Fujiwara *et al*, that while TOC reduces lipid peroxidation in EFA supplemented cells to levels approaching those in unsupplemented cells, it only partially reverses the growth inhibitory effects of the EFAs. It is thus likely that while lipid peroxidation might be involved in the mechanism of EFA mediated cell growth inhibition, another mechanism(s) is also likely to be involved. Other suggested mechanisms, some of which have already been mentioned in this study, include intracellular accumulation of EFAs (173,189,194-197) and alterations in membrane lipid content and fluidity (30,165,192,193).

The results of this study also provide further evidence of the effectiveness of TOC as a free radical scavenger and anti-oxidant protecting cells against lipid peroxidative damage. Supplementary TOC was found to reduce the levels of free radicals and lipid peroxides produced by cells, at all levels of EFA supplementation, to levels approaching those measured in unsupplemented cells.

The involvement of certain enzyme systems in the production of free radicals and lipid peroxidation were also examined in this study. The reactions catalysed by cyclooxygenase, lipoxygenase and

cytochrome P_{450} have been reported to generate free radicals and lipid peroxides (202). In this study however, inhibitors of these pathways, namely IM, CA and MPP, were found not to influence free radical production or lipid peroxidation in EFA supplemented cells. It is thus likely that general peroxidation reactions rather than the reactions catalysed by these enzymes are involved in the production of lipid peroxides and free radicals in the cells used in this study. Similar observations have been reported by Morisaki *et al* (202) in studies using guinea pig smooth muscle cells.

In conclusion therefore, the growth inhibitory effects of EFAs were found to be more pronounced in the malignant cells than the benign cells used in this study. The growth inhibitory effects were found to increase with increasing unsaturation of the EFAs and were found to be reversible upon removal of the EFA containing medium. While lipid peroxidation might have been involved in the the mechanism of cell growth inhibition by the EFAs, it appears unlikely that enhanced synthesis of PGs or LTs was involved.

Chapter 6

INFLUENCE OF ASCORBIC ACID ON CELL GROWTH

6.1.Introduction

Although Asc does not appear to be a requirement for growth of many cell lines (358), certain cells grow very poorly in the absence of Asc (346,355,358). A number of studies have shown that tumour cell growth is inhibited by supplementary Asc (312,344,345,347,348,350), while the growth inhibitory effects on normal cells are more selective being dependant on cell type and on the level of Asc added (344,357).

The present study was carried out to determine the effect of Asc on the growth of cultured BL6 and LLCMK cells. Since one of the main aims of this dissertation was to investigate the mechanisms of the effect of Asc on cell growth and possible interactions between Asc and EFAs, this study also examined whether enhanced PG synthesis or lipid peroxidation were involved in the growth inhibitory effects of Asc on tumour cells. These two mechanisms were examined since previous studies using cultured cells, cell free systems and tissue homogenates have reported that Asc influences PG synthesis (366-372) and lipid peroxidation (276,334-342). The possible role played by these mechanisms in cell growth inhibition has previously been discussed (section 1.5).

6.2.Materials and Methods

Materials

Ascorbic acid (SAARCHEM,Krugersdorp,RSA) was dissolved in 0,1M sodium phosphate buffer pH 7,2 to a final concentration of 2000µg/ml. Fresh solutions were made up immediately prior to use since Asc is unstable in aqueous solution.

Experimental medium and cells were as previously described in section 4.2 except that the experimental medium contained no Asc.

Dichlorophenolindophenol (DCIP) (Merck) was made up to a final concentration of 0,1µg/ml in distilled and deionised water.

Metaphosphoric acid (SAARCHEM) was made up to a final concentration of 10% in distilled and deionised water.

Citrate-acetate buffer was made up by dissolving 22g trisodium citrate dihydrate in 40ml water, adjusting the pH to 4,15 with glacial acetic acid and diluting to a final volume of 100ml with water. 200mg *p*-chloromercuribenzoate was then added, and the solution thoroughly mixed and then centrifuged to remove excess *p*-chloromercuribenzoate.

[1-^{14}C]-LA and [1-^{14}C]-AA. Details of these radiochemicals have previously been described in section 5.2.

Silver nitrate ($AgNO_3$) (10%) was prepared in 80% aqueous ethanol. Since $AgNO_3$ is light sensitive it was stored in a brown bottle in a dark cupboard. This and all subsequent work utilising $AgNO_3$ was performed in a darkroom using a photographic safety light.

Thin layer chromatography (tlc)plates (silica gel F254, 20cm x 20cm) were purchased from Merck, Darmstadt, West Germany.

Octadecylsilyl silica (ODS) cartridges. SEP-PAK C_{18} ODS cartridges were purchased from Waters Associates, Milford, Massachusetts, USA.

2,7-Dichlorofluoroscein (0,4%) was dissolved in ethanol.

IM, CA, TOC and other reagents used in the TBA and NBT assays for measurement of lipid peroxidation and free radical formation were made up as described in section 5.3.

Methods

Influence of ascorbic acid on cell growth. Asc was added to cultures at final concentrations of 0 (control), 25, 50, 75, 100 or 200µg/ml. Control cultures contained an appropriate volume of 0,1M sodium phosphate buffer pH 7,2. These concentrations have been used in numerous studies, several of which are listed in table 2.2, which examined the influence of Asc on cell growth. Cells were then incubated and enumerated as described in section 4.2. To determine whether the effects of Asc on cell growth were reversible, cells were incubated for 24 hours in the presence of 200µg/ml Asc whereafter the medium was replaced with fresh Asc free medium and the cells again incubated and enumerated as previously described. Since Asc is unstable in culture medium (378,379), in separate experiments Asc was added daily to cell cultures at 0-200µg/ml. At higher Asc concentrations this resulted in crystal formation on the flask surface which interfered with cell growth and made enumeration of cells difficult. The medium was thus replaced daily with fresh Asc containing experimental medium rather than sequential additions of Asc to the same medium. (Note : This was the only experiment in which

daily supplementation with Asc was carried out. All other experiments involved single Asc supplementation.)

Preincubation experiments. Due to the instability of Asc in culture medium it was possible that the effects of Asc on cell growth might have been due to a breakdown product of Asc rather than Asc itself. To investigate this possibility Asc supplemented (0-200µg/ml) medium was preincubated for 24 hours at 37°C in the absence of cells and the effect of this medium on cell growth was then determined.

Ascorbic acid content of cells and medium. The Asc content of the medium was determined before commencement of the experiment and at 24 hour intervals after incubation at 37°C with or without cells. The Asc content of cells was determined at termination of the study. Asc was measured by its ability to reduce DCIP as described by Omaye et al (380). Cells were trypsinised and centrifuged and the resultant cell pellet rinsed 3 times in PBS before being resuspended in 1ml of PBS. To the cell suspension (or 1ml of medium) was added 1ml of ice cold phosphoric acid and the cells were then homogenised using a Dounce homogeniser (30 strokes with the tight plunger) and centrifuged at 1000g (Hettich Universal K2S) for 20 minutes. Further analysis was then performed on 1ml of the supernatant to which was added 0,5ml DCIP and 0,5 ml citrate/acetate buffer. Exactly 30 seconds after mixing the absorbance of the solution at 532nm was determined using a Bausch and Lomb 1001 spectrophotometer. Distilled and deionised water was used as the blank. A few crystals of Asc were then added to the solution to reduce the remaining DCIP and the absorbance at 532nm again measured. The concentration of Asc was then determined using the following equation:

$$\Delta A = (RB - RB_b) - (S - S_b)$$

where: RB = absorbance of reagent blank (contained 5% phosphoric acid instead of sample); RB_b = absorbance of reagent blank after adding Asc crystals; S = absorbance of sample; S_b = absorbance of sample after adding Asc crystals.

The Asc concentration was then determined from a standard curve (appendix 2) of ΔA versus Asc concentration.

Effect of indomethacin, caffeic acid and α-tocopherol on Asc mediated cell growth inhibition. Asc (0, 50, 100 or 200µg/ml) was added to cultures together with either IM, CA or TOC (all 5µM) and the effect of these compounds on cell growth determined as before.

Measurement of free radical formation and lipid peroxidation. Free radical formation and lipid peroxidation were measured using the NBT and TBA assays (described in section 5.3) in cultures containing 0, 50, 100 or 200µg/ml Asc.

Measurement of PG synthesis. Cells were seeded at a density of $1x10^6$ cells per $25cm^2$ flask and incubated at 37°C until confluent. 0,5µCi of either ^{14}C-LA or ^{14}C-AA was then added to the flasks and the cells incubated as before for 24 hours to allow for uptake of the radiolabelled EFAs. Fresh experimental medium containing Asc (0-200µg/ml) was then added to the flasks. Cultures were incubated for a further 3 hours at 37°C before trypsinisation. The cells and medium from each culture were combined and PGs isolated from these samples.

Isolation of PGs. PGs were extracted according to the method of Powell (406). Cells were homogenised in the medium (10ml) using a Dounce homogeniser (30 strokes with the tight plunger). The lysate was then added to 15ml cold ethanol (95%) and shaken for 10 minutes. The sample was diluted to 100ml with cold water, vortexed for 1 minute and then centrifuged at 1000g (Hettich Universal K2S) for 20 minutes. The pH of the supernatant was adjusted to 3,00 using 1N HCl and the sample was then passed through a SEP-PAK cartridge, which had previously been wet by passing 20 ml of 80% aqueous ethanol through it and then washed with 20ml water to remove excess ethanol. The cartridges were then washed with 10ml water and 10ml petroleum ether before elution of the PGs using 5ml methyl formate. The eluant was dried under a stream of nitrogen at 25°C and redissolved in 50ul acetonitrile. 25ul of this sample was used to determine radioactivity of total PGs and the other 25ul used for separation of PGE_1 and PGE_2 by argentation tlc. The SEP-PAK cartridges were regenerated for reuse by washing with 20ml of 80% ethanol followed by 20ml water.

Preparation of argentation thin layer chromatography plates. Tlc plates were immersed in $AgNO_3$ for 10 seconds and then air dried. They were then activated by heating at 70°C for 1 hour.

Separation of prostaglandins E_1 and E_2. Separation of PGE_1 and PGE_2 was achieved by using a slight modification of the method of Bomalski et al (399). Unlabelled PGE_1 and PGE_2 (25µl of a 1mg/ml solutions) were added to samples to allow visualisation of samples. Samples were spotted onto the plates using glass capillary columns and air dried. The plates were then developed in the mobile phase which consisted of chloroform:methanol:acetic acid:water (90:7,5:7,5:0,8). After developement the plates were air dried, dipped in iodine vapour for 30 seconds and then sprayed with dichlorofluoroscein. The plates were visualised under a UV light and spots corresponding to PGE_1 and PGE_2 (identified by co-chromographing with standards) were carefully scraped into scintillation vials and mixed with 10ml scintillation fluid before counting the radioactivity in a Beckman LS 3801 scintillation counter.

Statistical analysis. One way analysis of variance and Scheffe's multiple range test were used for statistical analysis.

6.3.Results

Influence of ascorbic acid on cell growth. Asc was found to significantly ($p < 0,001$) inhibit growth of BL6 cells in a dose dependant manner while no significant effects on the growth of LLCMK cells were observed (figure 6.1). The growth inhibitory effects on BL6 cells are further illustrated by the growth curves obtained using the counting plate (figure 6.2). Growth curves were not determined for LLCMK cells since Asc did not influence growth of these cells at the concentrations used in this study. The growth inhibitory effects of Asc on BL6 cells appear not to be reversible since cells grown for 24 hours in medium containing 200µg/ml Asc, and subsequently in Asc free medium, continued to grow at the same rate as cells grown in medium containing 200µg/ml Asc throughout the experimental period (figure 6.2). In a separate experiment, cells treated with Asc for 24 hours and subsequently grown in Asc free medium were found to grow at the same rate as cells treated with 200µg/ml Asc for a period of two weeks, whereupon the experiment was terminated (results not shown).

Preincubation studies. Inhibition of both BL6 and LLCMK cell growth was not significant when the cells were grown in Asc containing medium which had been preincubated for 24 hours at 37°C (table 6.1).

Ascorbic acid content of medium. The Asc content of the medium prior to the commencement of the experiments correlated well with the level of added Asc (table 6.2). After 24 hours incubation at 37°C, with or without cells, the Asc content of the medium was found to have fallen significantly at all levels of added Asc (table 6.2). While the level of Asc was slightly lower in medium incubated with cells, no significant difference was evident when compared with medium incubated without cells. This suggests that the decline in the Asc levels was due mainly to auto-oxidation of Asc, rather than to Asc uptake by the cells. In view of the rapid loss of Asc from the medium, the medium in the cultures was replaced daily with fresh Asc containing medium and the influence on cell growth investigated. Daily Asc supplementation was found to significantly ($p < 0,05$) increase inhibition of BL6 cell growth when compared with single Asc supplementation at the commencement of the experiment (figure 6.1). In LLCMK cells daily Asc supplementation was found to inhibit cell growth slightly but non-significantly when compared with single Asc supplementation (figure 6.1).

Ascorbic acid content of cells. Supplementary Asc was found to significantly ($p < 0,001$) increase the Asc content of both BL6 and LLCMK cells in a dose dependant manner (figure 6.3). No significant difference between the Asc content of the two cell lines was evident however.

Effect of indomethacin, caffeic acid and α-tocopherol on ascorbic acid mediated cell growth inhibition. IM, CA and TOC were found to have no effect on BL6 cell growth inhibition by Asc (table 6.3).

Free radical formation. A significant ($p < 0,01$) increase in the production of free radicals by both BL6 and LLCMK cells was evident with increasing Asc supplementation (figure 6.4). While free radical production by LLCMK cells was greater, the difference was not significant when compared with BL6 cells.

Lipid peroxidation. Asc supplementation was found to significantly ($p < 0,05$) increase the level of lipid peroxidation by both BL6 and LLCMK cells (figure 6.5). While lipid peroxidation by LLCMK cells was greater, the difference was not significant when compared with BL6 cells.

PG synthesis i)From ^{14}C-LA. (tables 6.4 and 6.5) Supplementary Asc was found to significantly increase synthesis of PGE_1 from ^{14}C-LA by BL6 cells ($p < 0,05$ at 100 and 200μg/ml) and LLCMK cells ($p < 0,05$ at 200μg/ml).

When comparing data from tables 6.4 and 6.5 it was found that significantly more ($p < 0,05$) PGE_1 was produced in BL6 cells than in LLCMK cells at all levels of Asc supplementation.

Radiolabelled PGE_2 was synthesised, albeit in small quantities, from ^{14}C-LA in both BL6 and LLCMK cells indicating that these cells possess activity of the enzyme delta-5-desaturase which catalyses the desaturation of dihomo-gamma-linolenic acid to AA (see figure 1.1). No significant difference in the level of PGE_2 produced in the two cell lines was evident.

While total PG synthesis from ^{14}C-LA was increased by Asc supplementation in both BL6 and LLCMK cells, the increase was not significant. LLCMK cells were found to produce significantly more ($p < 0,001$) total PGs from ^{14}C-LA than BL6 cells.

ii)From ^{14}C-AA (table 6.4 and 6.5). Asc supplementation was found to significantly ($p < 0,05$) increase synthesis of PGE_2 and total PGs from ^{14}C-AA in both BL6 and LLCMK cells at all concentrations. LLCMK cells were found to produce significantly ($p < 0,05$) more PGs from ^{14}C-AA than BL6 cells at all levels of Asc supplementation.

While the increased synthesis of total PGs and of PGE_1/E_2, from both ^{14}C-LA and ^{14}C-AA, observed upon Asc supplementation were fairly large, they were not always significant due to the wide variation in the levels of PG synthesis in individual cultures at the same level of Asc supplementation.

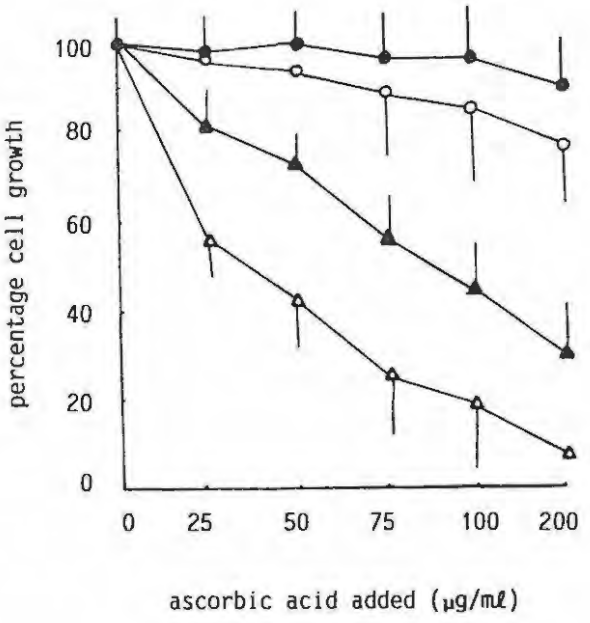

Figure 6.1. Effect of increasing concentrations of supplementary Asc on cell growth. ● LLCMK cells; ▲ BL6 cells (Asc supplemented once at beginning of experiment); o LLCMK cells; ▲ BL6 cells (Asc supplemented daily). Results are mean and SEM of 5 cultures. Asc was found to significantly ($p < 0,01$) inhibit BL6 cell growth at all concentrations, whether supplemented daily or once at the beginning of the experiment. LLCMK cell growth was not significantly inhibited by Asc using either method of supplementation.

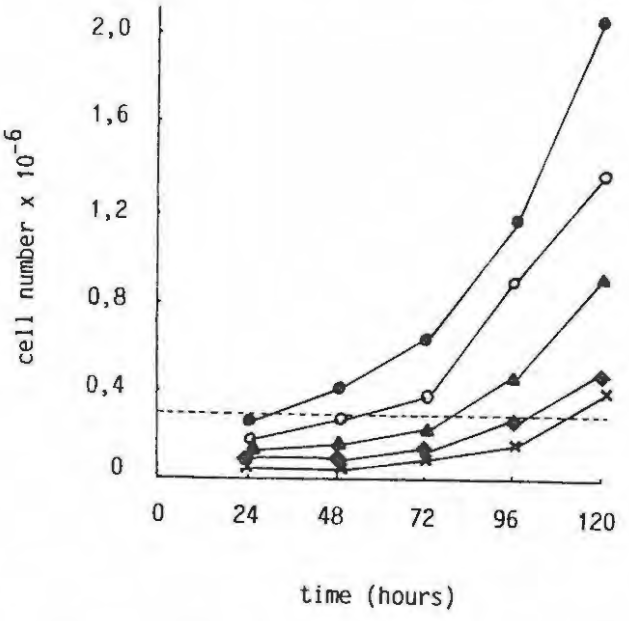

Figure 6.2. Growth curves of BL6 cells at different concentrations of supplementary Asc. ● control; o 50µg/ml Asc; ▲ 100µg/ml Asc; ◆ 200µg/ml Asc; ✖ cells grown in medium supplemented with 200µg/ml Asc for 24 hours and thereafter in unsupplemented medium. Results are mean and SEM of 5 cultures.

Table 6.1. Effect of preincubated ascorbic acid containing medium on in vitro cell growth.

	BL6 cells (cell number x 10^{-6})		LLCMK cells (cell number x 10^{-6})	
Asc added (µg/ml)	non-preincubated medium	preincubated medium	non-preincubated medium	preincubated medium
0	1,82±0,16	1,82±0,17	1,29±0,20	1,29±0,14
50	1,34±0,12	1,73±0,20	1,31±0,16	1,26±0,17
100	0,82±0,009	1,76±0,23	1,27±0,15	1,24±0,21
200	0,58±0,009	1,73±0,26	1.20±0,19	1,27±0,24

Results are mean ±SEM, n = 5. Preincubated medium was incubated at 37°C for 24 hours before being added to cells while non-preincubated medium was added to cells without prior incubation. Cell growth in cultures containing preincubated compared to non-preincubated medium was not significantly different.

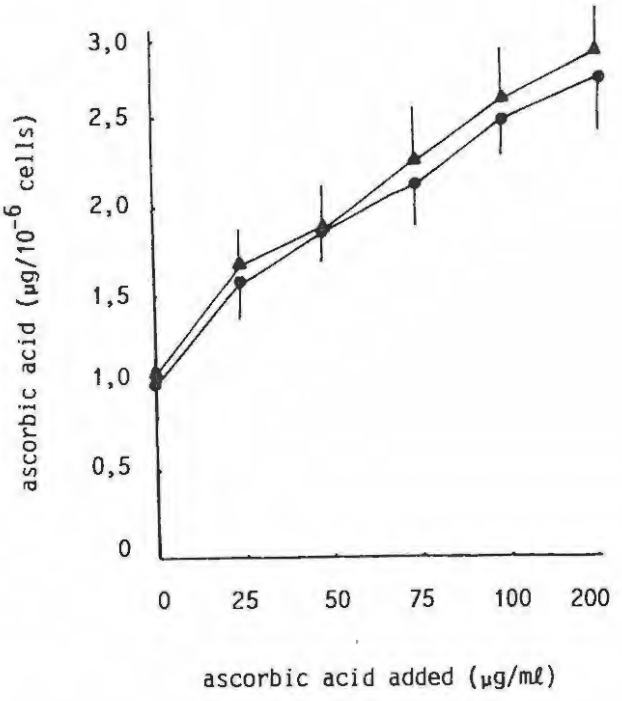

Figure 6.3. Effect of supplementary Asc on the Asc content of cells. ● LLCMK cells; ▲ BL6 cells. Results are mean and SEM of 5 cultures. Asc supplementation was found to significantly ($p < 0,01$) increase the Asc content of both cell lines in a dose dependant manner. No significant difference was evident between the Asc content of the two cell lines at all levels of Asc supplementation.

Table 6.2. Change in ascorbic acid content of the medium with time at 37°C in the presence or absence of cells.

Asc added (µg/ml)	Asc content of medium (µg/ml)					
	without cells			with cells		
	0 hours	24 hours	48 hours	0 hours	24 hours	48 hours
0	0,6±0,006	0,4±0,007	0,5±0,009	0,7±0,006	0,6±0,003	0,3±0,004
25	25,8±0,008	4,8±0,10	1,9±0,02	25,6±0,008	3,7±0,09	1,6±0,12
50	51,6±0,16	8,3±0,14	2,7±0,13	50,9±0,19	7,7±0,08	1,9±0,18
75	76,7±0,11	11,7±0,12	4,2±0,19	77,0±0,14	11,5±0,15	3,7±0,23
100	102,4±0,21	14,4±0,20	6,9±0,24	104,9±0,18	13,3±0,23	6,4±0,19
200	205,0±0,26	26,9±0,24	10,4±0,28	203,7±0,22	23,6±0,21	8,7±0,29

Results are mean ±SEM, n = 5. The Asc content was measured in individual flasks. Asc content was not significantly different in medium incubated with or without cells. By 24 hours the Asc content of all flasks had fallen significantly ($p < 0,001$) in comparison to the Asc content at 0 hours in that particular flask.

Table 6.3. Effect of indomethacin, caffeic acid and α-tocopherol on ascorbic acid mediated inhibition of BL6 cell growth.

Asc added (µg/ml)	cell number $x10^{-6}$			
	Asc only	Asc + IM	Asc + CA	Asc + TOC
0	1,84±0,10	1,81±0,13	1,85±0,16	1,88±0,21
50	1,29±0,11	1,32±0,14	1,30±0,15	1,34±0,15
100	0,74±0,14	0,78±0,20	0,70±0,18	0,78±0,16
200	0,56±0,11	0,59±0,16	0,53±0,13	0,60±0,18

Results are mean ±SEM, n = 5. IM, CA and TOC (all 5µM) were added to culture flasks together with the appropriate concentration of Asc. IM, CA and TOC did not significantly influence BL6 cell growth inhibition by Asc.

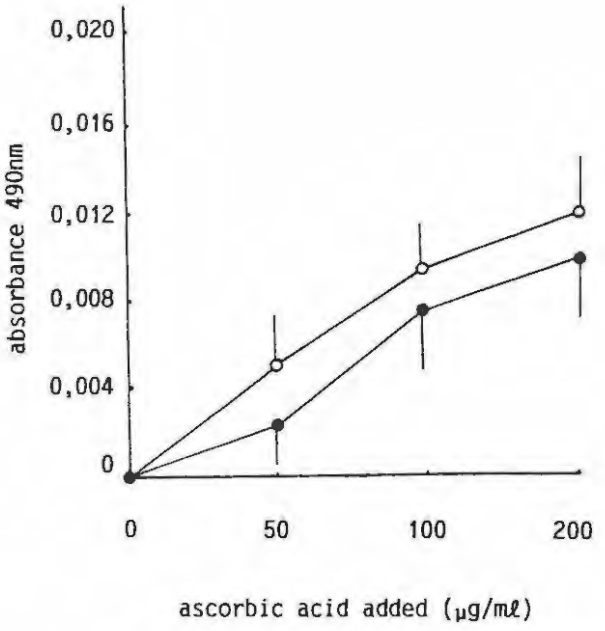

Figure 6.4. Effect of supplementary Asc on free radical production by cultured cells. o LLCMK cells; ● BL6 cells. Results are mean and SEM of 5 cultures. Asc was found to significantly ($p < 0,01$) increase free radical formation in both cell lines at all levels of supplementation. No significant difference was evident between free radical production by the two cell lines at all levels of Asc supplementation.

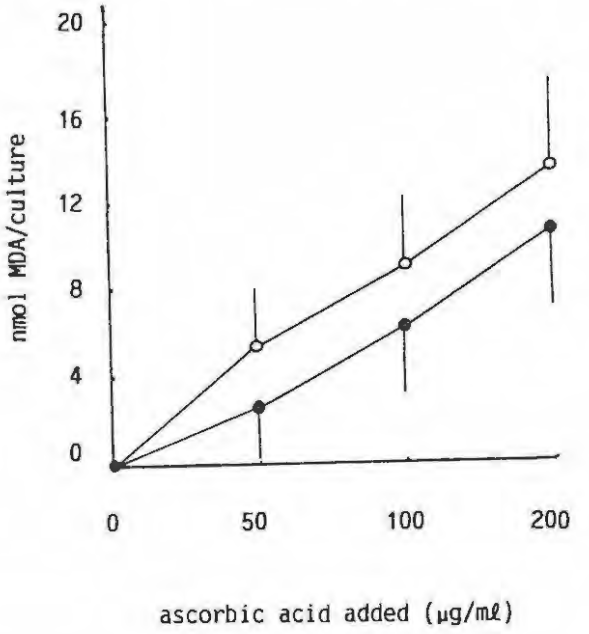

Figure 6.5. Effect of supplementary Asc on lipid peroxidation in cultured cells. o LLCMK cells; ● BL6 cells. Results are mean and SEM of 5 cultures. Asc was found to significantly ($p < 0,01$) increase lipid peroxidation in both cell lines at all levels of supplementation. No significant difference was evident between lipid peroxidation by the two cell lines at all levels of Asc supplementation.

Table 6.4. Effect of ascorbic acid on prostaglandin synthesis by BL6 cells.

Asc added (µg/ml)	cpm PGE_1	cpm PGE_2	total PG cpm
i) From ^{14}C-LA			
0	869,3±124,9	212,8±43,6	8345,9±856,7
50	1444,6±213,9	321,4±55,1	9239,9±886,0
100	2005,9±243,8[a]	403,6±81,8[a]	11831,7±684,7
200	2935,1±577,7[a]	478,2±82,1[a]	11925,9±863,8
ii) From ^{14}C-AA			
0	-	969,7±138,1	8593,3±691,2
50	-	1886,2±169,7[a]	11487,3±977,8[a]
100	-	2169,7±247,9[a]	13741,9±1767,0[a]
200	-	2364,7±293,2[a]	14681,8±1394,7[a]

Results are mean ± SEM, n = 5. PG synthesis by cells was determined in combined fractions of cells and the medium of cells incubated in the presence of radiolabelled EFAs for 24 hours prior to the commencement of the experiment.
[a]Significantly different ($p < 0,05$) to control cultures containing no supplementary Asc.

Table 6.5. Effect of ascorbic acid on prostaglandin synthesis by LLCMK cells.

Asc added (µg/ml)	cpm PGE_1	cpm PGE_2	total cpm PGs
i) From ^{14}C-LA			
0	635,1±94,3	269,7±49,9	16779,8±1357,0
50	749,7±183,1	305,8±41,8	18646,5±1718,1
100	809,6±138,9	396,9±60,0[a]	19204,7±1326,2
200	1008,7±118,6[a]	502,7±77,8[a]	20169,3±1495,4
ii) From ^{14}C-AA			
0	-	849,7±61,0	16638,4±1016,5
50	-	1574,4±170,5[a]	23106,9±1872,3[a]
100	-	2145,4±246,3[a]	24387,8±1868,8[a]
200	-	2382,8±239,1[a]	25621,3±1279,5[a]

Results are mean ± SEM, n = 5. PG synthesis by cells was determined in combined fractions of cells and the medium of cells incubated in the presence of radiolabelled EFAs for 24 hours prior to the commencement of the experiment. [a]Signigicantly different ($p < 0,05$) to control cultures containing no supplementary Asc.

6.4.Discussion

The growth inhibitory effects of Asc, at the concentrations used in this study, appear to have been confined to the malignant BL6 cell line and were not observed in the benign LLCMK cell line even upon daily supplementation with Asc. While Asc has been reported to inhibit growth of both malignant and benign cells at high concentrations (312,344,345,347-354,356,357), the results of this study are consistent with reports by Prasad *et al* (345) that high concentrations of Asc potentiate the growth inhibitory effects of certain chemicals against murine neuroblastoma cells while having no effect on the growth inhibitory effects in rat glioma cells. Poydock *et al* (312) reported that a mixture of vitamin B_{12} and Asc inhibited mitotic activity in murine sarcoma cells at a concentration which had no effect on division of normal murine fibroblasts. Lonn and Lonn (347) showed that Asc inhibited DNA synthesis and, in the presence of Cu^{+} ions, caused DNA fragmentation in human melanoma cells at concentrations which did not significantly influence DNA synthesis in normal human fibroblasts. Several studies have also examined the effects of Asc on tumour cell growth only. Bram *et al* (344) reported inhibition of human melanona cell growth, Bishun *et al* (348) reported inhibition of human carcinoma cell growth while Park (350) observed growth inhibitory effects in human leukaemic cells. The growth inhibitory effects of Asc, observed in BL6 cells in this study, did not appear to be reversible since replacement of Asc containing medium with Asc free medium did not result in an increased rate of growth of the BL6 cells.

The Asc content of the medium was found to be reduced to virtually negligible levels by incubation at 37^{o}C. This result is consistent with reports by Mohberg and Johnson (378) and Feng *et al* (379) who found that the stability of Asc in culture medium decreased with increasing temperature. At 20^{o}C Asc was found to have a half life of 15,5 hours in commercial Waymouth's culture medium (379). It was also reported that Asc decomposed to products other than dehydroascorbic acid or diketogulonic acid (378). The decline in the Asc level of the medium was probably due to auto-oxidation and to a lesser extent to cellular uptake, since in the present study no significant difference in Asc content was evident between medium incubated with or without cells. A similar observation has been reported by Mohberg and Johnson (378).

In view of the rapid decomposition of Asc in the culture medium and since the growth inhibitory effects of Asc in melanoma cells are reportedly due to increased uptake of Asc by melanoma cells (344), it was decided to examine the Asc content of the cells. A significant, dose dependant increase in intracellular Asc concentration was observed in both BL6 and LLCMK cells upon Asc supplementation. It is therefore apparent that Asc uptake by the cells must have been rapid and that sufficient Asc was taken up by cells, before decomposition of Asc in the medium occured, to increase the intracellular Asc content to the levels observed in this study. A correlation between increased

intracellular Asc content and increased BL6 cell growth inhibition is also evident. Whereas Bram *et al* (344) have proposed that Asc is preferentially taken up by melanoma cells, thus making them more susceptible to Asc mediated growth inhibition, no significant difference in the Asc content of the two cell lines was evident in this study. Some other mechanism must therefore have been responsible for the selective growth inhibitory effects of Asc observed in this study. The Asc present in unsupplemented BL6 (1,08µg/ml) and LLCMK (1,03µg/ml) cells was probably a basal level of Asc found within the cells since they were usually maintained in medium containing 50µg/ml Asc prior to the commencement of these studies.

Due to the rapid oxidation of Asc at 37°C the possibility arose that the growth inhibitory effects of Asc in BL6 cells might have been due to a breakdown product of Asc rather than Asc itself. Asc containing medium was thus preincubated at 37°C for 24 hours, to allow for Asc decomposition, before being added to cells. No significant inhibition of either BL6 or LLCMK cell growth was observed in these cultures. The growth inhibitory effects of Asc on BL6 cells were obviously then due to an effect of Asc itself, and not one of its breakdown products as has been reported by Peterkofsky and Prather (354).

It has been proposed that the mechanism of Asc mediated cell growth inhibition may involve increased free radical formation and lipid peroxidation, particularly since Asc treated cultures are known to produce H_2O_2 which can decompose in the presence of certain metal ions to produce hydroxyl radicals (315,354,356). Asc has also been shown to enhance lipid peroxidation in the presence of metal ions in certain cell free systems and subcellular fractions (340-342,361). While a small but significant increase in both free radical formation and lipid peroxidation were observed as a result of Asc supplementation in this study, it is unlikely that lipid peroxides and free radicals were involved in the inhibition of BL6 cell growth by Asc since supplementation of the antioxidant TOC to Asc containing medium did not influence the observed growth inhibition. Furthermore Asc also increased free radical production and lipid peroxidation in LLCMK cells but did not influence the growth of these cells. The observed increase in free radical production may have been be due to formation of the Asc free radical, which is relatively unreactive toward biological molecules but would presumeably reduce NBT, the indicator used in the determination of free radical concentrations.

A second mechanism whereby Asc has been proposed to inhibit cell growth involves increased PG production by cells upon Asc supplementation since Asc has been reported to enhance synthesis of PGs in various cells and isolated tissues (366-368,370 -372), as was found in this study in both BL6 and LLCMK cells. It appears unlikely however that the growth inhibitory effect of Asc on BL6 cells was mediated via this mechanism since IM, an inhibitor of PG synthesis, was unable to reverse the growth inhibitory effects of Asc observed in this study. Furthermore LLCMK cells which were not inhibited by supplementary Asc, were found to synthesise significantly more PGs from radiolabelled EFA

precursors than the BL6 cells. It is interesting to note that while Asc stimulated synthesis of total PGs to a greater extent in LLCMK cells, synthesis of PGE_1 from ^{14}C-LA was enhanced more significantly in BL6 cells than in LLCMK cells upon Asc supplementation. This result suggests that Asc may exert different effects on the individual PG synthesising enzymes in different cells.

Enhanced synthesis of LTs and other lipoxygenase products were also unlikely to be involved in the inhibition of cell growth by Asc as CA, a LT synthesis inhibitor, did not influence growth of Asc treated BL6 cells.

In conclusion therefore, it was found that Asc inhibited growth of the malignant BL6 cells but not of the benign LLCMK cells. Inhibition of the BL6 cells was not reversible upon removal of the Asc containing medium. As previously mentioned however, the benign LLCMK cells used in this study were transformed cells and would therefore have had some characteristics of malignant cells. They did however divide more slowly than the malignant BL6 cells allowing some comparison between the effects of Asc on malignant and benign cells in this study. The mechanism of growth inhibition did not appear to involve preferential accumulation of Asc by melanoma cells, nor was a product of Asc auto-oxidation involved in this mechanism. While production of free radicals, lipid peroxides and PGs by both cell lines were increased by Asc supplementation, these compounds were unlikely to have been involved in the mechanism of BL6 cell growth inhibition by Asc. It is therefore apparent that a mechanism other then those investigated in this study must have been involved in the Asc mediated inhibition of BL6 cell growth. Suggested mechanisms include increased H_2O_2 production in cells (315,354,356,357), DNA scission (315,362-365) and alterations in general tumour cell metabolism (304).

Chapter 7

INTERACTIONS BETWEEN ESSENTIAL FATTY ACIDS AND ASCORBIC ACID.

7.1.Introduction

Asc has been reported to stimulate PG synthesis (366-368,370-372) and to both promote and inhibit lipid peroxidation (335,340,341) from exogenous EFAs in cultured cells, cell free systems and in isolated tissues. Although the growth inhibitory effects of Asc in BL6 cells appeared not to involve enhanced PG synthesis or lipid peroxidation from endogenous EFAs (see chapter 6), this does not preclude the possibility of Asc interacting with exogenous EFAs by these mechanisms to inhibit cell growth. This study was carried out to investigate the influence of Asc on the growth inhibitory effects of exogenous EFAs and to examine the possible involvement of enhanced PG synthesis or lipid peroxidation in the mechanism of any possible interaction between the two nutrients in inhibiting cell growth.

7.2.Materials and Methods

Cells

The BL6 and LLCMK cells used have been described previously (section 4.2).

Reagents.

Essential fatty acids were prepared as previously described (section 5.2).

Ascorbic acid was made up as previously described (section 6.2).

Indomethacin, caffeic acid, α-tocopherol and reagents used in the NBT and TBA assays for free radical formation and lipid peroxidation were prepared as before (section 5.3).

^{14}C-linoleic acid and ^{14}C-arachidonic acid. Details of the radiochemicals have been provided previously (section 5.2).

Methods.

Effect of ascorbic acid on essential fatty acid cell growth inhibition. Ten culture flasks were prepared as before (section 4.2) at each EFA concentration (0-50μg/ml). In initial experiments, Asc was added

to half the flasks at a concentration of 200µg/ml, an appropriate volume of 0.1M sodium phosphate buffer pH 7,2 being added to the other half (controls). This treatment was however found to be totally cytotoxic to both BL6 and LLCMK cells at higher EFA concentrations. Therefore in subsequent experiments Asc was added to EFA treated cultures at a final concentration of 100µg/ml. Cells were then incubated and enumerated as before.

Effect of ascorbic acid on essential fatty acid uptake. Asc (100µg/ml) was added together with 0,5µCi ^{14}C-LA or ^{14}C-AA to confluent cultures which were incubated for 24 hours at 37^{o}C before being harvested by trypsinisation. Cells were pelleted by centrifugation (Hettich Universal K2S for 10 minutes at 1000g) and resuspended in 1ml of PBS. Cells were then homogenised using a Dounce homogeniser (30 strokes with the tight plunger) and the cell suspension centrifuged at 2000g to pellet cell debris. The supernatant was added to 10ml scintillation fluid (Beckman EP readysolv) in a glass counting vial, thoroughly mixed and the radioactivity counted using a Beckman model LS 3801 scintillation counter.

Effect of indomethacin, caffeic acid and α-tocopherol on cell growth. Inhibitors of PG and LT synthesis (IM and CA) and the anti-oxidant TOC, were used to determine whether enhanced PG or LT synthesis or lipid peroxidation were involved in any possible interaction between Asc and EFAs in inhibiting cell growth. These reagents (5µM) were added to cultures, containing EFAs (0-50µg/ml) and Asc (100µg/ml), which were then incubated and enumerated as before. Control cultures contained EFAs, Asc and an appropriate concentration of ethanol.

Free radical formation and lipid peroxidation were determined in cultures containing EFAs and Asc using the NBT and TBA assays as previously described (section 5.3).

Statistical analysis. One way analysis of variance and Scheffe's multiple range test were used for analysis of results.

7.3.Results

Since Asc was found in this study to inhibit BL6 cell growth (chapter 6), all BL6 cultures containing EFAs and Asc would be expected to exhibit decreased growth when compared with cultures containing EFAs only. In order to correct for any contribution of Asc to cell growth inhibition exerted independantly of any interaction with the EFAs, results from growth inhibition studies are expressed as percentage cell growth of the relevant controls. In cultures containing only EFAs, cell growth is expressed as a percentage of the growth observed in cultures containing no supplementary EFAs, while in cultures containing EFAs and Asc, growth is expressed as a percentage of the growth observed in cultures containing the relevant concentration of EFAs but no Asc.

Effect of ascorbic acid on cell growth inhibition by essential fatty acids. Growth of EFA supplemented BL6 and LLCMK cells was found to be significantly reduced, especially at higher EFA concentrations, by supplementation with Asc when compared with cells treated with EFAs only (figures 7.1-7.3). The reduction in growth inhibition in LLCMK cells was significant ($p < 0,05$) at 50µg/ml for all three of the EFAs while in BL6 cells it was significant ($p < 0,05$) at 25 and 50µg/ml supplementary LA and at 10, 25 and 50µg/ml of supplementary AA or GLA. The reduction in growth inhibition upon Asc supplementation to EFA treated cells was particularly evident in BL6 cells as can be seen from the above statistics. A combination of 50µg/ml AA and 100µg/ml Asc was in fact found to be totally cytotoxic to BL6 cells.

Essential fatty acid uptake. Asc was found not to influence the uptake of either ^{14}C-LA or ^{14}C-AA in either BL6 or LLCMK cells (figure 7.4).

Influence of indomethacin, caffeic acid and α-tocopherol on cell growth inhibition by essential fatty acids and ascorbic acid. IM and CA were found not to influence growth inhibition in EFA and Asc treated LLCMK or BL6 cells, while TOC was found to slightly but non-significantly reverse the growth inhibitory effects of these nutrients (tables 7.1 and 7.2). No cell growth was evident however, even in the presence of TOC, in BL6 cells treated with 50µg/ml AA and 100µg/ml Asc. In addition, increasing the concentration of TOC to 10µM in these cultures did not protect cells from the cytotoxic effects of AA and Asc at these concentrations (results not shown). The effect of TOC on lower concentrations of AA and Asc, namely 25µg/ml AA and 50µg/ml Asc, was thus examined in both BL6 and LLCMK cells. While a slight protective effect of TOC against the growth inhibitory effects of AA and Asc at the latter concentrations was evident, this effect was not significant (results not shown).

Free radical formation and lipid peroxidation. As Asc was found to increase the levels of free radicals and lipid peroxidation in cells (chapter 6), the increased levels of free radicals (tables 7.3 and 7.4) and lipid peroxides (tables 7.5 and 7.6) measured in both BL6 and LLCMK cultures, containing exogenous EFAs and supplemented with Asc, were not unexpected. When the contribution of Asc alone was taken into account, a small increase in lipid peroxidation and free radical formation in both cell lines was however still evident in Asc and EFA containing cultures when compared with cultures containing EFAs only. This increase was significant ($p < 0,05$ for LLCMK cells and $p < 0,01$ for BL6 cells) in cultures treated with 50µg/ml GLA and AA and was more pronounced with increasing unsaturation of the EFAs (AA > GLA > LA).

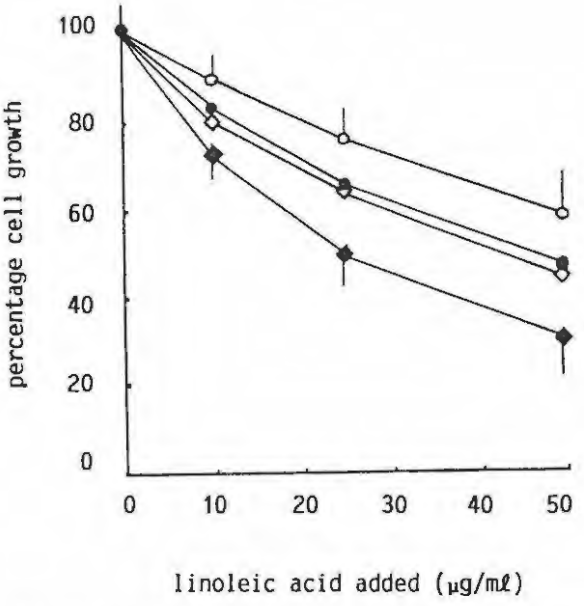

Figure 7.1. Effect of supplementary Asc on cell growth inhibition by LA. ○ LA only LLCMK cells; ● LA + 100µg/ml Asc LLCMK cells; ◇ LA only BL6 cells; ◆ LA + 100µg/ml Asc BL6 cells. Results are mean and SEM of 5 cultures. Asc significantly increased growth inhibition by LA in both cell lines ($p < 0,05$ at 50µg/ml supplementary LA for LLCMK cells; $p < 0,01$ at 25 and 50µg/ml supplementary LA for BL6 cells).

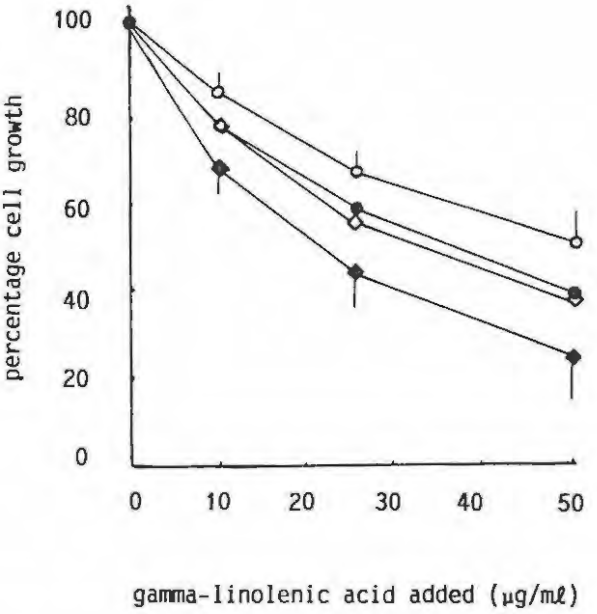

Figure 7.2. Effect of supplementary Asc on cell growth inhibition by GLA. ○ GLA only LLCMK cells; ● GLA + 100µg/ml Asc LLCMK cells; ◇ GLA only BL6 cells; ◆ GLA + 100µg/ml Asc BL6 cells. Results are mean and SEM of 5 cultures. Asc significantly increased growth inhibition by GLA in both cell lines ($p < 0,05$ at 50µg/ml supplementary GLA for LLCMK cells; $p < 0,05$ at 10 and 25µg/ml and $p < 0,01$ at 50µg/ml GLA for BL6 cells).

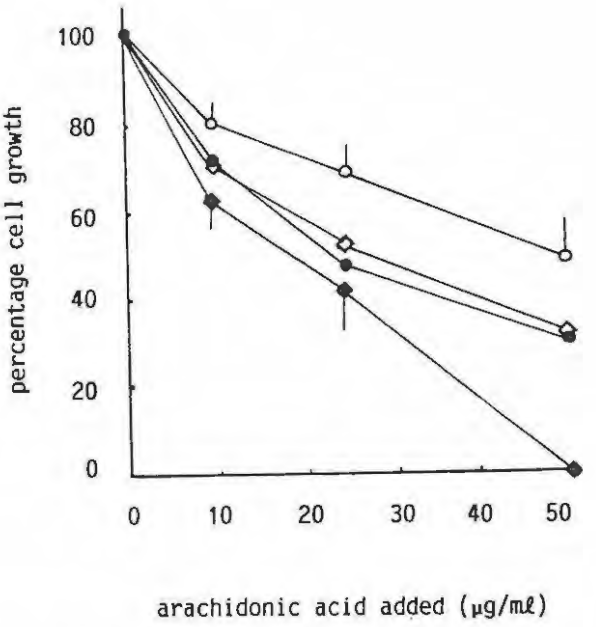

Figure 7.3. Effect of supplementary Asc on cell growth inhibition by AA. ○ AA only LLCMK cells; ● AA + 100µg/ml Asc LLCMK cells; ◇ AA only BL6 cells; ◆ AA + 100µg/ml Asc BL6 cells. Results are mean and SEM of 5 cultures. Asc significantly increased growth inhibition by AA in both cell lines ($p < 0,01$ at 25 and 50µg/ml supplementary AA for LLCMK cells; $p < 0,05$ at 10µg/ml and $p < 0,01$ at 25 and 50µg/ml supplementary AA for BL6 cells.

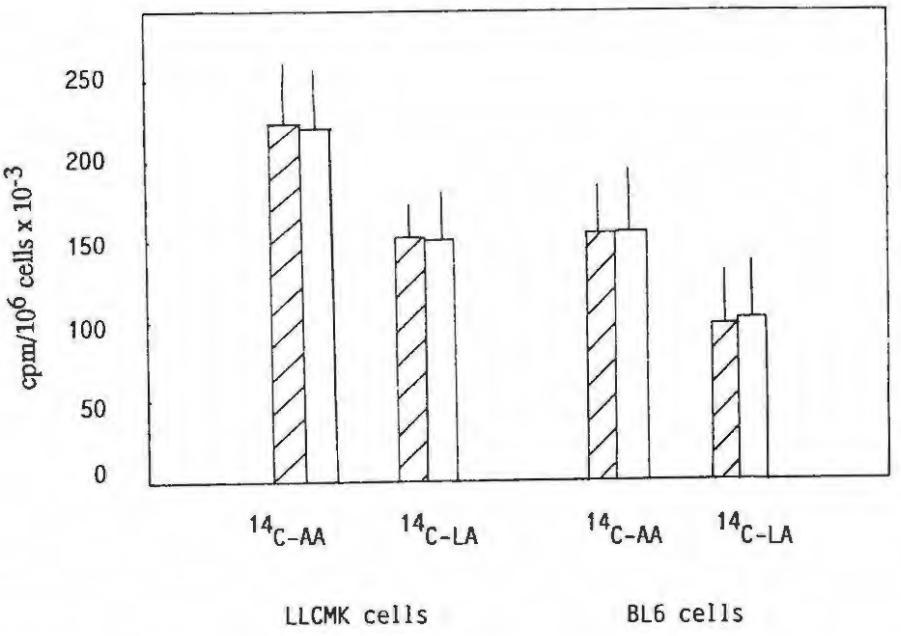

Figure 7.4. Effect of supplementary Asc on uptake of ^{14}C-LA and ^{14}C-AA in cultured cells. ▨ control cells (no supplementary Asc); □ cells supplemented with 100µg/ml Asc. Results are mean and SEM of 5 cultures. Supplementary Asc was found not to significantly influence uptake of either ^{14}C-LA or ^{14}C-AA by either cell line.

Table 7.1. Effect of indomethacin, caffeic acid and α-tocopherol on LLCMK cell growth inhibition by essential fatty acids and ascorbic acid.

EFA added (μg/ml)	Percentage cell growth* EFA+Asc	EFA+Asc+IM	EFA+Asc+CA	EFA+Asc+TOC
i)Linoleic acid				
10	85,2±3,7	86,3±3,1	84,6±2,7	88,7±2,0
25	68,7±4,4	67,2±4,7	69,7±3,3	73,4±5,1
50	52,9±5,6	54,8±4,3	51,8±3,7	57,4±4,6
ii)Gamma-linoleic acid				
10	80,1±2,8	81,6±3,5	82,6±4,1	82,5±3,3
25	62,3±4,4	65,1±2,8	61,4±3,9	66,3±4,5
50	43,2±4,7	44,3±4,3	44,2±3,7	48,7±3,1
iii)Arachidonic acid				
10	73,2±3,8	74,7±3,9	75,8±4,2	78,2±3,6
25	48,7±3,2	49,1±4,5	46,6±3,8	54,1±3,6
50	36,9±4,8	34,9±5,2	35,8±4,2	42,3±4,7

Results are mean ±SEM of 5 cultures. *Results are expressed as percentage growth of cultures containing EFAs and the relevant concentration of the compound being examined to take into account the growth inhibitory effects of Asc in the absence of EFAs. Asc was added to cultures at a concentration of 100μg/ml while IM, CA and TOC were added at a concentration of 5μM. Cell growth inhibition by the EFAs and Asc was not significantly affected by IM, CA or TOC.

Table 7.2. Effect of indomethacin, caffeic acid and α -tocopherol on BL6 cell growth inhibition by essential fatty acids and ascorbic acid.

EFA added (ug/ml)	Percentage cell growth* EFA+Asc	EFA+Asc+IM	EFA+Asc+CA	EFA+Asc+TOC
i)Linoleic acid				
10	76,3±3,8	76,8±4,1	73,7±2,7	77,8±3,6
25	52,7±5,0	55,3±4,1	53,7±2,9	56,3±3,5
50	34,0±4,7	36,2±4,2	36,9±4,3	39,1±4,5
ii)Gamma-linolenic acid				
10	70,4±3,3	71,2±2,9	69,7±4,1	72,3±3,7
25	44,8±4,8	42,1±3,6	44,4±4,2	47,3±4,5
50	29,8±4,8	30,3±3,7	31,8±5,1	35,1±4,2
iii)Arachidonic acid				
10	64,2±5,1	66,7±3,7	63,7±4,5	66,3±4,1
25	41,3±2,7	44,0±4,9	43,5±3,6	47,0±4,2
50	0	0	0	0

Results are mean ± SEM, n = 5. * Results are expressed as percentage cell growth of cultures containing EFAs and the relevant concentration of the compound being examined to take into account the growth inhibitory effects of Asc in the absence of EFAs. Asc was added to cultures at a concentration of 100μg/ml while IM, CA and TOC were added at a concentration of 5μM. Cell growth inhibition by the EFAs and Asc was not significantly affected by IM, CA or TOC.

Table 7.3. Free radical production in LLCMK cells supplemented with essential fatty acids and ascorbic acid.

EFA added (µg/ml)	Free radical production (absorbance 490nm) EFA alone	EFA + Asc	EFA + Asc - Asc alone*
i)Linoleic acid			
10	0,004±0,001	0,015±0,004	0,005
25	0,007±0,002	0,019±0,006	0,009
50	0,011±0,004	0,025±0,006	0,015
ii)Gamma-linolenic acid			
10	0,006±0,002	0,018±0,006	0,008
25	0,011±0,003	0,023±0,007	0,013
50	0,017±0,003	0,033±0,010	0,023[a]
iii)Arachidonic acid			
10	0,009±0,003	0,029±0,011	0,019
25	0,019±0,006	0,032±0,012	0,022
50	0,024±0,008	0,041±0,016	0,031[a]

Results are mean ±SEM, n = 5. *Cultures treated with 100µg/ml Asc alone and no supplementary EFAs were found to produce free radicals having an absorbance of 0,010 in LLCMK cells (section 6.3.). [a]Significantly different ($p < 0,05$) to cultures containing EFA only.

Table 7.4. Free radical production in BL6 cells supplemented with essential fatty acids and 100µg/ml ascorbic acid.

EFA added (µg/ml)	Free radical production (absorbance 490nm) EFA alone	EFA + Asc	EFA + Asc - Asc alone*
i)Linoleic acid			
10	0,007±0,003	0,014±0,005	0,007
25	0,010±0,002	0,020±0,007	0,013
50	0,014±0,003	0,026±0,009	0,019
ii)Gamma-linoleic acid			
10	0,009±0,003	0,016±0,005	0,010
25	0,013±0,003	0,022±0,006	0,015
50	0,021±0,008	0,036±0,011	0,029[a]
iii)Arachidonic acid			
10	0,012±0,003	0,021±0,007	0,014
25	0,024±0,005	0,038±0,014	0,031
50	0,035±0,013	0,051±0,018	0,044[a]

Results are mean ± SEM, n = 5. *Cultures treated with 100µg/ml Asc alone and no supplementary EFAs were found to produce free radicals having an absorbance of 0,007 in BL6 cells (section 6.3.). [a]Significantly different to cultures containing EFAs only.

Table 7.5. Lipid peroxidation in LLCMK cells supplemented with essential fatty acids and 100ug/ml ascorbic acid.

EFA added (ug/ml)	lipid peroxidation (nmol MDA/culture) EFA alone	EFA + Asc	EFA + Asc - Asc alone*
i)Linoleic acid			
10	8,1±1,4	17,5±3,8	8,7
25	10,4±2,4	20,7±4,2	11,9
50	20,6±4,6	33,5±6,6	24,7
ii)Gamma-linolenic acid			
10	9,9±2,5	19,4±3,7	10,6
25	13,1±1,6	24,0±5,4	15,2
50	24,7±4,1	38,7±4,7	29,9[a]
iii)Arachidonic acid			
10	13,0±2,8	22,4±3,6	13,6
25	23,2±5,7	33,6±5,3	25,8
50	37,7±6,7	52,9±9,2	44,1[a]

Results are mean ±SEM, n = 5. *LLCMK cultures supplemented with Asc only and no supplementary EFAs were found to produce lipid peroxides equivalent to 8,8nmol/MDA (section 6.3). [a]Significantly different ($p < 0,05$) to cultures containing EFA only.

Table 7.6. Lipid peroxidation in BL6 cells supplemented with essential fatty acids and 100µg/ml ascorbic acid.

EFA added (µg/ml)	lipid peroxidation (nmol MDA/culture) EFA alone	EFA + Asc	EFA + Asc - Asc alone*
i)Linoleic acid			
10	8,9±1,6	17,0±3,5	10,6
25	13,4±1,5	22,1±3,2	15,7
50	20,9±3,9	42,1±6,8	25,7
ii)Gamma-linolenic acid			
10	13,1±3,1	22,6±3,7	16,2
25	17,6±2,8	26,4±4,1	20,0
50	31,3±4,7	44,9±7,2	38,5[a]
iii)Arachidonic acid			
10	16,8±3,5	25,1±4,7	18,7
25	29,5±5,1	39,2±6,6	32,8
50	53,6±8,3	65,7±8,4	59,3[a]

Results are mean ±SEM, n = 5. *BL6 cultures supplemented with Asc only and no supplementary EFAs were found to produce lipid peroxides equivalent to 6,4nmol MDA/culture (section 6.3). [a]Significantly different ($p < 0,01$) to cultures containing EFA only.

7.4.Discussion

As already discussed in previous chapters, both Asc and the EFAs have been found in this and other studies to inhibit growth of cultured cells and in particular of malignant cells. While Asc has been reported to influence PG synthesis from EFAs in cells and isolated tissues (366-372) and lipid peroxidation in in vitro and in vivo systems (276,334-342), no reports could be found in the relevant literature relating to possible interactions, and the possible mechanisms thereof, between the two nutrients in malignant cells. In this study Asc was found to enhance PG synthesis from endogenous EFAs and to enhance peroxidation of endogenous lipids (chapter 6). While the enhanced PG synthesis and lipid peroxidation appeared not to be involved in the mechanism of Asc mediated inhibition of BL6 cell growth, possible interactions between Asc and exogenous EFAs in inhibiting cell growth may have involved these mechanisms. The influence and interactions of Asc and EFAs on growth of both malignant and benign cells and the possible involvement of enhanced PG synthesis and lipid peroxidation in any interaction were thus examined in this study.

Asc was found to significantly enhance EFA mediated growth inhibition in both BL6 and LLCMK cells, the effect being particularly evident in BL6 cells. Since Asc itself was found in this study to inhibit BL6 cell growth, this effect was expected in BL6 cells. However having taken into account the growth inhibitory effects of Asc in the absence of EFAs, an increase in growth inhibition was still evident in cultures treated with EFAs and Asc. This observation suggests that a synergistic interaction might have been occuring between the two nutrients in inhibiting growth of both the BL6 and LLCMK cells used in this study. This result is particularly interesting in view of the fact that Asc alone, while inhibiting BL6 cell growth, was found not to influence LLCMK cell growth (chapter 6). Since Asc was found not to influence uptake of radiolabelled EFAs by cells in this study, it is unlikely that the effect of Asc on EFA mediated cell growth inhibition was the result of increased EFA uptake by these cells. An intracellular interaction between the two nutrients was thus more likely.

Peroxidation of lipids, which is thought to play a role in the control of cell proliferation (159,165,168,169,197,198,200-202), is initiated by free radical attack of intracellular PUFAs/EFAs, and in particular of membrane PUFAs/EFAs, leading to the oxidative destruction of the PUFAs/EFAs. The lipid peroxidation can result in lipid-lipid, lipid-protein or protein-protein cross-linking leading to changes in the characteristics and functioning of the cell membrane and membranes of subcellular organelles. These changes are thought to influence the metabolism and growth of cells.

Since Asc has been reported to inhibit peroxidation of lipids in solution (335) and in rat brain microsomes (344) and to enhance lipid peroxidation in solution (341,342) and in liposomes (340), the influence of Asc on lipid peroxidation and on the production of free radicals, the initiating agents of

lipid peroxidation, were thus examined in EFA supplemented cells in this study. Since Asc alone was found to elicit a small but significant increase in the level of free radicals and of lipid peroxidation in the cells used in this study (chapter 6), this fact had to be taken into account when examining the influence of Asc on free radical production and lipid peroxidation in EFA supplemented cultures. As expected, Asc supplementation to EFA containing cultures increased the levels of free radicals and lipid peroxidation in both BL6 and LLCMK cells. A small but significant increase in free radical levels and lipid peroxidation was still evident however in cultures supplemented with Asc and 50µg/ml of GLA and AA once the contribution of Asc alone (in the absence of EFAs) had been taken into account. TOC was found in this study to partially reverse the growth inhibitory effects of supplementary EFAs in the absence of supplementary Asc (section 5.3) suggesting that the mechanism of EFA mediated cell growth inhibition might have involved lipid peroxidation. However in cells supplemented with EFAs and Asc, TOC was found not to influence cell growth inhibition by these compounds. It therefore appears unlikely that enhanced lipid peroxidation was involved in the mechanism whereby Asc increased inhibition of the cells by EFAs.

Asc has been found to enhance synthesis of PGs in this (chapter 6) and other studies using human platelets (366,368), human lung fibroblasts (371,372), human lung parenchyma (367), human smooth muscle and epithelial cells (371), murine macrophages (370) and rat aortic rings (368). Since the growth inhibitory effects of both endogenous and exogenous PGs in cultured cells are well documented (156,164,169,175-182), it was possible that the mechanism whereby Asc increased cell growth inhibition by the EFAs might have invoved enhanced PG synthesis from the precursor EFAs. However as found in cultures supplemented with either Asc or EFAs only (chapters 5 and 6), increased PG synthesis does not appear to have played a role in the interaction between the EFAs and Asc in inhibiting cell growth since the PG synthesis inhibitor, IM, was found not to influence growth inhibition of cells treated with a combination of the EFAs and Asc. Enhanced LT synthesis was apparently also not involved in the mechanism of growth inhibition since the LT synthesis inhibitor, CA, was found not to influence growth of cells treated with EFAs and Asc.

In conclusion therefore it was found that Asc enhanced EFA mediated cell growth inhibition in both the malignant and normal cells used in this study. Increased lipid peroxidation and PG or LT synthesis did not appear to be involved in the mechanism whereby Asc influenced EFA growth inhibition nor did Asc influence uptake of radiolabelled EFAs by the cells.

Chapter 8

EFFECT OF DIETARY ESSENTIAL/POLYUNSATURATED FATTY ACIDS ON TUMOUR GROWTH IN MICE

8.1.Introduction

Numerous studies have reported that PUFAs, and in particular EFAs, promote tumour growth in experimental animals (57,64-66,69-74,76,79-85,87-92). Various mechanisms, including enhanced PG synthesis (101,105-118) and lipid peroxidation (93,100,139) have been proposed for the tumour promoting effects of dietary PUFAs/EFAs. In the present study the EFAs, LA, GLA, and AA, were found to inhibit in vitro growth of the BL6 melanoma cells by a mechanism which appeared to involve lipid peroxidation as well as another as yet unidentified mechanism (chapter 5). Since in vitro systems involve far less variables which might be influenced by the EFAs than in vivo studies, it was decided to examine the effect of dietary PUFAs/EFAs on the growth of the melanoma cells in mice. The possible involvement of enhanced PG or LT synthesis in the mechanism of action of the PUFAs/EFAs on tumour growth was examined by using inhibitors of the pathways involved in synthesis of these compounds. The antioxidant TOC was used to determine the possible involvement of lipid peroxidation in the mechanism of tumour promotion by the PUFAs/EFAs. The influence of dietary EFA/PUFA manipulation on tissue and tumour EFA levels was also examined. Various vegetable oils were used as a source of dietary EFAs/PUFAs. While the lipids in these oils consist mainly of EFAs, they do contain other PUFAs which might contribute to the observed effects on tumour growth. These dietary lipids will therefore be referred to as PUFAs/EFAs.

8.2. EFFECT OF DIETARY ESSENTIAL/POLYUNSATURATED FATTY ACIDS ON TUMOUR GROWTH

8.2.1. Materials and Methods

Mice

All mice used in this and subsequent studies were approximately six week old C57 black female mice purchased from the South African Medical and Research Council, Cape Town, RSA. Mice were housed in plastic cages fitted with stainless steel bases and covers. Water and a stock pelleted diet (Epol, Port Elizabeth, RSA) were provided *ad lib* until use.

Rations

The basal semi-purified diet consisted of 67% corn starch, 20% dried egg albumin, 8% fat, 4% salt mix

(appendix 3) and 1% vitamin mix (appendix 4). In this study the PUFA/EFA composition of the diets was manipulated by using different plant seed oils as a source of PUFAs/EFAs. Four different oils were used, these being sunflower oil (SNO) and safflower oil (SFO) which contain predominantly LA, evening primrose oil (EPO) which contains both GLA and LA, and coconut oil (CO) which is EFA free. The EFA content of these oils was measured by gas-liquid chromatography and is provided in appendix 5. Six diets were prepared containing either 8% EPO, 8% SFO, 8% SNO, 8% CO, 4% EPO + 4% SFO or 4% SFO + 4% CO. The diets were stored at $4^{o}C$ in the powdered form until use. Attempts to feed the mice the diets in the powdered form resulted in considerable wastage and contamination of the diets with urine and faeces. Consequently the diets were pelleted after the addition of a minimum volume of water. Due to the instability of certain nutrients in aqueous solution, the pelleted diet was prepared in batches sufficient for one week of feeding and stored at $4^{o}C$.

Cells

Relevant information about the BL6 cells has been provided previously (section 4.2).

Reagents

Essential fatty acid methyl esters (FAMEs). Methyl esters of LA, GLA and AA were purchased from Sigma. DGLA was methylated using the procedure described in this section. A mixture of the four FAMEs, at final concentrations of approximately 3,4 µmole/ml of each FAME was prepared in petroleum ether. The preparation was stored at $-20^{o}C$ under nitrogen and protected from light until use.

Methanolic KOH was prepared in 25% water (v/v) and 75% methanol.

BF_3-methanol reagent. was purchased from Merck, Darmstadt, West Germany.

Methods

Effect of dietary essential/polyunsaturated fatty acids on tumour growth. Each of the experimental diets was fed to two groups of mice with one group from each diet being used for tumour implantation. Mice were fed these diets for one week and weighed before implantation of tumours.

Tumour implantation. One group of mice fed each of the diets were given a subcutaneous injection in the abdominal area of 1×10^4 BL6 cells (from cell culture) in approximately 50µl PBS. Mice were fed the diets for a further four weeks before sacrifice of the tumour bearing and tumour free mice by

cardiac puncture (to remove blood samples). Tumours and livers were excised, rinsed in cold PBS and weighed before all samples were rapidly frozen in liquid nitrogen. Samples were stored at -20° C until fatty acid analysis could be performed.

Fatty acid extraction was performed by a slight modification of the method described by Skeef (377). Tumour (0,5g), liver (0,5g) and blood (0,1ml) samples were homogenised in 3ml methanolic KOH and 2ml PBS using a Dounce homogeniser (30 strokes with each plunger). Lipids were saponified by heating in the KOH solution under reflux in a stream of nitrogen at 85°C for 45 minutes. They were then acidified with 1ml of 7N HCl to liberate fatty acids and the fatty acids were then extracted twice (for 3 minutes and 1 minute respectively) in 3ml petroleum ether with vigourous shaking. The petroleum ether extracts were pooled and evaporated to dryness under a stream of nitrogen at 60°C before methylation. Fatty acid extraction was performed on tissues from mice fed the EPO, SFO and CO diets. Since the EFA composition of SNO is essentially the same as that of SFO, EFA analysis was not carried out in tissues from SNO fed mice.

Fatty acid methylation. The fatty acids were reconstituted in 0,3ml BF_3-methanol reagent and methylated by heating under reflux in a nitrogen atmosphere at 100°C for 5 minutes. The fatty acid methyl esters were then extracted twice (3 minutes and 1 minute respectively) in 1ml petroleum ether with vigorous shaking. The extracts were pooled, evaporated to dryness under a stream of nitrogen at 60°C and reconstituted in the desired volume of petroleum ether. The FAMEs were stored at -20°C under nitrogen and protected from light until FAME separation was performed by gas liquid chromatography.

Separation of fatty acid methyl esters by gas liquid chromatography (glc). Fatty acid methyl ester separation was achieved by glc in a Hewlett Packard model 5890A gas chromatograph using a Durabond DB 225-30N column (J&W Scientific, Rancho Cordoba, California, USA.) of film thickness 0,25µm. Operating conditions employed were: oven temperature 200°C; injection port temperature 250°C; detector (flame ionisation) temperature 250°C. Individual FAMEs were identified by their retention time. Separation of FAME standards achieved using this system and a typical chromatograph of a sample are depicted in appendix 6.

Statistical analysis was performed using analysis of variance, followed by Scheffe's multiple range test. In certain instances a comparison of data from different tables and figures is required. To avoid complicating the tables and figures the relevant statistics are mentioned in the text only.

8.2.2. Results

Effect of dietary essential/polyunsaturated fatty acid manipulation on tumour growth. (table 8.1) Tumour growth was significantly influenced by manipulation of the dietary EFA/PUFA content. Tumours in mice fed the EPO diet were found to be 37% larger than those of mice fed the SFO diet, 39% larger than those of mice fed the SNO diet and 142% larger than those of mice fed the CO diet. All groups except the SNO and SFO groups were found to be significantly ($p < 0,05$) different. No significant difference was found in the tumour mass of mice fed the 8% SFO diet compared to those fed the 4% SFO + 4% CO diet, while tumours in mice fed the 4% EPO + 4% SFO diet were found to be of intermediate size between those of mice fed the 8% EPO or 8% SFO diets.

Body mass gain and liver mass. (table 8.2) The increase in the body mass of mice during the experimental period and the liver mass were not influenced by changing the PUFA/EFA composition of the diets. Body mass gain and liver mass were not significantly different when comparing tumour free or tumour bearing mice (after taking into account tumour mass).

Effect of the essential fatty acid deficient coconut oil diet on animal health. While the CO diet is known to be EFA deficient, no apparent symptoms of EFA deficiency were observed during the experimental period in mice fed this diet. Mice were thus fed this diet for longer periods of time to investigate whether or not this diet was in fact EFA deficient. Animals maintained on the CO diet for longer periods were found to develope typical symptoms of EFA deficiency, symptoms becoming apparent at about two months and progressively worsening. Readily apparent symptoms, which are clearly visible in figure 8.1, included hair loss, lesions and scaliness of the tail and skin and weight loss. EFA deficient mice also appeared listless and weak when compared with mice maintained on the SFO, SNO or EPO diets.

Tissue essential fatty acid composition. Tissue EFA composition is expressed as a percentage of total fatty acids.

i)Liver (table 8.3) The EFA composition of the liver was found to reflect dietary EFA composition in both tumour-bearing and tumour free mice. A significant ($p < 0,01$) difference between the liver LA content of mice fed the three diets was evident. The liver LA content was found to be highest in mice fed the SFO diet followed by mice fed the EPO diet, while it was lowest in mice fed the CO diet.

The liver GLA content was found to be significantly ($p < 0,01$) higher in mice fed the EPO diet than in mice fed the SFO or CO diets, no GLA in fact being detectable in the livers of mice fed the CO diet.

The DGLA content of mice fed the EPO diet was also found to be significantly ($p < 0,01$) higher than that of mice fed the other two diets. While the DGLA content of mice fed the SFO diet was higher than that of mice fed the CO diet, the difference was not significant.

No significant difference was evident in the liver AA content of mice fed any of the diets.

The presence of tumours was found not to influence liver EFA composition, no significant difference in the EFA composition being evident between mice with or without tumours.

ii)Blood (table 8.4). Blood EFA levels were also found to reflect dietary EFA composition in both tumour-bearing and tumour-free mice . The LA content of mice fed the SFO and EPO diets was found to be significantly ($p < 0,01$) higher than that of mice fed the CO diet while the LA content of SFO and EPO fed mice was found not to be significantly different.

The GLA content in the blood from mice fed the EPO diet was found to be significantly ($p < 0,001$) higher than that in SFO and CO fed mice, no GLA being detectable in the blood of CO fed mice.

While the DGLA content of EPO and SFO fed mice was found to be higher than that of CO fed mice, a significant difference ($p < 0,001$) was only evident between EPO and CO fed mice.

No significant difference was evident in the blood AA content of mice on any of the diets.

The presence of tumours was found not to influence the EFA composition of the blood in mice fed any of the diets.

iii)Tumour (table 8.5). The EFA composition of tumours was found to be more susceptible to change by dietary EFA manipulation than that of liver or blood and was also found to reflect dietary EFA levels. LA, AA, GLA and DGLA levels were all found to be significantly ($p < 0,001$) different in tumours from mice fed the EPO, SFO or CO diets. LA levels were highest in SFO fed mice, followed by EPO fed mice and lastly by CO fed mice. AA, GLA and DGLA levels were highest in EPO fed mice, followed by SFO fed mice and lastly by CO fed mice.

iv)Comparison of liver and tumour EFA content. While the LA content of tumours was found to be lower than that of livers from mice fed any of the diets, this difference was only significant ($p < 0,001$) in CO fed mice.

The tumour GLA content was found to be significantly ($p < 0,05$) higher than that of the liver in EPO fed mice, significantly ($p < 0,001$) lower in CO fed mice while no significant difference was evident in SFO fed mice.

The tumour DGLA content was found to be significantly ($p < 0,.05$) higher than that of the liver in EPO fed mice, while no significant difference was evident between tumour and liver DGLA levels in mice fed either of the other two diets.

The AA content of tumours was found to be significantly lower than that of livers from mice fed any of the diets ($p < 0,05$ for the EPO fed mice, $p < 0,001$ for SFO and CO fed mice).

Table 8.1. Effect of dietary manipulation of polyunsaturated/ essential fatty acids on melanoma growth in mice.

diet	tumour mass (g)
EPO	1,843±0,286[a]
SFO	1,343±0,202[b]
SNO	1,323±0,223[b]
CO	0,760±0,173
EPO + SFO	1,563±0,204
SFO + CO	1,298±0,213[b]

Results are mean ± SEM, n = 6 except for the EPO diet where n = 5 (1 mouse died). [a]Significantly greater ($p < 0,01$ in comparison to CO fed mice and $p < 0,05$ in comparison to SFO and SNO fed mice) than tumour mass of mice fed any of the other diets. [b]Significantly greater ($p < 0,05$) than tumour mass of CO fed mice.

Table 8.2. Effect of dietary manipulation of essential/ polyunsaturated fatty acids on body mass gain and liver mass of mice.

diet	body mass gain (g)	liver mass (g)
EPO	2,944±0,410	0,932±0,116
SFO	2,898±0,327	0,927±0,083
SNO	2,981±0,432	0,935±0,102
CO	2,773±0,368	0,905±0,127

Results are mean ± SEM, n = 6 except for the EPO diet where n = 5 (1 mouse died). No significant difference was evident in body mass gain or liver mass of mice fed any of the diets.

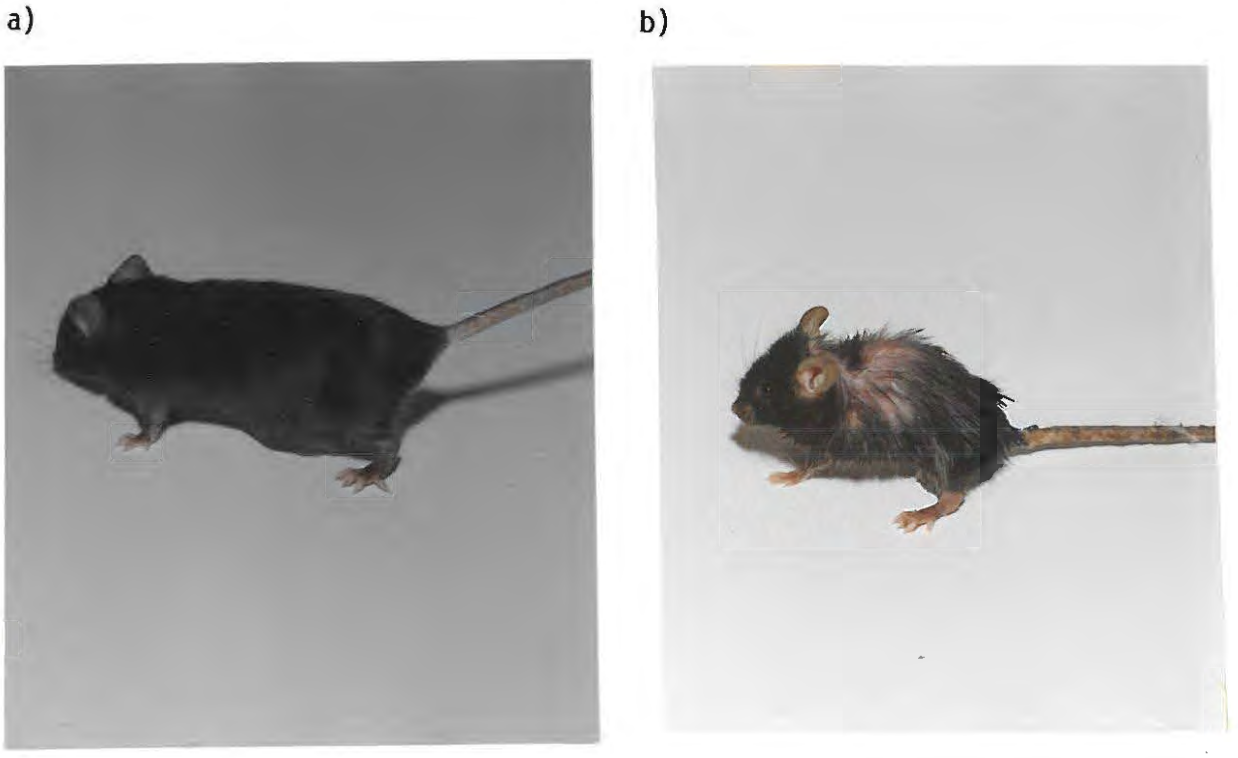

Figure 8.1. Photographs of mice fed (a) an essential fatty acid containing diet (sunflower oil) and (b) an essential fatty acid deficient diet (coconut oil) for a period of twelve weeks. Readily apparent symptoms of essential fatty acid deficiency include hair loss, lesions and scaliness of the skin and tail and weight loss.

Table 8.3. Effect of dietary essential/polyunsaturated fatty acid manipulation on the essential fatty acid composition of mouse liver.

	Essential fatty acid composition*							
	linoleic acid		gamma-linolenic acid		dihomo-gamma-linolenic acid		arachidonic acid	
diet	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour
EPO	$19,95 \pm 1,23^{a}$	$19,76 \pm 1,41^{a}$	$0,36 \pm 0,04^{b}$	$0,39 \pm 0,12^{b}$	$1,60 \pm 0,05^{c}$	$1,67 \pm 0,11^{c}$	$9,24 \pm 0,39$	$8,83 \pm 0,78$
SFO	$23,63 \pm 1,17^{a}$	$23,04 \pm 1,58^{a}$	$0,27 \pm 0,03^{b}$	$0,28 \pm 0,09^{b}$	$1,38 \pm 0,14$	$1,31 \pm 0,16$	$8,62 \pm 0,96$	$8,69 \pm 0,92$
CO	$16,70 \pm 1,28^{a}$	$16,66 \pm 1,24^{a}$	$0,23 \pm 0,03^{b}$	$0,21 \pm 0,07^{b}$	$1,31 \pm 0,12$	$1,27 \pm 0,17$	$8,73 \pm 0,92$	$8,67 \pm 0,83$

Results are mean $\pm$SEM, n = 6 except for tumour-bearing mice fed the EPO diet where n = 5 (1 mouse died). *Values are percentage composition of total fatty acids. [a] or [b] all groups significantly different ($p < 0,01$) to each other. [c] Significantly different to the DGLA content of livers of mice fed either of the other diets. No significant difference in the liver AA content was evident between mice fed any of the diets. No significant difference in the liver EFA content of mice with and without tumours was evident.

Table 8.4. Effect of dietary essential/polyunsaturated fatty acid manipulation on the essential fatty acid composition of blood in mice.

	Essential fatty acid composition*							
	linoleic acid		gamma-linolenic acid		dihomo-gamma-linolenic acid		arachidonic acid	
diet	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour
EPO	20,09±1,04[a]	20,01±0,63[a]	0,99±0,11[b]	0,97±0,14[b]	1,42±0,23[c]	1,42±0,16[c]	11,48±0,73	11,44±0,68
SFO	20,90±1,22[a]	21,32±1,58[a]	0,16±0,04	0,15±0,08	1,20±0,18	1,25±0,17	11,02±0,66	10,94±0,84
CO	10,86±0,86	10,67±0,72	0	0	1,05±0,06	0,94±0,16	10,73±0,77	10,86±0,63

Results are mean ±SEM, n = 6 except for tumour-bearing mice fed the EPO diet where n = 5 (1 mouse died). *values are percentage composition of total fatty acids. [a]Significantly different ($p < 0,01$) to CO fed mice. [b]Significantly different ($p < 0,001$) to the blood GLA content of mice fed either of the other two diets. [c]Significantly different ($p < 0,001$) to the blood DGLA content of CO fed mice. No significant difference was evident in the AA content of mice fed any of the diets. No significant difference in the EFA content of mice with or without tumours was evident.

Table 8.5. Effect of dietary essential/polyunsaturated fatty acid manipulation on the essential fatty acid composition of melanomas in mice.

diet	essential fatty acid composition* linoleic acid	gamma-linolenic acid	dihomo-gamma-linolenic acid	arachidonic acid
EPO	$18,52^{+}1,08^{a}$	$1,16^{+}0,13^{b}$	$2,94^{+}0,11^{c}$	$7,56^{+}0,52^{d}$
SFO	$25,44^{+}1,72^{a}$	$0,16^{+}0,02^{b}$	$1,37^{+}0,15^{c}$	$4,22^{+}0,32^{d}$
CO	$3,76^{+}0,57^{a}$	0^{b}	$0,85^{+}0,12^{c}$	$2,07^{+}0,28^{d}$

Results are mean $^{+}$SEM, n = 6 except for the EPO diet where n = 5 (1 mouse died). *Values are percentage composition of total fatty acids. ^{a,b,c}or^{d} all groups significantly ($p < 0,001$) different to each other.

8.3. MECHANISM OF ESSENTIAL/POLYUNSATURATED FATTY ACID TUMOUR GROWTH PROMOTION

8.3.1. Materials and Methods

Materials

Mice and cells were as previously described.

Diets. The diets containing 8% EPO, SFO, SNO or CO were prepared as described in section 8.2. To investigate the possible involvement of PG or LT synthesis in the mechanism of PUFA/EFA mediated tumour promotion, these diets were supplemented with either 0,004% IM or 0,004% CA (these levels of IM and CA have been used in previous studies and are sufficient to inhibit the relevant pathways (64)). In a separate experiment to investigate the possible involvement of lipid peroxidation in tumour promotion by the PUFAs/EFAs the TOC level of the diets was manipulated. A TOC deficient diet containing no added TOC, a diet containing the normal level (1 x 10^6IU TOC/kg diet) of TOC and a TOC supplemented diet containing 1 x 10^7IU TOC/kg diet were prepared for each of the oils used to supplement the diets.

Prostaglandins E_1 and E_2 (Sigma) were diluted and treated as previously described (section 5.3).

Methods

Effect of prostaglandins E_1 and E_2 on tumour growth. In this experiment mice were fed commercial pellets instead of one of the fatty acid containing semi-purified diets (since the effect of exogenous PGs which could not be administered via the diet was being examined) and tumours were implanted as before. Mice were fed this diet for a further four weeks and given a daily injection of 5 or 10µg of PGE_1 or PGE_2 at the site of tumour implantation. Mice were then sacrificed and the tumours excised, rinsed in cold PBS and weighed.

Effect of dietary indomethacin or caffeic acid supplementation on tumour growth promotion by dietary essential/polyunsaturated fatty acids. The possible involvement of enhanced PG or LT synthesis as a mechanism whereby dietary PUFAs/EFAs promoted tumour growth was examined by adding the PG synthesis inhibitor, IM, or the LT synthesis inhibitor, CA, to the diets. Mice were fed these diets for one week prior to implantation of tumours as previously described. Tumours were allowed to grow for four weeks before mice were sacrificed and the tumours excised, rinsed in PBS and weighed.

Effect of dietary α-tocopherol on tumour growth promotion by essential fatty acids. The possible involvement of lipid peroxidation in the mechanism of tumour growth promotion by dietary PUFAs/EFAs was examined by manipulation of dietary TOC levels. Mice were fed the diets containing one of the three TOC levels described under the materials section for one week before tumour implantation which was carried out as before. Mice were then fed the diets for a further four weeks before being sacrificed. Tumours were excised, rinsed in cold PBS and then weighed.

Statistical analysis was carried out using analysis of variance followed by Scheffe's multiple range test.

8.3.2.Results

Effect of prostaglandins E_1 and E_2 on tumour growth (table 8.6). PGE_1 was found to significantly ($p < 0,05$) inhibit tumour growth at a dose of 10μg while PGE_2 was found not to significantly influence tumour growth.

Effect of indomethacin and caffeic acid on tumour growth (table 8.7). Dietary IM and CA were found not to influence tumour growth in mice fed any of the diets.

Effect of dietary α-tocopherol manipulation on tumour growth (table 8.8). The level of dietary TOC was found to significantly influence tumour growth, an effect which appeared to depend on the PUFA/EFA content of the diets. Tumours in mice fed the TOC deficient EPO and SFO diets were found to be significantly ($p < 0,01$ and $p < 0,05$ respectively) smaller than those of mice fed the TOC control diets. This effect was not evident in CO fed mice. Tumours in mice fed the TOC supplemented diets were slightly but non-significantly larger than those of mice fed the TOC control diets.

The reduction in tumour growth by TOC deficiency in EPO and SFO fed mice was significantly ($p < 0,05$) greater than that of CO fed mice. No significant difference between reduction in tumour growth in SFO or EPO fed mice was apparent.

Table 8.6. Effect of prostaglandins E_1 and E_2 on melanoma growth in mice.

PG dose (µg/day)	tumour mass (g) PGE_1	PGE_2
0 (control)	1,405±0,213	1,405±0,221
5	1,268±0,167	1,376±0,204
10	1,141±0,231[a]	1,452±0,217

Results are mean ± SEM, n = 6 for mice injected with 5µg/day PGE_1 and 5µg/day PGE_2 while n =5 for mice injected with 10µg/day PGE_1 and n = 4 for mice injected with 10µg/day PGE_2.
[a]Significantly ($p < 0,05$) different to control.

Table 8.7. Effect of dietary indomethacin and caffeic acid supplementation on melanoma growth in mice.

diet	tumour mass (g) no added IM or CA	0,004% IM	0,004% CA
EPO	1,975±0,301	2,004±0,271	1,963±0,216
SFO	1,476±0,217	1,504±0,188	1,482±0,214
CO	0,827±0,173	0,825±0,141	0,834±0,197

Results are mean ± SEM, n = 6 except for mice fed the EPO and SNO diets where n = 5 (1 mouse died on each diet). Dietary IM or CA did not significantly influence tumour growth in mice fed any of the diets.

Table 8.8. Effect of dietary α-tocopherol manipulation on melanoma growth in mice.

	tumour mass (g)		
diet	TOC deficient	TOC control	TOC excess
EPO	1,479±0,313[a]	1,946±0,179	2,160±0,216
SFO	1,076±0,274[b]	1,465±0,158	1,507±0,234
CO	0,785±0,192	0,810±0,185	0,831±0,173

Results are mean ±SEM, n = 6. Significantly smaller than mice fed TOC control diets [a]$p < 0,01$, [b]$p < 0,05$. The reduction in tumour growth was significantly ($p < 0,05$) more pronounced in SFO and EPO fed mice than in CO fed mice. No significant difference was evident between the reduction in tumour growth in EPO or SFO fed mice.

8.4.Discussion

It is often difficult to draw conclusions from results obtained from studies involving in vivo systems due to the large number of variables found in these systems. Investigations using dietary manipulation often complicate this problem even more, as changes in the level of a single nutrient might influence the effects of another nutrient. The effect of dietary TOC manipulation can be used as an example to illustrate this point. Whereas dietary PUFAs/EFAs were found in this study to promote tumour growth in mice fed diets containing a normal level of TOC, tumour growth was restricted in mice fed TOC deficient PUFA/EFA containing diets. The mechanism whereby tumour growth was restricted in mice fed the TOC deficient diets appeared to involve dietary PUFAs/EFAs as the growth inhibitory effects of TOC deficiency were only apparent in mice fed diets containing PUFAs/EFAs. The possibility that the TOC deficient diets reduced tumour growth by a mechanism which involved another nutrient(s) or pathway(s) not examined in this study cannot however be discounted. Since this study involved manipulation of a number of dietary nutrients, it was particularly difficult to to make definitive statements on the basis of the results obtained. Of necessity therefore a certain amount of speculation is contained in this discussion.

Numerous studies have reported that diets high in PUFAs/EFAs promote tumour growth in experimental animals (see table 1.2). The studies of Carroll and associates (80,83,84,87,96,97) in particular, which examined the effects of dietary PUFAs/EFAs on DMBA induced mammary tumourigenesis, have provided evidence of the tumour promoting effects of PUFAs/EFAs. Whereas initial studies in that and other laboratories examined the effects of high fat diets on tumour growth without much regard for the PUFA/EFA content of the diets, later studies provided evidence that both the type and amount of dietary fat were important in promotion of tumour growth. Diets containing 3% SNO (which contains ± 70% LA) and 17% CO (EFA deficient, containing mainly SF) were found to promote tumour growth to the same extent as diets containing 20% SNO. Since 3% SNO by itself did not promote tumour growth, it was evident that a small amount of PUFA and a high fat diet were required to enhance tumour growth. Subsequent studies provided evidence that EFAs played an important role in tumour promotion. Diets containing 3% LA and 17% CO were found to promote tumour growth as effectively as diets containing 3% SNO and 17% CO, whereas diets containing 3% oleic acid, which is not an EFA, and 17% CO did not influence tumour growth. It was proposed on the basis of these results that once the EFA requirement of the tumour had been met, tumour promotion was dependant on the type and not amount of fat in the diet.

In the present study the effect of dietary PUFAs/EFAs, in the form of EPO, SNO and SFO, on tumour growth were compared to the effects of a PUFA/EFA deficient, SF containing diet (CO diet). In this study all the diets containing PUFAs/EFAs were found to promote growth of the BL6 melanoma when compared with the diet containing SF. Diets containing 4% SFO and 4% CO were found to be equally

as effective as diets containing 8% SFO in promoting melanoma growth. These results appear to support the observations of Carroll and colleagues that once the PUFA/EFA requirement of the tumour had been met, the promotion of tumour growth was dependant on the amount and not the type of dietary fat.

Several previous investigations (392-396) have reported that that many malignant cells are unable to metabolise LA to GLA due to the absence of delta-6-desaturase activity in the cells (see figure 1.1 for EFA metabolic pathway). It was thus proposed that loss of delta-6-desaturase activity might be an important factor in the malignant process (175) as it would result in a reduction in the synthesis of GLA and its EFA metabolites DGLA and AA. In order to examine whether provision of GLA in the diet would influence tumour growth in mice, the effect of supplementation to the diets with EPO, which contains GLA and LA, was thus compared to dietary supplemention with SFO or SNO which contain predominantly LA. The increased tumour growth observed in EPO fed mice when compared with SNO and SFO fed mice suggests that the BL6 melanomas may lack activity of delta-6-desaturase and that the effectiveness of the GLA containing diet as a tumour promotor might be the result of a specific growth requirement for GLA or one of its metabolites by the tumour. Further evidence is provided for this possiblity by the observation that tumours in mice fed the 4% EPO + 4% SFO diet were intermediate in size to those in mice fed the 8% EPO diet or the 8% SFO diet. These results appear to be in contrast to the studies mentioned above, in which loss of delta-6-desaturase activity, which would result in a reduction in GLA synthesis, is thought to be an important step in the malignant process allowing tumours to grow more rapidly. A number of earlier investigations have in fact reported that EPO inhibited growth of certain tumours, including DMBA induced and transplantable mammary tumours (68,86). The effect of EPO on tumour growth may thus be dependant on the type of tumour. The role played by delta-6-desaturase in tumour cell growth will be dealt with in more detail in chapter 11 and related to the effects of the various diets on tumour growth.

Examination of the EFA composition of tissues of mice fed the diets with different EFA/PUFA content revealed some interesting data. While dietary EFA/PUFA manipulation was found to influence the EFA composition of liver and blood, the EFA content of the tissues being found to reflect dietary EFA levels, it was the tumour EFAs which were particularly susceptible to modification. LA levels of all tissues including tumours were found to be significantly higher in mice fed the SFO diet, which contained the highest concentration of LA, followed by mice fed the EPO diet and lastly by mice fed the EFA deficient CO diet. The liver and blood AA content was not significantly altered by any of the diets including the EFA deficient CO diet, while the GLA and DGLA content of these tissues was significantly higher in mice fed the GLA containing EPO diet when compared with mice fed the SFO and CO diets. The tumour AA, GLA and DGLA content on the other hand was significantly influenced by dietary EFA /PUFA manipulation, being highest in mice fed the GLA

containing EPO diet followed by those fed the SFO diet and lastly by those fed the EFA deficient CO diet.

A possible explanation for these results may be provided if the EFA content of the diets is examined. The SFO diet and the EPO diets provided a significant amount of LA, which would presumeably have been taken up by the tissues. Although the level of EFAs/PUFAs in cell membranes is closely regulated and LA is not the major EFA/PUFA in cell membranes (21), the excess LA not incorporated into the cellular membranes could possibly have been stored in the cells in the form of triglycerides and lipid droplets thereby increasing the LA levels of the tissues. Several previous studies have reported that excess intracellular PUFAs/EFAs are stored in cells in this form (173,189,194-197). The EFA deficient CO diet on the other hand would not have provided LA, resulting in lower levels of LA in the tissues of mice fed this diet, than in the tissues of EPO and SFO fed mice.

A comparison of LA levels in tumour and normal (liver and blood) tissues in mice fed the EFA deficient CO diet revealed that LA levels in the tumours were significantly lower. An explanation for these results may be provided if one bears in mind that the tumours were only introduced into the mice after they were being fed the EFA deficient CO diet which then would not be able to supply the tumour with EFAs. The liver and blood on the other hand would presumeably have contained some LA from the diet fed prior to the CO diet.

While animals are able to synthesise GLA by desaturation of LA in the reaction catalysed by delta-6-desaturase (see figure 1.1), they are unlikely to do so in excess of their requirements. It would therefore be expected, as was found in this study, that GLA levels would be higher in the tissues of mice fed the GLA containing EPO diet than in those of mice fed either of the other two diets. Since DGLA is synthesised by elongation of GLA, in the reaction catalysed by elongase, a diet providing GLA, such as the EPO diet, would probably, as found in this study, increase tissue DGLA levels.

A comparison of the GLA and DGLA levels of tumours and normal tissues (liver and blood) revealed that the GLA and DGLA levels of tumours were significantly higher in EPO fed mice. This sequestration of GLA and DGLA by the tumours may indicate a specific requirement for GLA, DGLA or one of their metabolites by the tumours, particularly since tumours in mice fed this diet were found to grow much more rapidly than those of mice fed either of the other diets. Furthermore the GLA and DGLA content of tumours from EPO fed mice was found to be higher than that of SFO and CO fed mice. In contrast to EPO fed mice, the tumour GLA levels of SFO and particularly CO fed mice were found to be lower than liver and blood GLA levels. The lower tumour GLA levels in mice fed the SFO diet, which provides LA, suggest that the tumours may have reduced delta-6-desaturase activity.

The situation regarding AA is a little more complex since AA is the major EFA found in cell membranes and is tenaciously retained in these membranes even during EFA deficiency (19,22). Mice fed the EFA deficient CO diet would therefore be expected to contain an appreciable concentration of AA, obtained from the diets fed before the mice were fed the EFA deficient diet, in their normal tissues such as the blood and liver. Since AA is also unlikely to be synthesised in excess of requirements, it was therefore not unexpected that while the AA levels of the liver and blood in mice fed the EFA containing EPO and SFO diets were found to be higher than those in mice fed the EFA deficient CO diet, this difference was not significant. Furthermore while no detectable EFAs were present in the CO diet, during the period of the study EFA deficiency symptoms were not evident in mice fed the CO diet. The mice being examined were thus presumeably not severely EFA deficient as they were probably utilising EFAs from tissue stores. Tumour AA levels on the other hand were significantly lower in CO fed mice than in EPO and SFO fed mice. An explanation for this may be provided by a similar argument to that used to explain the lower LA levels observed in tumours from CO fed mice. The rapidly growing tumour was only introduced into the mice after they were being fed the EFA deficient CO diet which then would not have been able to supply the EFA requirements of the rapidly dividing tumour. Therefore since the tumour cells did not have the opportunity to store AA prior to the mice being fed the EFA deficient diet, tumour AA levels would be expected to be lower in mice fed the CO diet than in mice fed the EPO or SFO diets. The tumour AA levels were also significantly lower in SFO fed mice than in EPO fed mice which may be the result of a lack of delta-6-desaturase activity in the tumour cells.

A comparison of the AA levels in the different tissues reveals that the AA content was significantly lower in the tumour tissue than in normal tissues such as the liver and blood. The decreased AA content is in fact a well documented feature of tumours or tumour cells (94,134-136). It must be noted however that in this study the most rapidly growing tumours, namely those from EPO fed mice followed by those from SFO fed mice, were found to contain the highest level of AA which suggests that AA may play an important role in promotion of melanoma growth.

From these results involving an investigation of tumour EFA content it is apparent that dietary EFA/PUFA manipulation significantly influenced the EFA composition of the tumours. Several previous studies have reported that the EFA composition of tumours was susceptible to alteration by dietary EFA/PUFA manipulation (93,138). The increases in the EFA composition of the tumours could have played a role in the mechanism of tumour promotion by the PUFAs/EFAs since alterations in membrane EFA/PUFA composition may also have been involved, resulting in changes in the fluidity and physical and physiological properties of the membranes which could have influenced tumour growth. Furthermore the increased EFA/PUFA content of the tumours could have influenced lipid peroxidation or PG synthesis.

The possible involvement, in the mechanism of tumour promotion, of lipid peroxidation was investigated by manipulation of dietary TOC levels, since TOC is an inhibitor of lipid peroxidation (144), while the PG synthesis inhibitor, IM, was used to investigate the possible involvement of PGs. While the use of TOC and IM did not involve actual measurements of the level of lipid peroxidation or PG synthesis they are frequently adopted to investigate the role of lipid peroxidation or PG synthesis in tumour growth. At present no suitable method for the measurement of in vivo lipid peroxidation is available (144) while the methods employed to measure in vivo PG synthesis are expensive and sometimes inaccurate. Demonstration of an effect of dietary TOC or IM on tumour growth should provide evidence that lipid peroxidation or PG synthesis may be involved in the process of tumour growth promotion by the EFAs.

Since susceptibility to lipid peroxidation increases with increasing unsaturation of PUFAs/EFAs (144), the possibility arose that the tumour promoting effects of the SFO diet and particularly the EPO diet, which were found to increase the level of unsaturated tumour EFAs in comparison to the CO diet, may have involved lipid peroxidation. Further support for this possibility is provided by observations that the reduced levels of lipid peroxidation observed in tumours, in comparison to normal tissues, may be the result of substrate (PUFA/EFA) unavailability since tumour cells are known to contain lower levels of PUFAs/EFAs than normal cells (134,135,140-143). The EPO diet in particular and the SFO diet could therefore have promoted tumour growth by increasing tumour EFA levels thereby providing substrate for lipid peroxidation. As previously mentioned it has been suggested that enhanced lipid peroxidation plays an important role in promotion of tumour growth by PUFAs/EFAs (93,100,139). Other studies have however provided evidence that lipid peroxidation may play an important role in the control of proliferation of tissues and that the decreased level of lipid peroxidation observed in certain tumour cells when compared with normal cells may be an important factor in the uncontrolled growth of tumours (100,139).

While tumour cells have been reported to be more resistant to lipid peroxidation than normal cells (135,136,140,141) in the absence of dietary antioxidants, such as TOC, increased susceptibility of tumour cells to lipid peroxidative cellular damage may occur. Tumour cells may be particularly susceptible to lipid peroxidative damage during TOC deficiency since they have been reported to contain decreased activity of the free radical scavenging enzymes superoxide dismutase and glutathione peroxidase (134). In addition, since the lower levels of lipid peroxidation observed in tumour cells are thought to be due to the lower levels of the peroxidation substrate, viz.EFAs/PUFAs, in tumour cell membranes (134,135,140,141,142,143), dietary EFAs/PUFAs by providing EFAs/PUFAs to the tumour cells could lead to increased lipid peroxidation. While tumour cells have been reported to contain higher levels of TOC than normal cells (136,140,142), thereby presumeably also reducing the level of lipid peroxidation in the tumour cells, the TOC supply would probably be rapidly exhausted in mice fed TOC deficient diets since the diet would be unable to replenish the stocks of TOC utilised in

the defence of cells against free radical attack and lipid peroxidation. Previous studies have in fact reported that tissues are more susceptible to lipid peroxidation during TOC deficiency (144). A diet deficient in antioxidants has also been reported to inhibit the growth of Ehrlich ascites tumours in vivo (Baumgartner *et al* cited in 58). A number of in vitro studies have also provided evidence that in cells in which EFAs have growth inhibitory effects, the inhibition might involve lipid peroxidation since the growth inhibitory effects can be blocked by antioxidants including TOC (159,165,168,169,197,201,202).

In this study three diets, namely a TOC deficient, a TOC excess and a normal TOC diet, were prepared for each of the oils used to supplement the diets. While a deficiency or excess of dietary TOC did not significantly influence tumour growth in CO fed mice, some significant effects of dietary TOC manipulation were observed in EPO and SFO fed mice. Tumour growth was significantly inhibited by dietary TOC deficiency in EPO and SFO fed mice while excess dietary TOC slightly but non-significantly increased tumour growth in mice fed these diets when compared with tumour growth in mice fed diets containing a normal level of TOC. The growth inhibitory effects of TOC deficient diets were particularly evident in the EPO fed mice.

Dietary PUFAs/EFAs appeared to possibly enhance lipid peroxidation in tumours since dietary TOC deficiency in this study only reduced tumour growth in mice fed diets containing PUFAs/EFAs and not in mice fed a diet deficient in EFAs/PUFAs. The tumour cells however appeared resistant to lipid peroxidative damage in mice fed a diet adequate in TOC, since excess dietary TOC did not significantly influence the tumour growth promoting effects of the dietary EFAs/PUFAs in comparison to diets containing a normal level of TOC. In mice fed a TOC deficient diet however, the tumour cells appeared unable to cope with the possible increase in the peroxidation of PUFAs/EFAs in mice fed EFA/PUFA containing diets leading to reduced tumour growth when compared with mice fed diets containing normal or excess dietary TOC.

If lipid peroxidation was the mechanism whereby dietary EFAs/PUFAs promoted tumour growth, it would be expected that tumour growth would be promoted by TOC deficient diets and inhibited by TOC excess diets. In this study however tumour growth was reduced in mice fed TOC deficient PUFA/EFA containing diets. Increased lipid peroxidation did not therefore appear to be the mechanism whereby dietary EFAs/PUFAs promoted melanoma growth in nutritionally adequate animals as has previously been found in several other studies (93,100,139). Since many of the studies in which lipid peroxidation was found to play a role in promotion of tumour growth involved carcinogen induced tumours (134,140), it is possible that the effect of lipid peroxidation on tumour growth may depend on the type of tumour. Alternatively in those studies lipid peroxidation may have played a role in carcinogen metabolism or in the initiation of carcinogenesis. It must also be remembered however that while this study has confined its discussion of the effects of dietary TOC manipulation on tumour growth to the effects of TOC on lipid peroxidation, TOC deficiency could influence other pathways

which might affect tumour growth. Additionally since TOC is localised in cells in association with the cell membrane and intracellular membranes (144), it might play an important role in membrane function besides its role as an anti-oxidant. Therefore the earlier suggestion that lipid peroxidation did not appear to be involved in the mechanism of tumour promotion by dietary EFAs/PUFAs should be treated with caution.

Another mechanism whereby EFAs are thought to promote tumour growth involves enhanced synthesis of PGs from the precursor EFAs (101,105-118). This proposal was based on the results of a number of studies in which elevated PG synthesis (and in particular PGE) was reported in tumour-bearing animals and humans (105-114) and the results of studies in which inhibitors of PG synthesis were found to reverse the tumour promoting effects of dietary PUFAs/EFAs (101,105,106,112,115-117). In this study dietary EFAs/PUFAs were found to increase the levels of the PG precursors, DGLA and AA, in tumours and since dietary EFAs have been found to enhance synthesis of PGs (17,18,26,34-37) the possibility arose that enhanced PG synthesis may be involved in the mechanism of tumour promotion. In this study the effects of exogenous PGE_1 and PGE_2 and the effect of dietary supplementation of the PG synthesis inhibitor, IM, on tumour growth were thus examined.

While tumour growth inhibition by PGE_1 was not very large, the effect was significant at higher concentrations of PGE_1. PGE_2 on the other hand was found not to influence tumour growth. Thus while it was apparent that certain exogenous PGs were able to influence tumour growth, the possible involvement of endogenous PGs was investigated by supplementing IM to the diets of the mice. Due to the apparent lack of effect of IM on tumour growth in mice fed any of the diets in this study, it would appear unlikely that PGs played a role in the tumour promoting effects of the EPO and SFO diets. Whereas several earlier studies reported tumour growth inhibition by IM and other PG synthesis inhibitors (101,105,106,112,115,117), the results obtained here support those of a number of other studies which reported that IM and other PG synthesis inhibitors did not influence tumour growth and that PGs were apparently not involved in promotion of tumour growth by dietary EFAs (cited in 407). The lack of effect of the LT synthesis inhibitor, CA, on the promotion of tumour growth by the EFA/PUFA containing diets also appears to preclude an involvement of LTs in promotion of tumour growth by EFAs/PUFAs.

It would appear therefore that the mechanism of promotion of BL6 melanoma growth by dietary EFAs/PUFAs in this study did not involve enhanced PG or LT synthesis or lipid peroxidation. Several other mechanisms have been proposed for the tumour promoting effects of EFAs/PUFAs including changes in membrane lipid composition and fluidity (93,100,138), alterations in intercellular communication (100,138), hormonal changes (87,100,137), immunoinhibition (57,93,100,119,148-152) and caloric effects (100,146,147). Since all diets used in this study were isocaloric it would appear unlikely that a caloric effect was involved in the promotion of tumour growth by the EFAs/PUFAs.

Since dietary PUFA/EFA manipulation was found to readily alter the EFA content of tumours, it is possible that the tumour promoting effects of PUFAs/EFAs may have involved alterations in membrane PUFA/EFA content and fluidity.

While tumour growth was significantly influenced by dietary manipulation of PUFAs/EFAs, the growth of animals was not affected during the experimental period by dietary manipulation of the EFAs/PUFAs. The animals presumeably contained sufficient EFA reserves to maintain themselves in an apparently healthy state during the experimental period even when they were fed the EFA/PUFA deficient CO diet. Prolonged feeding of the CO diet did however induce typical symptoms of EFA deficiency in the mice, including hair loss, scaliness and lesions of the tail and skin, severe weight loss and listlessness. It is therefore apparent that while EFA deficient diets inhibit tumour growth, they also severely influence the health of animals and do not thus make a suitable form of treatment for cancer.

In conclusion therefore, it was found that diets containing PUFAs/EFAs promoted BL6 melanoma growth in mice. Promotion of tumour growth appeared to depend on the amount and not the type of dietary fat once the EFA requirements of the tumour were met. The mechanisms of tumour growth promotion by the PUFAs/EFAs appeared not to involve enhanced lipid peroxidation, enhanced endogenous synthesis of PGs or LTs or changes in caloric consumption. Since dietary manipulation of EFAs/PUFAs was found to influence tumour EFA content, the mechanism of tumour promotion by these nutrients may have involved changes in the intracellular or membrane EFA content of tumour cells.

Chapter 9

INFLUENCE OF ASCORBIC ACID ON TUMOUR GROWTH IN MICE

9.1.Introduction

The role played by Asc in the treatment of cancer remains a controversial issue. Therapeutic benefit in the treatment of terminal human cancer patients with large doses of Asc has been reported by Cameron and Pauling (241,288-291). Moertel and colleagues (278,279) in contrast have been unable to confirm these reports, no therapeutic benefit being evident in their studies examining the effect of large doses of Asc in the treatment of terminal human cancer patients. Several studies have also examined the effect of large doses of Asc on tumour growth in experimental animals, the majority of these studies reporting tumour growth inhibition as a result of Asc supplementation (299,312-314,316,317,319).

While mice are able to synthesise Asc and are therefore not the ideal animal models for use in studies involving Asc, facilities to house animals such as guinea pigs, which are unable to synthesise Asc, were not available to this laboratory. Furthermore a transplantable tumour cell line known to grow in guinea pigs could not be obtained. Mice are however still a useful model in which to study the effect of supplementation of large doses of Asc to a normal diet and have been widely used for this purpose. In this study the effect of supplementation of large doses of Asc, to the normal semi-purified diet of mice, on the growth of the transplantable BL6 melanoma was examined.

9.2.Materials and Methods

Rations

The composition of the semi-purified diet used in this study has been provided previously (section 8.2). In this investigation fat was provided in the diets in the form of SNO, which is the usual dietary fat source used in this laboratory. The above mentioned diet, which contained 50mg/kg Asc, was used as the control diet since Asc is usually added to the animal diets at this level. An Asc supplemented diet was prepared by adding a further 450mg/kg Asc to the control diet to provide a diet containing 500mg/kg Asc. The diets were prepared and stored as before (section 8.2). Since mice are able to synthesise Asc the effects of Asc deficiency on tumour growth could obviously not be examined.

Animals

The C57 mice used in this study have been described previously (section 8.2).

Cells

The transplantable BL6 murine melanoma cells used in this study have been described previously (section 4.2).

Methods

Diets. Mice were divided into four groups, two of which were fed the control diet and two the Asc supplemented diet. One group of mice from each of the diets was used for tumour implantation. Mice were fed these diets for one week and weighed before implantation of tumours.

Tumour implantation. Mice were given a subcutaneous injection in the abdominal area of $5x10^4$ BL6 cells in approximately 50µl PBS. Tumour bearing and tumour free mice were fed the semi-purified diets for a further four weeks and weighed before sacrifice by cardiac puncture (to remove blood samples). Tumours and livers were excised, rinsed in cold PBS and weighed before being rapidly frozen in liquid nitrogen. Tumour, liver and blood samples were then stored at -20°C until Asc analysis could be performed.

Ascorbic acid analysis. Tumour, liver and blood Asc levels were determined using the DCIP colourimetric method described previously (section 6.2). Tumour (0,5g), liver (0,5g) and blood (0,1ml) samples were homogenised in 1ml cold PBS and 1ml cold 10% metaphosphoric acid using a Dounce homogeniser (30 strokes with loose and tight plungers). Samples were then centrifuged at 1000g (Hettich Univeral K2S) for 10 minutes and Asc determination was then performed on the supernatant.

Statistical analysis was carried out using analysis of variance followed by Scheffe's multiple range test.

9.3.Results

Effect of supplementary ascorbic acid on melanoma growth. (Table 9.1). The diet containing supplementary Asc (500mg/kg Asc) was found to significantly ($p < 0,05$) inhibit melanoma growth when compared with the contol diet (50mg/kg Asc).

Effect of supplementary ascorbic acid and the presence of tumours on animal mass gain and liver mass. (Table 9.2). Supplementary Asc was found not to influence the body mass gain or the liver mass of the mice. While tumour bearing mice were found to have a slightly larger mass than non-tumour bearing mice, no significant difference in the mass of the animals was evident after taking into account the mass of the tumours. The presence of tumours was also found not to influence the liver mass.

Influence of supplementary ascorbic acid and the presence of tumours on tissue ascorbic acid content. (Table 9.3). i)Liver. The diet containing supplementary Asc was found to significantly ($p < 0,01$) increase the liver Asc content in both tumour bearing and tumour free mice when compared with the Asc control diet.

The liver Asc content was significantly ($p < 0,05$) reduced in tumour bearing mice fed either of the diets when compared with mice without tumours on the same diet.

ii)Blood. A non-significant increase in the Asc content of blood was evident in both tumour-bearing and tumour free mice fed the Asc supplemented diet when compared with mice fed the Asc control diet.

A non-significant reduction in blood Asc content was evident in tumour bearing mice fed either of the diets when compared with non-tumour bearing mice fed the same diet.

iii)Tumours. The diet containing supplementary Asc was found to significantly ($p < 0,05$) increase the melanoma Asc content when compared with the Asc control diet.

Table 9.1. Effect of dietary ascorbic acid manipulation on melanoma growth in mice.

dietary ascorbic acid (mg/kg diet)	tumour mass (g)
50	1,043±0,153
500	0,780±0,138[a]

Results are mean ±SEM, n = 6. [a]Significantly different ($p < 0,05$) to mice fed the diet containing 50mg Asc/kg diet.

Table 9.2. Influence of supplementary dietary ascorbic acid and the presence of tumours on body mass gain and liver mass of mice.

dietary ascorbic acid (mg/kg diet)	body mass gain (g)		liver mass (g)	
	with tumours	without tumours	with tumours	without tumours
50	2,646±0,267	2,621±0,320	0,906±0,087	0,916±0,092
500	2,623±0,324	2,761±0,338	0,924±0,104	0,924±0,104

Results are mean ±SEM, n = 6. No significant difference was evident in the body mass gain or liver mass of mice from any of the groups.

Table 9.3. Influence of dietary ascorbic acid manipulation and the presence of tumours on tissue ascorbic acid content.

dietary ascorbic acid (mg/kg diet)	ascorbic acid content				
	liver (mg/100g)		blood (mg/100ml)		tumour (mg/100g)
	with tumour	without tumour	with tumour	without tumour	
50	17,39±1,27[a]	20,34±1,40	1,09±0,17	1,24±0,06	16,72±0,89
500	24,47±1,39[a,b]	30,13±1,23[b]	1,22±0,23	1,33±0,14	21,13±1,36[c]

Results are mean ± SEM, n = 6. [a]Significantly different ($p < 0,05$) to mice on the same diets without tumours. [b]Significantly different ($p < 0,01$) to mice fed 50mg Asc/kg diet. [c]Significantly different ($p < 0,05$) to mice fed 50mg Asc/kg diet. No significant difference was evident in blood Asc levels.

9.4.Discussion

Several reports have voiced concern about the possible toxic effects of large doses of Asc (182,203,244,280,281,284,285). However a number of investigations have been unable to detect any toxic effects of large doses of Asc in animals, including guinea pigs and rats, fed nutritionally balanced diets (381-383). Furthermore no toxic effects of large Asc intakes, with the possible exception of diarrhea, have been reported in humans (241,241,283). However Nandi *et al* (381) have reported that large doses of Asc were fatal to guinea pigs fed an unfortified wheat diet. Since this diet was deficient in lysine and since protein supplementation protected against the toxic effects of Asc, the effects of large doses of Asc observed in that study are thought to involve an interaction of Asc with proteins or essential amino acids. While this may not be a problem in humans or animals fed a nutritionally adequate diet, care should perhaps be exercised regarding large intakes of Asc in populations where the diet may be nutritionally inadequate and where the diet consists mainly of cereals. In this and other (366) studies, Asc supplementation was found not to influence the body mass gain or liver mass of mice, presumeably indicating a lack of toxicity of Asc at the dietary levels employed, at least as far as the growth of the animals is concerned. Furthermore no visible toxic symptoms were evident in the Asc supplemented mice.

Several studies have examined the effect of large doses (a number of which were of a similar level to those used in this study) of Asc on the growth of tumours in experimental animals. The majority of these investigations have reported tumour growth inhibition by supplementary Asc. While a more comprehensive list of these studies is provided in table 2.2, a few of those involving murine subjects will be mentioned here. Piersen and Meadows (384) reported that supplementary Asc enhanced the growth inhibitory effects of cardipolevodopa against murine melanoma growth, while Poydock *et al* (313) found that a mixture of Asc and vitamin B_{12} prolonged survival of mice bearing Ehrlich carcinoma or leukaemia. Pauling *et al* (316) reported that supplementary Asc inhibited growth and incidence of spontaneous mammary tumours in mice, while Dunham *et al* (317) found that Asc reduced the incidence and growth of UV induced malignant skin tumours in mice. In support of these results, supplementary dietary Asc was found to significantly inhibit growth of the BL6 murine melanoma in this study. Since supplementary Asc appeared not to be toxic to mice, at least regarding the body weight of the animals and the lack of any visible toxic symptoms, Asc would appear to be suitable for use in cancer therapy. However as previously mentioned care should be exercised in the use of large doses of Asc in humans whose diet may be nutritionally inadequate.

While a number of mechanisms including immunostimulation (203,296,304,323-332), inhibition of the enzyme hyaluronidase (304,333) and enhanced accumulation of H_2O_2 in tumour cells (248,315,354,356) have been proposed for the tumour growth inhibitory effects of Asc, they are beyond the scope of this study and will require further investigation. Certain observations can however be

made regarding the level of Asc in tumours from mice fed the two diets. Whereas several studies have reported accumulation of Asc in melanotic and other tumour cells (298,304,344), the Asc levels of the BL6 melanomas measured in this study were found not to be higher than those previously reported for many other murine tissues (298,383) and in this study were found to be lower than the liver Asc values in mice fed either of the diets. It would thus appear unlikely, as was the case in the in vitro studies examining the effect of Asc on the growth of cultured cells, that the inhibition of melanoma growth by supplementary Asc observed in this study is due to preferential accumulation of Asc by the melanoma cells as has been reported in other studies (cited in 344). The absence of Asc accumulation in melanomas from animals fed increased dietary Asc has also previously been reported by Dyer and Ross in a study using Hardey-Passing melanomas (298). However since supplementary dietary Asc was found to increase the melanoma Asc content in this study, the mechanism of tumour growth inhibition by supplementary Asc may have involved changes in the Asc concentration of the tumours.

Several investigations involving both humans and experimental animals have reported depletion of tissue and blood Asc levels in tumour bearing animals and human patients when compared with healthy controls (241,247,296,299-304). The significant decrease in liver Asc content, as well as a decrease (although non-significant) in blood Asc levels, observed in tumour-bearing mice in this study support these earlier reports. The Asc levels are presumeably reduced as a result of increased Asc requirement and utilization for the mobilisation of the natural defence mechanisms of the body as well as to meet the Asc requirements of the rapidly dividing tumour tissue. As in several previous studies (320,381-383) supplementary Asc was found in this study to increase tissue, including liver, tumour and blood (although non-significant in blood) Asc levels.

Since changes in caloric intake are thought to influence tumour growth (89,92,146,147), in studies involving dietary manipulation it is important to investigate the possible contribution of any changes in caloric intake to the observed effects. Supplementary Asc at the level used in this study was however unlikely to make a significant contribution to the energy intake of the mice. Since supplementary Asc was also found not to influence the body weight gain of the mice it is unlikely that the mechanism of tumour growth inhibition by Asc involved changes in the caloric intake of mice.

In conclusion therefore it was found that large doses of supplementary Asc inhibited the growth of the murine BL6 melanoma. The mechanism of growth inhibition did not appear to involve preferential accumulation of Asc in the tumour cells although the increased tumour Asc levels observed upon Asc supplementation may have played a role in the mechanism of growth inhibition. Tissue Asc levels were found to be reduced in tumour bearing animals while supplementary Asc was found to increase tissue Asc levels. Supplementary Asc, at the level used in this study, did not appear to be toxic to mice.

Chapter 10

EFFECTS AND INTERACTIONS OF ESSENTIAL/POLYUNSATURATED FATTY ACIDS AND ASCORBIC ACID ON TUMOUR GROWTH IN MICE

10.1.Introduction

In this and other studies dietary EFAs/PUFAs have been found to promote tumour growth (57,64-66,69-74,76,79-85,87-92) and supplementary dietary Asc to inhibit tumour growth (299,334-342) in experimental animals. In the present study possible interactions between dietary EFAs/PUFAs and supplementary Asc in influencing tumour growth were investigated. Asc has previously been reported to influence PG synthesis (366-372) and lipid peroxidation (276,334-342), as was found in cultured BL6 and LLCMK cells in this study. Since PGs (105-114) and lipid peroxides (100,144,145) have been reported to influence tumour growth, the role of these mechanisms in possible interactions between EFAs/PUFAs and Asc were also examined in this study by using the anti-oxidant,TOC, and the PG and LT synthesis inhibitors,IM and CA. Furthermore Asc has been reported to regenerate TOC from the tocopheroxyl radical allowing a single TOC molecule to react with several free radicals (270,271,276).The influence of Asc on tumour growth in TOC deficient animals was therefore examined to investigate this possible interaction and any effect it may have on tumour growth.

10.2.INFLUENCE OF ESSENTIAL/POLYUNSATURATED FATTY ACIDS AND SUPPLEMENTARY ASCORBIC ACID ON TUMOUR GROWTH

10.2.1.Materials and Methods

Mice

Relevant information about the C57 female mice used in this study has been provided previously (section 8.2).

Diets

The composition of the semi-purified diets has been provided previously (section 8.2). In this study the PUFA/EFA composition of the diets was again manipulated by using different plant seed oils as a source of PUFAs/EFAs. Four different oils were used to supplement the diets, namely EPO which contains both GLA and LA, SFO and SNO which contain LA, and CO which is EFA free. Two levels of Asc supplementation were also used, namely 50mg Asc/kg diet (control diet) or 500mg Asc/kg diet (Asc supplemented diet). Four diets were prepared at each level of Asc supplementation. These diets contained either 8% EPO, 8% SFO, 8% SNO or 8% CO.

Cells

Relevant information about the BL6 cells has been provided previously (section 4.2)

Reagents

Essential fatty acid methyl esters (FAMEs). Relevant details about the FAMEs have been provided previously (section 8.2).

Radiochemicals. The relevant details about the [1-^{14}C]-LA and [1-^{14}C]-AA have been provided previously (section 4.2).

Methanolic KOH. The composition of the methanolic KOH has been provided previously (section 8.2)

BF_3-methanol reagent. was purchased from Merck, Darmstadt, West Germany.

Prostaglandins E_1 and E_2 were prepared as previously described (section 5.3).

Methods

Effect of dietary polyunsaturated/essential fatty acids and ascorbic acid on tumour growth. Mice were divided into 16 groups and fed the semi-purified diets (two groups per diet) for one week and weighed before implantation of tumours was carried out as before (section 8.2). One group of mice from each diet was used for tumour implantation. Mice were fed the diets for a further four weeks before sacrifice of the tumour bearing and tumour free mice by cardiac puncture (to remove blood samples). Tumours and livers were excised, rinsed in cold PBS and weighed before all samples were rapidly frozen in liquid nitrogen. Samples were stored at -20^{o}C until fatty acid analysis could be performed.

Fatty acid extraction, methylation and separation was carried out as previously deseribed (section 8.2).

Statistieal analysis was performed using analysis of variance followed by Scheffe's multiple range test.

10.2.2. Results

Effect of polyunsaturated/essential fatty acids and ascorbic acid on tumour growth (table 10.1). Tumour growth in mice was significantly influenced by Asc supplementation to diets containing EFAs/PUFAs but not in mice fed the EFA/PUFA deficient CO diet. Supplementation with 500mg

Asc/kg diet resulted in a significant ($p < 0,01$) reduction of 304% in the tumour mass of mice fed the EPO diet, a reduction of of 172% ($p < 0,05$) in the tumour mass of SFO fed mice and a reduction of 164% ($p < 0,05$) in SNO fed mice when compared with the relevant Asc control diets.

Influence of supplementary Asc on body mass gain and liver mass of mice (table 10.2). The increase in the mass of mice during the experimental period as well as the liver mass were not influenced by the Asc or EFA/PUFA composition of any of the diets.

Influence of supplementary Asc on essential fatty acid composition. Supplementary dietary Asc was found not to influence the EFA composition of the liver (table 10.3), blood (table 10.4) or tumour (table 10.5) tissue in mice fed any of the diets when compared with mice fed the relevant Asc control diets.

Table 10.1. Influence of dietary essential/polyunsaturated fatty acid and ascorbic acid manipulation on melanoma growth in mice.

diet	tumour mass (g) Asc control	Asc supplemented
EPO	1,843±0,286	0,605±0,163[a]
SFO	1,343±0,202	0,779±0,139[b]
SNO	1,323±0,223	0,862±0,153[b]
CO	0,760±0,189	0,782±0,184

Results are mean ± SEM, n = 6 except for mice fed the EPO Asc control diets where n = 5 (1 mouse died on each diet). Asc control diets contained 50mg Asc/kg diet while Asc supplemented diets contained 500mg Asc/kg diet. Significantly different to mice fed the Asc control diets : $^{a}p < 0,01$, $^{b}p < 0,05$.

Table 10.2. Influence of dietary essential/polyunsaturated fatty acid and ascorbic acid manipulation on body mass gain and liver mass of mice.

diet	body mass gain (g)	liver mass (g)
EPO Asc control	2,94±0,41	0,932±0,116
EPO Asc supplemented	2,96±0,31	0,939±0,127
SFO Asc control	2,89±0,32	0,927±0,083
SFO Asc supplemented	2,86±0,29	0,920±0,101
SNO Asc control	2,98±0,43	0,935±0,102
SNO Asc supplemented	2,91±0,36	0,924±0,113
CO Asc control	2,86±0,29	0,920±0,112
CO Asc supplemented	2,69±0,41	0,901±0,133

Results are mean ± SEM, n = 6 except for mice fed the EPO Asc control diet where n = 5 (1 mouse died). Asc control diets contained 50mg Asc/kg diet while Asc supplemented diets contained 500mg Asc/kg diet. No significant difference was evident in the liver mass or body mass gain in mice fed any of the diets.

Table 10.3. Influence of dietary manipulation of essential/polyunsaturated fatty acids and ascorbic acid on liver essential fatty acid composition of mice.

diet	essential fatty acid composition*							
	linoleic acid		gamma-linolenic acid		dihomo-gamma-linolenic acid		arachidonic acid	
	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour
EPO Asc control	19,76±1,41	19,95±1,23	0,39±0,12	0,36±0,04	1,67±0,11	1,60±0,05	8,82±0,78	9,24±0,39
EPO Asc supplemented	20,04±0,97	19,88±0,66	0,41±0,09	0,38±0,08	1,71±0,19	1,64±0,12	8,71±1,02	9,24±0,39
SFO Asc control	23,04±1,58	23,63±1,14	0,28±0,08	0,27±0,03	1,31±0,16	1,38±0,14	8,69±0,92	8,62±0,96
SFO Asc supplemented	22,17±1,36	23,56±1,73	0,26±0,04	0,23±0,02	1,24±0,13	1,29±0,16	8,78±0,63	8,75±0,94
CO Asc control	16,66±1,24	16,70±1,78	0,21±0,07	0,23±0,02	1,32±0,17	1,41±0,12	8,67±0,83	8,73±0,92
CO Asc supplemented	17,03±0,77	16,54±0,51	0,26±0,04	0,23±0,03	1,27±0,14	1,26±0,18	8,63±0,77	8,55±0,81

Results are mean ±SEM, n = 6 except for mice fed the EPO control diet where n = 5 (1 mouse died). *Values are percentage composition of total fatty acids. Supplementary Asc did not affect liver EFA levels in mice fed any of the diets. (Note : while dietary manipulation of EFAs/PUFAs significantly influenced liver EFA levels as shown in table 8.3, these statistics are not listed here as this section of the study was concerned primarily with the influence of dietary Asc supplementation on liver EFA levels.)

Table 10.4, Influence of dietary essential/polyunsaturated fatty acid and ascorbic acid manipulation on blood essential fatty acid composition of mice.

diet	essential fatty acid composition*							
	linoleic acid		gamma-linolenic acid		dihomo-gamma-linolenic acid		arachidonic acid	
	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour	with tumour	without tumour
EPO Asc control	20,01±0,63	20,09±1,04	0,99±0,11	0,97±0,14	1,42±0,23	1,43±0,16	11,44±0,68	11,48±0,73
EPO Asc supplemented	20,21±0,87	19,71±0,94	0,94±0,16	1,00±0,13	1,53±0,28	1,46±0,25	11,37±1,01	11,42±0,93
SFO Asc control	21,32±1,58	20,90±1,22	0,15±0,08	0,16±0,04	1,25±0,17	1,20±0,18	10,74±0,84	11,02±0,62
SFO Asc supplemented	20,98±1,36	21,70±1,41	0,14±0,06	0,16±0,02	1,22±0,21	1,25±0,21	10,58±0,96	10,98±1,10
CO Asc control	10,67±0,72	10,86±0,86	-	-	0,94±0,16	1,05±0,06	10,72±0,63	10,73±0,97
CO Asc supplemented	11,32±0,77	10,84±0,77	-	-	0,93±0,19	0,96±0,15	10,85±0,78	10,84±0,77

Results are mean ±SEM, n = 6 except for mice fed the EPO Asc control diet where n = 5 (1 mouse died). *Values are percentage composition of total essential fatty acids. Ascorbic acid supplementation did not significantly influence blood EFA content in mice fed any of the diets. (Note : while dietary manipulation of EFAs/PUFAs significantly influenced blood EFA levels as shown in table 8.4, these statistics are not listed here as this section of the study was concerned primarily with the influence of dietary Asc supplementation on blood EFA levels.)

Table 10.5. Influence of dietary essential/polyunsaturated fatty acid and ascorbic acid manipulation on the essential fatty acid composition of melanomas in mice.

diet	essential fatty acid composition* linoleic acid	gamma-linolenic acid	dihomo-gamma-linolenic acid	arachidonic acid
EPO Asc control	18,52±1,08	1,16±0,13	2,94±0,11	7,56±0,52
EPO Asc supplemented	18,84±0,28	1,24±0,25	2,76±0,27	7,71±0,46
SFO Asc control	25,44±1,72	0,16±0,02	1,37±0,15	4,22±0,32
SFO Asc supplemented	25,68±1,88	0,21±0,02	1,47±0,09	4,38±0,57
CO Asc control	3,76±0,57	-	0,85±0,12	2,07±0,28
CO Asc supplemented	3,83±0,20	-	0,77±0,03	2,37±0,19

Results are mean ±SEM, n = 6 except for mice fed the EPO Asc control diet where n = 5 (1 mouse died). *Values are percentage composition of total fatty acids. Ascorbic acid supplementation did not significantly influence melanoma EFA content in mice fed any of the diets. (Note : while dietary manipulation of EFAs/PUFAs significantly influenced tumour EFA levels as shown in table 8.5, these statistics are not listed here as this section of the study was primarily concerned with the influence of dietary Asc supplementation on tumour EFA levels.)

10.3.MECHANISM OF THE INTERACTION BETWEEN ESSENTIAL/POLYUNSATURATED FATTY ACIDS AND ASCORBIC ACID IN INHIBITION OF TUMOUR GROWTH

10.3.1.Materials and Methods

Materials

Mice and cells were as previously described.

Diets. The diets containing 8% EPO, 8% SFO, 8% SNO or 8% CO and 50 or 500mg Asc/kg were prepared as described in section 8.2. The possible involvement of enhanced PG or LT synthesis as a mechanism whereby Asc interacted with EFAs in inhibiting tumour growth was examined by adding the PG synthesis inhibitor IM (0,004%), or the LT synthesis inhibitor CA (0,004%) to each of the diets. The possible involvement of lipid peroxidation in inhibition of tumour growth was examined by preparing TOC deficient (diet containing no TOC), TOC control (diet containing 1×10^6 IU TOC/kg diet) and TOC supplemented (diet containing 1×10^7 IU TOC/kg diet) diets.

Methods

Mechanism of polyunsaturated/essential fatty acid and ascorbic acid mediated tumour growth inhibition. Mice were fed the diets containing IM, CA or the different levels of TOC for one week before tumour implantation was carried out as before. Mice were fed the diets for a further four weeks before sacrifice. Tumours were excised, rinsed in cold PBS and weighed.

Statistical analysis was carried out using analysis of variance followed by Scheffe's multiple range test.

10.3.2.Results

Influence of indomethacin and caffeic acid on tumour growth. (table 10.6). Dietary IM and CA were found not to influence tumour growth in mice fed any of the normal or Asc supplemented diets.

Influence of α-tocopherol on tumour growth. (table 10.7). The dietary level of TOC was found to significantly influence tumour growth in all diets except the EFA deficient CO diets as shown in chapter 8 (levels of significance are shown in table 8.8 and not in table 10.7 since the present study is concerned primarily with the effect of supplementary Asc on tumour growth in mice fed diets containing different EFAs/PUFAs or SF and different levels of TOC).

Supplementary Asc did not appear to influence the significant tumour growth inhibitory effects of the TOC deficient EFA/PUFA containing diets (EPO and SFO diets) or the slight but non-significant stimulation of tumour growth observed in mice fed the EFA/PUFA containing TOC supplemented diets. Thus while supplementary Asc was still found to inhibit tumour growth in mice fed PUFA\EFA containing diets, the reduction in tumour size by Asc in mice fed TOC deficient or TOC supplemented diets was the same as that in mice fed diets containing a normal level of TOC regardless of the EFA/PUFA content of the diets.

Table 10.6. Influence of dietary indomethacin and caffeic acid supplementation on melanoma growth in mice.

diet	tumour mass (g) no IM or CA	0,004% IM	0,004% CA
EPO Asc control	1,975±0,301	2,004±0,271	1,963±0,217
EPO Asc supplemented	0,732±0,213	0,758±0,196	0,749±0,175
SFO Asc control	1,476±0,217	1,504±0,188	1,482±0,139
SFO Asc supplemented	0,897±0,176	0,937±0,215	0,879±0,214
CO Asc control	0,827±0,173	0,826±0,141	0,834±0,197
CO Asc supplemented	0,815±0,159	0,819±0,173	0,803±0,163

Results are mean ±SEM, n = 6 except for mice fed the CA supplemented CO Asc control diet where n = 5 (1 mouse died). Asc control diets contained 50mg Asc /kg diet while Asc supplemented diets contained 500mg Asc/kg diet. Dietary IM and CA supplementation did not significantly influence melanoma growth in mice fed any of the diets.

Table 10.7. Influence of dietary α-tocopherol manipulation on melanoma growth in mice.

diet	tumour mass (g) TOC deficient	TOC control	TOC excess
EPO Asc control	1,479±0,313	1,946±0,179	2,160±0,196
EPO Asc supplemented	0,373±0,215	0,629±0,231	0,697±0,214
SFO Asc control	1,076±0,274	1,465±0,158	1,507±0,234
SFO Asc supplemented	0,592±0,163	0,863±0,262	0,917±0,174
CO Asc control	0,785±0,192	0,810±0,185	0,831±0,173
CO Asc supplemented	0,803±0,178	0,812±0,136	0,824±0,211

Results are mean ± SEM, n = 6. Asc control diets contained 50mg Asc/kg diet while Asc supplemented diets contained 500mg Asc/kg diet. TOC deficient diets contained no supplementary TOC while TOC control diets contained 1 x 10^6 IU TOC/kg diet and the TOC excess diets contained 1 x 10^7 IU TOC/kg diet. Asc supplementation did not significantly influence the growth inhibitory effects of TOC deficiency. (Note : while manipulation of dietary TOC significantly influenced tumour growth as shown in table 8.8, these statistics are not listed here as this section of the study was primarily concerned with the influence of Asc supplementation on the effects of dietary TOC manipulation on tumour growth.)

10.4.Discussion

Whereas Asc has been found to increase PG synthesis from EFAs (366-372)and to both inhibit and enhance lipid peroxidation (276,334-342) in a number of in vitro and a few in vivo studies, no reports could be found in the relevant literature regarding interactions of these nutrients in tumour cells. Some beneficial effects, including prolonged survival times and some clinical improvement, in the treatment of human patients suffering from terminal liver cancer with Asc and certain EFAs have however been reported by van der Merwe *et al* (156,375,376).

In this study dietary Asc supplementation was found to significantly reduce tumour growth in mice fed the EFA/PUFA containing EPO, SNO and SFO diets but not in the mice fed the EFA deficient CO diet. These results suggest that the growth inhibitory effects of Asc might have involved an interaction with dietary EFAs/PUFAs. The greater tumour growth inhibition observed in EPO fed mice upon Asc supplementation, when compared with SFO fed mice, suggests that Asc may have been interacting with GLA or one of its metabolites in inhibiting tumour growth. As already mentioned certain tumour cells have been reported to lack delta-6-desaturase activity (392-396) and would be unable to synthesise GLA from LA. It is possible therefore that the melanoma cells were unable to synthesise GLA from the LA provided by the SFO and SNO diets, while the EPO diet provided GLA with which Asc may have been interacting to inhibit tumour growth.

Since supplementary dietary Asc was found not to influence tumour EFA composition a more specific interaction between Asc and EFAs appears to have been involved in the tumour growth inhibitory interaction between Asc and the EFAs/PUFAs. As Asc has previously been reported to inhibit lipid peroxidation in in vivo (276,337-339) and in vitro (334-336) studies and to promote lipid peroxidation, particularly in the presence of transition metals, in in vitro studies (276,340-342), there was a possibility that the apparent interaction between Asc and the EFAs in inhibiting tumour growth may have involved lipid peroxidation. Furthermore Asc was found in this study (chapter 7) to enhance lipid peroxidation in EFA treated BL6 and LLCMK cells grown in culture. While manipulation of dietary TOC was found to influence tumour growth in mice fed PUFA/EFA containing diets as previously discussed (chapter 8), increasing the level of dietary Asc from 50mg/kg to 500mg/kg did not influence the tumour growth inhibitory effects of the TOC deficient diets or the slight increase (non-significant) in tumour growth in mice fed the TOC excess diets. It would therefore appear unlikely that lipid peroxidation played a role in the interaction between Asc and EFAs in inhibiting tumour growth. It is interesting to note that several studies utilising in vivo (276) and in vitro (270,271,276) systems have reported an interaction between Asc and TOC. Asc has been found to regenerate TOC from the tocopheroxyl radical produced from the reaction of TOC and free radicals (270,271,276). This interaction allows a single TOC molecule to scavenge a number of free radicals. Although this interaction was not examined in detail in this study, supplementary Asc did not appear to regenerate

TOC in the melanoma used in this study since regeneration of TOC by supplementary Asc would presumeably have resulted in increased tumour growth in mice fed the TOC deficient diet.

A further mechanism whereby Asc may have interacted with EFAs in inhibiting tumour growth could have involved enhanced PG synthesis. Asc has previously been reported to enhance PG synthesis in studies using cultured cells and isolated tissues (366-372), as was found in this study using cultured BL6 and LLCMK cells (chapter 7). The possible involvement of enhanced PG synthesis in the tumour growth inhibitory effects of Asc supplementation to PUFA/EFA containing diets was examined by using the PG synthesis inhibitor, IM. The LT synthesis inhibitor, CA, was used to examine the possible involvement of LTs in the tumour growth inhibitory effects. Neither IM nor CA were found to influence tumour growth in mice fed any of the diets suggesting that the mechanism of the interaction between Asc and PUFAs/EFAs in inhibiting tumour growth did not involve enhanced synthesis of PGs or LTs from EFA precursors.

It would therefore appear that the mechanism of the interaction between Asc and the EFAs/PUFAs did not involve enhanced lipid peroxidation or eicosanoid synthesis. Further studies are required to carry out a more detailed investigation of the mechanism(s) involved in the interaction between the two nutrients. Furthermore studies involving a larger variety of tumours and different levels of Asc and the dietary PUFAs/EFAs need to be performed before any definitive statements can be made regarding the possible therapeutic use of supplementatation of Asc, to EFA/PUFA containing diets, in the treatment of cancer.

While tumour growth was significantly influenced by dietary manipulation of Asc in mice fed EFA/PUFA containing diets, the growth of animals was not affected during the experimental period by dietary Asc manipulation. Supplementary Asc, while inhibiting tumour growth in PUFA/EFA containing diets, did not therefore appear to have been visibly toxic to the animals. Supplementary Asc thus appears to be suitable for use in the treatment of cancer as an important prerequisite for any substance to be used in cancer therapy is that while it must be toxic to tumours, side effects involving toxicity to the normal tissues of the host should be as small as possible. Several clinical trials have in fact reported some beneficial effects of large doses of Asc in the treatment of terminal patients suffering from primary liver cancer without any apparent side effects except a few cases of diarrhea (241,287-293). Furthermore as already mentioned Asc has been used together with GLA, with some success, in the treatment of terminal liver cancer patients (156,375,376). While the greatest growth inhibition in this study was observed in mice fed supplementary Asc and the EPO diet, when compared with mice fed supplementary Asc and the SNO or SFO containing diets, care should be exercised in using this diet in cancer therapy, as EPO in a diet containing a normal level of Asc was found to be a more potent promoter of tumour growth than the other vegetable oils used in this study.

In conclusion therefore, it was found that while diets containing PUFAs/EFAs and a normal level of Asc promoted BL6 melanoma growth in mice, supplementary dietary Asc appeared to interact with PUFAs/EFAs in inhibiting growth of this tumour. The growth inhibitory effects of Asc appeared to have involved an interaction with EFAs and more particularly with GLA or one of its metabolites. The mechanisms of tumour growth inhibition by Asc did not appear to have involved alterations in the level of tumour EFA levels, enhanced lipid peroxidation or eicosanoid synthesis.

Chapter 11

DELTA-6-DESATURASE ACTIVITY IN CULTURED CELLS AND MICROSOMES

11.1. Introduction

The metabolism of EFAs in mammalian cells proceeds via the pathway illustrated in figure 11.1.

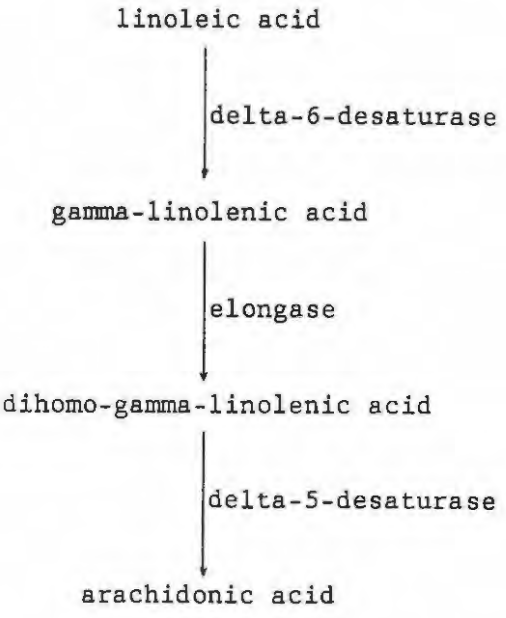

Figure 11.1. Metabolism of EFAs in mammalian cells (1).

LA is first converted by acyl-CoA-synthetase to linoleoyl-CoA, which is then desaturated by delta-6-desaturase to yield gamma-linoleoyl-CoA (GLA). Gamma-linoleoyl-CoA then acquires a 2C unit under the influence of elongase to yield DGLA, which can then be desaturated to AA by delta-5-desaturase (385-388). The rate limiting step in this pathway is thought to be the desaturation of LA to GLA by delta-6-desaturase (385,388).

Delta-6-desaturase is part of a multicomponent enzyme system called linoleoyl-CoA desaturase located in the endoplasmic reticulum membrane and consisting of cytochrome b_5 reductase, cytochrome b_5 and delta-6-desaturase (385-388). The other components of this system are responsible for transferring electrons from NADH to delta-6-desaturase, the terminal enzyme in the system, which then desaturates LA to GLA. The structure of this EFA desaturating system, which is located in the membrane lipid bilayer, is illustrated in figure 11.2. Although the principle electron donor for this pathway is NADH, both NADPH and Asc can act as electron donors, albeit less efficiently than NADH (389,390). A number of studies have shown that Asc can reduce cytochrome b_5 in a reaction

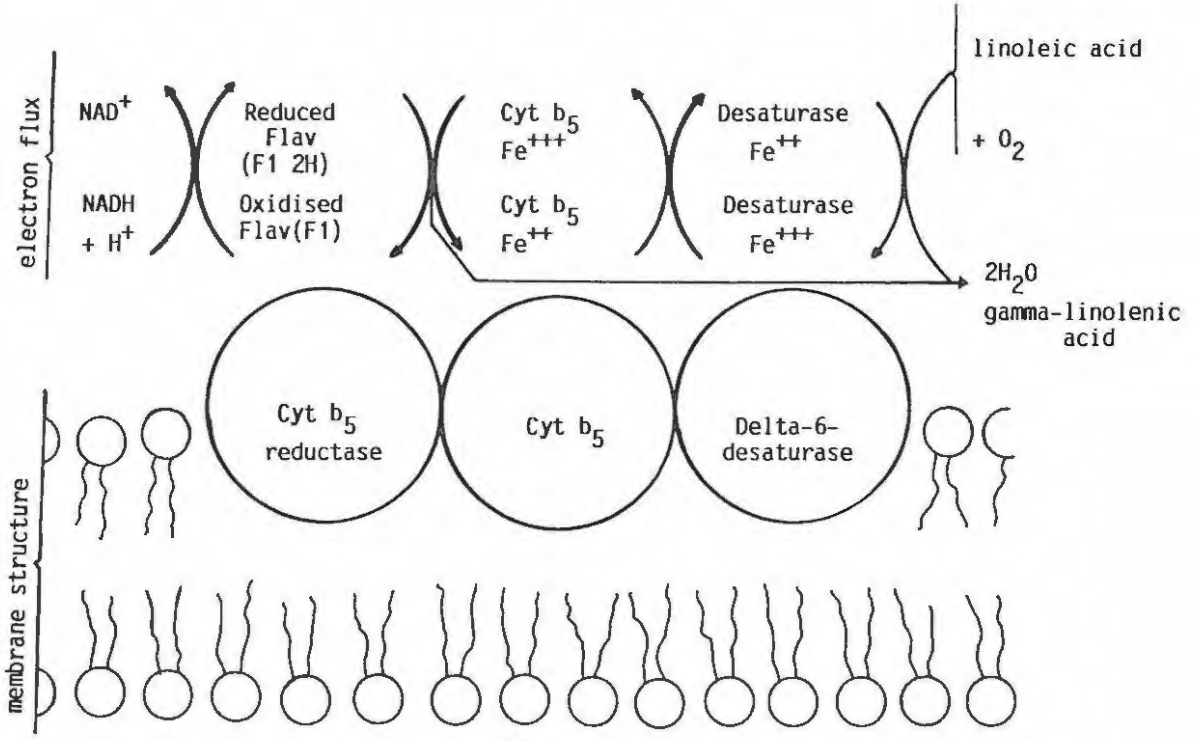

Figure 11.2. Structure of the delta-6-desaturase system in the membrane lipid bilayer (adapted from 388).

catalysed by the enzyme ascorbic acid : ferricytochrome b_5 oxidoreductase (390,391). Depletion of cytochrome b_5 levels has previously been reported in scorbutic guinea pigs (391) indicating that Asc may play an important role in the metabolism of EFAs by this pathway.

A number of malignant cells have been reported to lack, or to have reduced activity of delta-6-desaturase (392-396). Loss of delta-6-desaturase is suggested to be a characteristic feature of malignant transformation, the malignant cells thus being unable to synthesise GLA, DGLA or AA from the precursor LA (175,394,396). The decrease in the content of PUFAs/EFAs and in particular AA, observed in these malignant cells is thought to be due to this lack or reduction in activity of the enzyme (393). It has been proposed by Horrobin (175) that provision of GLA to tumour cells may be an important step in the normalisation of tumour cell growth. Several earlier studies supported this hypothesis since GLA was found to be a more effective inhibitor of cultured tumour cell growth than LA (156,162). More recently however several studies have reported that several tumour cell lines do possess delta-6-desaturase activity (395,397). It is therefore apparent that loss of delta-6-desaturase activity appears to be confined to certain types of malignant cells.

The most commonly employed systems for the study of delta-6-desaturase are cultured cells and microsomes. Microsomes are formed by homogenisation of cells resulting in the disruption of the endoplasmic reticulum into vesicles, which are called microsomes, in which delta-6-desaturase activity is retained (398). Activity is usually measured by conversion of radiolabelled substrate (LA) to products (GLA and metabolites of GLA) (392-395).

In view of the nearly equal effectiveness of LA and GLA in inhibiting BL6 cell growth (section 5.2) and the more significant promotion of tumour growth observed in EPO fed mice than in SFO fed mice (section 8.2) in this study, it was decided to examine the activity of delta-6-desaturase in the malignant BL6 cells. Furthermore, since analysis of tumour EFA levels suggested that tumour cells grown in mice might lack, or have reduced, activity of the enzyme, the delta-6-desaturase activity of tumour microsomes was also examined.The benign LLCMK cells and liver microsomes were used as normal controls. Since Asc has been reported to act as an electron donor to delta-6-desaturase (389,390), its effect on the activity of the enzyme was also examined.

11.2.DELTA-6-DESATURASE ACTIVITY IN CULTURED CELLS

11.2.1.Materials and Methods

Reagents

(1-^{14}C)-linoleic acid. The relevant information about this reagent has been provided previously (section 5.2).

Ascorbic acid. Relevant information regarding Asc preparation has been provided previously (section 6.2).

Fatty acid methyl ester mixture. Relevant information about the composition of this mixture has been provided previously (section 8.2).

Thin layer chromatography (tlc) plates. Precoated Merck F_{254} silica gel plates (0,25mm film thickness, 20 x 20cm) tlc plates were used.

Silver nitrate solution. Preparation of and procedures used in the preparation of the $AgNO_3$ solution have been described previously (section 6.2).

2,7-dichlorofluoroscein (0,4%). was prepared in 95% ethanol.

Cells

Relevant information regarding the BL6 and LLCMK cells has been provided previously (section 4.2).

Methods

Delta-6-desaturase assay. Experimental cell cultures were set up as before (0,3 x 10^6 cells per 25cm^2 culture flask) and allowed to grow for 3 days (nearing confluency). Fresh experimental medium together with supplementary Asc (0 (control), 25, 50, 100 or 200µg/ml) were added to cultures together with 0,25µCi ^{14}C-LA. Cells were incubated at 37^{o}C for 24 hours before harvesting as done before. Cells were resuspended in 2ml PBS and counted by haemocytometry before the isolation of EFAs. Delta-6-desaturase activity was measured as the conversion of radiolabelled LA to products, namely GLA, DGLA and AA.

Isolation and methylation of essential fatty acids. EFAs were isolated and methylated as described in section 8.2. Carrier FAMEs were added after methylation and the EFAs separated by argentation tlc..

Preparation of argentation thin layer chromatography plates. The tlc plates were immersed in the $AgNO_3$ solution for 10 seconds, air dried and activated by heating in an oven at 70°C for 1 hour. Plates were allowed to cool at room temperature and were then used for separation of FAMEs. As mentioned previously all procedures involving $AgNO_3$ were performed in a darkroom using a photographic safety light. Due to the photosensitivity of $AgNO_3$, plates were prepared immediately before use.

Separation and visualisation of samples. The FAME extracts were spotted at least 1,5cm apart onto the tlc plates using glass capillary tubes and then air dried. Separation of FAMEs was achieved using a slight modification of the solvent system of Bomalski *et al* (399). The solvent system used consisted of chloroform/methanol/acetic acid/water (90:7,5:7,5:0,8). After developement plates were air dried and sprayed with 2,7-dichlorofluoroscein to allow visualisation of the FAMEs under UV light. Zones corresponding to individual FAMEs were identified by co-chromatography with FAME standards. (The Rf values of FAME standards achieved using this solvent system are depicted in appendix 7). These zones were carefully scraped into glass scintillation vials and thoroughly mixed with 10ml of Beckman EP-readysolv scintillation fluid. Samples were left undisturbed for 24 hours before counting in a Beckman LS 3801 scintillation counter.

Statistical analyses were performed using analysis of variance followed by Scheffe's multiple range test.

11.2.2.Results

Delta-6-desaturase activity. (table 11.1) Both cell lines, namely BL6 and LLCMK cells, were found to contain delta-6-desaturase activity. Activity of the malignant BL6 cells was found to be significantly ($p < 0,001$) higher than that of the benign LLCMK cells.

Effect of ascorbic acid on delta-6-desaturase activity. (figure 11.3) Supplementary Asc was found to significantly ($p < 0,05$ at all Asc concentrations) increase delta-6-desaturase activity in BL6 cells while a slight but non-significant stimulation of activity was observed in the LLCMK cells. Results are expressed as cpm GLA + DGLA + AA formed/10^6 cells as Asc was found in this study to inhibit BL6 cell growth (chapter 6).

Table 11.1. Delta-6-desaturase activity of cultured cells.	
cells	delta-6-desaturase activity cpm GLA + DGLA + AA/10^6 cells
BL6	28693,4 ± 5136,8
LLCMK	18610,7 ± 4367,2

Delta-6-desaturase activity in BL6 cells was significantly ($p < 0,01$) higher than in LLCMK cells.

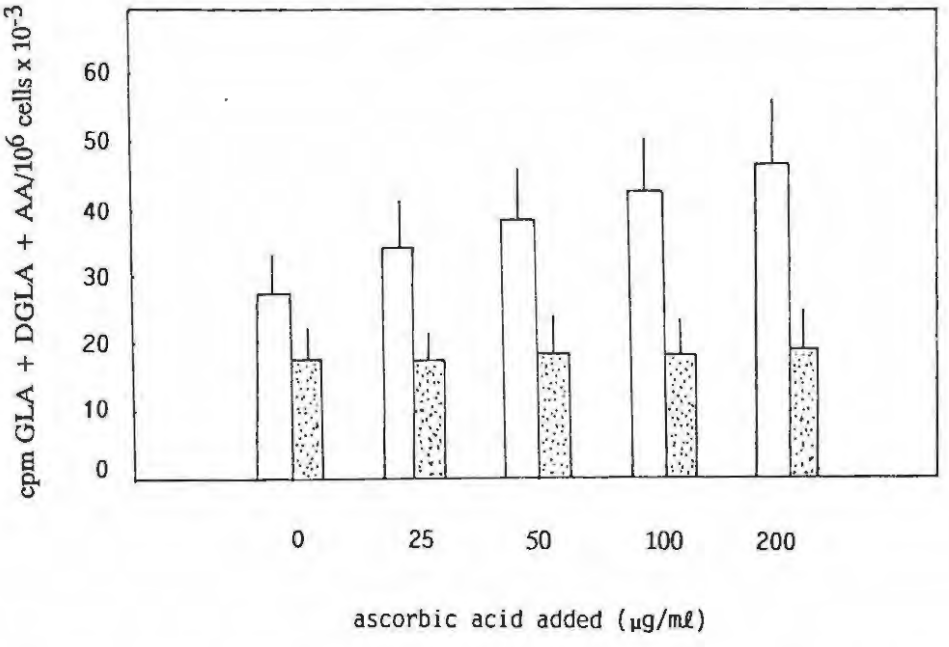

Figure 11.3. Effect of supplementary Asc on delta-6-desaturase activity of cultured cells. ⁘ LLCMK cells; □ BL6 cells. Results are mean and SEM of 5 cultures. Delta-6-desaturase activity in BL6 cells was significantly ($p < 0,05$) increased at all levels of Asc supplementation while activity of the enzyme in LLCMK cells was not significantly affected by Asc supplementation.

11.3.DELTA-6-DESATURASE ACTIVITY IN MICROSOME PREPARATIONS

11.3.1.Materials and Methods

Reagents

A number of reagents used in this study were prepared as described in Section 11.2. These include [1-^{14}C]-LA, $AgNO_3$ (10%), the FAME standard mixture, argentation tlc plates and 2,7-dichlorofluoroscein.

Homogenisation buffer consisted of sucrose (250mM), $MgCl_2$(5mM), KCl (150mM), reduced glutathione (1,5mM) and EDTA (1,0mM) prepared in potassium phosphate buffer (50mM, pH 7,4).

Assay buffer consisted of NADH (1,2mM), coenzyme A (0,3mM), ATP (5mM), reduced glutathione (1,5mM) and $MgCl_2$ (5mM) prepared in potassium phosphate buffer (50mM, pH 7,4).

Protein standard solutions (0-200µg/ml) were prepared by dissolving bovine serum albumin (Sigma) in a 1 : 100 dilution of the homogenisation buffer.

Protein solution A was prepared by dissolving 2,0g of sodium potassium tartrate and 100g of Na_2CO_3 in 500ml of 1,0M NaOH and diluting to a final volume of 1000ml with water.

Protein solution B was prepared by dissolving 2,0g of sodium potassium tartrate and 1,0g of $CuSO_4.5H_2O$ in 90ml water and diluting to 100ml with 1,0M NaOH.

Protein solution C was prepared by making a 1 : 10 dilution of commercial Folin and Ciocalteau's phenol reagent (SAARCHEM) (equivalent to 0,2N).

Cells

Relevant information concerning the BL6 cells is provided in section 4.2.

Mice

Relevant data concerning the mice used is provided in section 8.2.

Diets

Preparation of experimental diets containing EPO, SFO or CO and 50 or 500mg Asc/kg was carried out as described in section 8.2.

Methods

Tumour implantation. Mice were fed the experimental diets for one week before tumour implantation was carried out as described previously (section 8.2). Mice were fed these diets for a further four weeks before being sacrificed. Tumour and liver samples were excised, rinsed in cold PBS, rapidly frozen in liquid nitrogen and then stored at -20^{o}C until microsome preparation was carried out.

Microsome preparation. Microsomes were prepared from tissues by the method of Skeef (377). Tumour and liver samples from mice fed on the individual diets were pooled and weighed before homogenisation using a Dounce homogeniser (30 strokes with each plunger) in three volumes of homogenisation buffer. The homogenate was centrifuged (Beckman L8-80M ultracentrifuge) for 30 minutes at 12000g and 4^{o}C to pellet the mitochondrial fraction and cell debris. The supernatant was then centrifuged at 120000g and 4^{o}C for 90 minutes to pellet the microsomal fraction which was then resuspended in a volume of homogenisation buffer equivalent to the mass of starting tissue. The protein concentration of the microsome fractions was then determined.

Protein determination. The protein concentration of the preparations was determined by the Hartree (400) method for protein determination using a 1 : 100 dilution of the microsome preparations. A protein standard curve (appendix 8) was prepared from the protein standards. A 1 : 100 dilution of the homogenisation buffer was used for the protein assays since glutathione contained in the buffer was found to interfere with the assay at higher concentrations. The content of homogenisation buffer was thus kept constant in all samples and standards. The microsomal preparations were then diluted to a final protein concentration of 10mg/ml with homogenisation buffer and stored at -20^{o}C until use.

Delta-6-desaturase assay. A slight modification of the method of Skeef (377) was used to measure delta-6-desaturase activity, which was determined as the rate of conversion of substrate (^{14}C-LA) to products (^{14}C-GLA + ^{14}C-DGLA + ^{14}C-AA). To 2ml of assay buffer, through which O_2 had been bubbled for 10 seconds, was added 0,5µCi of ^{14}C-LA and an appropriate volume of microsome suspension containing 4mg protein. The samples were rapidly and thoroughly mixed and were then incubated in a water bath at 37^{o}C for 30 minutes. The reaction was stopped by adding 3ml of 10% methanolic KOH. Fatty acids were then extracted, methylated and separated by tlc as described in section 11.2. Bands corresponding to individual FAMEs were carefully scraped into scintillation vials and the radioactivity determined as before (section 11.2).

Statistical analyses were determined using analysis of variance followed by Scheffe's multiple range test.

11.3.2.Results

Delta-6-desaturase activity in tumour and liver microsomes (figure 11.4). Delta-6-desaturase activity in microsomes prepared from BL6 melanomas was negligible when compared with that measured in liver microsomes. Dietary manipulation of PUFAs/EFAs and Asc did not influence activity of the enzyme in the tumour or liver microsomes. While activity of the enzyme in liver micrososmes was highest in mice fed the EFA deficient CO diet followed by SFO fed mice and lastly by mice fed the GLA containing EPO diet, these differences were not significant.

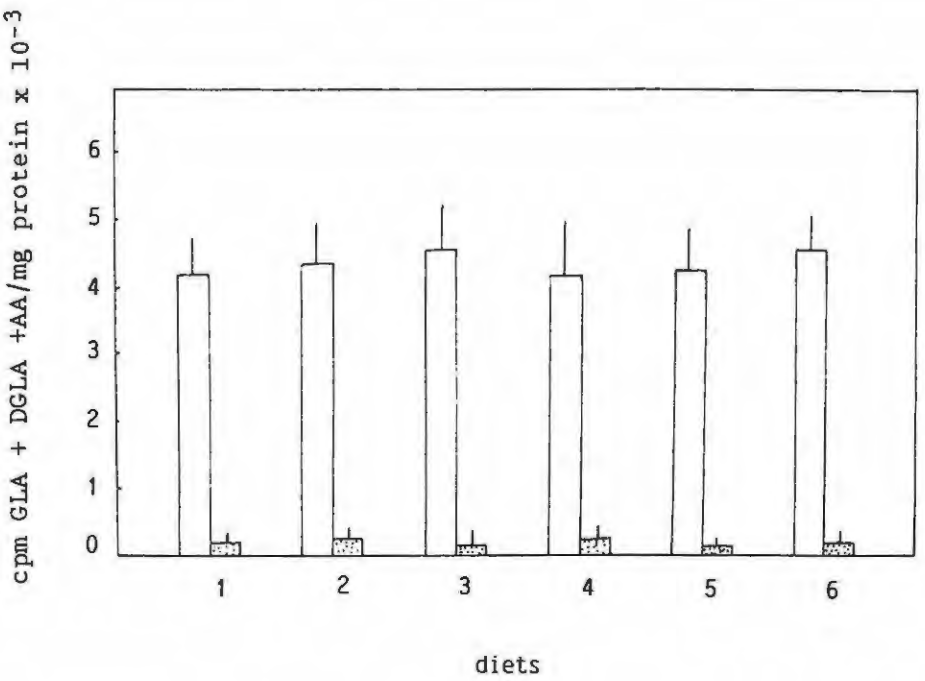

Figure 11.4. Effect of dietary manipulation of PUFAs/EFAs and Asc on delta-6-desaturase activity of microsome preparations. ☐ liver microsomes; ⁙ tumour microsomes. Results are mean and SEM of 5 assays. Delta-6-desaturase activity was not significantly affected by dietary EFA/PUFA or Asc manipulation. 1 - EPO Asc control diet; 2 - SFO Asc control diet; 3 - CO Asc control diet; 4 - EPO Asc supplemented diet; 5 - SFO Asc supplemented diet; 6 - CO Asc supplemented diet.

11.4. Discussion

Previous reports have suggested that loss of delta-6-desaturase activity might be a characteristic of malignant transformation (175,392-396). The results of this study however indicate that the cultured BL6 melanoma cells contain significant activity of the enzyme. The activity of the enzyme was in fact higher than that observed in the non-malignant LLCMK cells, even though the uptake of the substrate, ^{14}C-LA, was found in this study (chapter 5), to be higher in the LLCMK cells than the BL6 cells. Since delta-6-desaturase activity has previously been reported in murine mammary and murine leukaemic cells (395) and in cultured HeLa and murine neuroblastoma cells (397), it would appear that possible loss of delta-6-desaturase activity upon malignant transformation may depend on cell type.

Asc was found to stimulate delta-6-desaturase activity in BL6 cells in a dose dependant manner while not influencing activity in the LLCMK cells. Since Asc was found not to influence ^{14}C-LA uptake by either cell line in this study (chapter 7), the observed increase in desaturation of LA by BL6 cells is likely to involve a direct effect of Asc on delta-6-desaturase or one of the components of linoleoyl-CoA-desaturase. It is also evident therefore that the influence of Asc on delta-6-desaturase activity is dependant on cell type. Further studies are required to determine whether this effect is confined to malignant cells. Since previous studies have reported that Asc can act as an electron donor to cytochrome b_5 (389,390), a component of linoleoyl-CoA-desaturase which is involved in the transfer of electrons from NADH to delta-6-desaturase, it is possible that Asc increased the flow of electrons to delta-6-desaturase thus stimulating activity of the enzyme. Additionally other studies have reported the existence of the enzyme ascorbate : ferricytochrome b_5 reductase, which is responsible for the transfer of electrons from Asc to cytochrome b_5, in a number of microsomal fractions from different organs (390). Additionally previous studies have demonstrated that Asc can act as an electron donor to the fatty acid desaturating system, although it is not as effective as NADH (390). A schematic representation of this proposed pathway of electron flux from Asc to delta-6-desaturase is represented in figure 11.5. It has however been reported that the electron flux provided to fatty acid desaturases from NADH is many times greater than the electron requirement of the desaturase enzymes (402). It is therefore possible that the stimulation of delta-6-desaturase activity by Asc may involve a mechanism other than that discussed above.

In view of the significant level of delta-6-desaturase activity observed in cultured BL6 cells, the finding that microsomes from tumours grown in mice lacked activity of the enzyme was particularly surprising. These results have however been confirmed in a recent study by a colleague, in which the effect of zinc on delta-6-desaturase activity of cultured BL6 cells and BL6 microsomes was examined (377). It is unlikely that enzyme activity was lost during microsome preparation as liver microsomes, which were

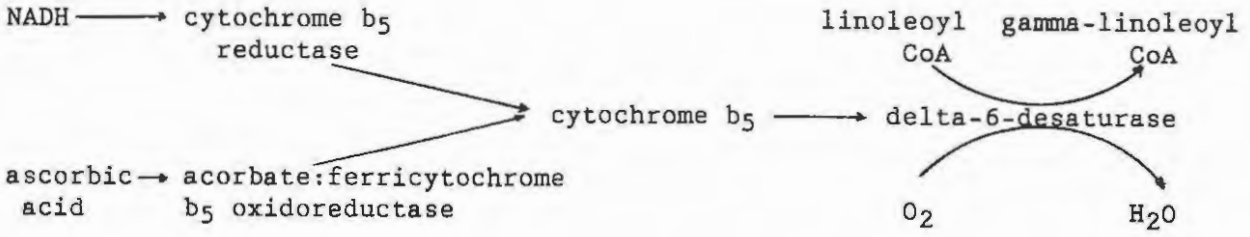

Figure 11.5. Schematic representation of the proposed electron flow from ascorbic acid to delta-6-desaturase (adapted from 390).

subjected to the same preparation technique, contained significant activity of the enzyme. It is however possible that the enzyme might be less stable in tumour cells than liver cells and would therefore be more susceptible to denaturation by the isolation procedures used. While it is difficult with the available data to provide an alternative explanation for the apparent loss of delta-6-desaturase activity in tumour cells grown in vivo, tumour cell metabolism was presumeably altered by the host enviroment, leading to metabolic changes which may have involved changes in the regulation of expression or activity of certain enzymes including delta-6-desaturase. Further studies are required to examine this phenomenon, particularly since loss of delta-6-desaturase activity is suggested to be an important event in the malignant process (175).

Investigation of liver microsome delta-6-desaturase activity reveals that dietary manipulation of PUFAs/EFAs did not significantly influence activity of the enzyme in the microsomal fractions. Previous studies using rat liver microsomes have reported that EFA deficient diets stimulate delta-6-desaturase activity (402,403), presumeably as a compensatory mechanism to increase the unsaturation of membrane PUFAs/EFAs to maintain membrane fluidity (403). In the present study the EFA deficient CO diet was found to slightly but non-significantly stimulate delta-6-desaturase activity in liver microsomes. However since this diet was found not to significantly reduce the EFA content of the mouse livers, presumeably due to the fact that the mice were not severely EFA deficient at termination of the experiments (chapter 8), it is possible that stimulation of delta-6-desaturase activity might require a longer period of exposure to an EFA deficient diet than that employed in this study. Since the EPO diet was found in this study to increase liver GLA levels (chapter 8), a decrease in activity of delta-6-desaturase may have been expected in mice fed on this diet. However no significant difference was evident in the activity of the enzyme in liver microsomal fractions from SFO or EPO fed mice.

While supplementary Asc was found in this study to increase liver Asc levels (chapter 9), dietary Asc manipulation was found not to influence liver delta-6-desaturase activity. As delta-6-desaturase activity was found to be stimulated by Asc in the malignant BL6 cells but not in the non-malignant LLCMK cells or in liver microsomes, it is tempting to speculate that stimulation of activity of the enzyme may be confined to malignant cells. Obviously further studies utilising a wide range of malignant and

normal, or benign, cells and cellular fractions are required to more fully examine this possibility. Unfortunately this observation could not be extended to the tumour microsomes as they were found to contain negligible delta-6-desaturase activity in this study.

The presence of delta-6-desaturase activity in cultured BL6 cells and the lack of activity observed in tumour microsomes could clarify a number of observations made in previous chapters of this dissertation. These include the nearly equipotent effects of LA and GLA in inhibiting growth of cultured cells, the increased tumour growth observed in EPO fed mice when compared with SFO fed mice and could provide an explanation for the decreased levels of GLA, DGLA and AA observed in tumours from mice fed diets lacking GLA. The relevance of the delta-6-desaturase activity observed in cells and in the microsomal fractions to these effects will be discussed in more detail in the final discussion (chapter 12).

In conclusion therefore it is apparent that while BL6 cells grown in culture exhibited delta-6-desaturase activity, microsomal fractions from BL6 cells grown in mice did not. Asc supplementation stimulated delta-6-desaturase activity in cultured BL6 cells but not in LLCMK cells. Dietary manipulation of PUFAs/EFAs and Asc did not appear to influence liver microsome delta-6-desaturase activity.

Chapter 12

FINAL DISCUSSION AND CONCLUSIONS

The term cancer is a general name (often substituted by the terms tumour, malignant, transformed and neoplasm) which refers to cells which have acquired characteristics not found in their normal cells of origin. A number of characteristics are common to most types of cancer (with some exceptions) including an enhanced rate of cell proliferation, loss of contact inhibition of cell growth, loss of morphological differentiation and the acquisition of the ability to invade the normal tissues of the host. Since the transformation of normal cells to the malignant state can be induced by various cancer causing agents or carcinogens, which include chemical carcinogens, radioactivity, UV irradiation and the oncogenic viruses, and since these malignant transformations can be induced in a wide variety of host tissues, it is obvious that the term cancer, which encompasses all these transformations, refers to a broad spectrum of diseases. As a result of the wide variety of cancers it is highly unlikely that a single cure will be found for these diseases. It has however recently been appreciated that diet and manipulation of certain dietary components may play an important role in the prevention and treatment of cancer or at least of certain types of cancer.

Although it has been known for decades that tumour incidence in experimental animals can be influenced by nutritional manipulations, it is only more recently that that the possiblity that diet may play an important role in the cause or in the prevention of cancer in humans has received major attention (309). This possibilty has stimulated an enormous amount of research into the influence of dietary factors on tumour growth, several approaches having been used in these studies. These include the use of randomized clinical trials, epidemiological investigations and laboratory experiments involving experimental animals and cultured cells. As a consequence of the ethical principles involving experimentation on humans, the majority of these studies have been performed in experimental animals and cultured cells. Obviously the use of experimental animals more closely simulates the human situation than cultured cells do and would thus be the system of preferance to be used in studies of this nature. Cell culture does however provide greatly simplified systems, involving fewer variables than in vivo systems, making it easier to study the influence of compounds on cell growth.

Two of the nutrients which have been identified to influence tumour growth are dietary fat and Asc (56). The Committee on Diet, Nutrition and Cancer of the USA National Research Council in Diet, Nutrition and Cancer concluded on the basis of experimental evidence, that of all the dietary nutrients it examined, the greatest frequency of direct associations with increased cancer occurence was provided by dietary fat. On the basis of this evidence the committee recommended that consumption of dietary fat, both saturated and unsaturated, be reduced from the current average of 40%, to 30% of total calories (56). A nutrient identified by the committee as being associated with a lower risk of

certain types of cancer in human populations is Asc. Several clinical trials involving terminal cancer patients have also reported that increased Asc intake resulted in increased survival time and some clinical improvement of the patients (241,288-291). A recent clinical trial involved the use of both GLA and Asc in the treatment of terminal liver cancer patients (156,375,376). Increased survival time and some clinical improvement were reported. In the present study the influence and interactions of EFAs/PUFAs and Asc on cell and tumour growth in cultured cells and in experimental animals were examined. Certain mechanisms which may have played a role in the observed effects on cell or tumour growth were also investigated.

In initial studies the influence and interactions of the nutrients were examined in cultured cells. The transplantable BL6 murine melanoma cell line was used as the malignant cell line since it can be grown in culture and can be transplanted into mice. It would therefore be possible to examine the growth of these cells in two different systems. Since the aim of any possible cancer therapy is to destroy or normalise tumour cells while minimising damage to the normal tissues of the host, it is also important to examine the effects of these nutrients on normal cells. The ideal situation in which to compare the effects of nutrients on the growth of normal and malignant cells would be to obtain a primary culture of cells and malignantly transform some of these cells. The effectiveness of a compound in selectively inhibiting malignant cell growth, at least in vitro, could then be reliably assessed using these cells, as the normal and malignant cells would be of identical origin. Unfortunately at the time of these studies cells of this nature were not available to this laboratory. Due to the difficulty experienced in obtaining a more suitable cell line, the LLCMK (monkey kidney) cells were used as the normal or benign cell line. While these cells are transformed and are of different origin to the BL6 cells, they are non-malignant. As in numerous other studies (157,158,162,166,168,171,198,199,344,345,347) a comparison of the effectiveness of nutrients in selectively inhibiting tumour cell growth using cells from different origins is however possible. Most tumour cells divide more rapidly than normal cells, and in this study the BL6 cells were found to divide significantly faster than the LLCMK cells. A comparison of the effectiveness of EFAs and Asc in selectively inhibiting tumour cell growth could thus be made. Obviously a more accurate assessment of the effects of these compounds on tumour growth could however be provided by in vivo studies which were subsequently performed. The cell culture studies did nevertheless provide an opportunity for a direct investigation of the influence of Asc and the EFAs on cell growth without any interference of other possible influences of these nutrients on different host systems, such as the immune response, which may mask direct effects of the nutrients on the tumour.

All three EFAs examined in this study, namely LA, GLA and AA, were found to inhibit in vitro growth of the two cell lines used. Inhibition of the malignant BL6 cells was however significantly more pronounced than in the benign LLCMK cells despite the fact that the uptake of radiolabelled LA and

AA was found to be significantly higher in the LLCMK cells. It is therefore apparent that the malignant BL6 cells were more susceptible to the growth inhibitory effects of the EFAs, an effect which may have been related to the more rapid division and growth of the BL6 cells. A second possibility is that since tumour cell membranes are reported to contain less PUFAs/EFAs than those of normal cells (137,191), they would have been more susceptible to changes in their PUFA/EFA composition upon EFA supplementation. The growth inhibitory effects of the EFAs were found to require the presence of the EFAs in the growth medium and were found to be reversible upon removal of EFA containing medium, suggesting that the mechanism of growth inhibition did not involve permanent damage to the cells.

The growth inhibitory effects of the EFAs observed in this study are in agreement with numerous other studies (156-166,168,169,171) some of which have reported that GLA and AA are more potent inhibitors of cell growth than LA. Since certain malignant cells have been reported to lack activity of the enzyme delta-6-desaturase (175,394,396), it has been suggested that the reduced growth inhibitory effects of LA may have been related to the inability of the cells examined in those studies to desaturate LA to GLA. It has been proposed on the basis of those results that the provision of GLA to tumour cells may be an important step in the normalisation of malignant cells (175). While the growth inhibitory effects of the EFAs were found to increase with increasing unsaturation (AA > GLA > LA) in this study, the difference in cell growth inhibition by the three EFAs was only significant at the highest level of supplementation used, namely 50μg/ml. Upon examination of the delta-6-desaturase activity of the cells, it was found that the malignant BL6 cells in fact possessed higher activity of the enzyme than the benign LLCMK cells. The presence of delta-6-desaturase activity in the cells may provide an explanation for the small difference in the cell growth inhibitory effects of the EFAs examined. These differences in the growth inhibitory effects of the EFAs are consequently also likely to be the result of some other mechanism. Since the presence of delta-6-desaturase activity has previously been reported in other malignant cells (395,397), it would appear that loss of activity of the enzyme upon malignant transformation is confined to certain cells and is not a characteristic feature of malignant transformation as has previously been suggested (175,394,396). The effect of different EFAs on the growth of different cells may also therefore be dependant on the activity of this enzyme in the cells.

Several mechanisms have been proposed for the growth inhibitory effects of the EFAs in cultured cells, including enhanced PG synthesis (156,164,169,175-182), enhanced lipid peroxidation (159,168,186,198,199), alterations in cell membrane lipid composition (137,165,191-193) and intracellular accumulation of EFAs (173,189,194-197). In this study the possible involvement of lipid peroxidation and PG synthesis in the mechanism of cell growth inhibition by the EFAs was examined.

Since the antioxidant TOC was found to reduce the growth inhibitory effects of the EFAs, while not significantly influencing growth of cells in medium not supplemented with EFAs, it was apparent that lipid peroxidation might be involved in the mechanism of growth inhibition. Further support for this possibility was provided by the observation that while LLCMK cells grown in unsupplemented medium produced more lipid peroxides than BL6 cells grown in unsupplemented medium, upon EFA supplementation, BL6 cells, which were more susceptible to EFA mediated growth inhibition, produced significantly more lipid peroxides. Since susceptibility to lipid peroxidation is known to increase with increasing unsaturation (144), it was not unexpected to find that production of lipid peroxides in EFA supplemented cells increased with increasing unsaturation of the EFAs (AA > GLA > LA). Levels of free radicals, which are the initiating agents in peroxidation (144) and are also formed by degradation of lipid peroxides (144), were also found to increase with increasing EFA unsaturation in cells grown in EFA supplemented medium. Since cell growth inhibition was also found to increase with increasing EFA unsaturation, a correlation appeared to exist between the degree of cell growth inhibition by the individual EFAs and the level of lipid peroxidation induced by them in cells. However no correlation in the reversal of EFA mediated cell growth inhibition and the inhibition of lipid peroxidation by the antioxidant TOC was apparent. While TOC reduced the level of free radicals and lipid peroxides in cells grown in EFA supplemented medium to levels approaching those found in cells grown in unsupplemented medium, it was found to only partially reverse inhibition of cell growth by the EFAs. Furthermore while TOC is known to be an important anti-oxidant in cell membranes (271) and would therefore play an important role in the prevention of lipid peroxidation, the possibility exists that the effects of TOC on EFA mediated cell growth inhibition observed in this study might involve a mechanism which is unrelated to lipid peroxidation. For example, since TOC is localised in cells in association with the cell membrane and intracellular membranes (144), it may play an important role in membrane structure and function besides its function as an anti-oxidant. The lack of direct correlation between the effect of TOC on EFA mediated cell growth inhibition and lipid peroxidation suggests that while lipid peroxidation may have been involved in the inhibition of cell growth by the EFAs, other mechanisms were also involved. Several other studies have provided evidence that the mechanism of EFA mediated in vitro cell growth inhibition may involve lipid peroxidation (159,168,201). As in this study however a lack of correlation between the level of lipid peroxidation and the level of cell growth has been observed in other studies (168,186).

Since the level of free radical production and lipid peroxidation in EFA supplemented medium incubated at 37^{o}C in the absence of cells was negligible, it was apparent that the production of free radicals and lipid peroxides involved a cellular mechanism. Several enzyme systems are thought to play a role in the production of lipid peroxides in cells, including lipoxygenase, cyclooxygenase and cytochrome P_{450} oxidase (202). In this study however inhibitors of these enzymes did not influence free radical levels or lipid peroxidation in EFA supplemented cells. It is possible therefore that

general peroxidation reactions were responsible for lipid peroxidation in the cells used in this study. Several mechanisms have also been proposed for the decreased lipid peroxidation observed in tumour cells when compared with normal cells, as found in this study in cells grown in unsupplemented medium. These include reduced substrate (EFAs/PUFAs) availability (134,135,140,142,143), decreased activity of enzymes known to produce lipid peroxides (136,142,143) and increased levels of TOC in membranes of tumour cells (136,140,142). The dramatic increase in the level of lipid peroxidation observed in the BL6 cells upon EFA supplementation suggests that, in the BL6 cells at least, substrate availability may have been the limiting factor influencing lipid peroxidation.

The apparent lack of effect of the PG synthesis inhibitor, IM, and the LT synthesis inhibitor, CA, on cell growth inhibition by the EFAs appears to preclude enhanced synthesis of PGs or LTs as the mechanism whereby EFAs inhibited cell growth in the two cell lines used in this study. Whereas a number of studies have provided evidence that PGs may be involved in the inhibition of cell growth by EFAs (156,164,169,175-182), other studies are in agreement with the results obtained in this investigation (164,169,171,190,202).

An examination of the effect of supplementary Asc on cell growth in this study revealed that Asc inhibited growth of the malignant BL6 cells while not influencing growth of the benign LLCMK cells. Several previous studies have also reported that the growth inhibitory effects of Asc are more pronounced in malignant cells than in benign cells (312,345,347). The growth inhibitory effects of Asc on the melanoma cells were found not to be reversible upon removal of the Asc containing medium from the cells, suggesting that the mechanism of growth inhibition may have involved permanent damage to the cells or cellular processes. Despite the fact that Asc was rapidly degraded in the culture medium, supplementary Asc was found to significantly increase Asc levels in both BL6 and LLCMK cells suggesting that Asc was rapidly absorbed by the cells. Since no difference was apparent in the Asc levels of BL6 and LLCMK cells at each level of Asc supplementation, it was evident that the BL6 cells were more susceptible to the growth inhibitory effects of intracellular Asc. Due to the rapid decomposition of Asc in the culture medium, the possibility arose that the growth inhibitory effects of Asc on the melanoma cells might in fact have been due to a degradation product of Asc rather than Asc itself. However medium which had been preincubated at 37^{o}C for 24 hours, resulting in the decomposition of the majority of the Asc in the medium, did not influence growth of the BL6 cells. It was therefore clear that inhibition of the BL6 cells was due to Asc and not one of its degradation products.

Several mechanisms have been proposed for the growth inhibitory effects of Asc in cultured cells, including enhanced H_2O_2 formation (315,354,356,357), DNA strand cleavage (315,362-365) and alterations in cellular metabolism (304). This study was however concerned primarily with the

influence of Asc on EFA mediated cell growth inhibition. Since Asc has previously been reported to enhance PG synthesis (366-368,370-372) and to both inhibit and enhance lipid peroxidation in in vitro systems (276,334-342,361), the possible involvement of these two mechanisms in the growth inhibitory effects of Asc on the BL6 cells was examined.

Since Asc was found to enhance free radical production and lipid peroxidation in both BL6 and LLCMK cells in this study it appeared possible that the growth inhibitory effects of Asc on BL6 cells might have involved enhanced lipid peroxidation or free radical production. However the lack of effect of TOC in protecting the BL6 cells from the growth inhibitory effects of Asc appears to preclude lipid peroxidation or free radical production from involvement in the mechanism whereby Asc inhibited BL6 cell growth. Furthermore Asc was found to increase lipid peroxidation and free radical production to a greater degree in LLCMK cells, which were not inhibited by Asc, than in BL6 cells.

The possible involvement of enhanced PG synthesis in the mechanism whereby Asc inhibited BL6 cell growth was investigated by measuring the synthesis of PGs from radiolabelled EFA precursors in cells supplemented with Asc and by utilising the PG synthesis inhibitor IM. Asc was found to stimulate synthesis of PGs, including PGE_1 and PGE_2, in the cultured cells. It is therefore apparent that the mechanism of Asc mediated BL6 cell growth inhibition might have involved enhanced PG synthesis, particularly since exogenous PGE_1 and PGE_2 were found to inhibit cell growth in this study. However the lack of effect of IM on cell growth inhibition in cells supplemented with Asc appears to preclude enhanced PG synthesis as the mechanism whereby Asc inhibited BL6 cell growth. The lack of effect of CA in reversing the cell growth inhibitory effects of Asc also appears to preclude enhanced LT synthesis from involvement in the mechanism of growth inhibition by Asc.

In EFA supplemented cells addition of Asc was found to increase growth inhibition by the EFAs in both LLCMK and BL6 cells. While Asc was found to inhibit BL6 cells in the absence of EFAs, an increase in growth inhibition in EFA supplemented cells was still evident after taking into account the growth inhibitory effects of Asc in the absence of EFAs. The increase in growth inhibition was more apparent with increasing unsaturation of the EFAs. A lack of effect of Asc supplementation on the uptake of radiolabelled EFAs by cells suggests that an intracellular interaction was involved between the two nutrients in inhibiting cell growth. While the mechanism of cell growth inhibition by Asc in cells not supplemented with EFAs did not appear to involve enhanced lipid peroxidation or PG synthesis, the possibility that these mechanisms could have been involved in the increased inhibition of cell growth by supplemention with both Asc and EFAs could not be discounted.

While Asc was found to increase lipid peroxidation and free radical formation at higher levels of EFA supplementation in particular, the lack of effect of TOC in protecting the cells from the growth

inhibitory effects of the two nutrients appears to rule out lipid peroxidation as the mechanism whereby Asc and the EFAs interacted to inhibit cell growth. It is interesting to note that TOC was able to block inhibition of cell growth by supplementary EFAs to a certain degree in the absence of Asc, but was unable to do so in the presence of supplementary Asc. These observations suggest that different mechanisms may have been involved in the inhibition of cell growth by the EFAs in the presence and absence of supplementary Asc.

The lack of effect of IM on cell growth inhibition in cells supplemented with EFAs and Asc appears to preclude enhanced PG synthesis as the mechanism whereby Asc interacted with the EFAs in inhibiting cell growth. The lack of effect of CA on the cell growth inhibitory effects of the two nutrients also appears to preclude enhanced lipoxygenase synthesis from involvement in the mechanism of growth inhibition by the nutrients.

It is tempting to speculate that stimulation of delta-6-desaturase activity by Asc in BL6 cells could have played a role in the inhibition of growth of these cells by Asc, particularly since Asc was found not to inhibit growth of LLCMK cells in which it was also found not to influence delta-6-desaturase activity. The increased activity of the enzyme upon Asc supplementation in BL6 cells would presumeably have increased the levels of the more highly unsaturated EFAs in these cells. Since tumour cell membranes have been reported to contain reduced levels of these EFAs when compared with membranes of normal cells (137,191), increased delta-6-desaturase activity could have increased the content of these EFAs in the tumour cell membrane which may have led to changes in membrane unsaturation and fluidity thereby inhibiting cell growth.

From the in vitro studies it was therefore apparent that both Asc and the EFAs inhibited melanoma cell growth while the EFAs also inhibited growth of the normal LLCMK cells although the growth inhibitory effects were not as great as those observed in the melanoma cells. In cells supplemented with both nutrients however, Asc was found to increase the growth inhibitory effects of the EFAs in both cell lines. While numerous studies have examined the influence of the EFAs and Asc on the growth of cultured cells, no studies examining the interactions of these two nutrients in tumour cells could be found in the relevant literature. This study has therefore provided an insight into the possible use of these nutrients in the control of malignant cell growth. However extreme care should be exercised in relating the results of in vitro studies to the in vivo situation as will be discussed in more detail later in this chapter. The in vivo studies involving dietary manipulation of the two nutrients did however provide further evidence for the existence of an interaction between the two nutrients in inhibiting melanoma growth. The in vivo interaction will also be discussed in more detail later in this chapter.

While the mechanism of the synergistic interaction between Asc and the EFAs in inhibiting in vitro cell growth remains unclear, this study did eliminate the possible involvement of enhanced eicosanoid

synthesis or lipid peroxidation in this mechanism. Furthermore this study provided further evidence for the involvement of lipid peroxidation in inhibition of in vitro tumour cell growth by EFAs. Further studies are required to investigate the mechanism of the interaction and growth inhibition of the two nutrients. A possible mechanism is the influence of Asc on the PUFA/EFA composition of tumour cell membranes since Asc was found in this study to stimulate delta-6-desaturase activity which would presumeably result in increased unsaturation of intracellular EFAs. The stimulation of delta-6-desaturase activity by Asc, as observed in the BL6 cells, has also has not previously been reported in the literature and could provide an interesting area for further research using different cell lines.

In contrast to the in vitro studies, dietary PUFAs/EFAs were found to stimulate BL6 melanoma growth in mice. These contradictory results, obtained using the same cell type in two different systems, emphasise the importance of utilising more than one system in biochemical studies of this nature. Obviously in vivo systems involve many more variables than in vitro systems. These variables may include the immune response which is known to be influenced by dietary PUFA/EFA manipulation (57,148,149). The requirements for EFAs/PUFAs in the two systems also appear to be different. While PUFAs/EFAs are known to be essential in animals, and also presumeably in tumours growing in animals, for functions such as eicosanoid synthesis (16,31), in membrane structure and function (19,21,22) and for maintenance of the epidermal water permeability barrier (43-46)), many cultured cells do not appear to require EFAs/PUFAs for normal growth (58,72). Consequently while certain cultured cells are not inhibited by an EFA deficiency, PUFA/EFA deficient diets inhibit growth of most,if not all,tumours. Furthermore, in in vitro systems a compound can be added directly to the enviroment of the tumour cells. In in vivo systems on the other hand, the concentration of the compound reaching the target or tumour tissue may be diluted by homeostatic control at the level of absorption, distribution or excretion of the compound. Other tissues may also compete with the tumour for uptake of the compound or nutrient under observation. It is therefore possible in the case of the PUFAs/EFAs that different levels of these compounds may be involved in the apparently opposite effects observed in the two systems, particularly in view of the fact that while higher concentrations of EFAs have been found to inhibit cell growth in nearly all cultured cells, lower concentrations of EFAs have been reported to stimulate growth of some cultured cells (58,167,170).

The observation that cultured BL6 cells contained a significant level of delta-6-desaturase activity while microsome preparations from BL6 tumours grown in mice did not, further emphasises the importance of utilising more than one system in biological studies. Since the mechanisms regulating delta-6-desaturase activity are at this stage largely unknown, it is difficult to provide an explanation for this phenomenon. Regulation of the enzyme activity and/or synthesis is presumeably influenced by interaction with the host enviroment. The presence of delta-6-desaturase activity in cultured BL6 cells and the lack of activity in microsomes from BL6 tumours grown in mice was also observed in a recent study by a colleague (377).

The contradictory results obtained regarding EFA/PUFA effects on tumour cell growth and delta-6-desaturase activity in cultured cells and in experimental animals also emphasise the danger in relating the results of in vitro studies to the in vivo situation. As previously mentioned the results of the studies using experimental animals are more likely to simulate those in the human body. The importance of the cell culture studies however lies in the ability in this system to relate the effects of the nutrients directly to the tumour cells without interference from effects of the host animal.

The tumour growth promoting effects of dietary PUFAs/EFAs observed in this study, when compared with an EFA/PUFA deficient diet containing mainly SF, have been documented in numerous studies involving a variety of tumours in experimental animals (64-66,69-74,76,79,85,87-92). Furthermore the observations of this study support those of Carroll and colleagues (81,84,87,93) that once the EFA/PUFA requirement of the tumour is met, promotion of tumour growth is dependant on the amount and not the type of dietary fat. In this study it was also observed that the melanomas appeared to have a specific requirement for GLA,or one of its metabolites, since tumours in mice fed the GLA and LA containing EPO diet were found to be significantly larger than those of mice fed the LA containing SFO diet. Presumeably the tumour cells were unable to synthesise GLA as they lacked delta-6-desaturase activity, thus provision of dietary GLA could have enhanced tumour growth by bypassing this metabolic block. It is difficult to provide an explanation for the loss of delta-6-desaturase activity in the melanoma cells in view of these results, since it would be very unusual for the tumour cells to lose the activity of an enzyme while they appear to require the products of that enzyme for growth. However the growth of these malignant cells in an animal obviously results in some change in the control of synthesis and/or activity of this enzyme.

Since all diets used in this study were isocaloric, increased caloric consumption was obviously not the mechanism whereby melanoma growth was stimulated by dietary PUFAs/EFAs. In an attempt to determine the mechanism whereby dietary PUFAs/EFAs promoted tumour growth, the effects on tumour growth of the antioxidant TOC and inhibitors of PG synthesis (IM) and LT (CA) synthesis were examined.

While diets deficient in antioxidants, which would presumeably result in increased lipid peroxidation, have previously been found to inhibit growth of Ehrlich ascites tumours (58), several studies have reported that the mechanism of tumour promotion by PUFAs/EFAs might involve lipid peroxidation (100,134,135,140,142,143). In this study manipulation of dietary TOC levels was found to significantly influence tumour growth. TOC deficiency was found to inhibit melanoma growth in mice fed the PUFA/EFA containing diets but not in mice fed the SF containing CO diet. PUFA/EFA containing diets supplemented with a high level of TOC on the other hand resulted in a slight but non-significant stimulation of tumour growth in the mice. On the basis of these results it would appear that the

melanoma was susceptible to growth inhibition by lipid peroxides in mice fed a diet containing PUFAs/EFAs and deficient in TOC, which is the major lipid soluble antioxidant in cell membranes (142,144).

Tumour cells and cell membranes have been found to contain lower levels of PUFAs/EFAs than those of normal cells in this and other (93,134-136) studies, presumeably resulting in the decreased lipid peroxidation which is known to occur in tumours when compared with normal cells or tissues (135,136,140,141). However dietary PUFAs/EFAs have been found in this and other (93,138) studies to increase the PUFA/EFA content of tumour cells, presumeably increasing the potential level of lipid peroxidation in the tumour cells. Tumour cells may be particularly susceptible to lipid peroxidation, particularly during TOC deficiency, since they are thought to contain reduced actvity of the free radical scavenging enzymes superoxide dismutase and glutathione peroxidase (134). An adequate supply of TOC on the other hand appeared sufficient to protect the melanoma cells from the apparently toxic effects of lipid peroxidation. From these results it is also apparent that lipid peroxidation was not the mechanism whereby PUFAs/EFAs promoted BL6 melanoma growth in mice since diets containing EFAs/PUFAs were found to reduce tumour growth during TOC deficiency, which would be expected to lead to increased lipid peroxidation, when compared with TOC sufficient or TOC supplemented EFA/PUFA containing diets.

Several studies have reported that tumour cell membranes contain higher levels of TOC than those of normal cells making them more resistant to lipid peroxidation than normal tissues (136,140,142). In the case of mice fed a TOC deficient, PUFA/EFA containing diet these tumour cell TOC stores would presumeably be utilised by interaction with free radicals formed in the tumour cells since further TOC to replenish these stores would not be provided by the diet. Furthermore since the tumours were only introduced into mice once they were being fed the TOC deficient diets, the tumour cells would not have had the opportunity to build up TOC stores. These tumour cells would therefore be susceptible to free radical attack and lipid peroxidative damage which may be the cause of the tumour growth inhibition observed in mice fed the TOC deficient, PUFA/EFA containing diets. It has previously been reported that subcellular fractions of tissues from animals fed TOC deficient diets readily peroxidised in vitro whereas the same fractions from animals fed control, TOC sufficient diets did not (144).

It must be noted however that the levels of GLA, DGLA and AA in tumours from EPO fed mice were significantly higher than those of tumours from SFO fed mice. During TOC deficiency, the tumours of EPO fed mice would therefore be expected to undergo more lipid peroxidation than those from SFO fed mice. Since the melanomas appear to be susceptible to growth inhibition by lipid peroxidation during TOC deficiency, it would be expected that tumour growth in EPO fed mice would be inhibited to a larger extent than in SFO fed mice during TOC deficiency. However in this study growth of

tumours in mice fed the EPO and SFO diets was reduced to the same extent by TOC deficiency. The growth inhibitory effects of TOC deficient EFA/PUFA containing diets may therefore have involved a mechanism other than lipid peroxidation particularly since, as previously mentioned, TOC has functions other than as an antioxidant.

Investigations of the effect of dietary Asc on tumour growth in this study also indicated that there is no interaction between TOC and Asc in the melanoma cells. Previous studies have reported that Asc regenerates TOC from the tocopheroxyl radical allowing one molecule of TOC to react with a number of free radicals (203,264-271). However in mice fed the TOC deficient diet, increasing the level of dietary Asc did not appear to protect the tumour cells from the tumour growth inhibitory effects of TOC deficiency. Despite the fact that no actual measurement of tumour TOC levels was made in this study, the lack of any observed protective effect of supplementary Asc against tumour growth inhibition in mice fed the TOC deficient diets would appear to preclude the occurence of any interaction between TOC and Asc. It must be noted however that while this discussion has confined itself to relating the effects of TOC deficiency to lipid peroxidative inhibition of tumour growth, the mechanism of tumour growth inhibition in mice fed the TOC deficient diets might have involved other mechanisms not related to lipid peroxidation. Since TOC is an important component of cell membranes (144) it may play an important role in membrane structure and function for example. The depletion of membrane TOC levels induced by TOC defiency might therefore influence membrane structure and function leading to inhibition of tumour growth.

IM and CA, which are inhibitors of PG and LT synthesis, when supplemented to the PUFA/EFA containing diets of mice did not influence tumour growth suggesting that PGs and LTs were not involved in the promotion of tumour growth by dietary PUFAs/EFAs. Several previous studies have reported that PGs may be involved in promotion of tumour growth by dietary PUFAs/EFAs (101,105,106,112,115,117), while others have reported that they are not (cited in 407). In view of the contradictory reports in the literature and the results of this study it would appear that the mechanism of promotion of tumour growth by PUFAs/EFAs may vary between different types of tumours. Further studies are consequently required to determine the mechanisms whereby PUFAs/EFAs promote tumour growth in tumours of different origin. Knowledge of the mechanisms involved in promotion of tumour growth would obviously be of benefit in the treatment and possible prevention of tumour growth.

Since it appears that neither eicosanoid synthesis nor lipid peroxidation were involved in the mechanism of PUFA/EFA tumour promotion, the EFA levels of the tumours were measured to determine whether or not dietary PUFA/EFA manipulation influenced tumour EFA composition thereby providing a possible mechanism for the tumour promoting effects of these nutrients. The EFA

composition of the tissues of the host mice were in fact found to be influenced by dietary PUFA/EFA manipulation with the tumour EFA composition being particularly susceptible to manipulation by changes in dietary PUFA/EFA composition. The EFA/PUFA deficient CO diet was found to significantly reduce the levels of LA, GLA, DGLA and AA in tumours. It would therefore appear that this diet which, when fed to mice, resulted in the smallest rate of tumour growth of the diets examined, did not supply the EFA requirements of the tumours resulting in reduced tumour growth. While this diet did not induce any apparent deletrious effects on the health of the mice during the experimental period, more prolonged maintenance of mice on this diet induced severe EFA deficiency symptoms. It is therefore apparent that EFA deficient diets should not be used in the treatment of cancer as the health of animals fed these diets is severely affected.

The lack of delta-6-desaturase activty of the tumour cells observed in this study became evident when the EFA levels in the tumours of SFO and EPO were examined. The levels of GLA and its more highly unsaturated metabolites, DGLA and AA, were significantly lower in tumours from mice fed the LA containing SFO diet than in mice fed the LA and GLA containing EPO diet. Thus it is apparent that changes in the EFA composition of the tumours induced by dietary EFA/PUFA manipulation might have been involved in the tumour promoting effects of these diets. The changes in EFA content of the tumours may have been accompanied by changes in tumour cell membrane composition which are known to influence the growth of tumours (93,134-136). Higher levels of the more highly unsaturated EFAs (GLA, DGLA and AA) in the cell membranes would obviously have increased membrane fluidity thereby possibly influencing tumour growth. Consequently it would appear that the EPO diet, in providing GLA to the tumours, was able to compensate for the loss of delta-6-desaturase activity in the tumour cells. Provision of GLA which could be further metabolised to DGLA, AA or more highly unsaturated EFAs could therefore have led to the more rapid growth of tumours observed in EPO fed mice.

In examining the effect of dietary Asc supplementation on melanoma growth in mice it was found that Asc, at a level 10 times greater than that normally added to the diet of mice, significantly inhibited tumour growth when compared with the usual level of dietary Asc supplementation. The tumour growth inhibitory effects of Asc have in fact been documented in numerous other studies (299,312-314,316-319). Several mechanisms have been proposed to account for the in vivo tumour growth inhibitory effects of Asc including immunostimulation (203,304,323), maintenance of collagen and proteoglycan integrity (241,304,333) and antioxidant action (334-339). Furthermore it has been proposed that the growth inhibitory effects of Asc may be mediated by one or more metabolites of Asc (304,343). Since this study was primarily concerned with possible interactions between Asc and dietary EFAs/PUFAs in inhibiting tumour growth and the possible mechanisms of any interaction, the role played by these suggested mechanisms in Asc mediated tumour growth inhibition was not investigated.

An examination of the tissues of the mice fed the Asc supplemented diet revealed that liver and tumour Asc levels were significantly increased by the supplementary Asc. Whereas several studies have reported that the mechanism of tumour growth inhibition of melanomas appeared to involve accumulation of Asc by the melanomas (298,304,344) the Asc levels of the BL6 melanomas measured in this study were found not to be higher than those previously reported for many other murine tissues (298,293) and in this study were found to be lower than the measured liver Asc levels in mice fed either of the diets. Thus while the mechanism of tumour growth inhibition did not appear to involve accumulation of Asc in the melanomas, supplementary Asc was found to increase the level of Asc in the melanomas thereby possibly inhibiting tumour growth. Alternatively the mechanism of inhibition might have involved an effect on the host such as immunostimulation.

Unlike the EFA deficient CO containing diet which inhibited tumour growth as well as severely impairing the health of mice, the Asc supplemented diet was found not to visibly effect the health of mice fed this diet. Since several previous studies have reported that Asc supplementation is not toxic to experimental animals or humans (241,244,283) it would appear that Asc supplementation could be used in the treatment of cancer without adversely affecting the health of patients.

Since the diet in which Asc was found to inhibit tumour growth contained EFAs/PUFAs in the form of SNO, the possible involvement of an interaction between Asc and the EFAs/PUFAs was investigated by manipulating the EFA/PUFA content of the diet. Supplementation of Asc to the diets of the mice at the same level as above, was found to significantly reduce tumour growth in mice fed PUFA/EFA containing diets but not in mice fed the EFA/PUFA deficient CO diet. The inhibition of tumour growth by Asc thus appeared to involve some interaction with PUFAs/EFAs. Since the tumour growth inhibitory effects of Asc were particularly evident in EPO fed mice, the inhibition may have involved interaction with GLA or one of its more highly unsaturated metabolites. The observation that the influence of dietary PUFAs/EFAs on tumour growth were dependant on the Asc content of the diet, and vice versa, highlights a potential problem facing nutritionalists in prevention or treatment of cancer by dietary manipulation. Whereas a nutrient might exert a particular effect in animals fed a specific diet, changing the level of another dietary nutrient might change the effect or mechanism of action of the nutrient. This problem would be particularly evident in humans as the composition of the human diet is difficult to control. While GLA and high levels of Asc have been used with some success in the treatment of terminal cancer patients (156,375,376), the results of this study suggest that care should be exercised in the utilisation of these nutrients in treating cancer as the influence of the GLA on tumour growth may depend on the level of dietary Asc.

Since Asc was found not to influence the EFA composition of the tumours it was evident that a more specific interaction between the two nutrients was involved in the inhibition of tumour growth. The

apparent lack of effect of dietary IM and CA supplementation and of dietary TOC manipulation on tumour growth in mice, fed the EFA/PUFA containing Asc supplemented diets when compared with mice fed the EFA/PUFA containing Asc control diets, appears to preclude the involvement of PG or LT synthesis or of lipid peroxidation in the interaction between the two nutrients in reducing tumour growth. Whereas this study examined possible mechanisms whereby the PUFAs/EFAs and Asc influenced tumour cell metabolism and growth, it must also be borne in mind that manipulation of the diet might also have affected host pathways or systems which may have influenced tumour growth. In this study the host immune system may be particularly relevant, especially since high levels of dietary PUFAs/EFAs have been reported to inhibit the immune system (57,148,149), while high levels of dietary Asc have been found to stimulate the immune system (304,323).

As found in the in vitro investigation, numerous studies have examined the influence of dietary PUFAs/EFAs and Asc on tumour growth in experimental animals. However except for one ongoing clinical trial involving the treatment of terminal cancer patients with GLA and Asc (156,375,376), no reports could be found in the relevant literature which examined the influence and interactions of combined dietary PUFA/EFA and Asc manipulation on tumour growth and the possible mechanisms of any interaction. The interaction between the two nutrients in inhibiting melanoma growth observed in this study therefore appears to be one of the first studies to examine the interactions of these nutrients in tumours and tumour cells. This study while not being able to identify the mechanism of the interaction, did eliminate the possible involvement of enhanced eicosanoid synthesis and lipid peroxidation. Furthermore Asc was found not to influence the EFA composition of tumour cells in vivo. Further studies examining the interactions of these nutrients, and the mechanisms thereof, in other types of cancer cells and at different levels of dietary Asc and PUFAs/EFAs are obviously required before any more conclusive statements about these interactions can be made.

In conclusion therefore, this study confirmed the results of previous studies of the effects of EFAs/PUFAs and Asc on the growth of malignant cells in cell culture and in experimental animals. Furthermore an interaction between the two nutrients in inhibiting malignant cell growth was observed. Whereas attempts were made to identify the mechanism(s) of action of the two nutrients on cell growth and the mechanism of interaction between the nutrients these were largely unsuccessful and resulted only in the elimination of a number of possibilities. Further studies are required before any conclusive deductions can be made regarding the possible utilisation of these nutrients in cancer therapy. The difficulty of drawing direct comparisons between in vitro and in vivo studies were also emphasised by the results of this study. Furthermore the danger of using nutrients in cancer therapy while not closely regulating the level of the nutrient used and of other nutrients was also apparent in this study.

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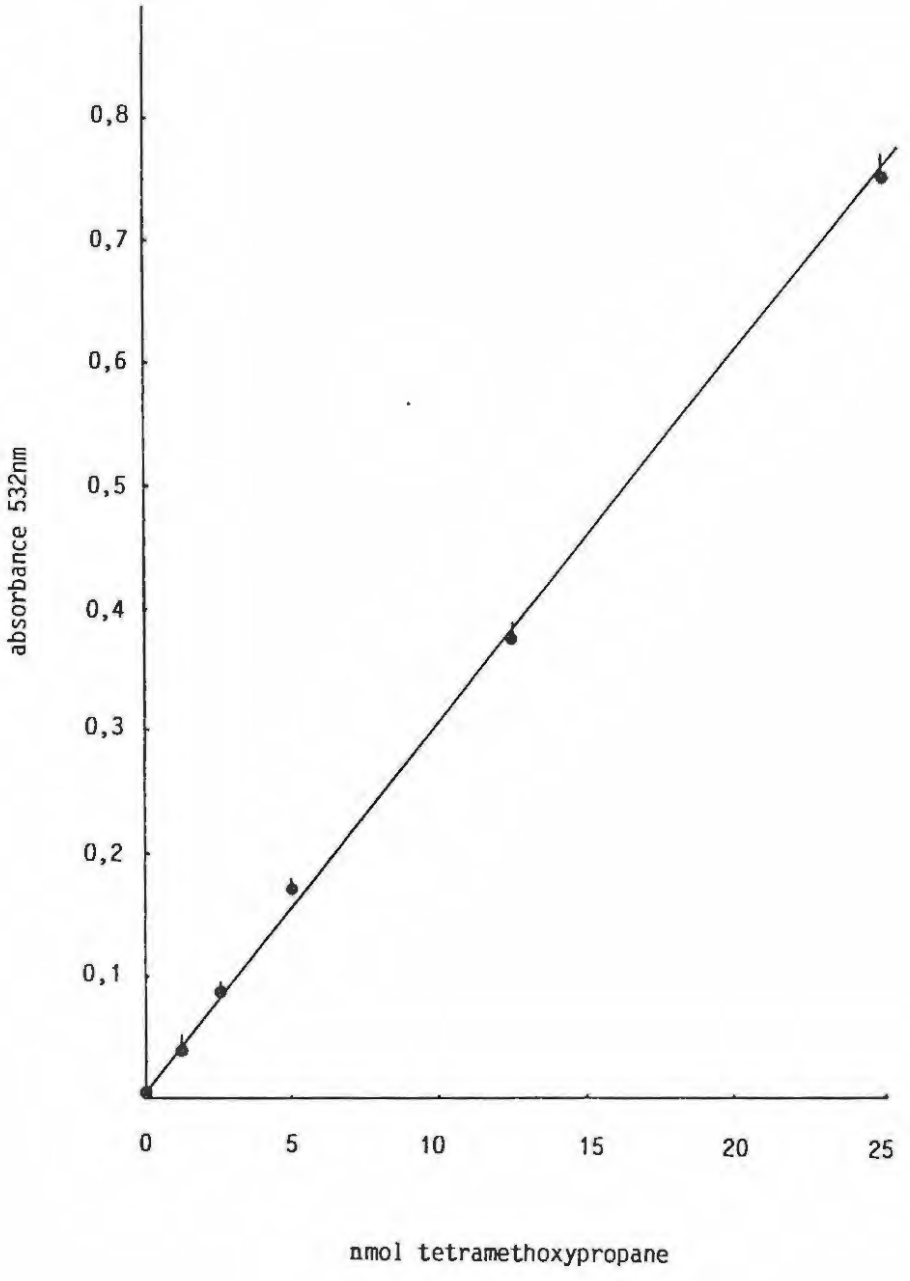
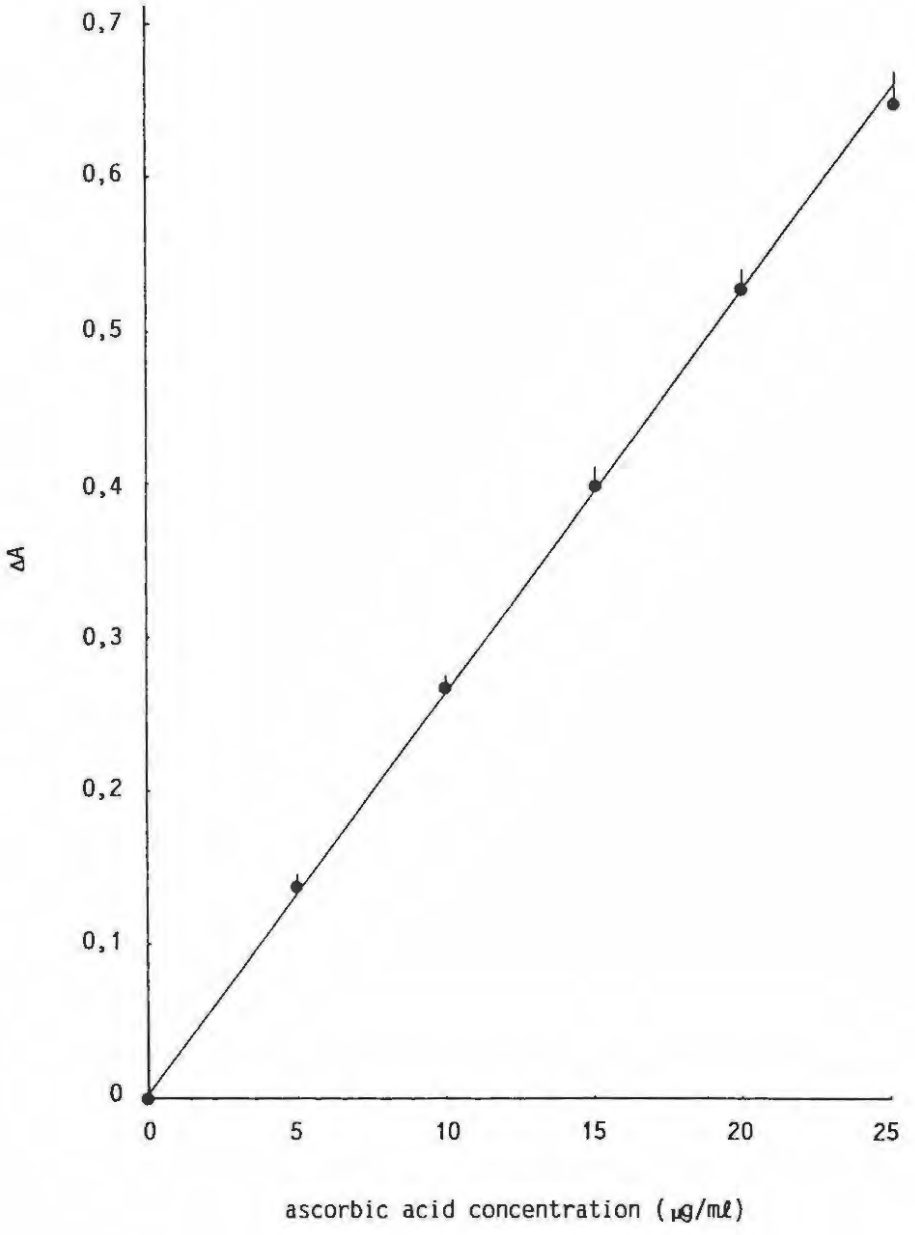
Appendix 1

Standard curve for the determination of lipid peroxidation using 1,1,3,3-tetramethoxypropane as a standard (201). Means and SEM of 5 samples.



Appendix 2

Standard curve for the determination of ascorbic acid concentration (380). Means and SEM of 5 samples.



Appendix 3

Composition of the salt mix used in the semi-purified diets (g/kg diet)	
$CaCO_3$	12,0
$CaHPO_4.2H_2O$	4,4
K_2HPO_4	8,8
$MgSO_4.7H_2O$	5,0
$FeSO_4.7H_2O$	1,0
$MnSO_4.4H_2O$	0,128
KI	0,032
$CuSO_4$	0,072
NaCl	8,57
$ZnSO_4.7H_2O$	0,218

Appendix 4

Composition of the vitamin mix used in the semi-purified diets (mg/kg diet)	
dextrose	8900
biotin	0,0125
folic acid	0,3
p-aminobenzoic acid	5,0
riboflavin	5,0
menadione	12,5
nicotinic acid	15,0
pyridoxine hydrochloride	15,0
thiamine hydrochloride	15,0
vitamin B_{12} (0,1%)	15,0
pantothenic acid	25,0
ascorbic acid	50,0
inositol	250,0
choline chloride	500,0
retinyl acetate	7×10^6 IU
calciferol	360000 IU
d-α-tocopherol	100000 IU

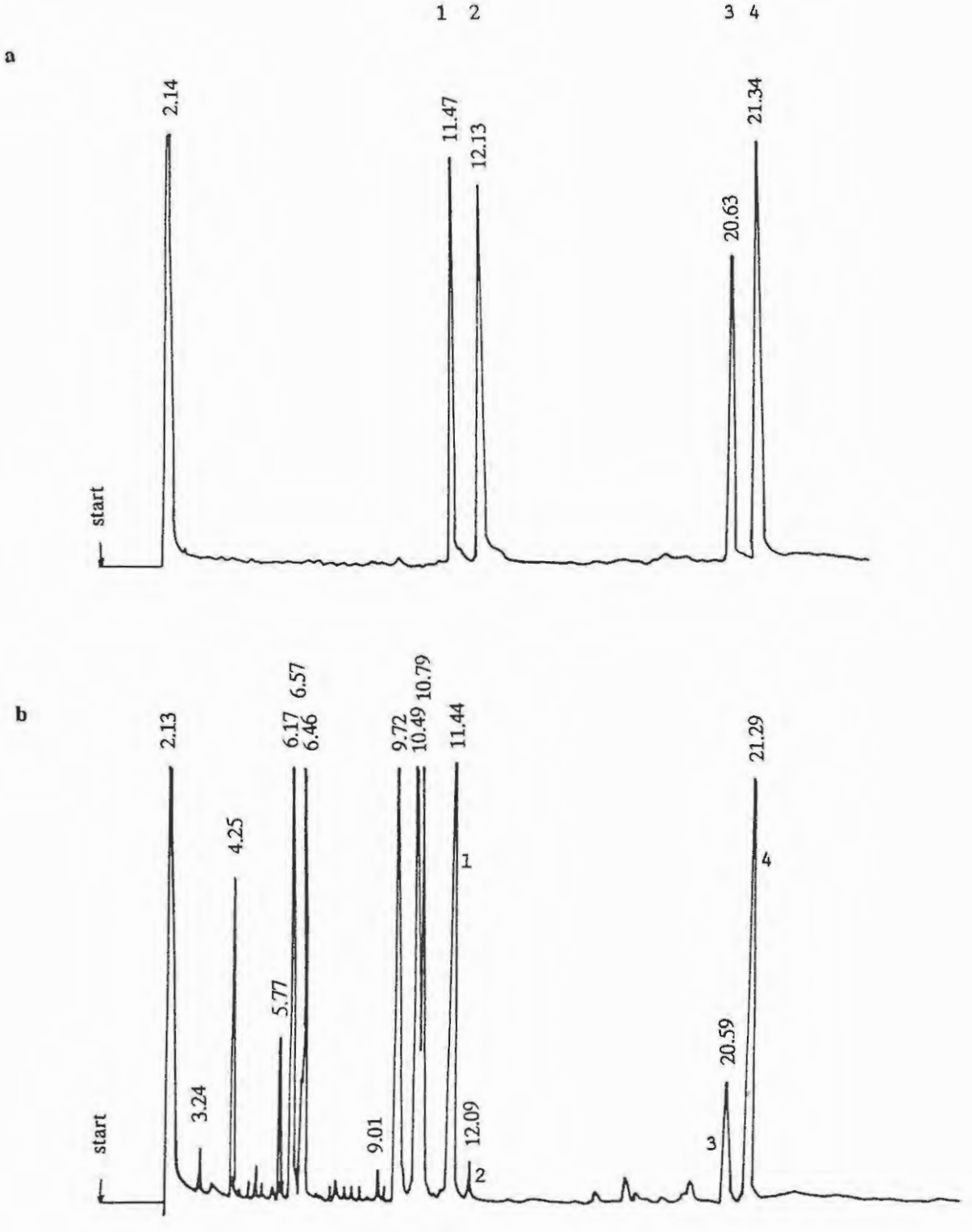
Appendix 5

Essential fatty acid composition of the oils used to supplement the diets (%) measured by gas liquid chromatography.

oil	linoleic acid	gamma-linolenic acid
evening primrose	70,41	8,93
safflower	78,75	-
coconut	-	-

Appendix 6

Separation of essential fatty acids by gas liquid chromatography. a)Standards. b)Typical tumour sample. 1 - LA; 2 - GLA; 3 - DGLA; 4 - AA.



Appendix 7

Separation of essential fatty acids by thin layer chromatography using a slight modification of the method of Bomalski *et al* (399).

EFA	R_f value
LA	0,89
GLA	0,79
DGLA	0,73
AA	0,71

Appendix 8

Standard curve for the determination of protein concentration according to the method of Hartree (400). Means and SEM of 5 readings.